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ABSTRACT

GIBBERELLIN METAHBOLISM AND APPLE FLOWERING

By

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The presence of seeds in 'Spencer Seedless' apple inhibited flower initiation. Fruit removal experiments showed that A critical time for inhibition began around 35 days after full bloom (DAFB). Seeded fruits were significantly larger than seedless ones at harvest. However, fruit weight did not differ significantly until 36 or 37 DAFB in both years.

^{14}C-GA_{12} was injected into seeds and apices of 'Spencer Seedless' and 'Spartan' apple to investigate the metabolism of GA(s) in relation to apple flowering. No qualitative or quantitative differences were found between metabolites in apices of bourse shoots on spurs bearing seeded vs. seedless fruits, or between cultivars ('Spencer Seedless' vs. 'Spartan'). However, the rate of metabolism was higher in apices of bourse shoots in the presence of seedless fruits. Seven major metabolites were detected in apices, six in seeds. Tissues external to the site of injection were oxidized to determine the transport of metabolites. Only a

small percentage of the ^{14}C injected (about 1.47 to 3.22% in 1992, 0.09 to 0.13% in 1993) was transported from seeds to other tissues, and only ca. 0.1 to 0.4% (1992) and 0.01 to 0.04% (1993) was detected in the tissues outside the fruit. With but one exception, no radioactivity occurred in the apex when ^{14}C-GA_{12} was injected into seeds, but one polar metabolite was found in the cluster base and two metabolites in the fruit flesh. When apices were injected, very low amounts of radioactivity (0.2 to 6%) were recovered from outside the injection site; one polar metabolites in cluster bases was found in the cluster bases.

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Introduction

The main objectives of growing fruit trees are to get reasonable yields and good quality fruit and to maintain the tree's productive life. How to control flowering is one of the key issues in reaching these goals. A common problem is biennial bearing -- cropping every other year--which reduces yield and quality. Many species from different families exhibit this tendency (Table 1).

Biennial bearing in fruit production

Biennial bearing affects fruit size, color, and quality. In general, half of the total yield for an "on year" plus an "off year" is less than the yield for one "on year" of a regular bearing cultivar. More branches are broken in the "on year" because of the excessive weight of fruits. Ey depletion of the reserve products in the "on year", the tree can become more susceptible to winter injury than regular bearing trees (Bomeke, 1955).

Much research has been done on biennial bearing, but the problem still exists. A better understanding of the mechanisms controlling flowering is essential to solving

Table 1. Families and species of plants in which alternate bearing has been reported.

Family	Species	Common name	Reference
Anacardiaceae	Mangifera indica	mango	Singh, 1971
	Pistacia vera	pistachio	Crane and Nelson, 1971
Corylaceae	Corylus avellana	hazel	Gardner, 1966
Ericaceae	Vaccinium macrocarpon	cranberry	Lenhardt, 1976 Eaton, 1978
Euphorbiaceae	Aleurites fordii	tung	Potter, et al., 1947
Juglandaceae	Carya illinoensis	pecan	Davis and Sparks, 1974
Oleaceae	Olea europaea	olive	Stutte and Martin, 1986

Table 1 (cont'd)

Rutaceae	Citrus sinensis	orange	Maggs & Alexander, 1969 Moss, 1969; West and Barnard, 1935
	Citrus reticulata	tangerine	Jones, et al.,1975
	Citrus unshui	satsuma	Iwasaki, et al., 1962
Sapindaceae	Litchi sinensis	litchi	Chandler, 1950
Rosaceae	Malus sylvestris	apple	Jonkers, 1979
	Pyrus communis	pear	Jonkers, 1979
	Prunus domestica	plum,prune	Couranjou, 1970
	Prunus armeniaca	apricot	Fisher, 1951

this problem.

Trees usually bear on alternate years because fruit set is excessive during the "on year". When the quantity of fruit on the tree in relation to the amount of foliage is excessive, flower bud formation is reduced or entirely prevented. Thus in the season following the " on year", the reduction in bloom results in a short crop; in the " off year ", too many fruit buds form. Once begun, such a fruiting pattern tends to continue. Practically all cultivars bear more heavily on alternate years, but the tendency is more pronounced in certain cultivars than in others. A typical cycle of biennial bearing is shown in Fig. 1.

Individual limbs or whole trees may exhibit this cyclic bearing pattern, depending upon the previous cropping and weather conditions.

In most cases, large crops inhibit flowering. However, in pistachio heavy cropping does not inhibit flower initiation, but induces abscission of the flower buds (Crane,et al., 1976).

Effects of defoliation and defruiting.

Early defoliation and defruiting experiments with apple, pear, and prune revealed that leaves promoted flower bud formation but subsequent setting of seeded fruit inhibited it. Davis (1957) removed prune flowers and

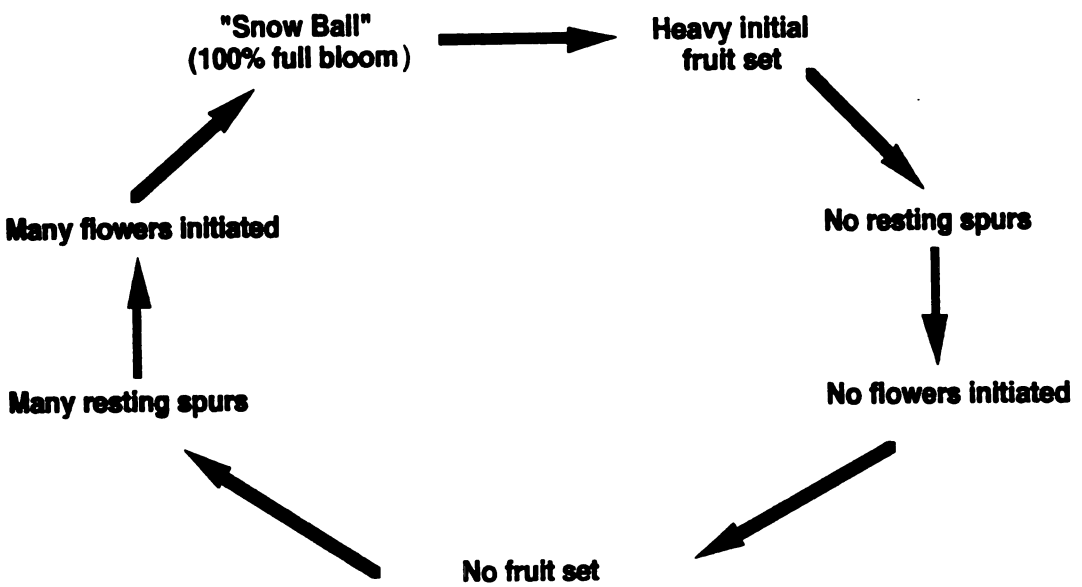


Fig.1. Cycle of biennial bearing of apple trees. (Williams, 1974).

fruitlets at 10-day intervals after full bloom. As treatment was delayed return bloom the following spring fell to nearly zero after 50 days and thereafter. When leaves on apple spurs were removed at weekly intervals after full bloom, no flower buds formed on those spurs that were defoliated within 6 to 10 weeks after full bloom, but flowering increased as treatment was delayed (Harley, et al., 1942).

Defoliation of rapidly growing shoots of 'Chico', a highly fruitful walnut cultivar, did not inhibit pistillate flower bud formation (Ryugo and Ramos, 1979). The flowering stimulus in this cultivar is seemingly omnipresent, being translocated to the dormant buds in the defoliated zone from older leaves below or new ones being formed above this zone. When shoots were pruned back to the fourth, eighth, or twelfth nodes from the apex in July, buds that developed into shoots from the fourth node bore some pistillate flowers while those at lower nodes had proportionately more flowers. Examination of comparable buds on unpruned shoots revealed only sepal primordia. Flower differentiation proceeded rapidly when buds were forced to grow.

Alternate bearing has been investigated in apples longer and more extensively than in any other fruit trees. Alternation has been and still is a problem of horticultural importance in many countries (Williams and Edgerton, 1974). In the United States, alternate bearing is less important

for apples than it used to be. This is due partly to selection of regularly bearing cultivars, but much more to the development of chemical thinning programs, which indirectly regulate flower production (Jonkers, 1979; Williams and Edgerton, 1974; Williams, 1979). However, thinning response is cultivar dependent; some cultivars are very difficult to thin, and thinning effectiveness varies with weather conditions. Moreover, synthetic chemicals represent a potential pollution problem. Therefore a better understanding of the flowering process is needed in order to control flowering naturally.

Effects of growth regulators on flowering.

The flowering response in some plants can be influenced by specific stimuli such as daylength or vernalization, but in others flowering is not controlled specifically by external factors. Most woody plants, including apple, fall into the second group and for this reason have not been examined in detail by workers involved in research on flowering. Exogenous factors include light (Tromp, 1984), pruning (Schupp, et al., 1992; Barden, 1989; Gao et al., 1992), shoot orientation (Jindal, 1990, 1992), ringing and scoring (Iwahori, 1990), rootstock (Schupp, 1992; Gao, et al., 1992), nitrogen nutrition (Grasmanis and Edwards, 1974; Gao, et al., 1992), phosphorus supply (Neilsen, et al., 1990; Taylor and Nichols, 1990), water supply (Jones, 1987),

temperature (Tromp, 1984; Osanai, et al., 1990) and growth regulators/growth substances (Tromp, 1973; McLaughlin and Greene, 1984). Given my research problem, I will confine my discussion to effects of natural and synthetic growth regulators on flowering, with emphasis on gibberellins.

The effects of GAs on flowering of herbaceous plants.

The most intensive research relating GA with flowering has been done with long-day herbaceous plants, as GA can induce such plants to flower under short-day. However, in some cases GA just induces bolting, i.e., stem elongation, without inducing flowering.

The effects of GAs on flowering of conifers.

For woody plants, there is increasing evidence that specific GAs and their metabolites are involved in flowering (Moriz, 1989; Pharis, 1991, cited by Bonnet-Masimbert and Webber, 1995). A mixture of the less polar GAs--GA_4 and GA_7--induces flowering in Pinaceae, whereas GA_3 is commonly used for species of Cupressaceae and Taxodiaceae. Doumas, et al. (urpublished results, cited by Pharis 1991) observed higher concentrations of GA_4 and GA_7 in shoots and primordia of flowering Douglas-fir as compared to vegetative plants. Primordia with a high potential to flower had 50 to 80 times more GA_7 than primordia on nonflowering Douglas-fir trees. Root pruning applied in the absence of an exogenous

application of $GA_{4/7}$ increased the amount of GA_7 present in the primordia. When both root pruning and $GA_{4/7}$ treatments were applied, a strong synergistic increase was observed. Therefore GA appears to have a direct influence on flowering in Pinaceae, Cupressaceae and Taxodiaceae species.

The effects of GAs on flowering of fruit trees.

GAs inhibit flowering of most fruit trees (Table 2). However, GA_4 reportedly promotes and GA_7 inhibits flowering of apple (Looney, et al., 1985). Depending on the species, the sites and modes of GA action may differ, and this may account for the contradictory responses observed between and within species. Nevertheless, there is no explanation at present why fruit trees respond differently from conifers to application of GAs.

Oliveira and Browning (1993) reported that GA_1, GA_4 and GA_5 inhibited floral initiation in Prunus avium by 9-17%, GA_7 by 43%, GA_3 by 65-71% and 2,2-dimethyl GA_4 by 78%. GA_9 and GA_{20} were inactive. Thus activity of GAs with a C-3 hydroxyl was increased markedly by a double bond in the C-1,2 or C-2,3 position, and activity increased with increasing hydroxylation. They found that juvenile and mature shoots differed in sensitivity to a C-1,2 or C-2,3 double bond and that phase change altered the GA complement, GA receptor or transduction mechanisms of P. avium. Floral initiation and growth probably have different requirements

Table 2. GA effects on flowering of tree fruits

Species	GA	Flower Resp.	Reference
Apple	GA_3	-	Hull and Lewis, 1959; Buban and Faust, 1982; Guttridge, 1962; Marcelle and Sironval, 1963; Dennis and Edgerton, 1966.
	$GA_{4/7}$	-	Marino and Greene, 1981; Wertheim, 1973.
	GA_4	+	Looney, et al., 1978; Looney, et al., 1985.
	GA_7	-	Hoad,1984; Tromp,1982.
Pear	GA_3	-	Griggs and Iwakiri, 1961.
Cherry,sweet	GA_3	-	Bradley and Crane, 1959.
Cherry,sour	GA_3	-	Bukovac, et al., 1986.
Plum	GA_3	-	Bradley and Crane, 1959.
Almond	GA_3	-	Bradley and Crane, 1959.
Peach	GA_3	-	Edgerton, 1966; Hull and Lewis, 1959; Gur, et al., 1993.
Citrus	GA_3	-	Guardiola et al., 1982; Goldschmidt and Goren, 1985.

in terms of GA transport and/or action. They proposed that flower initiation in fruit trees is related to the metabolism of GAs. Whether this control is primary or secondary is still not clear.

In most polycarpic angiosperms, applied gibberellin (GA_3) is associated with floral inhibition (Table 2). In contrast, applying growth retardants such as daminozide (SADH) increases flower initiation.

GA_3 inhibits or prevents flower bud formation if it is applied to shoots during the flower bud induction stage (Hull and Lewis, 1959; Griggs and Iwakiri, 1961). Dennis and Edgerton (1966) showed that GA sprays to apple trees at or before flower initiation inhibited flowering. In rare instances applied gibberellin induces or promotes flowering (See Table 2). Gibberellin promotes extension growth in many plants, and Jackson and Sweet (1972) suggested that in apple, flower initiation is prevented because GA stimulates vegetative growth. However, application of $GA_{4/7}$, known to be endogenous in apple seeds, inhibited flower initiation, yet shoot growth was not increased significantly (Wertheim, 1973). Marino and Greene (1981) noted that gibberellin treatment did not increase vegetative growth, hence the inhibition of flowering could not be attributed to gibberellin-induced growth. Rather, inhibition must have been a direct result of the gibberellin sprays. These results cast doubt on the idea that GA(s) inhibit flowering

by increasing shoot growth.

GA $_{4/7}$ inhibited flowering in 'Empire' apple (Greene, 1989). Repeat applications 19 and 34 days after full bloom were only slightly more inhibitory to flowering than one application of 50, 100, or 150 mg.liter^{-1} made 10 days after full bloom. McArtney (1994) showed that a single spray of either GA_3 or $GA_{4/7}$ at full bloom in the "off" year reduced the severity of the biennial bearing cycle of 'Braeburn' apples. The proportion of flowering spurs one and two years after application was linearly related to the concentration of GA applied -- negatively and positively, respectively.

Effects of growth retardants and/or GA synthesis inhibitors on flowering of tree fruits.

Flower bud formation in many fruit tree species is enhanced by application of growth retardants. Both daminozide (SADH) and cycocel (CCC) inhibit GA biosynthesis and favor flower bud initiation. Increased flowering has been reported in apple (Williams, 1972; Greenhalgh and Edgerton, 1965; Edgerton and Hoffman, 1966; Luckwill, 1970), pear (Griggs and Iwakiri, 1968), peach (Edgerton, 1966), plum (Couranjou, 1968), citrus (Monselise and Goren, 1977; Nir, et al., 1972), mango (Maiti, et al., 1971) and other species. Ryugo et al. (1972) found that SADH reduced the GA content of apices of upright-growing cherry branches. The GA content also decreased and flowering was promoted when

shoots were bent towards the horizontal position. Hoad and Monselise (1976) sprayed M 26 rootstocks with daminozide and measured gibberellin and ABA content of the stem tips. Within 2 days of spraying, ABA content of the tissue increased and 5 days after spraying a decrease in extractable GA-like substances was observed. Thus,daminozide could be affecting growth by altering the hormone balance in the stem tips. Marangoni, et al. (unpublished -- cited by Ryugo, 1986) reported that a flower cluster of 'Muscat of Alexandria' grape formed several weeks later on the terminal shoot when rapidly growing shoots were headed back to the eighth node and sprayed with CCC, whereas heading back alone did not induce flowering. Young SADH-treated cherry and pear trees bloomed at an earlier age than comparable untreated ones (Ryugo,et al., 1972).

Hence, any treatment that inhibits GA synthesis and limits shoot elongation apparently favors flower formation.

Endogenous factors affecting flowering.

Carbohydrates. Harley et al., (1942) concluded that an "intimate association between starch content and flower bud differentiation" existed in apple spurs. Following this report, a tremendous amount of work on utilization of carbohydrate reserves was carried out (Priestley, 1970; Jonkers, 1979), but its practical implications for the

understanding of flowering and alternation in apple were very meager. Jackson and Sweet (1972) found no unequivocal evidence to support the belief that carbohydrates play a direct, or even supportive, role in flower initiation in woody plants. The results of Stutte and Martin's experiment (1986) showed few and inconsistent differences in sucrose, fructose and mannitol concentrations in shoots of olive trees exposed to 850 $umols^{-1}m^{-2}$ vs. 150 $umols^{-1}m^{-2}$ PAR. The high level of starch in the former treatment had no effect on subsequent flowering of bearing or nonbearing trees. They concluded that an insufficient supply of carbohydrate reserves was not the primary factor limiting flower induction. Other reports on pistachio (Crane et al., 1976) and citrus (Jones et al., 1974) also showed that carbohydrates were not a limiting factor for flower bud differentiation. For citrus, however, contradictory results were also reported (Hilgeman et al., 1967; Goldschmidt and Golomb, 1982; Schaffer et al., 1985). Data from Goldschmidt et al. (1985) showed that autumn girdling and GA_3 treatment were both effective and additive in increasing starch contents of leaves and twigs of 'Shamouti' orange trees. However, GA_3 inhibited flowering whereas girdling promoted it.

Even studies of photosynthetic efficiency and carbohydrate utilization with intact and partly defoliated regular and alternating apple cultivars (Avery et al., 1979)

did not provide evidence that carbohydrate reserves were limiting, but rather that meristems differ in their capacity for carbohydrate mobilization. Thus reserve mobilization and flowering may be closely regulated, possibly by hormonal factors.

Seeds. In apples, strong inhibition of flower bud initiation occurs during the early stages of fruit development. The physiological processes responsible for this effect are unknown (Buban and Faust, 1982). After successful pollination fruits usually contain many developing seeds. In certain cases seeds inhibit flowering (apples) or induce flower bud abscission (pistachio).

Chan and Cain (1967) showed that seeded 'Spencer Seedless' fruits inhibited flower initiation whereas seedless ones did not. This suggested that presence of seeds was the main factor controlling biennial bearing, rather than depletion of nutrients by fruits. Seeds produce relatively large amounts of gibberellin that presumably inhibit flower initiation. Further confirmation of the role of seeds was provided by Huet (1972) using seeded and seedless fruits of 'Williams' pear. He demonstrated that seeded fruits were more inhibitory to flowering than were seedless ones. However, neither seeded nor seedless fruits inhibited flowering in 'Bartlett' pear trees in California (Griggs et al., 1970). Huet (1972) reported that spurs of 'Williams' pear bearing seeded fruits initiated flowers if

their leaf surface were sufficiently large. In California, growth was very vigorous and leaf surface may have been sufficient to overcome the inhibitory effects of the seeds. Chan and Cain (1967) reported that seeded fruits did not inhibit flowering when 'Spencer Seedless' apple trees were grown in sand culture in a greenhouse; under those conditions tree growth was vigorous. Neilsen (personal communication) evaluated flowering response in ' Spencer Seedless' apple vs. fruit weight, seed number, cropload, and bourse shoot length. Across all seed number categories greater than 4, shoots less than 2 mm never flowered and shoots longer than 16 mm almost always flowered. In shoots 2 to 4 mm long flowering decreased sharply as seed number increased. Flowering in shoots 5 to 9 mm long appeared to be inhibited as seed number increased, but this was less pronounced than in short shoots. Thus shoot length (leaf area?) overrode the seed's inhibition of flower initiation, confirming Huet's result. Neilsen also showed that fruit weight per spur and fruit density did not affect return bloom if fruit were seedless. This finding does not support the hypothesis that competition for carbohydrates is the mechanism whereby the fruit inhibits flowering.

Grochowska (1968a) found that surgical removal of apple seeds after 'June drop' (fruit size 1.7 to 2.0 cm in diameter) had no effect upon return bloom unless growth regulators were substituted for the seeds, GA_3 inhibiting,

IAA promoting flowering. Stutte and Martin (1986) did a series of experiments to determine the role of the seed and crop load on flower induction in olive. Killing the seed with a needle prior to endocarp sclerification promoted flower formation in two cultivars. Surprisingly, however, fruit removal did not stimulate flowering. Dry matter accumulation and partitioning in both seeded and seed-killed fruit were similar. Thus, carbohydrate demand did not appear to be a primary factor in flower formation. They postulated three hypotheses to explain why killing the seed promoted flowering while fruit removal did not. 1) The seed is a source of flower inhibiting compounds and the fruit is a source of flower promoting substances. 2) The fruit competes with potential flower buds for flower promotive compounds. 3) The seed sequesters flower promotive compounds originating from the fruit. Timing is critical here, for flowering of olive is favored if fruits are removed early in the season.

Gibberellins in apple tissues.

Nitsch (1958) first demonstrated GA-like activity, and Dennis and Nitsch (1966) tentatively identified GA_4 and GA_7 in methanol extracts of immature apple seeds. The identification was confirmed by MacMillan (1968) and Luckwill, et al. (1969) using gas chromatography-mass spectrometry (GC-MS); GA_9 was also found. Many more GAs

Table 3. Gibberellins identified in apple tissues

Tissue	Gibberellin(s)	Reference
Immature seeds	A_4; A_7	Dennis and Nitsch, 1966; MacMillan, 1968; Luckwill, et al., 1969; Hoad, 1978, 1980
	A_1; A_9;A_{12};A_{15};A_{17};A_{20}; A_{44};A_{51};A_{63};13-OH-A_{12}; 15-B-OH-A_7; 9,11-dehydro-A_4;iso-A_7	Hoad, 1978, 1980
	A_{61}; A_{62}	Kirkwood and MacMillan,1982
	A_{63}	Avanzi, et al., 1988
	A_1;A_3;A_4;A_7;A_8;A_9; A_{15};A_{17};A_{19};A_{20};A_{24}; A_{34};A_{35};A_{44};A_{51};A_{53}; A_{54};A_{61};A_{62};A_{68};	Lin, et al., 1991
	A_1;A_3;A_4;A_7;A_8;A_9;A_{12}; A_{15};A_{17};A_{19};A_{20};A_{24};A_{34};	Steffens,et al.,1991

Table 3 (cont'd)

	A_{35};A_{44};A_{51};A_{53};A_{54};A_{61}; A_{62};A_{63};A_{68};	
	A_1;A_4;A_9;A_{12};A_{17};A_{20};A_{44}; A_{53}; iso-GA_7	Ramirez, 1993
	A_1,A_3, A_4,A_7,A_9,A_{12},A_{15}, A_{17}; A_{19};A_{20};A_{25}; A_{34};A_{35}; A_{44};A_{45}; A_{53};A_{54};A_{61};A_{62}; A_{63};A_{68};A_{80}; A_{84}; 3-epi-A_{54};A_{88}	Hedden, et al., 1993
	A_{73}; 9,15-cyclo-GA_9; 1β,2β,3β,3α and 11β-hydroxy-9,15-cyclo-GA_9.	Ovama, et al., 1996
Immature fruit	A_3	Hayashi, et al. 1968; Dennis, unpublished
	A_{17}	Dennis, unpublished
Vege.tissue	A_{19}	Koshioka, et., 1983b
	A_{12}; A_{17}; A_{19}; A_{20}; A_{29}; A_{53}; 16,17-dihydro-17-	Saavedra, et al. 1989

Table 3 (cont'd)

OH-A_{53}; 2-OH-A_{53}; mono-hydroxy-A_{12}

have since been identified in extracts of seeds and other tissues of apple (Table 3).

The GAs identified in immature apple seeds represent GAs in the early 13-hydroxylation pathway and early non-hydroxylation pathway.

Seasonal changes of gibberellins in apple seeds.

Luckwill, et al., (1969) measured seasonal levels of GA-like substances - presumably a mixture of GA_4 and GA_7 - in seeds of 3 apple cultivars, using the lettuce hypocotyl assay. Maximum activity occurred 9 weeks after full bloom, when the embryo was approaching full length and the secondary endosperm had attained maximum volume. Two promoters of Avena mesocotyl section elongation, which were not GA-like, were also characterized and quantified, maximum activity occurring at 6 and 13 weeks, respectively. Dennis (1976) reported that levels of gibberellin-like substances in apple seeds and fruit flesh were at a maximum in early July, shortly after June drop. Bioassay of extracts prepared at this time indicated that over 95% of the activity occurred in the endosperm. Extracts of fruit flesh contained two GA-like components, both more polar than $GA_{4/7}$. Activity per unit fresh weight was approximately 3000-fold higher in seeds than in fruit tissue.

The change of GAs in vegetative tissues of apple.

Six GAs were found in extracts of shoots of standard and dwarf apple trees (Steffens and Hedden, 1992). They reported that levels of GA_1, GA_3 and GA_8 were similar in mid-April when both standards and dwarts started to develop. On June 2, standard shoots contained higher concentrations of GA_{19}, GA_{20} and GA_{29}. In both stardard and dwarf trees the content of GA_3 and GA_8 on May 12 and June 2 were similar to those on April 20, whereas the GA_{19}, GA_{20}, GA_1 and GA_{29} levels had declined. GA levels continued to decline between June 2 and 22 in standard shoots. During that time, shoot elongation stopped. No qualitative differences of gibberellins were found between standard and dwarf apples. However, GA_{19} was higher in dwarf apple trees than the standard ones during summer time.

The role of GAs in flower initiation.

The role of endogenous hormones in flower initiation in woody plants and the effects of applied growth regulators on this process have been studied extensively (Luckwill et al, 1978; Hoad 1978; Ramirez and Hoad, 1978; Ramirez, 1979).

The evidence that gibberellins inhibit flower initiation is both direct and indirect. Luckwill, et al., (1969) showed that the concentration of GA-like substances in seeds increased considerably about 5 weeks after full bloom -- the period when seeded fruitlets became inhibitory

to flower initiation (Luckwill 1970). Proof that seed gibberellins are responsible for inhibition requires demonstration that they can move from the fruit into the spur where flowers are initiated. Luckwill (1974) found more GA-like activity in spurs with fruits than in those without fruits, but there was no evidence that the compounds were moving from the fruit through the pedicel into the spur tissues. However, compounds, including GAs, injected into seeds or locules of apples can move into the bourse shoot (Grochowska, 1974). Higher concentrations of endogenous gibberellin-like substances have been observed in diffusates from pedicels of biennial-bearing cultivars of apple than in those from annual cultivars (Hoad, 1978). Gil (1973) reported greater GA-like activity in diffusates from pear fruit of the biennial cultivar 'Winter Nelis' than in those from fruit of the annual cultivar 'Bartlett'. Hoad (1978) obtained more gibberellin-like activity in diffusates from seeded 'Spencer Seedless' fruitlets than from seedless ones. As GA content of the diffusate was very low, the compounds could not be identified by GC-MS. However, their Rfs on thin layer chromatography resembled those of $GA_{4/7}$. He also (1978) investigated the diffusate collected from apple fruitlets during the period prior to flower initiation. More hormones (oat mesocotyl assay) moved out of the biennial flowering cultivar 'Laxton's Superb' than from the annual cv. 'Cox's Orange Pippin'. Ebert and Bangerth

(1981) investigated the levels of diffusible and extractable gibberellin-like substances from fruits of the alternate-bearing 'King of the Pippins' and the regularly fruiting 'Golden Delicious' by using the barley endosperm bioassay (Note: Golden Delicous is annual in Germany, but biennual in the U.S.). In 'King of the Pippins' a very steep increase in the level of activity occurred in both years during the second week after full bloom, reaching a maximum about 3 weeks after full bloom. Maximum levels in diffusates from control fruits were approximately 40 to 45 times higher than corresponding values from chemically thinned fruits. In later stages of fruit development, the contents of diffusible GA-like substances again increased, but this time no marked difference existed between control and treated fruits, with the exception of those treated with naphthaleneacetamide, in which the second peak occurred earlier. A different pattern was found in the regularly fruiting 'Golden Delicious'. With this cultivar, no increase in diffusible GAs from untreated fruits was observed until 4 weeks after full bloom, and peak values occurred during the 5th week. The thinning treatments caused no significant change in the amount of extractable GAs, which was very similar in fruits from both cultivars.

Marino and Greene (1981) noted that gibberellin-like activity in diffusates of flowers or fruits of 'Empire' was detected at bloom and continued to increase until 45 DAFB.

More GA-like activity was obtained from fruit-bearing spurs than from vegetative spurs. However, peak gibberellin activity in seeds and diffusates was not paralleled by elevated activity in fruiting spurs. Thus, inhibition of flowering may not be related solely to diffusion of gibberellin to the spurs during one short time period. In preliminary experiments, Pierson (personal communication, 1996) found more GA_7 in bearing shoots compared with non-bearing shoots.

Another way in which transport has been investigated is by introducing radiolabelled hormones into the seeds, and following their movement from the fruit. Several papers (Knight and Webster, 1986; Hoad, 1978; Green, 1987) indicated movement of GAs; however only a very small proportion of the labelled GAs moved out of the fruit. Knight and Webster (1986) applied GA_3 to a single flower per cluster of 'Conference' pear and 'Early Rivers' cherry at full bloom and 50% petal-fall, respectively, and to a single fruitlet per cluster of 'Victoria' plum four weeks after 50% petal-fall. They measured the distribution of label to flowers or fruitlets, either on the same spur or on adjacent spurs, but little or no radioactivity was detected outside the treated organ. When Hoad (1978) applied ^3H-GA_9 to seeds of intact fruit, no activity was detected in the spur tissue 72 hr later. However, he found that more radioactivity was detected in the spur tissue of the strongly biennial

cultivar 'Laxton' than the regular cultivar 'Cox' after applying ^{3}H-GA_3 to seeds. Green (1987) continued these experiments; transport was limited for all compounds used (^{14}C-GA_{12} aldehyde, ^{3}H-GA_4, ^{14}C-IAA). She found that there was no difference in GA content of seeds of the biennial cultivar 'Tremlett's Bitter' vs. the annual bearing one 'Dabinett' in terms of quantity and quality. She could not detect GA_4 or GA_7 in fruit diffusates, and only a small amount of GA_1 was identified by GC-MS.

Another hypothesis, proposed by Looney et al., (1978), is that seeds affect metabolism of GAs in the bourse shoot and thereby inhibit flowering. Looney, et al., (1985) demonstrated that GA_4 promotes flowering and GA_7 inhibits flowering of apple. They also sprayed ^{3}H-GA_4 on apple trees, and measured the rate of metabolism. Trees destined to flower metabolized GA faster than those in which flowering did not occur.

Summary

Some commercial apple cultivars ('Paulared', 'Golden Delicious', etc.) are strongly biennial, flowering and bearing fruits every other year. This problem was once attributed to a reduction in carbohydrate reserves in the bearing year, few flowers being formed when many fruits were competing with apical meristems for carbohydrates. But Chan and Cain (1967) reported that seedless fruit of the

facultatively parthenocarpic cultivar 'Spencer Seedless' did not inhibit flowering, whereas seeded fruit did. This finding cannot be explained by the carbohydrate hypothesis. Since apple seeds contain high concentrations of GAs, and exogenous application of GAs can inhibit flower formation in apple, GAs originating in the seeds may inhibit flower initiation. How GAs might influence flowering is not yet clear. One hypothesis proposes that gibberellins from the seeds move to the buds and inhibit flowering. Another hypothesis is that the seeds affect metabolism of GAs in the bourse shoot, resulting in an inhibition of flowering (Looney, et al. 1978). The purposes of my research were to determine if:

1. GAs move from apple seeds to the apex of the bourse shoot;
2. The presence of seeds in the fruit affects metabolism of GAs in the apex.

SECTION I **Effect of Time of Fruit Removal on Flower Initiation in 'Spencer Seedless' Apple, and Effect of Cutting Fruits on Retention and Growth of 'Spencer Seedless' and 'Paulared' fruits.**

ABSTRACT

Defruiting experiments confirmed that seeded fruit of 'Spencer Seedless' apple inhibited flower initiation and seedless ones did not. Significant inhibition began around 35 days after full bloom (DAFB). At that time, seed length was 7.6 mm (75% of final length) in 1992, and 7.2 mm in 1993 (64% of the final length). Embryos had not begun rapid growth at that time. Seeded fruits were significantly larger than seedless ones at harvest, but diameters did not differ significantly until 52 DAFB in 1992 and 41 DAFB in 1993, and fruit weight did not differ significantly until 36 or 37 DAFB in both years. Cutting fruits to expose the locules and seeds, then replacing the apical portion, had no effect on abscission of fruit of 'Spencer Seedless'. However, wounded fruits were smaller than controls at maturity. In contrast, seeded 'Paulared' fruits all abscised within 2 weeks when similarly treated.

INTRODUCTION

Biennial bearing is a common problem in fruit production. The presence of a heavy fruit crop inhibits flower initiation in the adjacent shoot buds (Greenhalgh and Edgerton, 1967; Parry, 1974; Ludders, 1978). Alternate bearing was once thought to be the result of nutritional deficiencies. However, there is no unequivocal evidence to support the belief that carbohydrates play a direct, or even supportive, role in flower initiation in woody plants (Jackson and Sweet, 1972). The level of carbohydrates had no effect on the flowering pattern of olive trees (Stutte and Martin, 1986). Chan and Cain (1967) found that seeded fruit of 'Spencer Seedless' and 'Ohio 3', both facultatively parthenocarpic cultivars, inhibited return bloom, whereas seedless fruits did not. Removal of seeded fruits at intervals after bloom indicated that 65% of the inhibition occurred within the first 3 to 5 weeks after pollination. Huet and Lemoine (1972) also found seeded pears to be more inhibitory to flower initiation than seedless ones. These results clearly show that seeds, rather than fruits, inhibit flower initiation.

Apple fruit retention is dependent upon seed development for several weeks following anthesis. Abbott (1958) demonstrated that early seed removal 4 weeks after

petal fall induced abscission of 'Cox's Orange Pippin' fruits. Replacement of the seeds with a lanolin paste containing naphthaleneacetic acid (NAA) permitted continued development. However, NAA did not prevent abscission of deseeded 'Crawley Beauty' fruits. After 7 weeks, seeds could be removed with little or no effect upon abscission. Southwick, et al. (1962) reported that growth of 'Golden Delicious' fruits was independent of seed development after 'June' drop, although 'McIntosh' fruit tended to abscise following seed removal. Gucci, et al., (1991) observed that 'Paulared' also had a high abscission potential following seed removal, even when fruits were half grown. The purpose of this research was to determine the effects of time of removal of seeded vs. seedless fruit of 'Spencer Seedless' on flower initiation, and to evaluate the effects of cutting the fruits (wounding) on fruit retention and growth of both seeded and seedless fruits.

MATERIALS AND METHODS

Plant materials: Two mature trees of 'Spencer Seedless' apple, an apetalous and facultatively parthenocarpic cultivar, and two mature 'Paulared' trees were used at the Horticultural Research and Teaching Center, Michigan State University, E. Lansing.

Clusters of flowers on individual branch units of 'Spencer Seedless' were pollinated with a mixture of

'McIntosh' and 'Delicious' pollen in 1992 and 1993 to obtain seeded fruits; flowers on the remaining branches were not pollinated, and served as a source of seedless fruits.

Effects of seeds and fruit removal on flower initiation in 'Spencer Seedless'. Fruits were removed from limbs bearing pollinated and non-pollinated flowers (25 spurs each at each date) at intervals between 25 and 120 days after full bloom (DAFB) in 1992 and 1993, and spurs were tagged to evaluate return bloom.

Diameter and weight of fruits were recorded, and seed and embryo length were measured.

Effects of cutting fruits on retention and growth. An additional 10 fruits (one per spur) of both seeded and seedless 'Spencer Seedless' and 10 fruits of 'Paulared' (all seeded) were cut in half with a sharp knife on each date, leaving the seeds intact. All non-treated fruits were removed fom the spurs. The apical half was then replaced, and parafilm used to cover the wound. An additional 10 non-treated fruits of both cultivars were also tagged on the first date of treatment (30 to 37 DAFB). The abscission rate and the diameter of the wounded fruit were recorded at harvest.

RESULTS

Effect of seeds and fruit removal on flower initiation in 'Spencer Seedless'. Seedless fruits had essentially no effect on flowering, even when left until harvest (Fig. 1). However, flowering of spurs bearing seeded fruit declined dramatically within about 25 DAFB, although approximately 50 (1992) and 25% (1993) of the spurs flowered even when fruits remained until harvest (Fig. 1). These results confirm those of Chan and Cain, except that they observed 6 to 27% flowering, depending upon year, when seeded fruits were left until harvest. The relatively high rate of return bloom when seeded fruits were removed late in 1992 was probably the result of the use on these dates of spurs with relatively long bourse shoots, which have a greater flowering potential (Neilsen, unpublished data).

Effect of seeds on fruit development in 'Spencer Seedless'. Seeded fruit were larger at harvest than seedless fruit. Differences in diameter did not become significant until 7 to 8 weeks after full bloom, but differences in weight were significant at 6 weeks after full bloom (Table 1, Fig. 2).

Seed had reached almost 70 percent of full length by 37 days after full bloom in 1992, and 50 percent of full length at 30 days in 1993 (Table 1, Fig. 2). The embryo became visible at 45 DAFB in 1992, 41 DAFB in 1993. Embryo lengths

were only 2 to 3 percent of mature length at this time (Table 1).

Effect of cutting fruit on fruit retention and growth. Abscission of 'Spencer Seedless' fruits following wounding ranged from 10 to 30 percent -- similar to the untreated control, whereas all wounded 'Paulared' fruits abscised within 10 to 14 days after cutting (Table 2). Wounding strongly inhibited fruit enlargement of both seeded and seedless fruits. Fruits treated 4 to 5 weeks after full bloom were considerably smaller than control fruits, and the earlier the fruits were wounded, the greater the effect (Table 1, Fig. 3). Seeded fruit did not grow appreciably more than seedless ones following treatment. Average fruit diameter increased only 3 to 8 mm following early treatment.

Figure 1 - The effect of removal of seeded vs. seedless fruit on flowering of 'Spencer Seedless' spurs the following year. NP-not pollinated (seedless) fruits. P-pollinated (seeded) fruits. Fruits removed in 1992 and 1993; flowering recorded in 1993 and 1994.

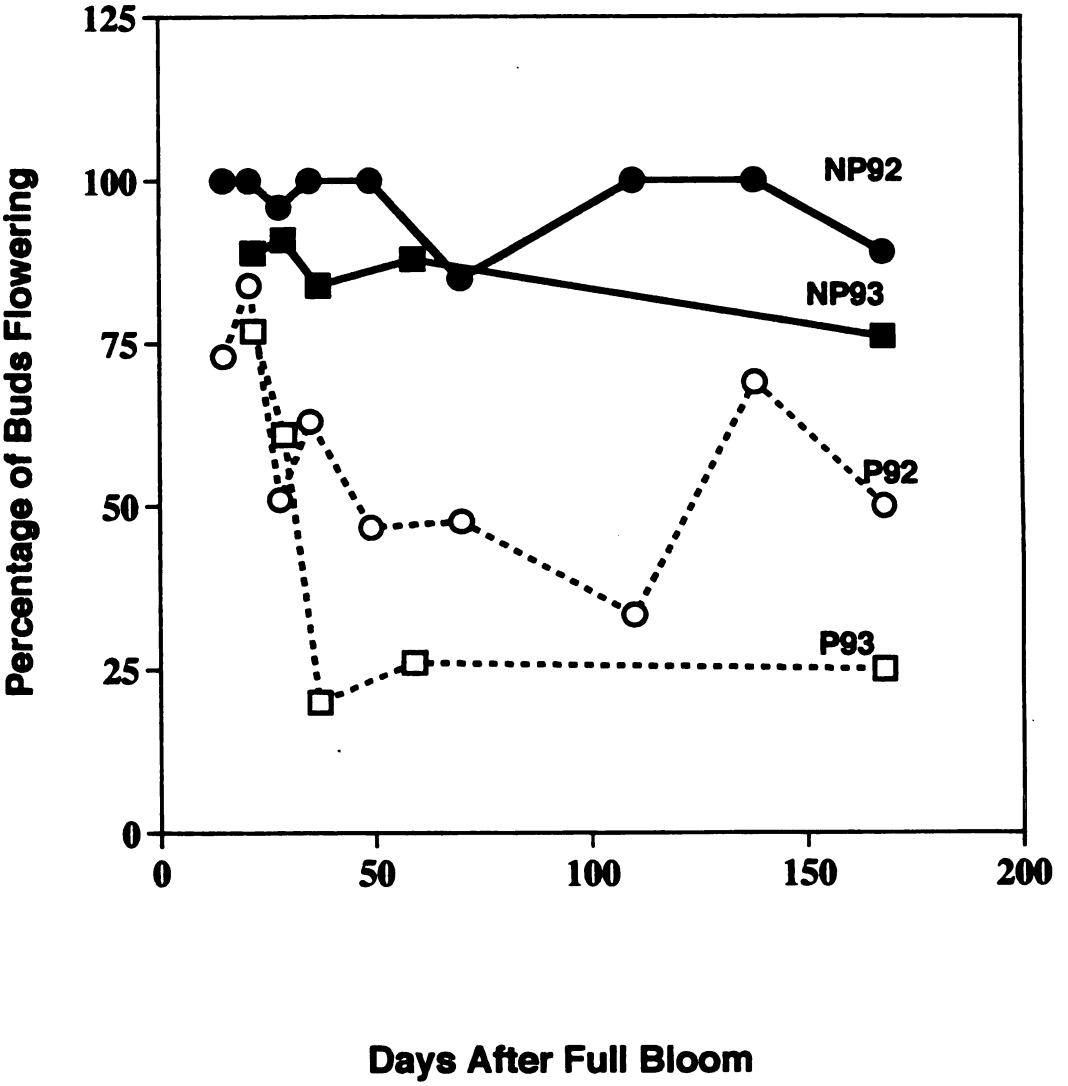

125
100
75
50
25
0
0
50
100
150
200
Percentage of Buds Flowering
Days After Full Bloom
NP92
NP93
P92
P93

Table 1. Diameter and weight of fruit, and length of seed and embryo of 'Spencer Seedless' at the time of sampling or wounding and diameter of wounded fruit at harvest (170 DAFB) in 1992 and 1993 (ten fruits per sample).

DAFB	Seed	Fruit diam. (mm)	Fruit weight (g)	Seed length (mm)	Embryo length (mm)	Final diam[x]. (mm)
			1992			
37	+	30	15	8	0	39
	-	29 n.s.	13 n.s.			37 n.s.
45	+	32	20	8	0.3	44
	-	29 n.s.	15*			40 n.s.
52	+	41	34	8	2	53
	-	38 n.s.	27*			49 n.s.
59	+	57	57	10	4	73
	-	49*	45*			63*
170	+	77	172	12	10	77
	-	68*	164*			68*
			1993			
30	+	25	8	6	0	29
	-	21 n.s.	6 n.s.			24 n.s.
36	+	28	12	7	0	36
	-	27 n.s.	8 n.s.			34 n.s.
41	+	34	21	8	0.2	45
	-	30 n.s.	14*			41 n.s.
48	+	40	30	8	0.8	55
	-	36*	22*			49 n.s.
170	+	74	168	11	10	74
	-	68*	158*			68*

*Significantly less than seeded control , based upon standard deviation of the mean.

[x]Final diameters at harvest of fruits cut at the same times, but not treated.

Figure 2 - Effects of seeds on growth of 'Spencer Seedless' apple in 1992 (A) and 1993 (B).

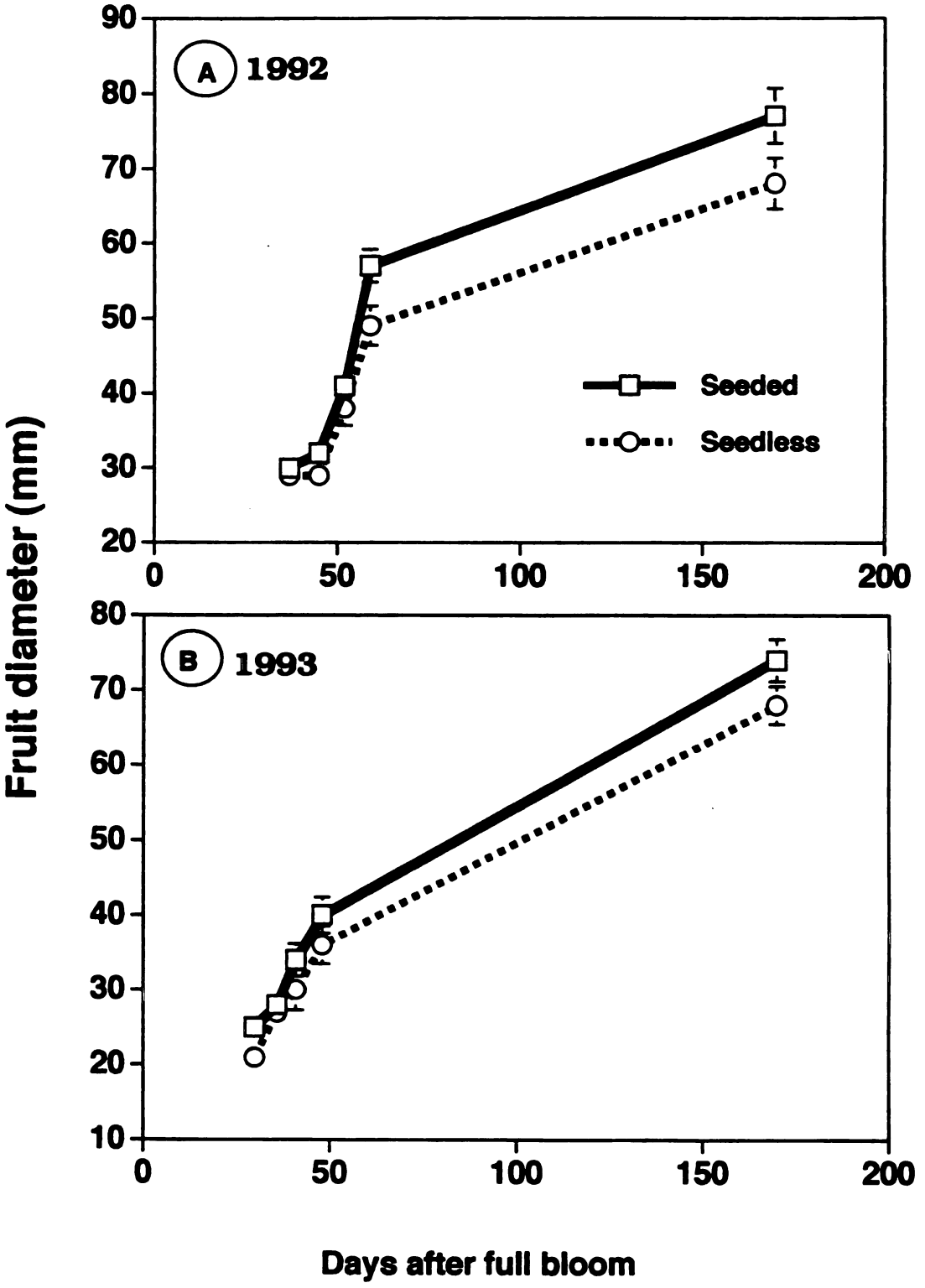
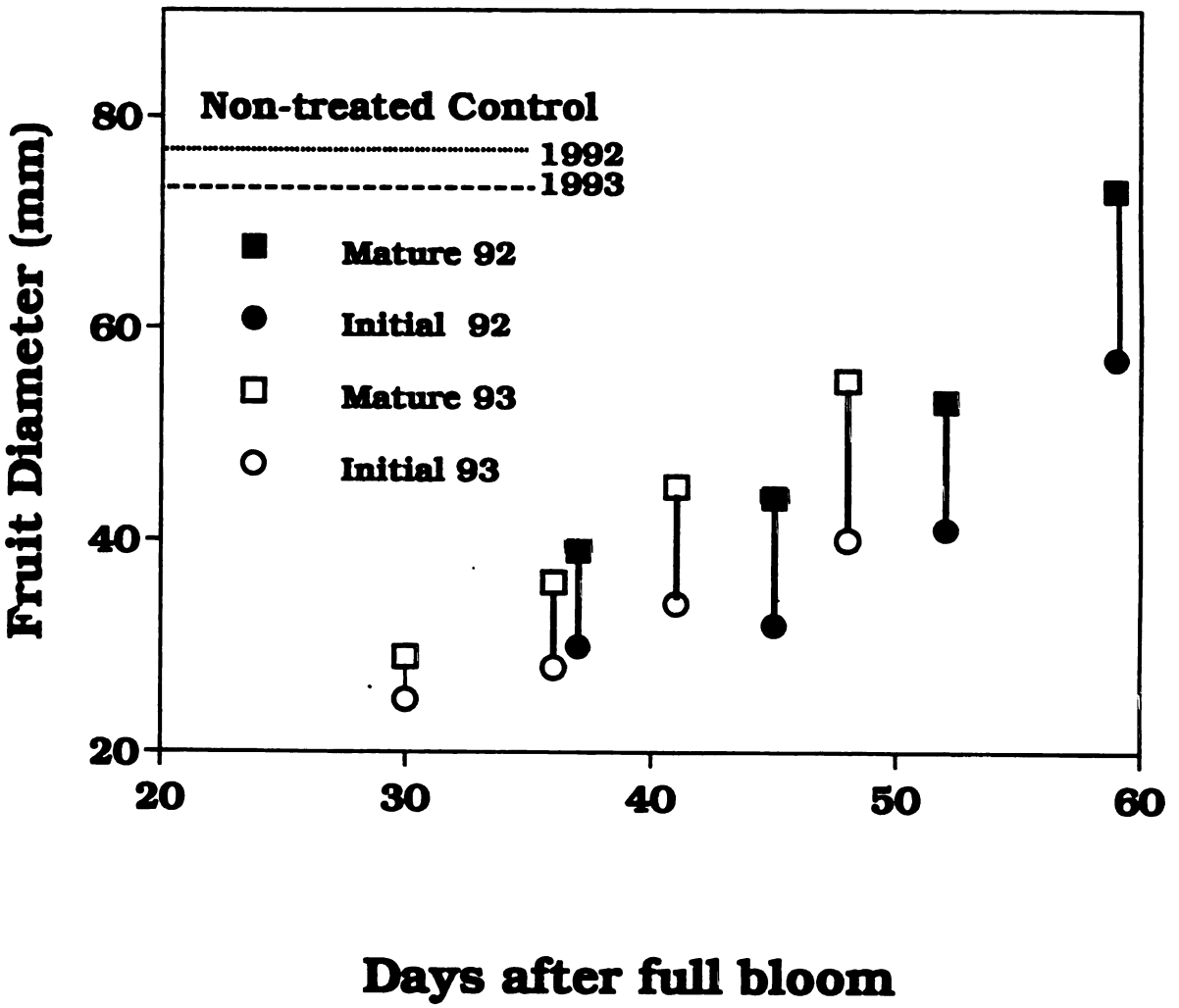

A 1992
B 1993
Seeded
Seedless
Fruit diameter (mm)
Days after full bloom
90
80
70
60
50
40
30
20
10
0
50
100
150
200

Table 2. Effect of wounding at various times on abscission of 'Spencer Seedless' apple fruit in 1992 and 1993 and 'Paulared' fruit in 1993. Untreated fruits (CK) were marked for comparison on the first date of treatment.

	Fruits retained (%)														
	Spencer Seedless										Paulared				
	1992 (DAFB)					1993 (DAFB)					1993 (DAFB)				
Seed	37	45	52	59	CK	30	36	41	48	CK	35	41	46	53	CK
+	60	80	80	90	80	70	60	80	80	80	0	0	0	0	60
-	70	70	80	60	70	60	70	80	70	80	-	-	-	-	-

Figure 3 - Diameters of seeded 'Spencer Seedless' apple fruits at time of wounding (initial) and at maturity (170 DAFB), and diameters of non-treated controls at maturity, in 1992 and 1993. Time of treatment (DAFB) is indicated on the ordinate.


Non-treated Control
1992
1993
Mature 92
Initial 92
Mature 93
Initial 93
Fruit Diameter (mm)
80
60
40
20
20
30
40
50
60
Days after full bloom

DISCUSSION

Fruit removal indicated that seeded fruits of 'Spencer Seedless' had inhibited return bloom by 50 to 70% five weeks after full bloom, whereas seedless fruits had no effect. The critical time for flower inhibition therefore was before 35 DAFB. My results parallel those of Chan and Cain (1967) except that they observed 6 to 27% flowering, depending on the year, when seeded fruits were left until harvest. Neilsen (personal communication) found similar results with 'Spencer Seedless'. Again, variation in degree of inhibition occurred from year to year, and seeds did not inhibit flower initiation completely. Even when fruits were left until harvest, about 30% of the bourse shoots flowered. These results imply that seeds are not the only factors involved in controlling flower initiation. Neilsen (unpublished data) found that both seed number and shoot length had quantitative effects on flowering response of 'Spencer Seedless', with flowering declining with seed number and increasing with bourse shoot length. Shoots 16 mm or longer flowered regardless of seed number. Chan and Cain (1967) observed that seeds had less effect on return bloom of 'Spencer Seedless' when trees were grown in a greenhouse, where bourse shoots grew vigorously, and had greater leaf area. Further confirmation was provided by

Huet and Lemoine (1972) with seeded and seedless fruits of 'Williams' pear. They reported that spurs bearing seeded fruit could flower if their leaf surface were sufficiently large. Thus some factor(s) coming from leaves apparently overcomes the inhibitory effects of the seed.

The critical time of inhibition was within five weeks after full bloom. At that stage, the embryos were very small, implying that the endosperm or nucellus is responsible for inhibition. Auxin levels in apple seeds rise within 35 DAFB (Luckwill, 1959); these compounds may be involved in controlling flower initiation, although gibberellins are believed to play a more prominent role (Luckwill, 1978).

Seeded fruit differed little in size from seedless fruit during the critical time for inhibition of flower initiation, and Neilsen (unpublished data) found no relationship between weight of fruits per spur and subsequent flowering of bourse shoots. Thus competition for carbohydrates cannot explain the effect.
Although wounding caused all 'Paulared' fruits to abscise, it did not affect fruit retention of 'Spencer Seedless' even when the fruits were relatively small; however, fruit size was reduced. The effect on size lessened as treatment was delayed. The data of Abbott (1958), Southwick, et al. (1962) and Gucci,et al. (1991) indicate that wounding reduced fruit size considerably, but that fruit grew much

more than did 'Spencer Seedless' fruits following wounding. Previous workers used lanolin pastes, rather than Parafilm to cover the wounds, and Southwick, et al. (1962) noted that cut fruits treated with lanolin grow more than those left open to the air.

Seed and fruit conditions were good after cutting and injecting 1 ul of 10% ethanol into the seeds (data not shown). Thus the experimental system could be used to study the metabolism of 14C-GA12 *in vivo* in subsequent research (Sections II, III).

No treatments were applied prior to 5 weeks after full bloom, therefore one cannot conclude that seedless fruits are less subject to abscission following wounding prior to this time than are seeded ones.

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SECTION II Metabolism of ^{14}C-GA_{12} and Characterization of Metabolites in Apple Seeds and Apices in Relation to Flower Induction.

ABSTRACT

Radioactive metabolites were analyzed in apple seeds and apices after injection of ^{14}C-GA_{12} during the flower induction period. No qualitative or quantitative differences were found between metabolites in apices of bourse shoots on spurs bearing seeded vs. seedless fruits, or between cultivars ('Spencer Seedless' vs. 'Spartan'). However, the rate of metabolism was higher in apices of bourse shoots in the presence of seedless fruits. Seven major metabolites were detected in apices, six in seeds. Four of the metabolites in seeds may be conjugated, as they were less polar after acid hydrolysis. [Abbreviations used: ATP=adenosine triphosphate; PEP=phosphoenolpyruvate; NADPH=nicotinamide adenine dinucleotide phosphate (reduced form); GA=gibberellin]

INTRODUCTION

Apple seeds inhibit flower initiation (Chan and Cain,1967; Neilsen , 1994; Ban, 1996). Because seeds contain high concentrations of gibberellins (GAs) and/or GA-like compounds during early phases of fruit growth (Luckwill, et al., 1969; Dennis, 1976) and because several GAs can inhibit flowering of apple (Buban and Faust, 1982; Guttridge, 1962; Marcelle and Sironval, 1963; Dennis and Edgerton, 1966; Marino and Greene, 1981; Wertheim, 1973; Looney et al., 1978, 1985; Hoad, 1984; Tromp, 1982), Luckwill (1970) suggested that seed GAs may inhibit flowering. More auxin and/or GA-like activity was observed in diffusates from seeds or pedicels of biennial vs. annual cultivars of apple (Grochowska and Karasewska, 1976; Hoad, 1978). However, Ebert and Bangerth (1981) found no differences in hormone profiles in diffusates from biennial vs. annual cultivars over several years. Injection of ^{14}C-GA_{12} resulted in very low radioactivity in tissues outside the fruit, and trace amounts or no radioactivity were detected in bourse shoot apices, where flower initiation occurs (Green, 1987). These results cast doubt on the hypothesis that GAs move from seeds to apices and there inhibit flower initiation.

Looney, et al.,(1978) suggested on alternative hypothesis. They reported a positive relationship between rate of ^{3}H-GA_4 metabolism in shoots and return bloom in

apple. They proposed that spurs initiating flowers have a greater capacity to metabolize GAs. Thus seeds might inhibit flower initiation by affecting GA metabolism in the apices. My objective was to investigate both of these hypotheses, as well as to determine how GA_{12} is metabolized by apple seeds and bourse shoots.

MATERIALS AND METHODS

Plant materials: Two mature trees of 'Spencer Seedless' apple, which is apetalous and facultatively parthenocarpic, and one annual bearing cultivar, 'Spartan', were used at the Horticultural Research and Teaching Center, Michigan State University, E. Lansing. Although 'Spartan' bears annual crops of fruit, the spurs alternate (personal observation). The inhibitory effect of seeds becomes evident 20 DAFB, based upon flowering response of defruited spurs (Ban, 1996), and reaches a maximum after 40 to 50 DAFB.

Clusters of flowers on individual branch units of 'Spencer Seedless' were pollinated with a mixture of 'McIntosh' and 'Delicious' pollen in 1992 and 1993 to obtain seeded fruits; flowers on the remaining branches were not pollinated, and served as a source of seedless fruits. One 'Spartan' tree was open-pollinated.

Biosynthesis of ^{14}C-GA_{12}. The methods used by Birnberg and Brenner (1986), as modified by J.A.D.Zeevaart (personal communication), were used to prepare ^{14}C-GA_{12}, the precursor of all the known gibberellins. Briefly, R,S-4,5-^{14}C-mevalonate lactone , specific activity 54 mCi/mmol (purchased from Amersham, Buckinghamshire, England) was hydrolyzed to mevalonic acid (^{14}C-MVA) with 0.5 M NaOH. Liquid endosperm from pumpkin (Cucurbita maxima) seeds was collected on an ice bath approximately 1 month prior to fruit maturity. The endosperm was homogenized in a hand-held glass homogenizer, and the homogenate was dialyzed against potassium phosphate buffer [1 M K_2HPO_4 : 1 M KH_2PO_4 : 0.5 M $MgCl_2.6H_2O$ (47.5 ml : 2.5 ml : 5.0 ml, diluted to 10 L)]. The dialysate was frozen as droplets in liquid nitrogen, and stored at -80°C. The enzyme preparation was thawed as needed, and incubated with ^{14}C-MVA for 3 hr at 30°C in 30 ml centrifuge tubes on a shaker. The reaction mixture contained: ^{14}C-MVA, 300 ul = 20 uCi; 0.5 M $MgCl_2$, 25 ul; 0.1 M $MnCl_2$, 25 ul; 0.5 M ATP, 25 ul; 0.5 M PEP, 25 ul; 0.05 M NADPH, 25 ul; 0.2 M 2,2'dipyridyl, 25 ul; and enzyme preparation, 2.075 ml. Acetone was added to give 50% acetone to stop the reaction, the solution was centrifuged to precipitate proteins, and the supernatant was partitioned 6 times against ethyl acetate. The aqueous layer was discarded. The ethyl acetate fraction was chromatographed on aluminum-backed silica gel TLC plates in chloroform :

ethyl acetate : acetic acid (75 : 25 : 1) and ^{14}C-GA_{12} was localized by autoradiography. The ^{14}C-GA_{12} band was eluted with acetone : methanol (1 : 1). HPLC was used for final purification (Fig. 1), the ^{14}C-GA_{12} being detected with a flow-through detector (β-Ram, IN/US System, Inc.). Specific activity of purified ^{14}C-GA_{12} was determined by GC-MS (Figs. 2-4). The final product contained a mixture of unlabelled GA_{12} plus GA_{12} with 1,2,3, or 4 labelled carbons. The specific activity of ^{14}C-GA_{12} was calculated according to the method of Browen and MacMillan (1972) (95 uCi/umol for ^{14}C-GA_{12} synthesized in 1992, 115 uCi/umol in 1993). ^{14}C-GA_{12} used in 1995 was bought from Dr. L. Mander (Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia) , with specific activity 56 mCi/mmol; this was labelled in only one carbon (Fig. 5).

Application of ^{14}C-GA_{12}. ^{14}C-GA_{12} was applied to seeds and bourse shoot tips *in vivo* (Table 1). On each date in 1992, fruits from hand-pollinated flowers were reduced to 2 per spur, and cut in half equatorially to expose the seeds in the 5 main locules. A total of 0.3 uCi of ^{14}C-GA_{12} was injected into 3 to 4 seeds of each fruit using a hypodermic syringe. The top half of the fruit was then replaced and held in place by wrapping the wound with a strip of Parafilm.

^{14}C-GA_{12} was also injected into bourse shoot tips on

Figure 1 - HPLC profile in methanol/water system for ^{14}C-GA_{12} biosynthesized from R,S-4,5-^{14}C-mevalonate (see text for the conditions of biosynthesis and chromatography)

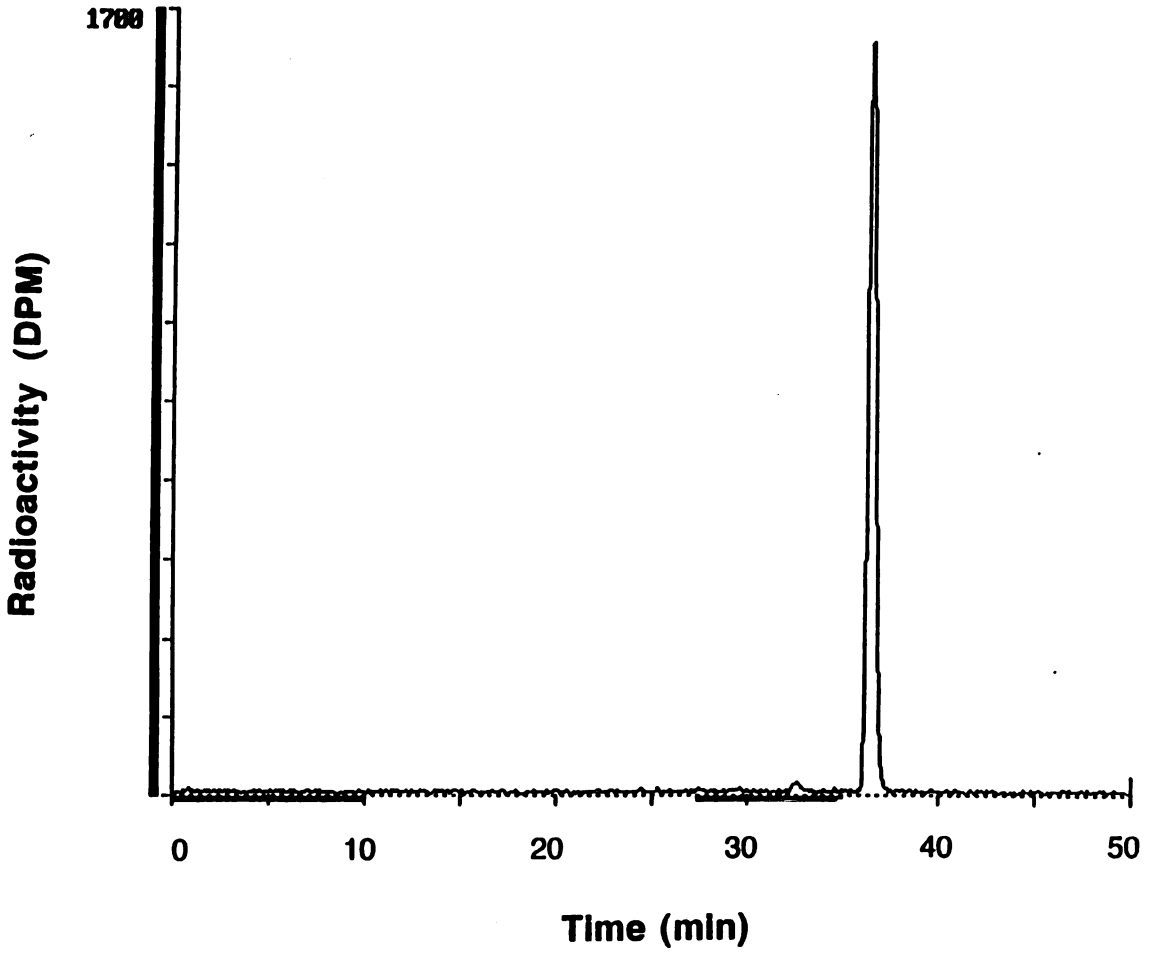

1700
Radioactivity (DPM)
0
10
20
30
40
50
Time (min)

Figure 2 - Aburdance of selected ions in mass spectrum of Me-TMS derivative of ^{14}C-GA_{12} synthesized from ^{14}C-MVA following GC-MS (see text for conditions).

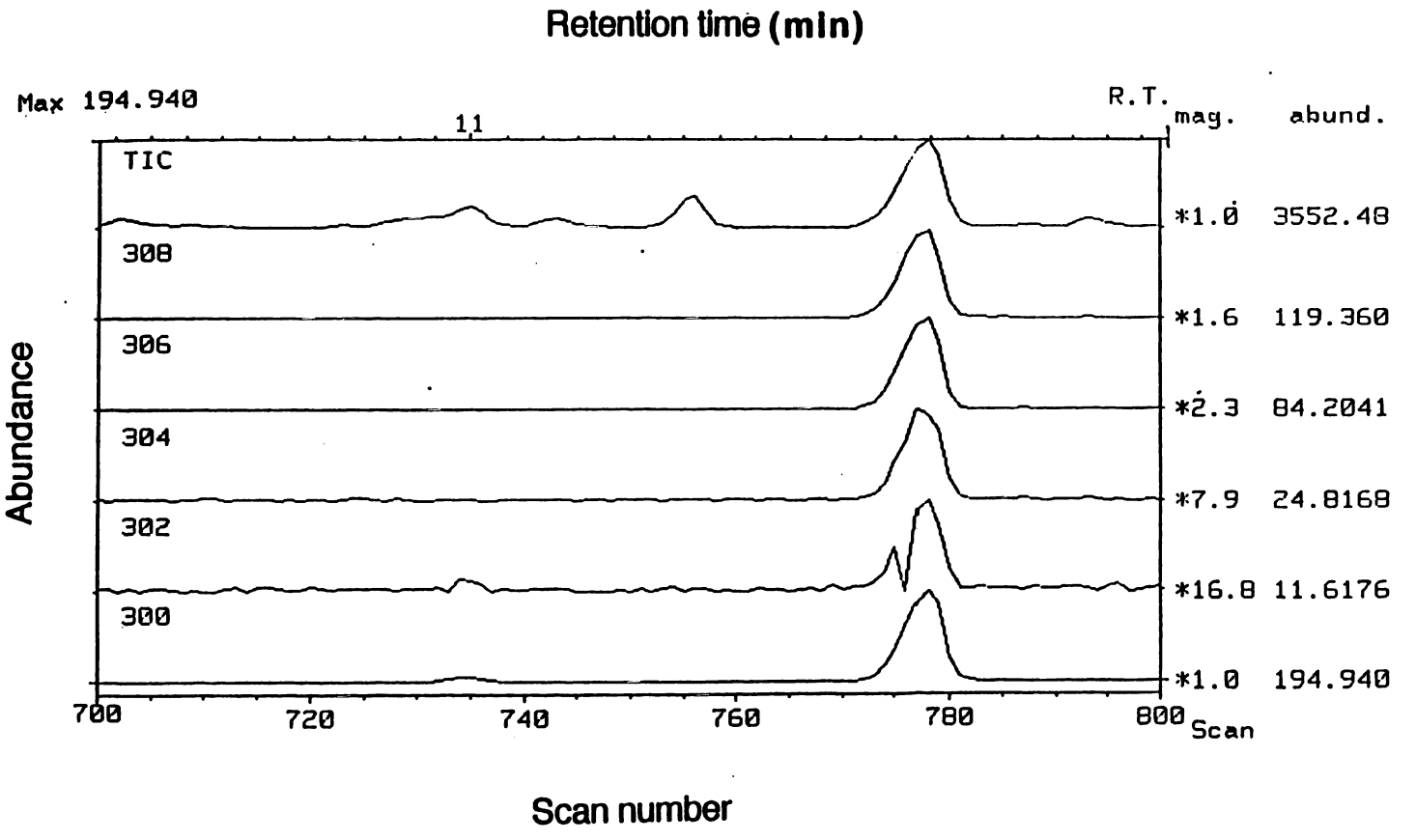

Retention time (min)
Max 194.940
11
R.T.
mag.
abund.
TIC
308
306
304
302
300
*1.0 3552.48
*1.6 119.360
*2.3 84.2041
*7.9 24.8168
*16.8 11.6176
*1.0 194.940
Abundance
700
720
740
760
780
800
Scan
Scan number

Figure 3 - Mass spectrum of Me-TMS GA_{12} synthesized from ^{14}C-MVA. Ions characteristic of GA_{12} are 360 (M+), 328, 300 (base peak=X) and 241. Additional ions are evident at X+2, X+4, X+6, and X+8, indicating presence of radioactive forms.

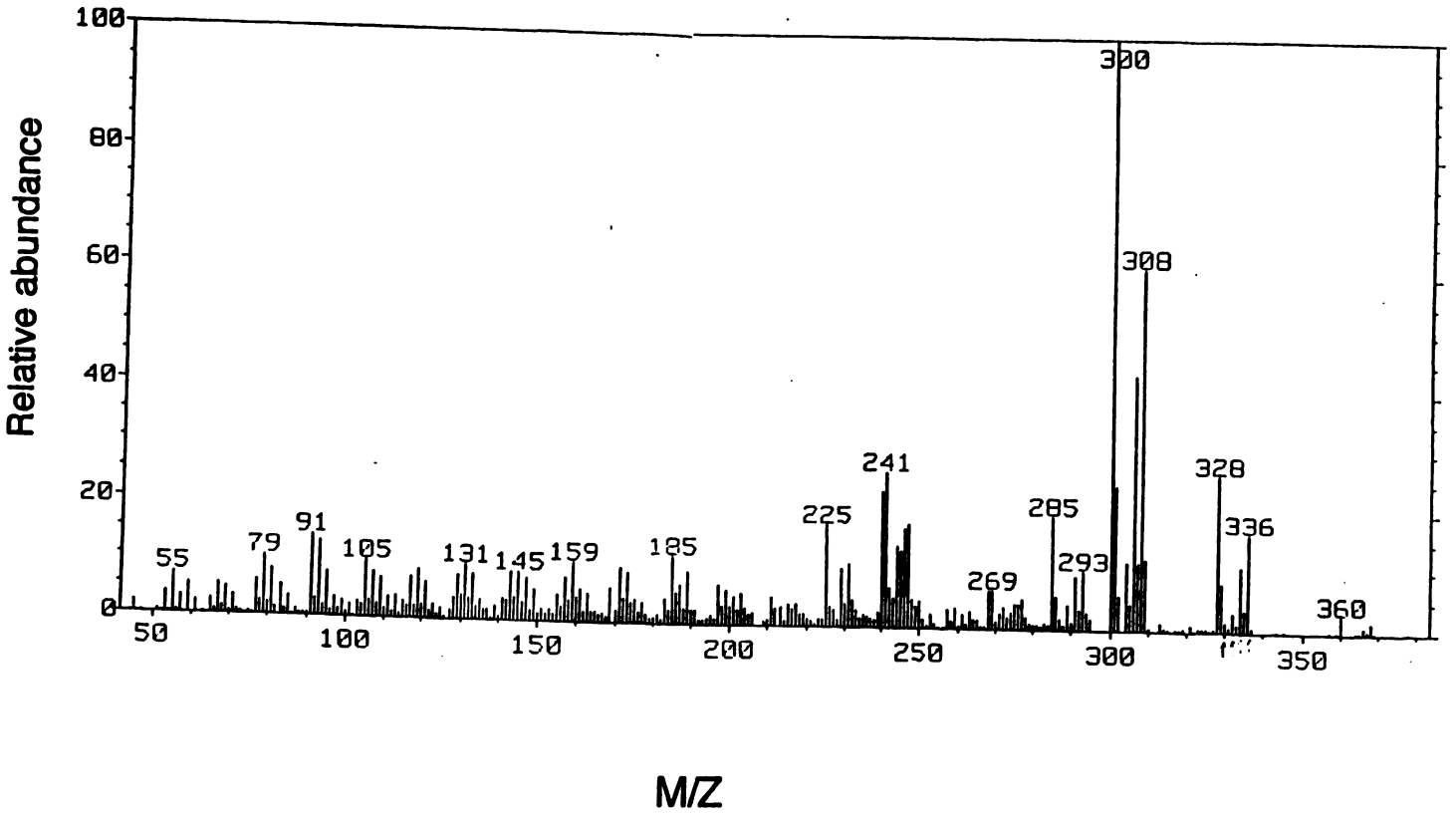

Relative abundance
100
80
60
40
20
0
50
100
150
200
250
300
350
M/Z
55
79
91
105
131
145
159
185
225
241
269
285
293
300
308
328
336
360

Figure 4 - Expanded mass spectrum of Me-TMS derivative of ^{14}C-GA_{12} (see Fig. 3), showing evidence of radioactive forms.

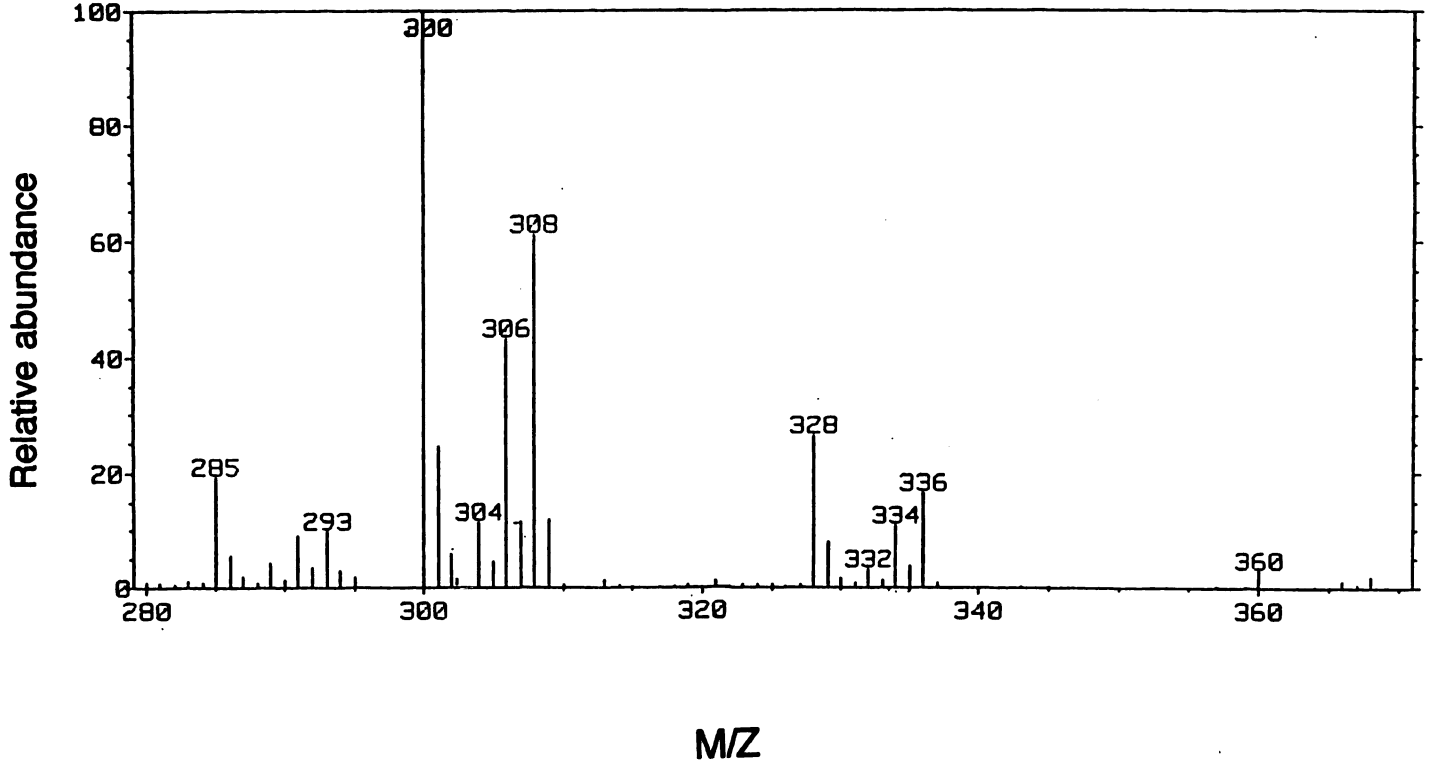
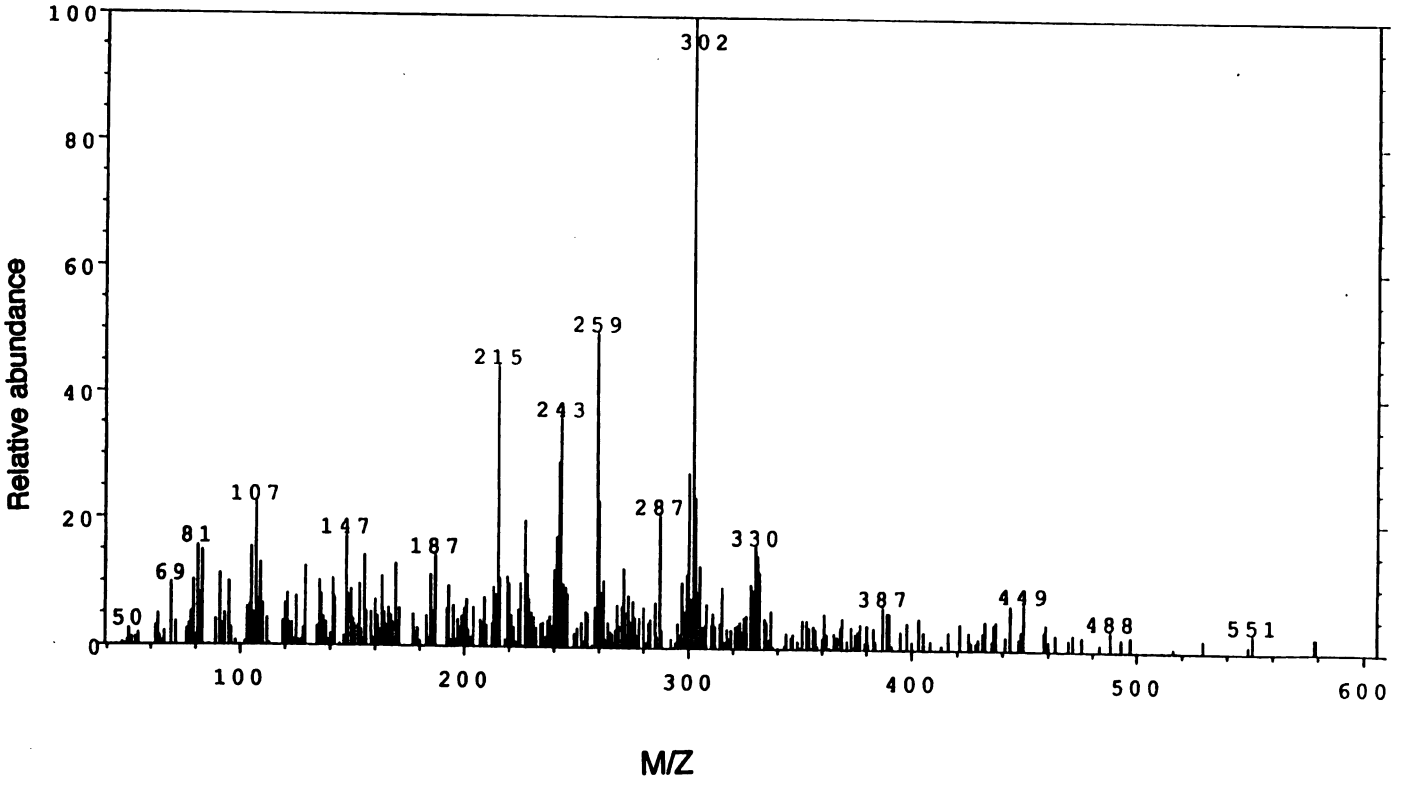

Relative abundance
100
80
60
40
20
0
280
300
320
340
360
300
308
306
285
293
304
328
336
334
332
360
M/Z

Figure 5 - Mass spectrum of Me-TMS ether of chemically synthesized ^{14}C-GA_{12} obtained from L. Mander. Relative heights of base peaks at 300/302 indicate that approximately 80% is 14C-GA12 with one labelled carbon.


Relative abundance
100
80
60
40
20
0
302
259
215
243
107
147
187
287
330
81
69
50
387
449
488
551
100
200
300
400
500
600
M/Z

Yea

'93

'93

'94

'95

'3 u

Trea

Table 1. No. of spurs and fruits (seed treatment) of 'Spencer Seedless' treated, 1992-1995

			Point of application of ^{14}C-GA_{12}		
			Seed	Bourse	Shoot[Z]
Year	Date	DAFB	Seed present : Yes	No	Yes
1992	June 16	37	1	6	6
	June 24	45	1	6	6
	July 1	52	1	6	6
	July 8	59	1	6	6
1993	June 16	30	8	6	6
	June 22	36	8	6	6
	June 27	41	8	6	6
	July 4	48	8	6	6
1994*	June 28	40	4	3	3
	July 1	45	4	3	3
1995	July 5	51	-	80	85

[Z] 0.3 uCi/spur, 0.1 uCi/apex (0.05 uCi/apex in 1995)

* Treatments applied to seeds and bourse shoots on excised branches

spurs bearing either seeded or seedless fruits, 0.1 uCi being injected with a hypodermic syringe approximately 5 mm below the apex. Transparent plastic sleeves, open at the end, were placed around all treated spurs and bourse shoots to prevent loss of radioactivity in case of rain.

An *in vitro* experiment was performed at 40 and 45 DAFB in 1994 to provide greater quantities of metabolites of 'Spencer Seedless' for qualitative analysis (Table 1). Branches (30 cm in length) bearing fruiting spurs were cut from the trees and their bases placed in water. On transfer to the laboratory, ^{14}C-GA_{12} was injected into the seeds or apices, using the same quantities as in 1992-1993 (0.3 uCi per fruit and 0.1 uCi per apex), and the branches were placed in a growth chamber (16 h light / 8 h dark) at 25°C (light) and 16°C (dark) for 72 hr.

In 1995, 80 bourse shoots on spurs bearing seedless fruits and 65 bourse shoots bearing seeded fruits were selected, then treated with ^{14}C-GA_{12} *in vivo* in order to obtain larger quantities of metabolites. Samples of tissues were collected and extracted as described below.

Sample collection. Spurs with treated apices were collected 24 and 48 hr after treatment in both 1992 and 1993. Spurs with treated fruits (seeds) were collected after 48 hr in 1992, and after 24 and 48 hr in 1993. Samples were collected 72 hr after treatment in both 1994 and 1995.

Samples were immediately dissected into treated seeds, remaining fruit tissue (inner and outer parts separately), pedicel, cluster base, bourse shoot(s), and leaves. When bourse shoots were treated, the apices (1 cm) were separated from the remainder of the shoot. The separated tissues were frozen at -20°C and lyophilized, and the dry tissues were stored at -20°C with a desiccant to prevent rehydration.

Sample extraction. 1. Small samples (1992-1994).
Lyophilized tissues were extracted with cold 80% methanol in a cold room (4°C) using a Polytron (PT-MR3000, Kinematica AG, Littau-Switzerland, Brinkmann Instruments Inc., Cantiague Rd., Westbury, New York) homogenizer. The extract was centrifuged at low speed to precipitate the tissue, the solvent decanted and the precipitate re-extracted with the same solvent. This was repeated three times for each extraction. After removal of an aliquot for radiocounting, the tissue was discarded, the combined extracts filtered, and the filtrate partially evaporated in a 'Speedvac' (Savant SC 200, Forma Scientific, Inc. Marietta, Ohio) centrifugal vacuum evaporator. The residual solvent was filtered by centrifugation using 400 ul microspin filters (Life Science Products, Inc.), and 0.2 to 0.5 ml of filtrate was injected into the HPLC. When more than one sample was available for each date, up to three separate samples were extracted and chromatographed.

2. Large samples (1995). Apex tissues were extracted in the same manner as for small samples, but the extracts were fractioned on a silicic acid column prior to HPLC. Columns were prepared as described by Powell and Tautvydas (1967). After stirring and shaking with distilled water, the suspension of silicic acid (100 mesh) was allowed to settle and the finer particles poured off. This was repeated until the silicic acid settled within a few minutes. The particles were then dried to constant weight at 100°C.

The silicic acid (20 g) was mixed thoroughly with 12.5 ml 0.5 N HCOOH, resulting in a free-flowing powder. This was slurried with hexane saturated with 0.5 N HCOOH and poured into a glass column 22 mm in diameter to form a column 20 cm in height. Additional hexane/HCOOH was forced through with air pressure to pack the column.

Extracts were dried on glass wool and the sample placed on the top of the column, which was then eluted stepwise with increasing concentations of ethyl acetate in hexane, both solvents having been saturated with 0.5 N HCOOH. The sequence used was: 0%; 10%; 30%; 50%; 80% and 100% ethyl acetate, and finally, 25% n-butanol in ethyl acetate. Thirty ml of each solvent were used except for 100% hexane (100 ml). The first two fractions contained little or no radioactivity, and were discarded. The remaining fractions were evaporated to dryness and the residues dissolved in 30% methanol for HPLC, as described for small samples.

HPLC. Samples were chromatographed on a C18 Bondapak reverse phase column (250 x 4 mm), using a Waters (Waters 600) HPLC. A discontinuous gradient of methanol in water was used, both solvents containing 0.1 N acetic acid or trifluoroacetic acid (TFA). The following protocol was used: 30% MeOH, 5 min., 30% to 100% in 25 min; 100%, 10 min, then 100% to 30% in 2 min. to complete the cycle. Flow rate was 1 ml/min, and pump pressure ranged from 400 to 2500 psi, depending upon the solvent being pumped. The radioactivity was measured by the flow-through radioactive detector (β-RAM). Retention times of available reference GAs and of additional GAs are given in Table 2.

Statistical analysis of data. Data were quantified by calculating both the DPM associated with each metabolite, and the same value as a percentage of the total radioactivity recovered. Analysis of variance was used on the data obtained in 1993 to determine the significance of quantitative differences due to time of treatment, time of sample collection (24 vs 48 hr), and the presence of seeds. Duncan's multiple range test at $p < 0.05$ was used when more than two means were compared.

Thin layer chromatography: Metabolites were dissolved in methanol, and chromatographed (ascending) on 'Eastman Chromagram' thin layer silica gel plates (20 x 20 cm), using

ethyl acetate / chloroform / acetic acid (15:5:1) as the developing solvent. The plates were exposed to X-ray film in darkness for 10 to 14 days, the film was processed, and Rf values were calculated for radioactive zones. Several standard GAs were also chromatographed for comparison of Rf values (Table 2).

Gas chromatography and mass spectrometry: Metabolites were transferred to 100 ul conical vials, reduced to dryness by vacuum centrifugation, and then dissolved in a small volume of methanol for methylation. Diazomethane in ether (ca. 50 ul) was added and the vials were sealed and left for 30 minutes. The ether was evaporated under nitrogen, and all samples were again dried by vacuum centrifugation. Methylated samples were further derivatized by adding 5 ul of TMS solution (trimethylsilyl reagent in pyridine, Sigma Sil.A, Sigma Chemical Company, St.Louis, MO), sealing the vials and heating them in an oven at 80°C for 10 minutes.

Samples were injected into a Hewlett-Packard 5890 A gas chromatograph attached to a JEOL JMS-AX 505H mass spectrometer. A DB-5 ms capillary column (30 m X 0.25 mm X 0.25 um film thickness) was used. Helium was the carrier gas at a flow rate of about 1 $ml.min^{-1}$ (at head pressure of 70 KPa). The samples were injected on-column with the initial column temperature at 50°C, followed by temperature programming at 40°$C.min^{-1}$ to 220°C and then 6°$C.min^{-1}$ to

Table 2. Rf values of some standard gibberellins on TLC with developing solvent ethyl acetate : chloroform : acetic acid (15:5:1)

GA	Rf value	
	Observed	Published[Z]
GA1		0.37
GA2	0.22	0.19
GA3	0.39	0.37
GA4	0.58	0.61
GA5		0.59
GA6		0.57
GA7	0.59	0.62
GA8		0.24
GA9	0.74	0.78
GA10		0.33
GA11		0.74
GA12	0.77	0.78
GA13		0.46
GA14		0.63
GA15		0.78
GA18		0.35
GA19		0.42

[Z]Takahashi (1983)

300°C. The column was connected directly to the ion source via a heated interface. The ion source temperature was 280°C, the electron ionization energy was 70 ev, and the emission current was 0.25 mA.

Hydrolysis of metabolites: Metabolites were dissolved in methanol (ca. 200 ul), and the volume adjusted to 1 ml with water. Hydrochloric acid (0.1 M, 5 ml) was added, and the solution was incubated in a water bath for at least 6 hr at 80°C. After incubation, the sample was allowed to cool to room temperature, then extracted 3 times with ethyl acetate (about 20 ml in total).

RESULTS

Characterization of metabolites.

Seed samples. Six major metabolites of ^{14}C-GA_{12} in both 'Spencer Seedless' and 'Spartan' seeds were eluted at 23, 26,28, 31, 34, and 36 min. (Fig. 6). For convenience these will be referred to as S23, S26, etc. All of the ^{14}C-GA_{12} (retention time 37 min) was metabolized within 24 hr.

A slightly different pattern of metabolites was observed when branches were held *in vitro* in 1994 and seeds were sampled 72 hr after treatment. On 26 June, some ^{14}C-GA_{12} (S37) remained in the tissues, and metabolites were

Figure 6 - HPLC profile of metabolites of ^{14}C-GA_{12} in extract of 'Spencer Seedless' apple seeds treated 36 DAFB in 1993. Numbers indicate approximate retention times (min).

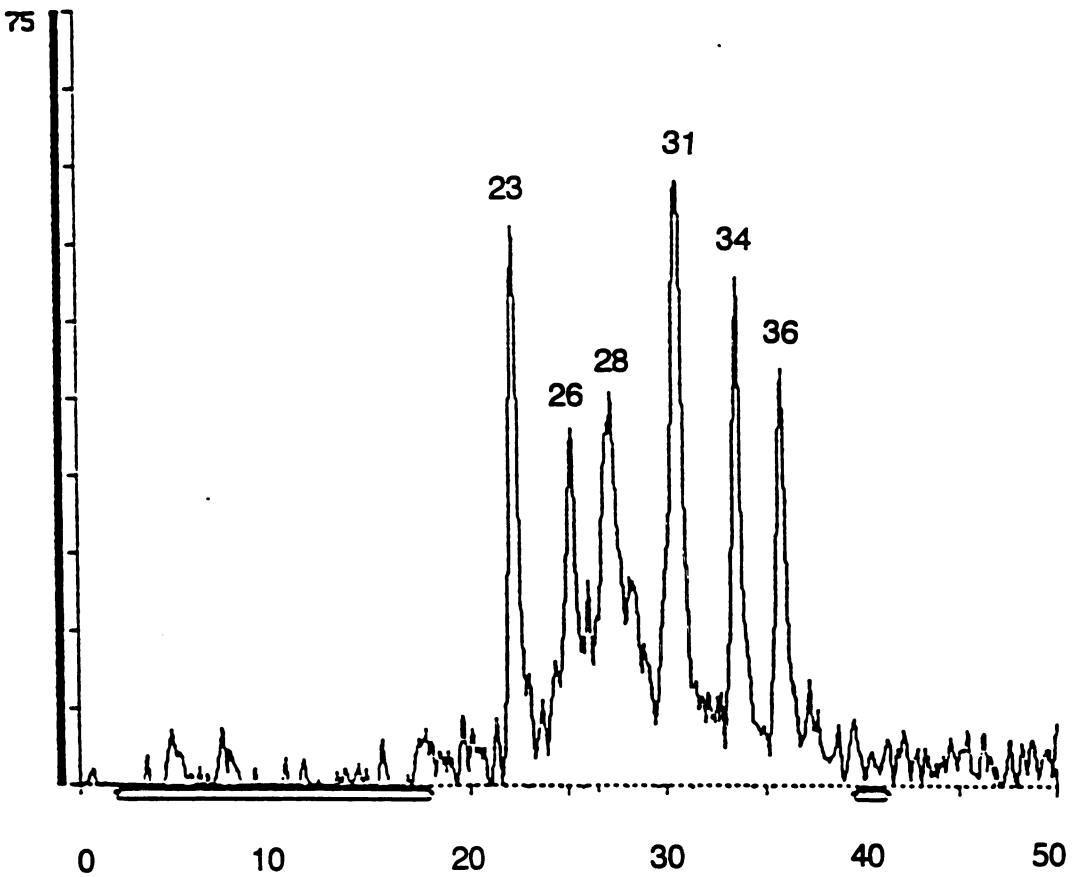

75
23
26
28
31
34
36
0
10
20
30
40
50
Time (min)

Figure 7 - HPLC profiles of metabolites of ^{14}C-GA_{12} in extracts of seed of 'Spencer Seedless' on branches held *in vitro* in 1994. Tissues sampled 72 hr after treatment. A. Treated 40 DAFB; B. Treated 45 DAFB.

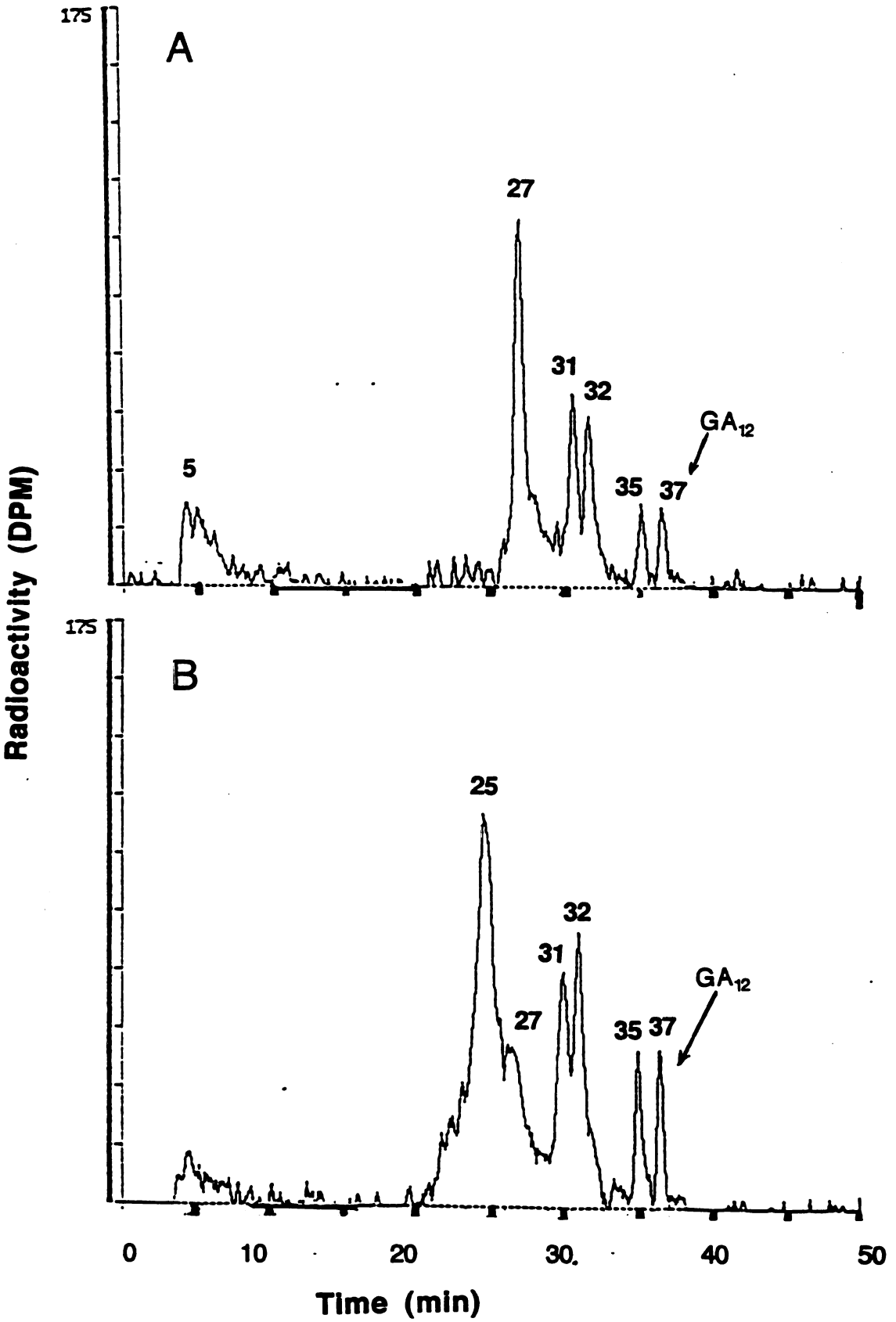

175
A
27
31
32
GA12
5
35
37
175
B
25
32
31
GA12
27
35 37
Radioactivity (DPM)
0
10
20
30.
40
50
Time (min)

Table 3. Retention times of some standard gibberellins on HPLC, using a methanol/water gradient (see text for conditions).

GA	Retention time (min)	
	Observed	Published[z]
GA_{55}		5
GA_{8}		5.1
GA_{29}	10.1	6.3
GA_{32}		6.5
GA_{33}		7
GA_{30}		7.5
GA_{23}		7.9
GA_{28}		8.2
GA_{38}		8.4
GA_{41}		9.2
GA_{3}	15.6	9.4
GA_{2}	16.0	15
GA_{1}	18.2	10.5
GA5	26.1	17.9
GA_{10}		18.1
GA_{20}	28.0	19
GA_{44}		21.4
GA_{63}		21.5
GA_{19}	29.0	22.4
GA_{54}		22.6
GA_{62}		23.5
GA_{7}	30.5	24.9

Table 3 (cont'd)

GA_4	31.6	26.1
GA_{53}		27.8
GA_{24}		28.9
GA_9	32.8	29.4
GA_{15}		29.8
GA_{12}	37.0	35

[z] Lin and Stafford (1991)

evident at retention times of 5, 22, 31, and 35 min (Fig 7). On 1 July, these metabolites were again evident, as well as a large quantity of an additional component at 25 min. The compound(s) at 5 min was low in quantity and poorly resolved; although not observed in other seed samples, it occurred in several extracts of treated apices (see below).

Available standards with similar retention times (Table 3) were GA_5 (26.1 min), and GA_{20} (27.95 min). The metabolites eluted at 34 and 36 min appeared to be less polar than GA_9 on HPLC.

The metabolites (combined from all samples of seeds based on the retention time on HPLC) were subjected to thin layer chromatography (Fig. 8), using ethyl acetate: chloroform: acetic acid (15:5:1) as the solvent. Radioactive zones were visualized by autoradiography. S23, S26, S28, and S34 were almost immobile in this solvent (Fig. 8), with Rfs of 0.05 to 0.1. S31 contained at least 8 components varying in Rf from 0 to 0.73. S36 was resolved into 4 compounds (Rfs 0.2, 0.27, 0.35, and 0.41).

S28, S34, and S36 were not affected by acid hydrolysis (Fig. 8), and S31 was affected little, if at all. However, many additional components were evident in S23 and S26, suggesting that these were conjugates. Several of the compounds in the hydrolysate of S23 had Rfs similar to, if not identical with, the components in S31, suggesting that S31 had been conjugated to give S23. Several additional,

less polar, compounds not observed in other samples were also evident.

Comparison of the Rf values (Table 4) and HPLC retention times of metabolites with those of standard GAs (Tables 2,3; Fig. 9) indicated that S26 could contain GA_{10}, although this has not been identified in apple tissue to date. No gibberellins or gibberellin derivatives could be identified in the extracts by GC-MS.

Apex samples. Eight major metabolites were observed following HPLC of extracts of apices treated with ^{14}C-GA_{12} (Fig. 10). These had retention times of 37, 36, 34, 32, 30, 29, 28, 27 and 5 min (hereafter referred to as AP37, AP36, etc.). AP5 occurred only in small amounts. AP27 was found only in samples collected relatively late (59 DAFB in 1992, 41 and 48 DAFB in 1993). AP37 was identified by GC-MS as the parent compound, ^{14}C-GA_{12} (Figs. 11-13). Comparison of retention times on HPLC (Table 3) indicated that AP28 could be GA_{20}, AP29 GA_{19}, and AP32 GA_4. Both AP34 and AP36 were apparently less polar than available GAs other than GA_{12}.

When silicic gel column fractions from large samples collected in 1995 were subjected to HPLC, AP37 was found in the 30% ethyl acetate fraction; AP36 and AP34 in 50%; AP32 and AP30 in 80%; and AP29, AP28 and AP27 in 100%.

The metabolites were chromatographed on TLC using ethyl acetate: chloroform: acetic acid (15:5:1) as the developing solvent (Fig. 13). The most polar metabolite (AP27)

Figure 8 - Autoradiogram of TLC plate following chromatography of metabolites of ^{14}C-GA_{12} from 'Spencer Seedless' apple seeds in ethyl acetate : chloroform : acetic acid (15:5:1). Numbers indicate elution times on HPLC. Non-hydrolyzed metabolites (left); acid hydrolyzed metabolites (right).

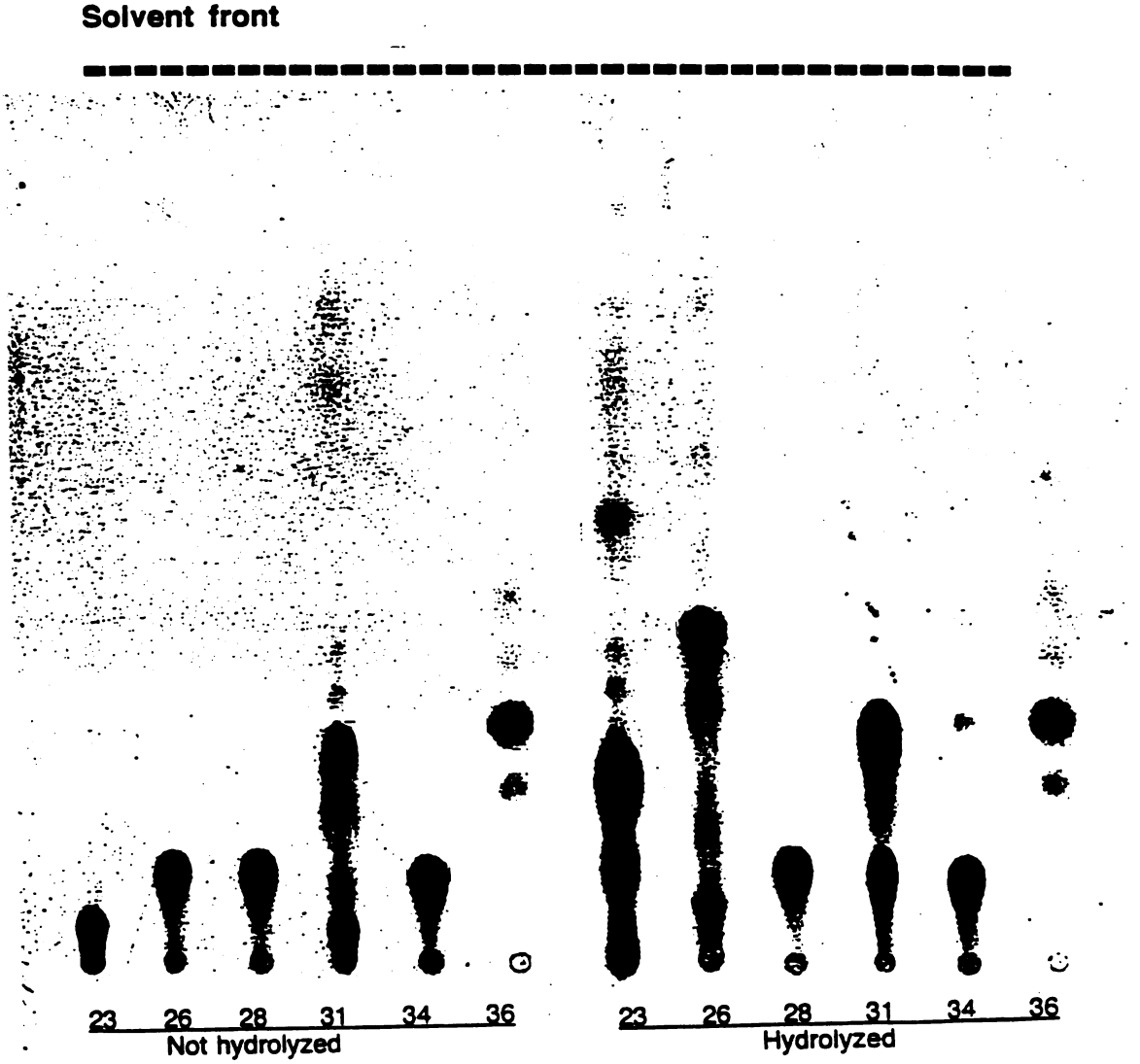

Solvent front
23 26 28 31 34 36
Not hydrolyzed
23 26 28 31 34 36
Hydrolyzed

Table 4. Rf values of metabolites of ^{14}C-GA_{12} in extracts of apple seeds and apices.

Metabolite (min)	Rf value	
	Non-hydrolyzed	Hydrolyzed
	Seeds	
23	0, 0.05	0, 0.1, 0.2, 0.3, 0.35, 0.49
26	0, 0.1	0, 0.07, 0.15, 0.29, 0.37
28	0, 0.1	0.1
31	0, 0.05, 0.08, 0.18, 0.23	0.1, 0.25
34	0, 0.1	0.1
36	0.2, 0.27, 0.33, 0.4	0.2, 0.27, 0.33, 0.4
	Apices	
27	0, 0.08, 0.13	0, 0.08, 0.13, 0.31
28	0, 0.15, 0.22	0, 0.15, 0.22
29	0.29	0.29
30	0.27	0.27
32	0.31	0.31
34	0.61	0.61
36	0.03, 0.69	0.03, 0.69
37	0.77	0.77

Figure 9 - Comparison of chromatographic properties of metabolites of ^{14}C-GA_{12} in seeds (A) and apices (B) of apple with those of known GAs (see Table 2,3,7). ○ - Metabolites in extracts; ■ - Known GAs; ◪ Known GAs, based upon data of Lin and Stafford (1991), adjusted for retention time.

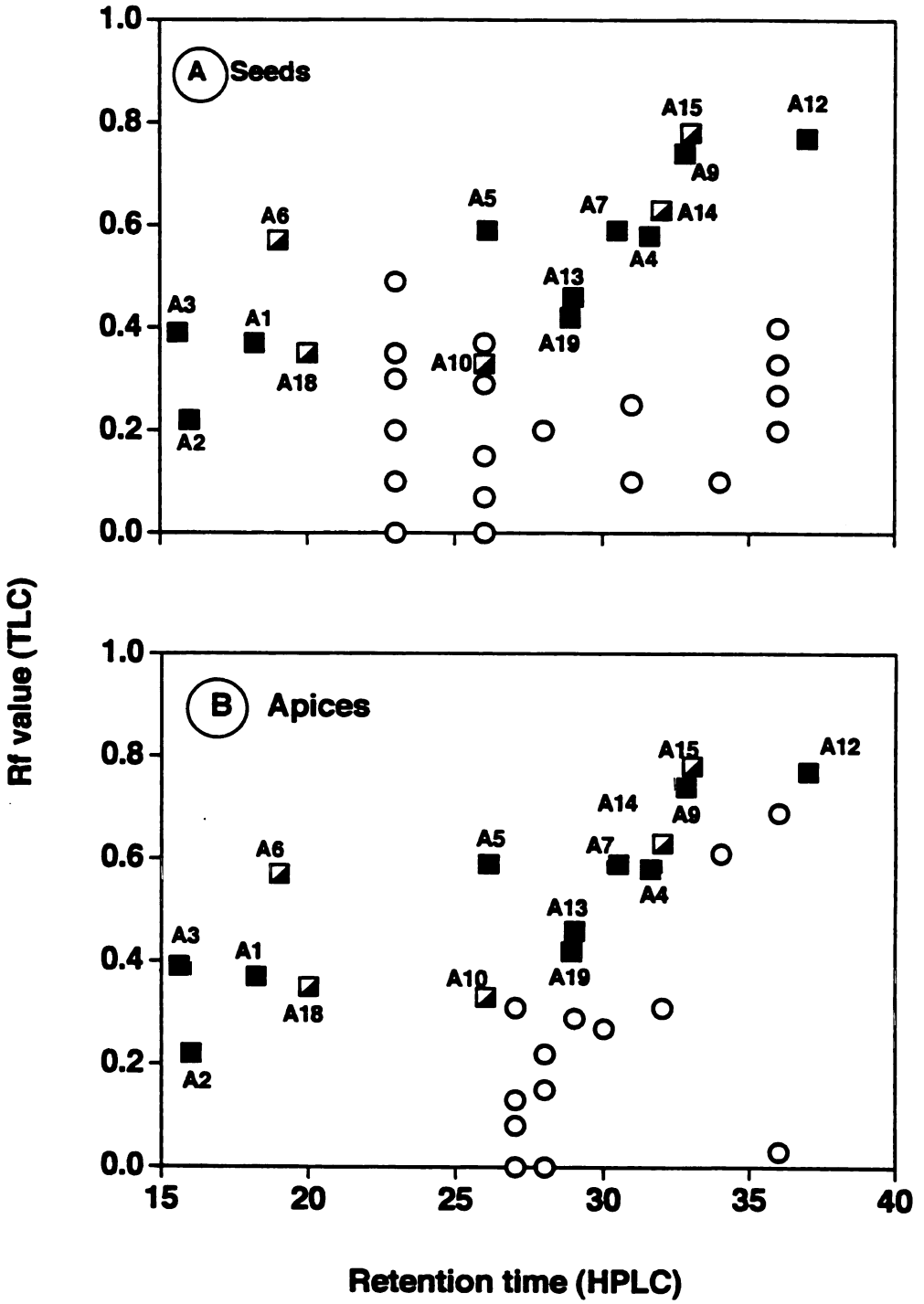

A Seeds
B Apices
Rf value (TLC)
Retention time (HPLC)
1.0
0.8
0.6
0.4
0.2
0.0
15
20
25
30
35
40
A1
A2
A3
A4
A5
A6
A7
A9
A10
A12
A13
A14
A15
A18
A19

Figure 10 - HPLC profiles of metabolites of ^{14}C-GA_{12} in extracts of apices of 'Spencer Seedless' apple treated 36 DAFB (A) and 48 DAFB (B) in 1993.

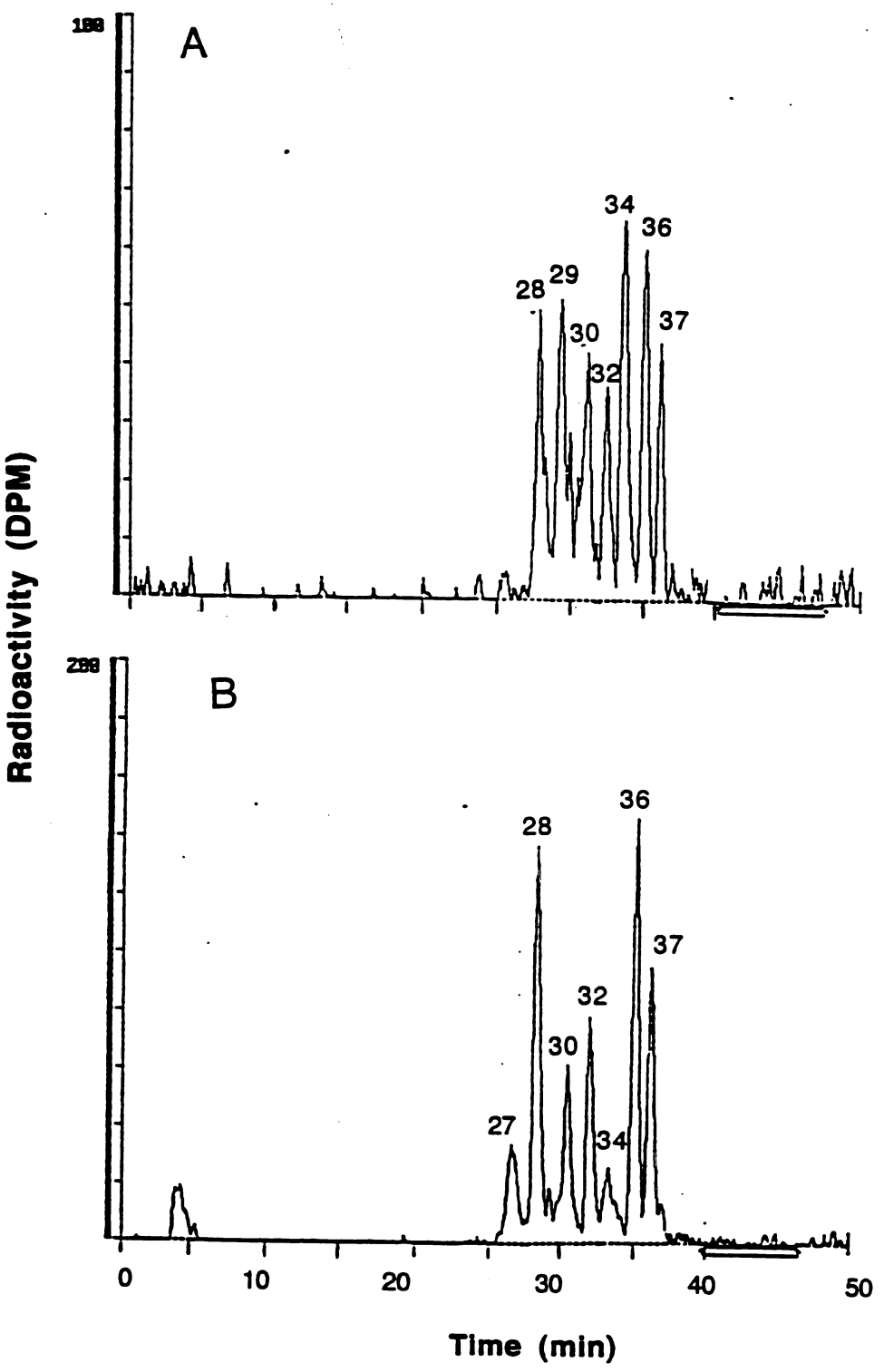

A
B
28
29
30
32
34
36
37
27
Radioactivity (DPM)
0
10
20
30
40
50
Time (min)

Figure 11 - Abundance of selected ions in mass spectrum of Me-TMS derivative of residual ^{14}C-GA_{12} (AP37) in apices of 'Spencer Seedless' in 1993 (TIC, ions 241, 300, 328).

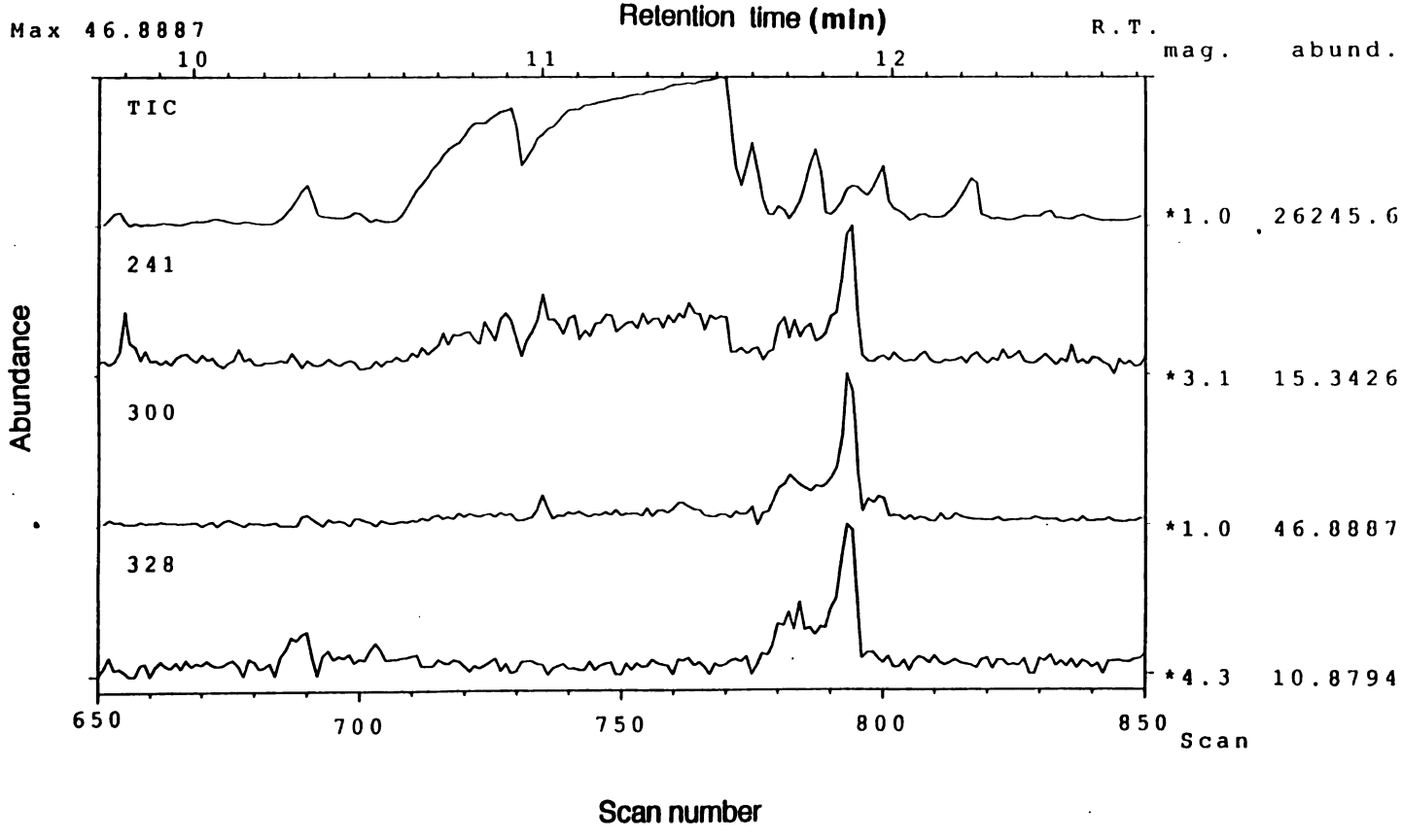

Max 46.8887
Retention time (min)
R.T.
mag.
abund.
10
11
12
TIC
241
300
328
*1.0
26245.6
*3.1
15.3426
*1.0
46.8887
*4.3
10.8794
Abundance
650
700
750
800
850
Scan
Scan number

Figure 12 - Mass spectrum of the Me-TMS derivative of the residual ^{14}C-GA_{12} (AP37) in extract of apices of 'Spencer Seedless' in 1993.

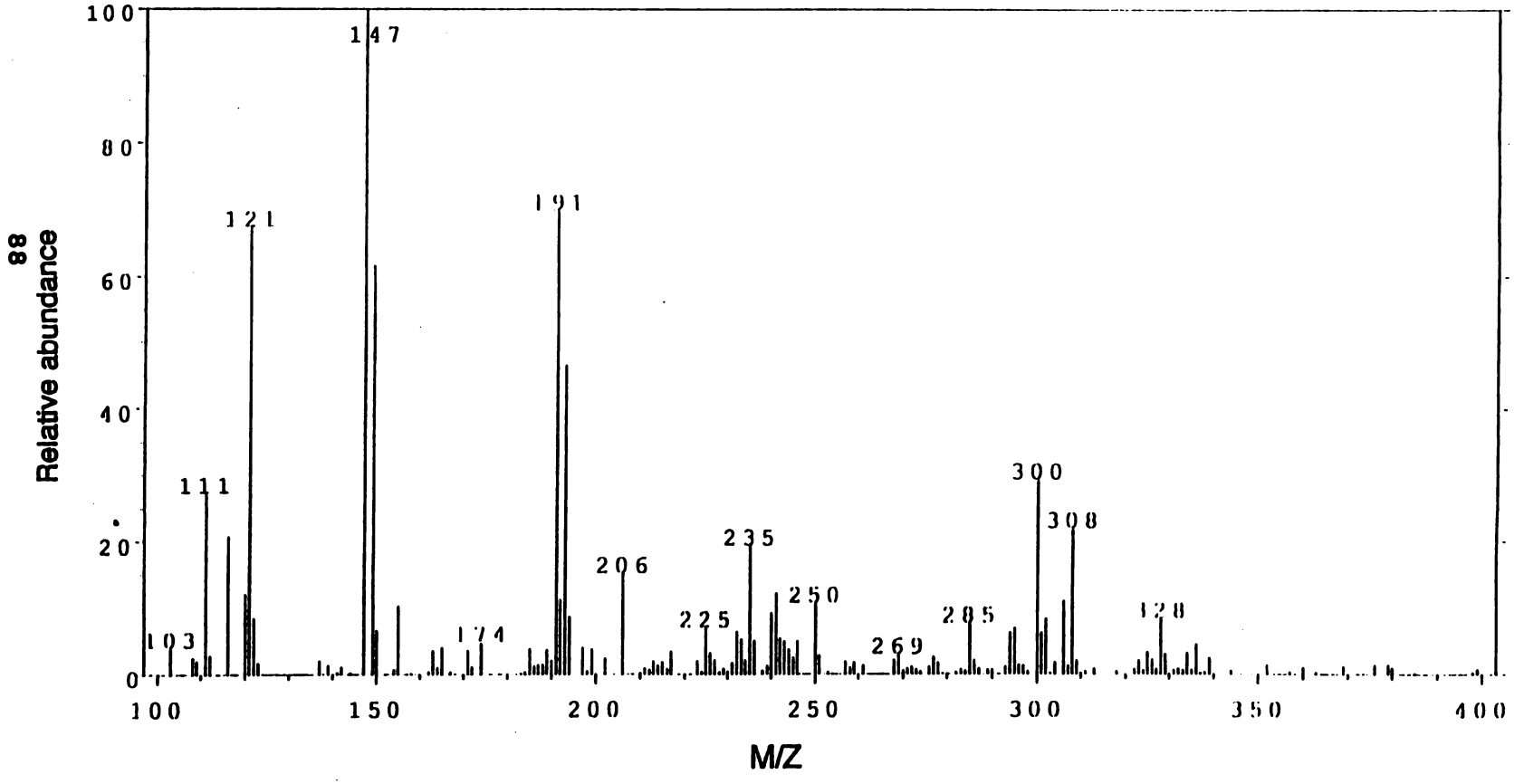
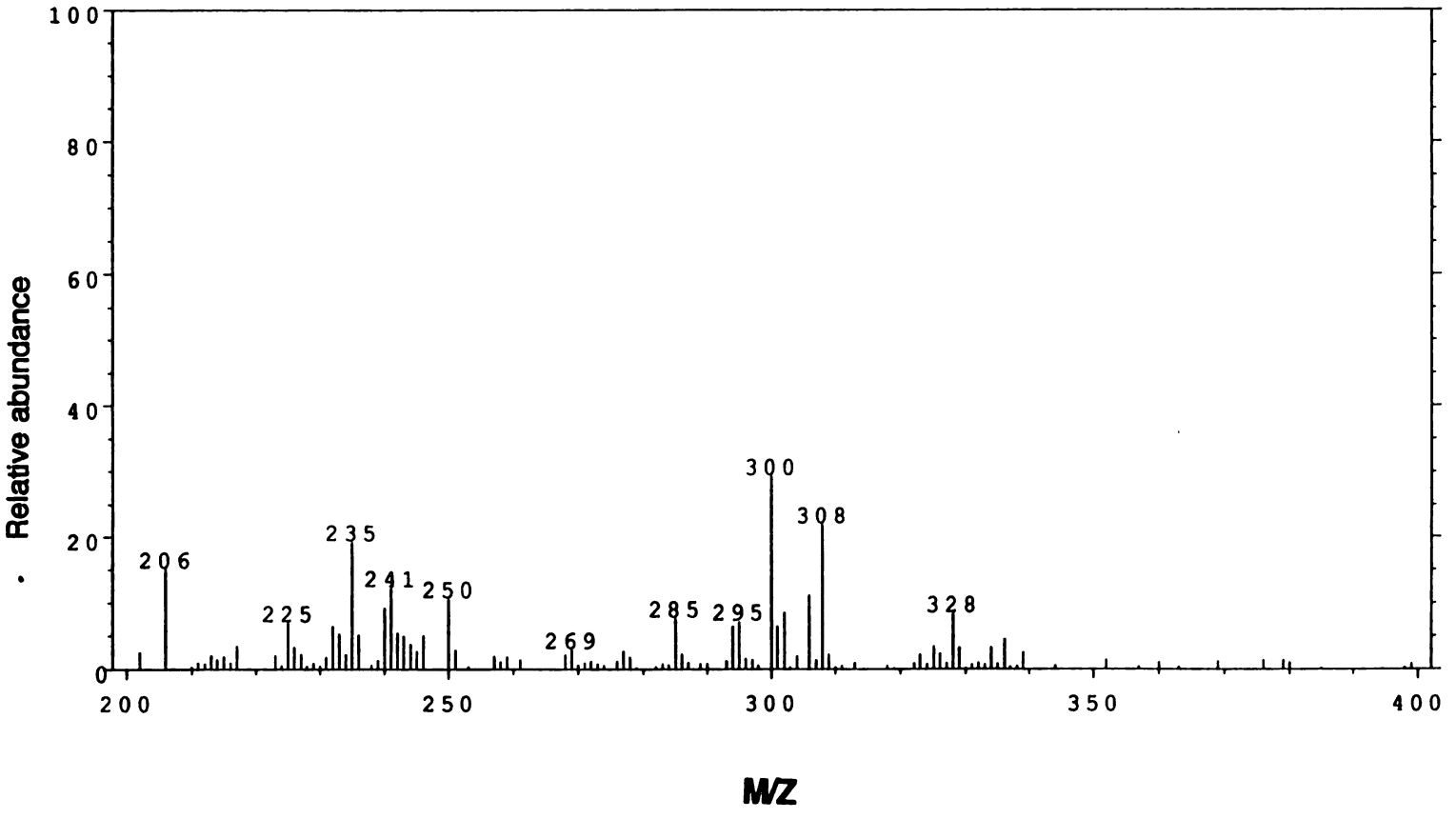

88
Relative abundance
100
80
60
40
20
0
147
121
191
111
103
174
206
225
235
250
269
285
300
308
128
100
150
200
250
300
350
400
M/Z

Figure 13 - Upper portion of mass spectrum of the Me-TMS derivative of residual ^{14}C-GA_{12} (AP37) in extracts of apices of 'Spencer Seedless' in 1993.


100
80
60
40
20
0
Relative abundance
206
225
235
241
250
269
285
295
300
308
328
200
250
300
350
400
M/Z

contained 2 to 4 components, the least polar of which had anRF of 0.12. AP28 was less polar, and two components appeared to be identical with those in AP27. The solubility of the remaining metabolites paralleled that observed on HPLC. None of the metabolites matched the standard GAs both in HPLC retention times and in Rf values on TLC (Fig.9). Acid hydrolysis had little effect on Rfs (Fig. 14), except that the least polar component in AP27 moved farther up the plate. Therefore, these metabolites, unlike those found in seeds, are probably not conjugated; if conjugated, they are not acid hydrolyzable. Quantification of metabolites.

Seeds. 'Spencer Seedless'. Only one sample was chromatographed on each sampling date in 1992, but 3 samples were analyzed on each date in 1993.

As noted above, no ^{14}C-GA_{12} remained after 24 (1993) or 48 hr (1992 and 1993). The data were quantified by determining the DPM represented by each metabolite and expressing this as a percentage of the total radioactivity recorded for each sample, after subtraction of background. This resulted in values (Table 5) that did not total 100 as radioactivity not included in peaks was included as part of the total.

In 1992 activity in S36 fell continuously from 37 to 59 DAFB (Table 5, Fig. 15), whereas levels of the other metabolites fluctuated, but showed no particular trends. The quantity of S34 was relatively high on all sampling

dates.

Values for S36 in 1993 tended to parallel those observed in 1992 (Fig. 15), although the initial level (30 DAFB) rose prior to dropping. Several differences between sampling dates were significant (S36, S34, S31, S23), but seldom paralleled values observed in 1992. Levels of both S36 and S34 were significantly lower after 48 hr than after 24 hr (Table 5), but these differeces were small, and were not accompanied by increases in more polar components.

'Spartan'. One sample of 'Spartan' was chromatographed on each sampling date in 1992. The only noticeable parallel between data for 'Spartan' and those for 'Spencer Seedless' was the steady decline in S36 from 37 to 60 DAFB (Table 5, Fig. 15).

Apices. *'Spencer Seedless'*. Three replicates were extracted for each individual treatment in 1992 and 1993. Rate of metabolism of AP37 (^{14}C-GA_{12}) consistently declined with treatment date, thus more was recovered in both years as treatment was delayed (Table 6, Figs. 16, 17). In 1992, metabolism was signifiantly more rapid in bourse shoots of spurs bearing seedless fruit than in those of spurs bearing seeded fruits (Fig. 16). This was also true for the 24 hour sample in 1993 (Fig. 17), but differences at 48 hr were small and non-significant in 1993. Rate of metabolism (24 vs. 48 hr) appeared to be affected by the presence of seeds (Tables 6, 7, Fig. 18), but these differences were not

consistent from year to year, and interactions between time of treatment and the presence of seeds were also apparent. However, AP28 declined significantly with sampling time in both years (Fig. 19). More metabolites were found in the apex of spurs with seedless fruits than in those with seeded fruits in the *in vitro* experiment (Fig. 20), but comparison with samples taken *in vivo* indicated fewer metabolites overall (compare Figs. 10 vs. 20). The rate of metabolism of ^{14}C-GA_{12} in the apex was higher *in vivo* than *in vitro* (Fig 21).

'Spartan'. Three samples of 'Spartan' were taken at each date in 1992. Rate of metabolism of ^{14}C-GA_{12} again declined with time (Table 4, Fig. 22), although differences were non-significant until 59 DAFB. Little difference between 24 and 48 hr was evident in the quantity remaining. Levels of AP29, AP32, and AP36 paralleled those for 'Spencer Seedless' sampled the same year, but changes in other metabolites differed.

Figure 14 - Autoradiogram of TLC plate following chromatography of metabolites of ^{14}C-GA_{12} in extracts of apices of 'Spencer Seedless' in ethyl acetate : chloroform : acetic acid (15:5:1). Numbers indicate elution times on HPLC. Non-hydrolyzed metabolites (left); acid hydrolyzed metabolites (right).

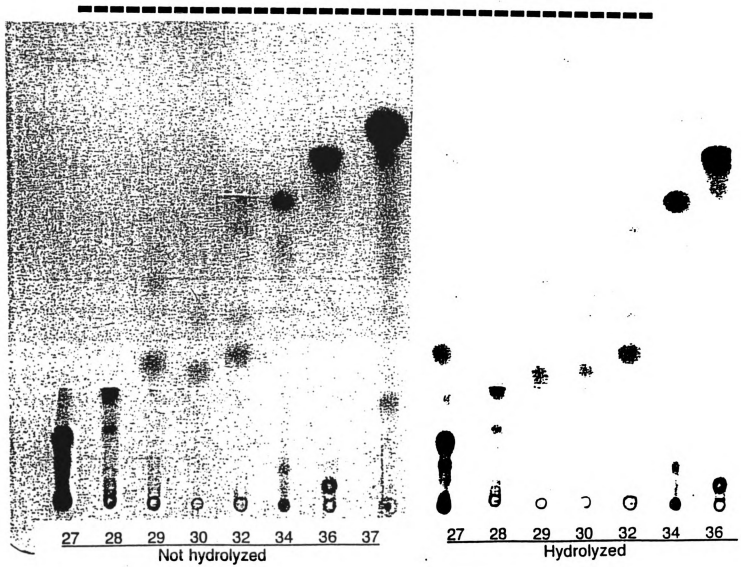

Solvent front
27 28 29 30 32 34 36 37
Not hydrolyzed
27 28 29 30 32 34 36
Hydrolyzed

Table 5. Relative amounts of metabolites of ^{14}C-GA_{12} in seed of 'Spencer Seedless' in 1992 and 1993 and 'Spartan' in 1992 as a percentage of the total radioactivity recovered by HPLC. Only one sample processed per culture and date in 1992, 3 samples in 1993.

Treat.		Retention time (min)					
DAFB	time(H)	36	34	31	28	26	23
		Spencer Seedless, 1992					
37	48	11	20	8	14	2	17
45	48	4	23	20	16	3	5
52	48	3	19	15	10	3	20
59	48	0	20	6	5	44	0
Mean		5	21	12	12	13	10
		Spencer Seedless, 1993					
30	24	11	13	12	10	8	15
	48	5	6	11	13	13	18
36	24	10	11	16	17	11	17
	48	11	10	21	17	10	16
41	24	15	13	16	17	12	12
	48	6	14	12	10	12	6
48	24	1	6	32	26	13	1
	48	0	3	30	32	12	1
Mean30		8 b	10 a	11 c	12 c	10 a	17 a
36		10 ab	11 a	19 b	17 b	10 a	17 a
41		11 a	13 a	14 c	14 bc	13 a	8 b
48		1 c	5 b	30 a	28 a	14 a	1 c
24		9	11	18	17	12	11
48		6***	8*	19	18	11	10

Table 5 (cont'd)

DAFB	Treat. time(H)	Retention time (min) 36	34	31	28	26	23
		Spartan, 1992					
37	48	12	24	6	16	7	6
45	48	10	18	10	8	9	9
52	48	8	14	8	13	8	14
59	48	4	20	9	38	4	1
Mean		9	19	8	19	7	8

Figure 15 - Effects of time of treatment on relative levels of metabolites of ^{14}C-GA_{12} recovered from seeds of 'Spencer Seedless' and 'Spartan' apple in 1992-93. Data points indicate radioactivity as a percent of total radioactivity recovered following HPLC. Values for 1 sample (1992) or means for 6 (2 times of sampling X 3 replicates) samples (1993). Vertical bar=lsd (p=0.05) for 'Spencer Seedless' in 1993.

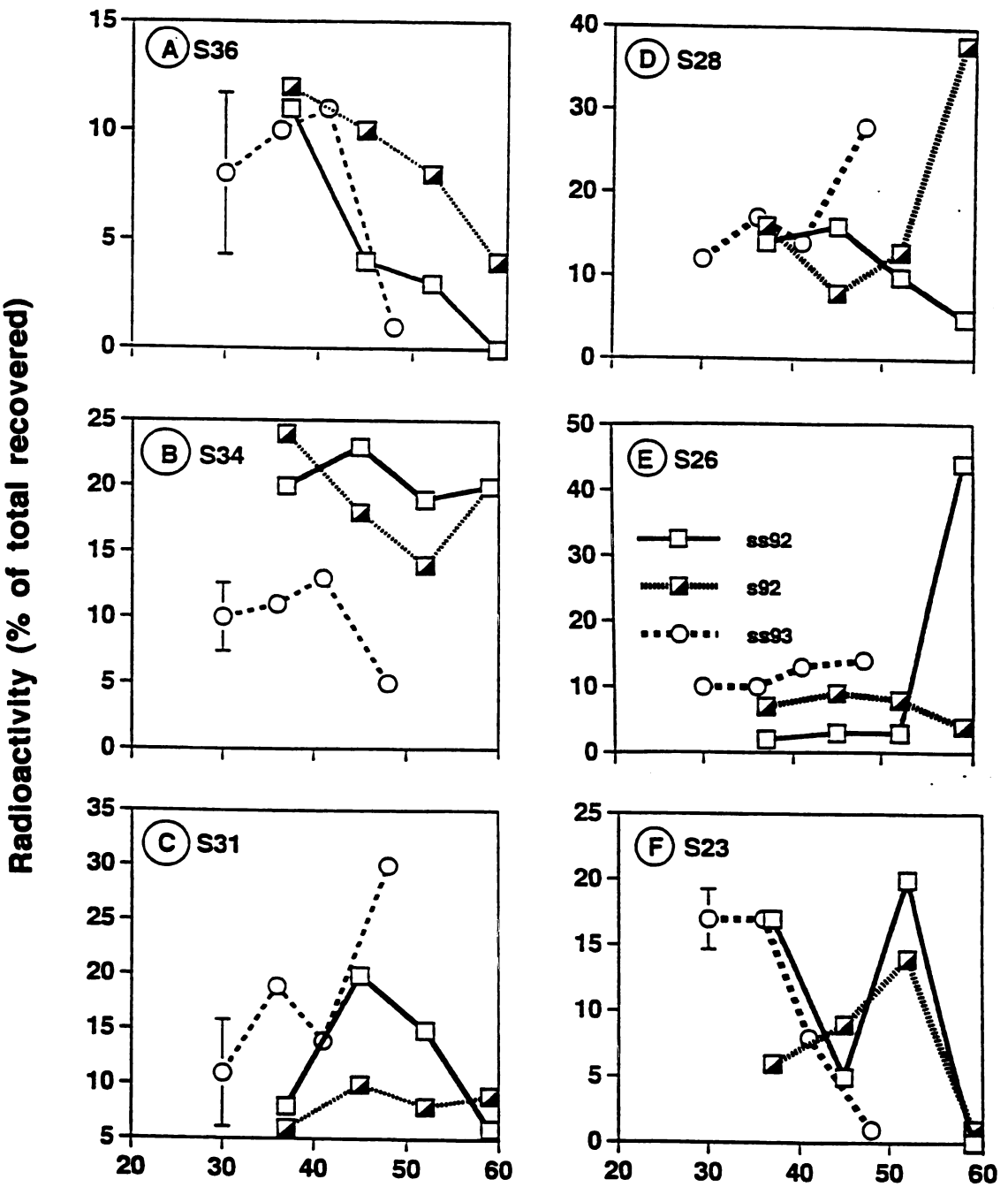

A S36
B S34
C S31
D S28
E S26
F S23
ss92
s92
ss93
Radioactivity (% of total recovered)
Days after full bloom

Table 6. Effect of seeds and time of treatment on levels of metabolites of ^{14}C-GA_{12} recovered from apices of 'Spencer Seedless' in 1992. Means for 3 replicate samples as a percentage of total DPM recovered following HPLC.

DAFB	Seed	Treat. time(H)	Retention time (min) 37	36	34	32	30	29	28	27
37	+	24	12	11	11	28	7	6	3	
	+	48	8	11	3	33	16	5	5.	
	-	24	7	12	11	25	6	8	19	
	-	48	0	13	2	17	6	11	29	
45	+	24	23	7	8	6	4	9	6	
	+	48	19	6	7	12	8	9	15	
	-	24	11	19	11	21	4	11	8	
	-	48	10	4	10	23	6	12	12	
52	+	24	31	11	8	9	3	17	5	
	+	48	23	16	10	10	4	18	5	
	-	24	15	9	18	7	7	5	6	
	-	48	15	9	17	8	6	5	7	
59	+	24	49	6	0	8	0	10	7	4
	+	48	48	5	2	6	0	14	10	3
	-	24	46	0	10	4	0	13	10	4
	-	48	26	10	15	5	4	6	4	13

Table 6 (cont'd)

DAFB	Seed	Treat. time(H)	Retention time (min) 37	36	34	32	30	29	28	27
Mean										
37			7d	13	7	33a	6a	7	14a	
45			16c	9	9	16b	5a	15	10ab	
52			21b	11	13	9bc	5a	12	6b	
59			42a	6	7	6c	2b	11	8b	
Main effect means										
	+		27	10	6	18	4	11	7	
	-		16***	10	12*	14	5	11	12*	
		24	24	10	10	17	4	12	8	
		48	19***	10	8	15	5	10	11	

Figure 16 - Effects of time of treatment and of incubation (24, 48 hr) on content of ^{14}C-GA_{12} (% of total recovered) in apices of spurs bearing seeded (P) vs. seedless (NP) fruits of 'Spencer Seedless' in 1992. Vertical bars: ± standard deviation.

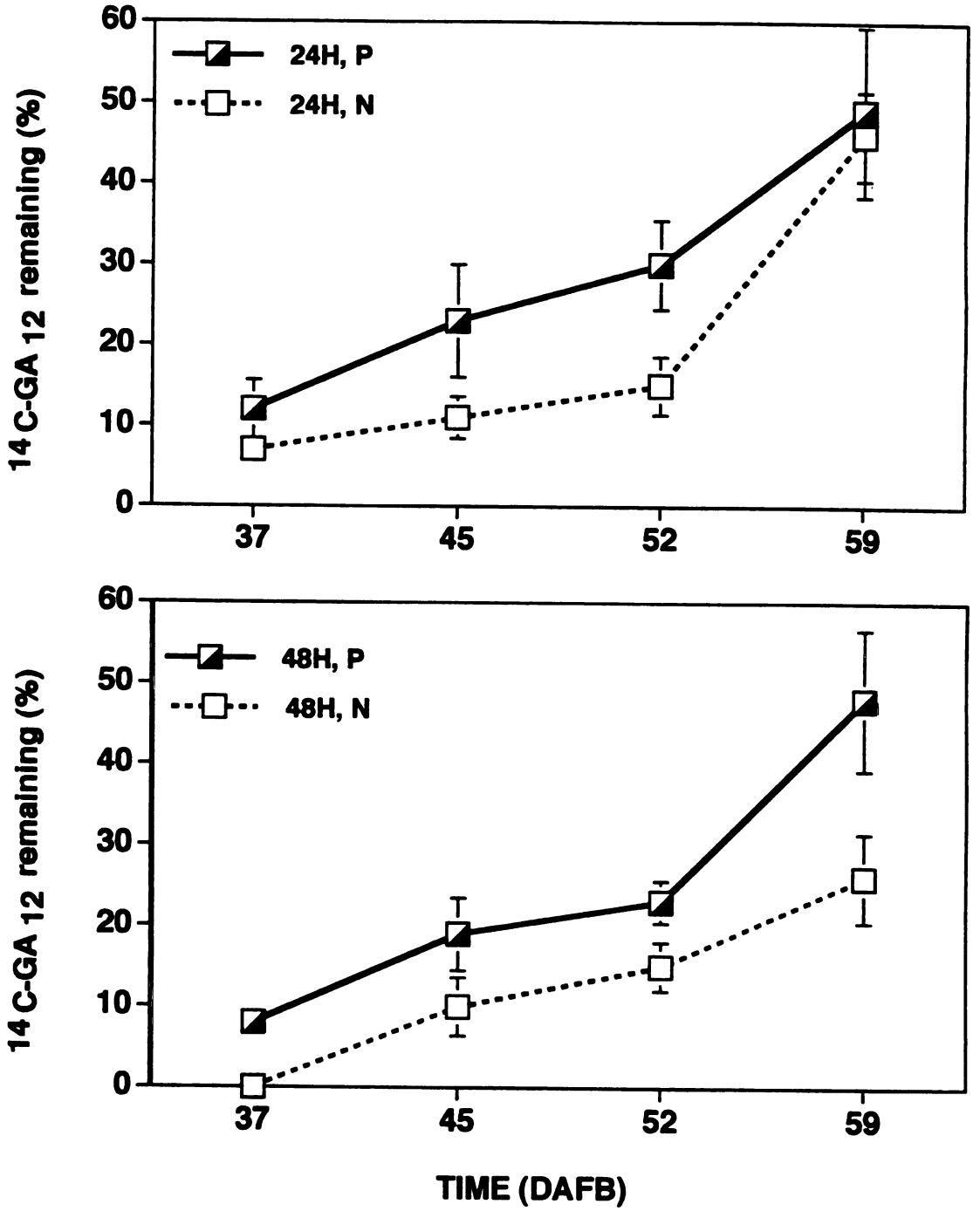

24H, P
24H, N
14 C-GA 12 remaining (%)
60
50
40
30
20
10
0
37
45
52
59
48H, P
48H, N
14 C-GA 12 remaining (%)
60
50
40
30
20
10
0
37
45
52
59
TIME (DAFB)

Figure 17 - Effects of time of treatment and of incubation (24, 48 hr) on content of ^{14}C-GA_{12} (% of total recovered) in apices of spurs bearing seeded (P) vs. seedless (NP) fruits of 'Spencer Seedless' in 1993. Vertical bars: ± standard deviation.

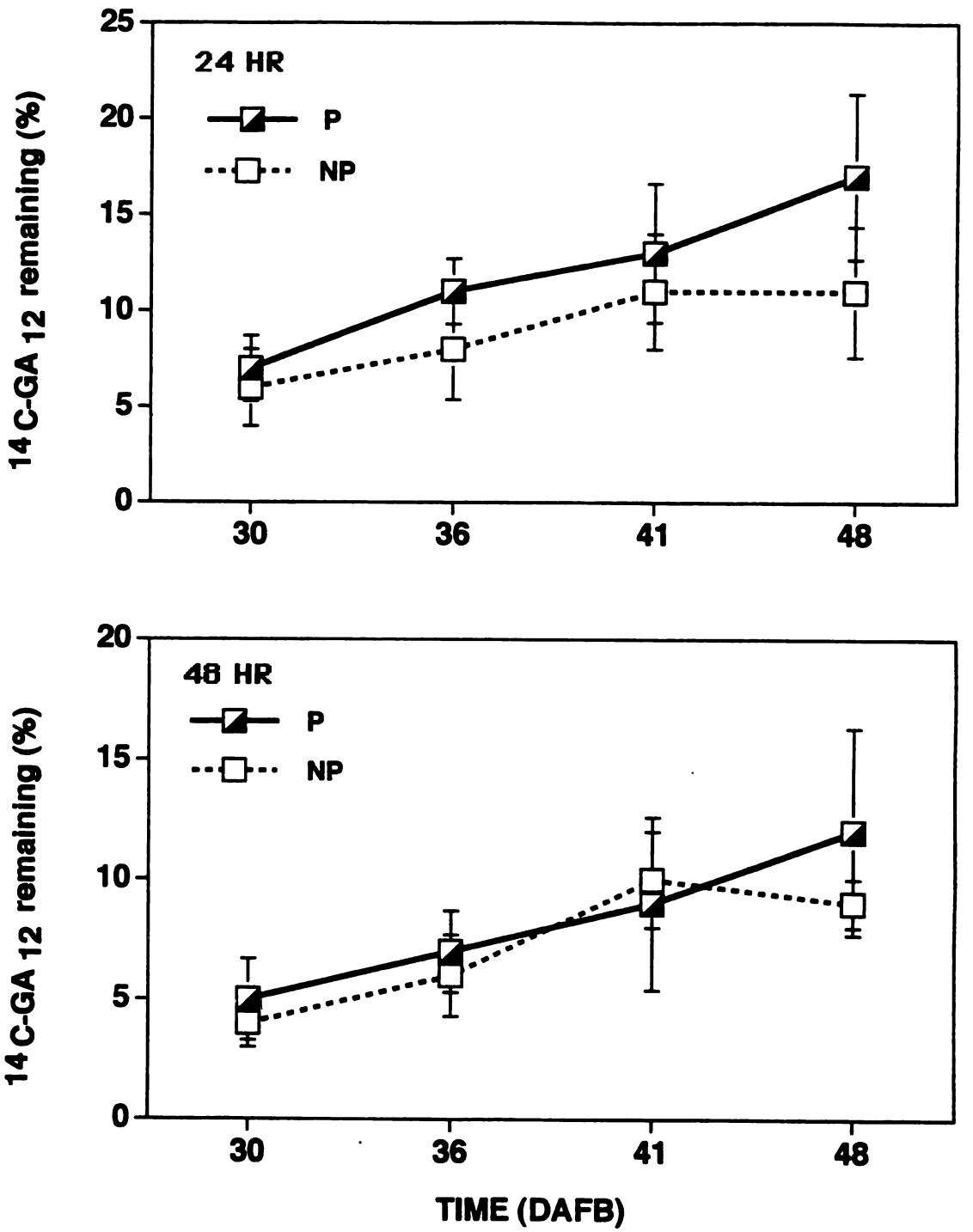

24 HR
P
NP
14C-GA 12 remaining (%)
25
20
15
10
5
0
30
36
41
48
48 HR
P
NP
14C-GA 12 remaining (%)
20
15
10
5
0
30
36
41
48
TIME (DAFB)

Table 7. Effect of seeds and time of treatment on levels of metabolites of ^{14}C-GA_{12} recovered from apices of 'Spencer Seedless' in 1993. Means for 3 replicate samples as a percentage of total DPM recovered following HPLC.

DAFB	Seed	Treat. time(H)	Retention time (min) 37	36	34	32	30	29	28	27
30	+	24	7	18	9	5	9	8	17	
	+	48	5	16	10	4	13	19	7	
	-	24	6	13	5	9	6	14	18	
	-	48	4	10	6	9	9	8	18	
Mean			6d	14b	8c	7bc	10a	12b	15a	
36	+	24	11	13	16	8	10	13	11	
	+	48	7	15	21	5	8	6	22	
	-	24	8	24	5	7	5	21	11	
	-	48	6	10	20	5	12	27	4	
Mean			8c	16b	16a	7c	9a	17a	12b	
41	+	24	13	18	14	7	15	5	15	-
	+	48	9	14	8	6	6	6	11	15
	-	24	11	17	5	12	9	21	7	5
	-	48	10	18	8	11	5	6	5	15
Mean			11b	17ab	8c	8b	9a	10c	10c	
48	+	24	17	21	17	10	4	5	5	4
	+	48	12	20	5	10	7	10	6	5
	-	24	11	20	6	13	9	13	6	6
	-	48	9	18	20	12	4	4	12	-
Mean			12a	20a	12b	11a	6b	8c	7d	

Table 7 (cont'd)

DAFB	Seed	Treat. time(H)	Retention time (min) 37	36	34	32	30	29	28	27
Mean			6d	14b	8c	7bc	10a	12b	15a	
			8c	16b	16a	7c	9a	17a	12b	
			11b	17ab	8c	8b	9a	10c	10c	
			12a	20a	12b	11a	6b	8c	7d	
Main effect means										
	+		10	17	12	7	9	9	12	
	-		8***	16	9***	10***	8**	14***	10**	
		24	11	18	9	9	8	12	11	
		48	8***	15*	12***	8*	8	11*	11	

Figure 18 - ^{14}C-GA_{12} remaining in apices on spurs with seedless and seeded fruit of 'Spencer Seedless' and on spurs of 'Spartan' 24 hr after treatment 1992. Vertical bars: ± standard deviation.

SSN=Spencer Seedless, not pollinated

SSP=Spencer Seedless, pollinated

SP=Spartan, pollinated

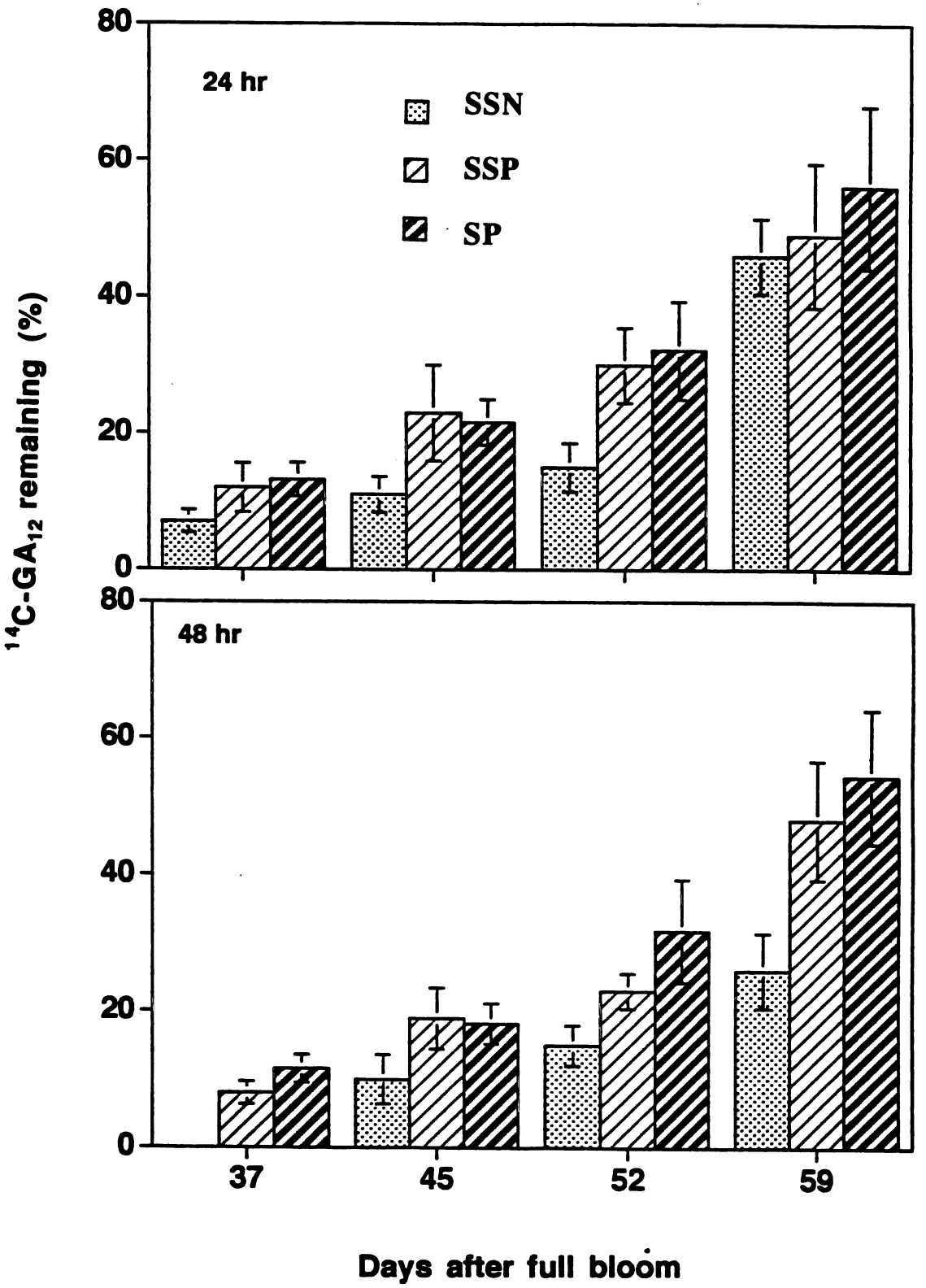

24 hr
48 hr
SSN
SSP
SP
^{14}C-GA_{12} remaining (%)
0
20
40
60
80
37
45
52
59
Days after full bloom

Figure 19 - Effects of time of treatment on relative levels of metabolites of ^{14}C-GA_{12} recovered from apices of 'Spencer Seedless' and 'Spartan' apple in 1992-93. Data points indicate radioactivity as a percent of total radioactivity recovered following HPLC. Values are means for 6 (2 times of sampling X 3 replicates) samples (1992 and 1993). Vertical bar = lsd (p=0.05) for 'Spencer Seedless' in 1993.

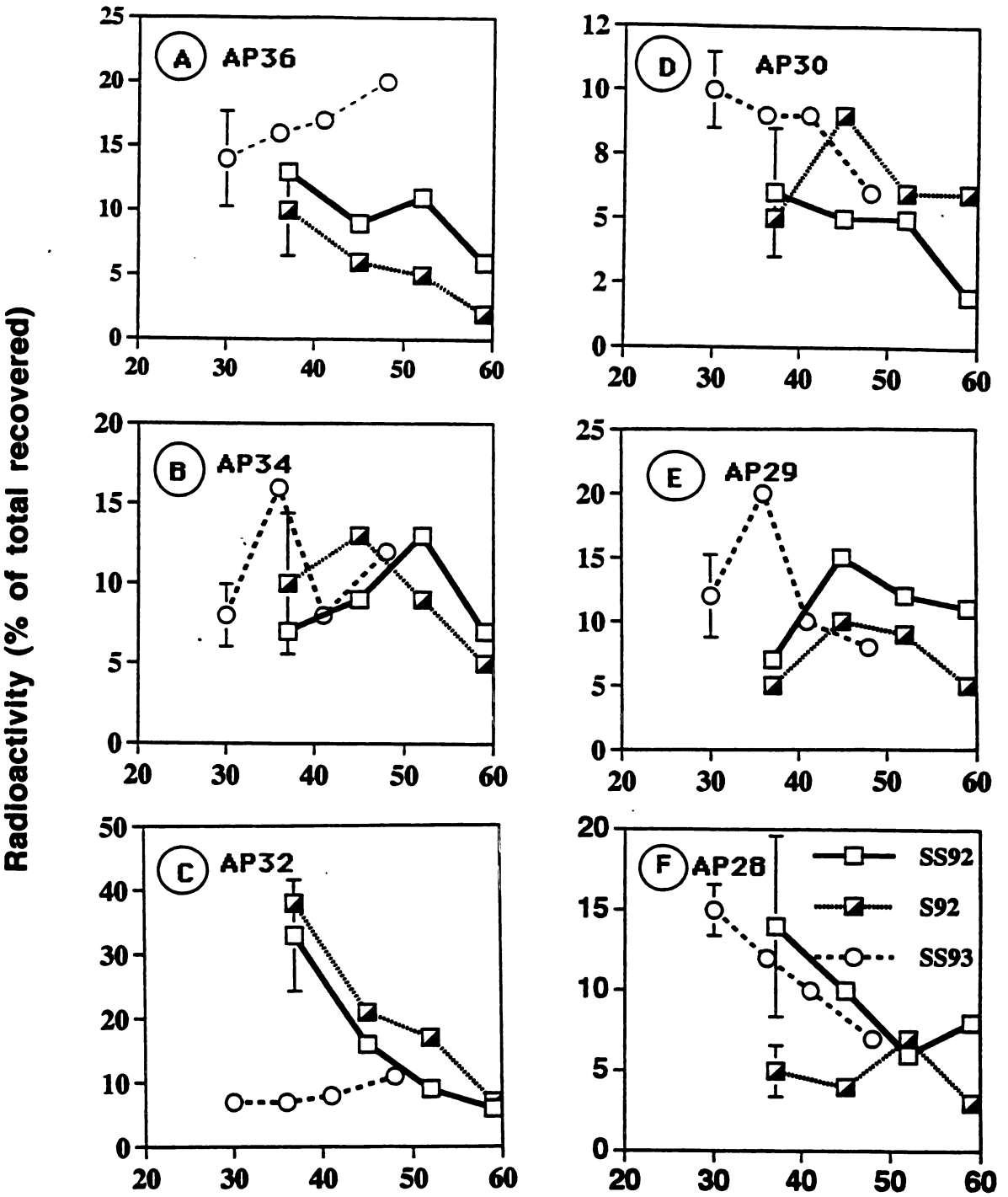

A AP36
B AP34
C AP32
D AP30
E AP29
F AP28
SS92
S92
SS93
Radioactivity (% of total recovered)
Days after full bloom

Figure 20 - HPLC profiles of metabolites of ^{14}C-GA_{12} in extracts of apices of 'Spencer Seedless' on branches held *in vitro* in 1994. Tissues sampled 72 hr after treatment.

A. and C. Treated 26 June (40 DAFB)

B. and D. Treated 1 July (45 DAFB)

A. and B. Spurs with seeded fruits

C. and D. Spurs with seedless fruits.

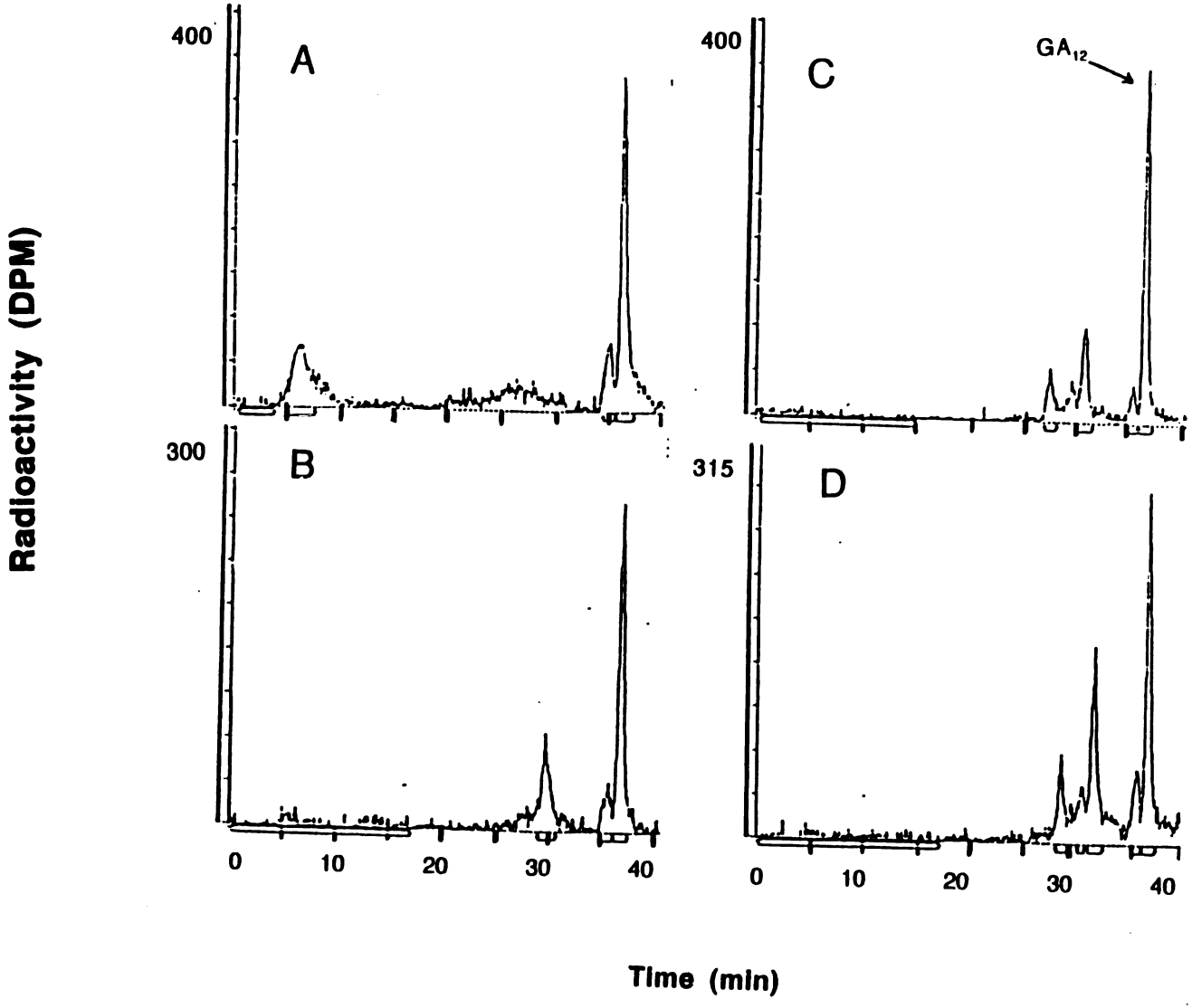

Radioactivity (DPM)
400
A
300
B
400
C
GA12
315
D
0
10
20
30
40
0
10
20
30
40
Time (min)

Figure 21 - ^{14}C-GA_{12} remaining (%) in seeds (A), and in apices of spurs with seedless (B) or seeded fruits (C) of 'Spencer Seedless' after 24 (1992 and 1993) or 72 hr (1994). (Treatments in 1994 were applied to fruits and apices on severed branches). Vertical bars: ± standard deviation.

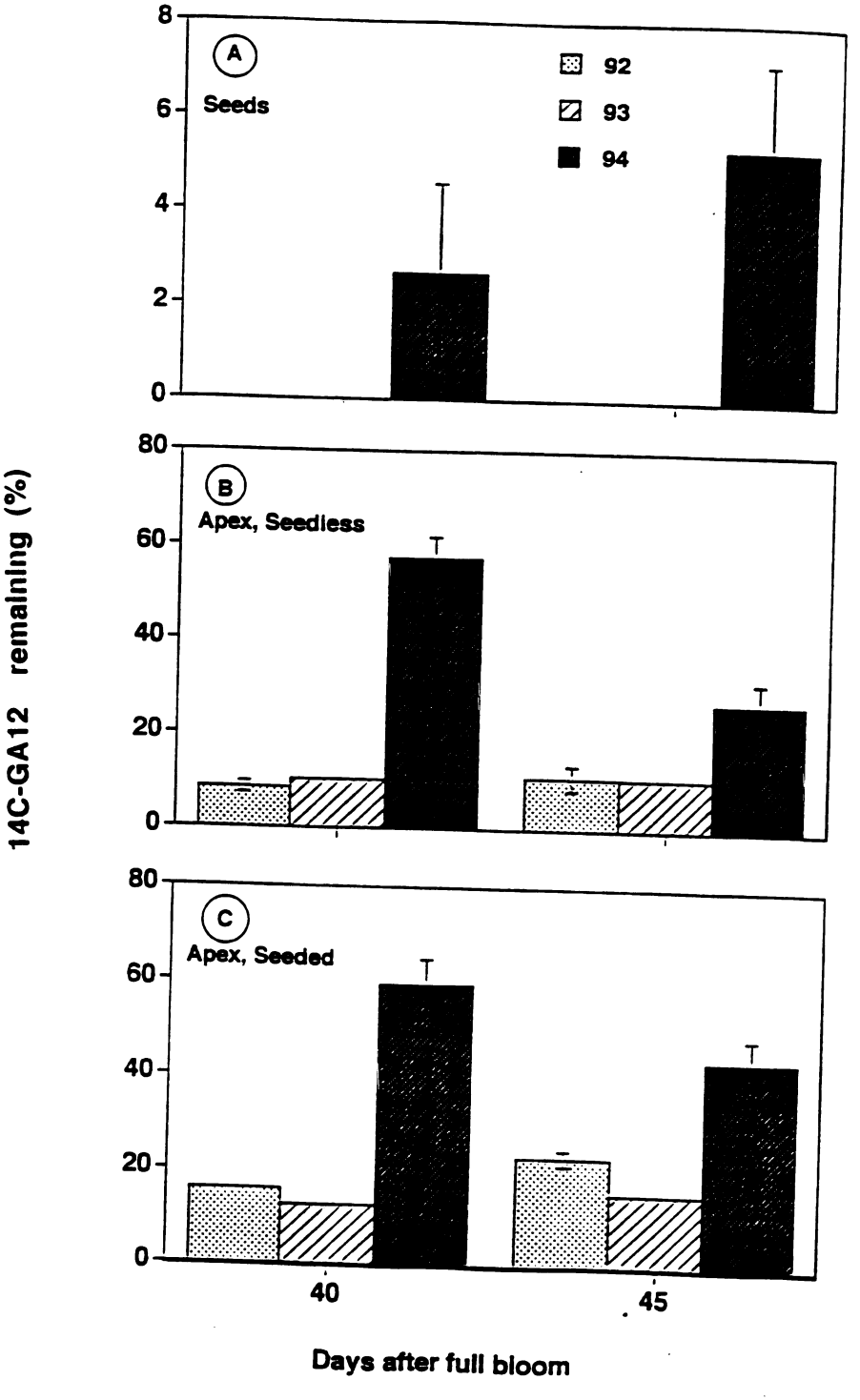
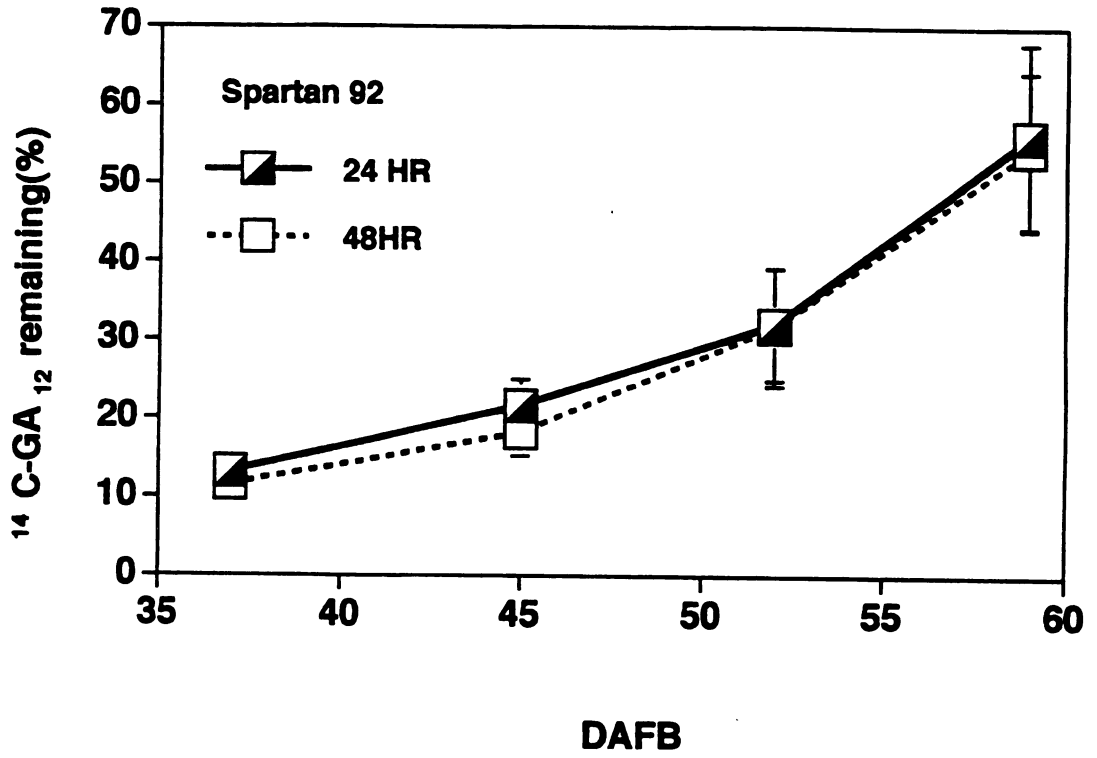

A
Seeds
92
93
94
8
6
4
2
0
B
Apex, Seedless
80
60
40
20
0
C
Apex, Seeded
80
60
40
20
0
40
45
14C-GA12 remaining (%)
Days after full bloom

Figure 22 - Effects of time of treatment and of incubation time on content of ^{14}C-GA_{12} (% of total recovered) in apices of spurs of 'Spartan' in 1992. Vertical bars: ± standard deviation.


Spartan 92
24 HR
48HR
^{14}C-GA$_{12}$ remaining(%)
DAFB
0
10
20
30
40
50
60
70
35
40
45
50
55
60

DISCUSSION

Several hypotheses have been proposed as to how seeds inhibit flowering in apple. The most popular hypothesis is that gibberellins produced (or accumulated ?) in the seeds are transported to the apex of the bourse shoot where they inhibit flowering (Luckwill, 1970). The second is that seeds somehow control metabolism of GAs in the bourse shoot, favoring the accumulation of those that inhibit flowering and/or reducing the concentrations of those that favor flowering (Looney, et al., 1978). A third possibility is that seeds compete for flowering hormones (florigen ?) produced by the leaves, thereby reducing the quantity available for the bourse buds.

My objective was to examine the metabolism of ^{14}C-GA_{12} in both (a) seeds and (b) bourse shoots bearing either seeded (inhibit flowering) or seedless (do not inhibit flowering) fruits. The most important findings were that ^{14}C-GA_{12} was metabolized more slowly in apices bearing seeded fruits than in those bearing seedless ones, and that the rate of metabolism declined during the period of treatment (June-July). Unfortunately, the quantities of metabolites available were insufficient for identification by GC-MS. Nevertheless, these results support the hypothesis of Looney, et al., (1978) that flower induction is associated

with a more rapid rate of metabolism of GAs by vegetative tissues. Whether the relatively small difference in rate observed (ca. 15 %) is physiologically significant cannot be determined at this writing.

The rate of metabolism of ^{14}C-GA_{12} was much more rapid in seeds than in apices, none remaining after 24 hours in seeds in experiments performed *in vivo*; however, a significant quantity remained even after 72 hr when excised branches, rather than trees, were used. The number of metabolites formed was also reduced *in vitro*. The slower rate *in vitro* may indicate that conditions were not optimum for metabolism, or that root factors (cytokinins ?) may affect metabolism of GAs in the aerial parts of the tree. Note that cytokinins can promote flowering in apple (Ramirez and Hoad, 1981).

No consistent pattern was evident in seeds or apices to indicate a step-wise conversion of one metabolite to another, as observed in the metabolism of ^{14}C-GA_{12}-aldehyde by mature apple embryos (Chilembwe, 1992). Although quantitative differences were evident over time in some instances, these were rare, and year-to-year and cultivar-to-cultivar variations were high. The most consistent change was the decline in the quantity of S36 with time (Fig. 15).

Little ^{14}C moved out of the fruit following treatment of seeds with ^{14}C-GA_{12} (Ban, 1996), confirming the observations

of Green (1987). However, both Green (1987) and Bangerth (personal communication) found that radio-labelled IAA was transported out of the fruit, although in small quantities. Conceivably, then, auxin produced by the seed could be the signal that controls GA metabolism in the apex.

A parallel situation exists in the control of growth of the pericarp by seeds of pea. Huizen, et al. (1995) studied the conversion of ^{14}C-GA_{19} to ^{14}C-GA_{20} in pea pericarp with or without seeds, and in deseeded pericarp treated with 4-chloroindole-3-acetic acid (4-Cl-IAA), which is endogenous in pea seeds (Marumo, et al., 1968). Seed removal essentially prevented conversion, whereas deseeded pods treated with the auxin continued to produce GA_{20}. The authors postulated that seeds produce 4-Cl-IAA that moves to the pericarp and there controls the metabolism of GAs.

The pea seed is attached directly to the pericarp, where growth occurs, whereas hormones from apple seeds must pass through the pedicel, the cluster base and the bourse shoot in order to reach the meristem, where flowering occurs. Nevertheless, the parallel is suggestive.

Four of the 6 major metabolites found in apple seeds were strongly polar on TLC, even though they were eluted from the HPLC column at retention times similar to those of "free" GAs. These are probably glycosides, although Rfs of 3 of the 4 did not change following acid hydrolysis. In contrast, 6 of the 8 metabolites in apices behaved as "free"

GAs on TLC (Fig. 14). However, none matched the standard GAs available in both retention time on HPLC and Rf value on TLC (Fig. 9).

Some seed and apex samples contained a polar metabolite which also occurred in the cluster base, although the amount of this metabolite was very low.

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SECTION III Transport of ^{14}C-GA_{12} Metabolites from Apple Seeds and Apices to the Fruit and Other Tissues, and Characterisation of the Metabolites.

ABSTRACT

^{14}C -GA_{12} was injected into apple seeds and bourse shoots *in vivo*, and radioactivity was detected by oxidizing tissues external to the site of injection. Only a small percentage of the ^{14}C injected (about 1.47 to 3.22% in 1992, 0.09 to 0.13% in 1993) was transported from seeds to other tissues, and only ca. 0.1 to 0.4% (1992) and 0.01 to 0.04% (1993) was detected in the tissues outside the fruit. Even lower transport was found following injection in an *in vitro* experiment. With one exception, no radioactivity occurred in the apex when ^{14}C-GA_{12} was injected into seeds, but one polar metabolite was found in the cluster base and two metabolites in the fruit flesh. When apices were injected, from 0.2 to 6% of the total radioactivity was recovered from tissue outside the point of injection; a major part of this occurred in leaves but one metabolite was found in the cluster bases. Metabolites in cluster bases and fruits

differed in chromatographic properties, but were more polar than GA_3 on TLC.

INTRODUCTION

Some commercial apple cultivars have serious alternate bearing problems. In 'Spencer Seedless' apple, a facultatively parthenocarpic cultivar, seeds inhibit flower initiation (Chan and Cain, 1967; Neilsen, unpublished data; Ban, 1996). Nitsch (1958) first demonstrated GA-like activity in immature apple seeds, and Dennis and Nitsch (1966) tentatively identified GA_4 and GA_7 in methanol extracts. Many GAs have since been identified in extracts of apple seeds (Luckwill, et al., 1969; Hoad, 1978; Hoad and Ramirez, 1980; Kirkwood and MacMillan, 1982; Steffens, et al., 1991; Ramirez, 1993; Hedden, et al., 1993). Luckwill, et al. (1969) showed that the concentration of GA-like substances in seeds increased during early stages of fruit development. He also reported that more GA-like activity occurred in spurs with fruits that in those without fruits, and proposed that GAs synthesized in seeds were transported to the bourse shoot, and there inhibited flower initiation. In support of this hypothesis, more GA-like activity was found in diffusates from pollinated 'Spencer Seedless' fruitlets than from non-pollinated ones (Hoad, 1978).

Marino and Greene (1981) detected gibberellin activity in diffusates of flowers or fruits of 'Empire' at bloom; this continued to increase until 45 DAFB. Fruit bearing spurs contained more GA-like activity than vegetative spurs. However, the changes in gibberellin activity in the seeds did not match those in fruiting spurs. Thus diffusion of gibberellin may not be the sole factor related to inhibition of flowering.

Looney, et al. (1978) proposed an alternative hypothesis: the presence of seeded fruits reduces the rate of metabolism of GAs in the bourse shoot, resulting in a higher concentration and therefore inhibition of flowering.

Hoad (1978) injected ^{3}H-GA_4, -GA_3 and -GA_9, as well as ^{14}C-IAA and ^{14}C-sucrose, into apple seeds in vivo, and measured the amount of radioactivity recovered from the bourse shoots on the same spurs. Following injection of sucrose and IAA, more ^{14}C was recovered from bourse shoots of a biennial cv. (Laxton's Superb) than from those of an annual cultivar (Cox's Orange Pippin); however, the differences between cultivars in amount of ^{3}H transported following application of GAs to seeds were not significant, and ^{3}H from GA_9 was not detectable in the bourse shoot.

Green (1987) continued these experiments with these and other compounds, but transport was very limited, regardless of the form of GA injected (^{14}C-GA_{12} aldehyde, ^{3}H-GA_4, ^{14}C-IAA). She reported that GA contents of seeds of a biennial

bearing cultivar (Tremlett's Bitter) and an annual bearing one (Dabinett) were similar qualitatively and quantitatively. In diffusates from fruits, no GA_4 or GA_7 was found, and only a small amount of GA_1 was identified by GC-MS.

My purpose in this research was to investigate transportation of the GAs and /or their metabolites following injection of ^{14}C-GA_{12} into the seeds and apices.

MATERIALS AND METHODS

Plant materials: Plant materials and procedures were identical to those used in section II of this thesis, with few exceptions. 'Spencer Seedless' was again used.

Oxidation of tissue to determine the distribution of ^{14}C. Weighed quantities of lyophilized samples were oxidized in an automatic oxidizer (Biological Oxidizer-OX 400, R.J. Harvey Instrument Corporation, Hillsdale, N.J.) and the CO_2 produced was collected in scintillation fluid. Radioactivity was recorded on a liquid scintillation analyzer (1500 TRI-CARB, Downers Grove, Illinois) and corrected for background. Percentage recovery of radioactivity in various tissues was calculated on the basis of the total recovered from all

tissues, including the portions injected (seeds or apex).

Statistical analysis: For seed samples, one sample was used per treatment in 1992, 3 in 1993 and 1994; for apex samples, 2 in 1993 and 1994, 3 in 1992. The data obtained in 1993 were subjected to analysis of variance, and significant differences determined by F value (2 treatments) or Duncan's Multiple Range Test (3 or more treatments).

RESULTS

Transport of GA metabolites following injection of ^{14}C-GA_{12} into seeds, 1992. Oxidation of tissues other than seed tissues following injection of ^{14}C-GA_{12} into the seeds indicated that most of the activity remained in the inner portion of the flesh, adjacent to the seeds (primarily ovary tissue), with much less activity in the outer portion (Table 1, Fig. 1). Only 1.2 to 3.2% of the radioactivity was transported from the seeds, and 80 to 96% of this remained in the fruit. Most of remaining activity was in the pedicel and cluster base, with less than 0.01% of the total injected being recovered from the bourse shoot. Radioactivities were found in the apex only on one date, 59 DAFB. Less radioactivity (based on total radioactivity in the tissues) was transported from seeds as fruit enlarged.

1993. In 1993, much less radioactivity was found outside the seeds (about 0.1% of the total injected), and only about 0.03% outside of the fruits (Table 2, Fig. 2). Transport to the inner portion of the fruits declined significantly as the season progressed , but differences in other tissues were non-significant. No radioactivity was found in the bourse shoots, bourse shoot leaves, or apex. Transport to the fruit, but not other tissues, increased as incubation time was prolonged from 24 to 48 hr.

1994. Again, very low percentages of the total radioactivity injected into seeds were translocated to adjacent tissues (0.13%, with only 0.04% being transported outside the fruits) (Table 3). None was recovered from vegetative tissues.

Transport of metabolites following injection of ^{14}C-GA_{12} into apices. 1992, When the ^{14}C-GA_{12} was injected into the apex, 1.9 to 6.0% of the radioactivity was transported; a major part of this (0.55 to 3.88%, or 40% of the total recovered) was found in the leaves (Table 4). More radioactivity was transported to bourse shoot leaves, cluster bases, and inner portions of fruits on spurs with seedless fruits than on those with seeded fruits. Data for percent recovery paralleled these data except that differences in fruit tissues were not significant (Fig. 3). With the longer treatment time (48 hr) less radioactivity was recovered in

Table 1. Effect of time of treatment on total radioactivity recovered in different tissues , and on distribution of total radioactivity ,following injection of ^{14}C-GA_{12} into seed of 'Spencer Seedless' apples in 1992. All samples (about 100 mg DW , one per treatment) oxidized and CO_2 collected.

DAFB[Z]	Inner	Outer	Ped.	CB	CL	BS	BL	Apex	Total
	Radioactivity recovered (DPM/100 mg DW)								
37	9204	655	653	567	531	58	0	0	
45	3984	286	511	151	162	25	0	0	
52	1680	175	421	242	506	45	0	0	
59	2076	89	32	28	43	40	76	128	
Mean	4236	301	404	247	310	42	19	32	
	Per cent of total radioactivity recovered[Y]								
37	2.23	0.61	0.05	0.25	0.09	0.01	0	0	3.22
45	1.25	0.27	0.04	0.06	0.03	<0.01	0	0	1.65
52	0.73	0.23	0.04	0.11	0.09	0.01	0	0	1.21
59	1.26	0.16	≤0.01	0.01	0.01	0.01	0.05	0.01	1.47
Mean	1.37	0.32	0.03	0.11	0.05	<0.01	<0.01	<0.01	1.89

[Y] – % of total injected

[Z] Abbreviations:

DAFB – Days after full bloom

Inner – Inner part of treated fruit

Outer – Outer part of treated fruit

Ped. – Pedicel

CL – Cluster base leaves

BS – Bourse shoot

BL – Bourse shoot leaves

CB – Cluster base

Figure 1 - Percentage of total radioactivity recovered from 'Spencer Seedless' apple tissues after injection of ^{14}C-GA_{12} into seeds in 1992 (A) and 1993 (B). Note that scales on vertical axes differ for the two years. Values for BS, BL and apex in 1992 are multiplied by 50.

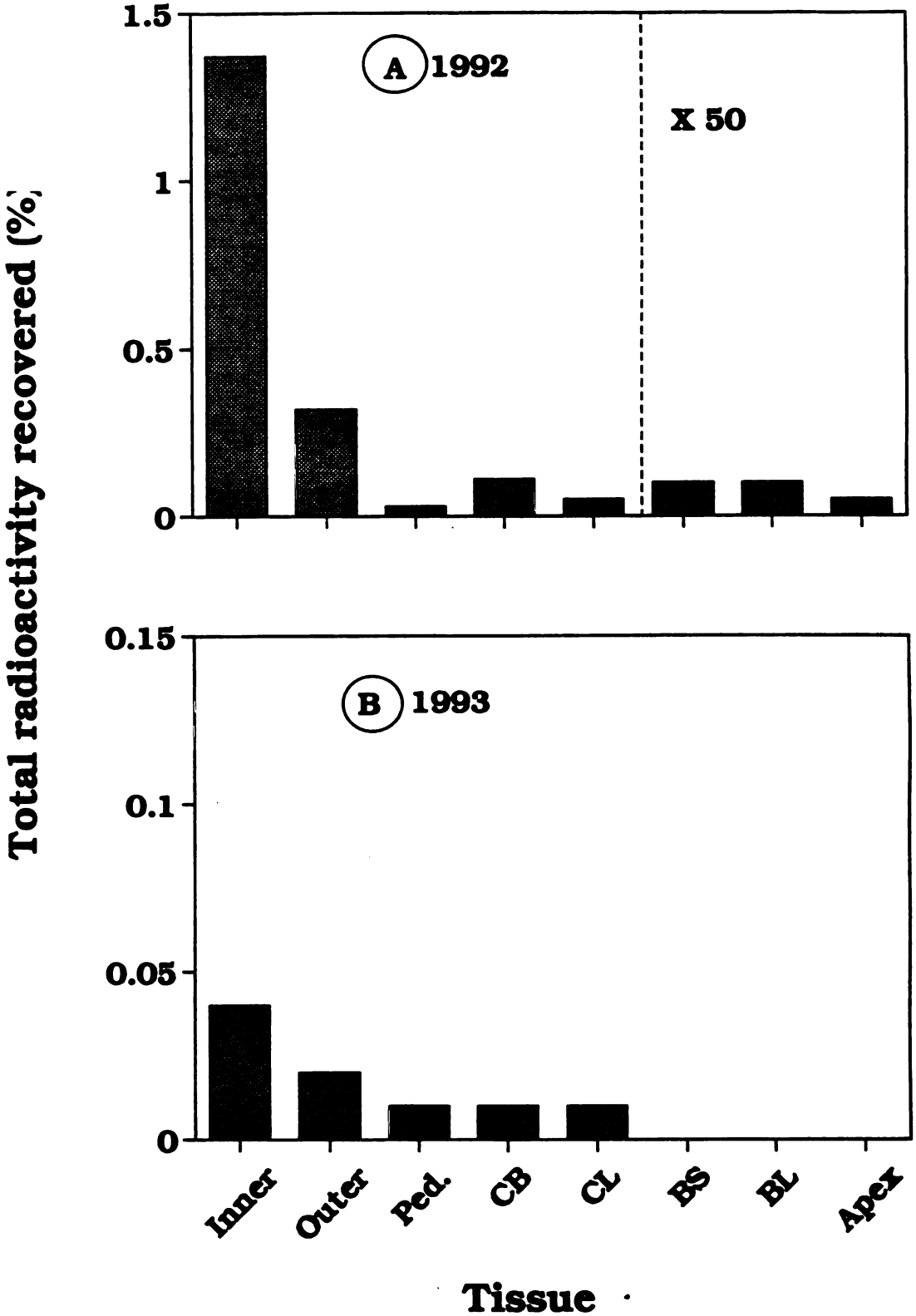

1.5
1
0.5
0
A 1992
X 50
0.15
0.1
0.05
0
B 1993
Inner
Outer
Ped.
CB
CL
BS
BL
Apex
Tissue
Total radioactivity recovered (%)

Table 2. Effect of time of treatment on percentage of total radioactivity recovered (DPM/100 mg DW) , and distribution of total radioactivity recovered, in other tissues following injection of ^{14}C-GA_{12} into seed of 'Spencer Seedless' apple in 1993. All samples (about 100 mg DW, 3 per treatment) oxidized and CO_2 collected.

DAFB	Time(h)	Total	Inner	Outer	Ped.	CB	CL
			Radioactivity recovered (DPM/100 mg DW				
30	24		224	31	151	42	0
	48		305	26	71	34	22
Mean			264a	29a	111a	38a	11a
36	24		141	24	93	31	14
	48		191	31	207	28	0
Mean			166b	28a	150a	30a	7a
41	24		89	29	121	15	11
	48		123	21	72	26	31
Mean			106c	25a	97a	21a	21a
48	24		71	16	91	21	20
	48		96	9	83	12	17
Mean			84c	13a	87a	17a	19a
Mean	24		123	25	114	27	11
	48		179**	22	108	25	18

Table 2 (cont'd)

			Per cent of total radioactivity recovered[Y]				
30	24	0.09	0.03	0.02	0.01	0.02	0
	48	0.08	0.05	0.01	≤0.01	0.01	0.01
Mean			0.04a	0.02a	0.01a	0.01a	0.01a
36	24	0.06	0.03	0.02	0.01	0.01	0.01
	48	0.10	0.04	0.03	0.01	0.01	0
Mean			0.04a	0.03a	0.01a	0.01a	<0.01a
41	24	0.08	0.03	0.03	0.01	0.01	0.01
	48	0.08	0.04	0.02	0.01	0.01	0.01
Mean			0.04a	0.02a	0.01a	0.01a	0.01a
48	24	0.08	0.03	0.02	0.01	0.01	0.01
	48	0.08	0.04	0.01	0.01	0.01	0.01
Mean			0.03a	0.02a	0.01a	0.01a	0.01a
mean	24		0.03	0.02	0.01	0.01	0.01
	48		0.04**	0.02	0.01	0.01	0.01

Abbreviations:

Inner – Inner part of treated fruits
Outer – Outer part of treated fruits
BS – Bourse shoot
Ped – pedicel
CB – Cluster base
CL –Cluster base leaf
BL –Bourse shoot leaf

[Y] % of total injected

abc Mean separation within columns and sets by DMRT $p < 0.05$

* Significantly different from 24 hr. sample by ANOVA $p < 0.05$

Figure 2 - Radioactivity recovered (DPM/100 mg DW) from inner part of fruits of 'Spencer Seedless' apple 24 and 48 hr after injection of ^{14}-GA_{12} into seeds at different dates in 1993.

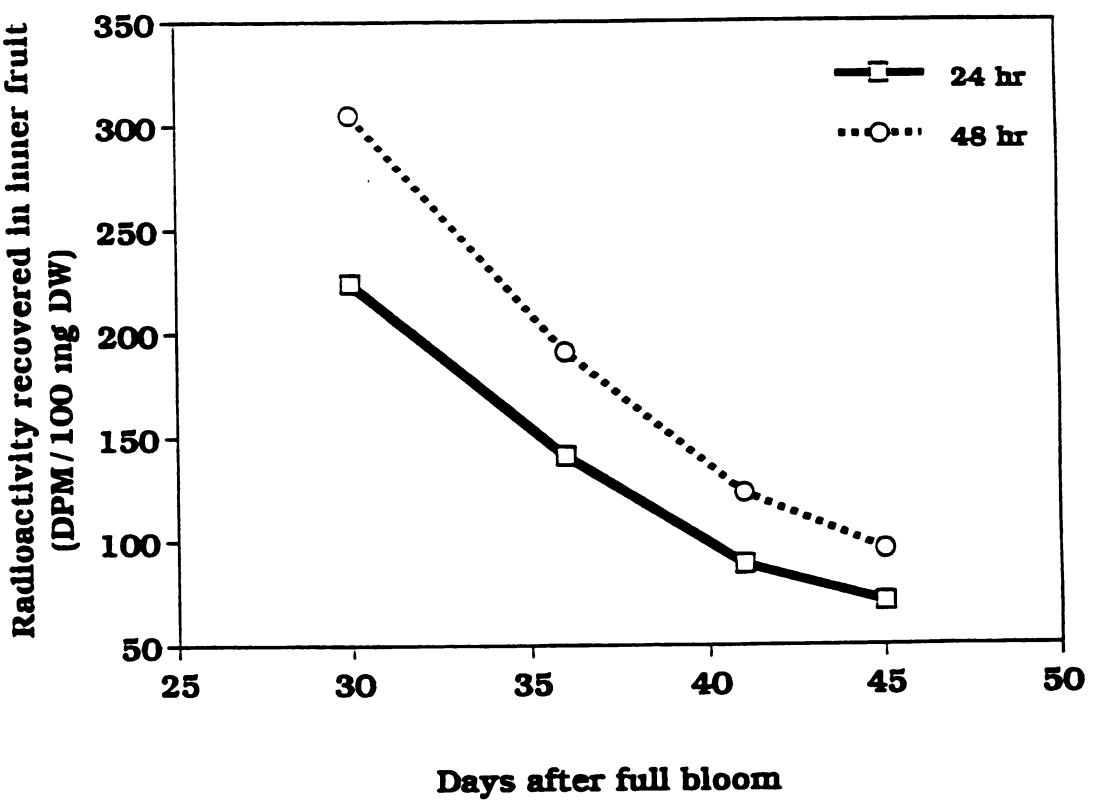

Radioactivity recovered in inner fruit
(DPM / 100 mg DW)
350
300
250
200
150
100
50
25
30
35
40
45
50
24 hr
48 hr
Days after full bloom

Table 3. Percentage of total recovered radioactivity and distribution of radioactivity 72 hr after injection of ^{14}C-GA_{12} into seeds of 'Spencer Seedless' in vitro. 1994

DAFB[Z]	Inner	Outer	Ped
	Radioactivity recovered (DPM/100 mg DW)		
40	188	43	28
45	155	16	22
Mean	172	30	25
	Per cent of total radioactivity[Y]		
40	0.08	0.06	<0.01
45	0.09	0.02	<0.01
Mean	0.09	0.04	<0.01

[Z]Abbreviation: **DAFB**– Days after full bloom

Inner – Inner part of fruit

Outer – Outer part of fruit

Ped – Pedicel

[Y] % of total injected

Table 4. Effects of time of treatment and presence of seeds on total radioactivity recovered in different tissues[Z] , and on distribution of radioactivity following injection of ^{14}C-GA_{12} into bourse shoots of 'Spencer Seedless' apple in 1992. All samples (about 100 mg DW , 3 per treatment) oxidized and $^{14}CO_2$ collected.

DAFB	Seed	Time(h)	BS[Z]	BL	CB	F	Total
			Radioactivity recovered (DPM/100 mg DW)				
37	-	24	4120	3410	670	260	
		48	2810	4050	1080	420	
	+	24	3260	1080	880	180	
		48	1780	1140	710	230	
Mean			2393a	2420a	835a	273a	
45	-	24	1610	2130	660	100	
		48	1450	2530	1670	230	
	+	24	3320	1060	380	180	
		48	2710	890	430	310	
Mean			2273b	1653b	535b	205b	
52	-	24	1050	800	350	130	
		48	2630	850	850	120	
	+	24	2140	1220	460	90	
		48	1120	650	560	90	
Mean			1735c	879c	555b	108c	
59	-	24	1450	970	680	70	
		48	1150	320	680	80	
	+	24	1670	780	520	80	
		48	980	470	820	65	
Mean			1313c	635c	546b	74d	
Mean	-		2034	1883	703	176	
	+		2122	911***	533***	153*	
		24	2327	1431	575	136	
		48	1829***	1362	660	193***	
Interaction			*	ns	**	ns	

Table 4 (cont'd)

			Per cent of total radioactivity recovered[X]				
37	-	24	1.35	3.47	0.73	0.20	5.76
		48	0.75	3.88	1.10	0.28	6.01
	+	24	0.93	1.48	0.99	0.13	3.53
		48	0.52	1.63	0.81	0.17	3.12
Mean			0.89a	2.55a	0.89a	0.19a	
45	-	24	0.51	2.53	0.81	0.09	3.97
		48	0.44	3.54	0.71	0.17	4.87
	+	24	1.12	1.77	0.44	0.15	3.48
		48	0.89	1.65	0.49	0.23	3.25
Mean			0.73b	2.36a	0.60b	0.16b	
52	-	24	0.37	1.48	0.36	0.15	2.37
		48	0.97	1.41	1.01	0.13	3.51
	+	24	0.64	2.06	0.52	0.10	3.32
		48	0.35	1.13	0.64	0.09	2.21
Mean			0.58c	1.50b	0.62b	0.12c	
59	-	24	0.60	1.88	0.83	0.12	3.43
		48	0.40	0.55	0.82	0.13	1.91
	+	24	0.64	1.47	0.64	0.15	2.90
		48	0.39	0.83	0.56	0.12	1.89
Mean			0.49c	1.15b	0.71b	0.13c	
Mean -			0.66	2.29	0.78	0.16	
	+		0.68	1.48***	0.63***	0.14	
		24	0.76	2.00	0.65	0.14	
		48	0.59***	1.78	0.76*	0.16***	
Interaction			*	ns	**	ns	

[Z] – Excluding point of injection

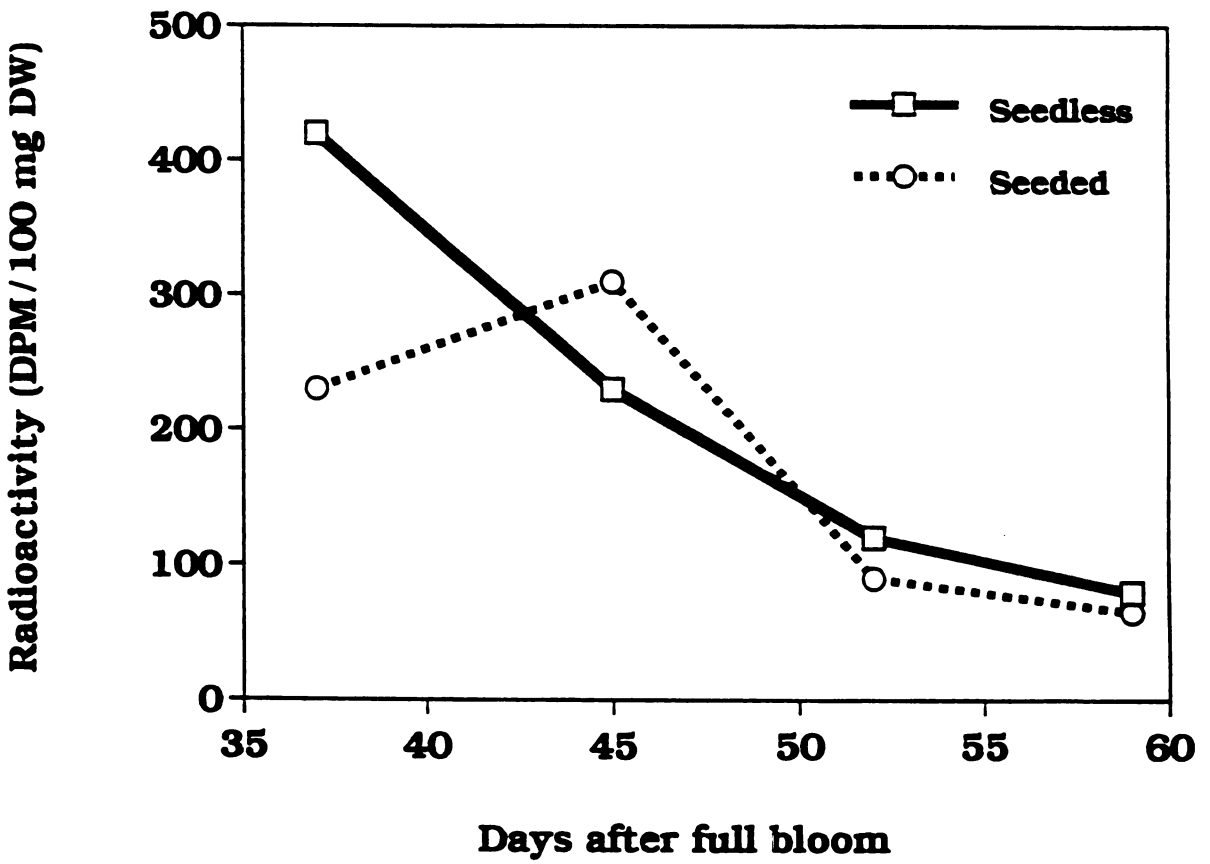
Abbreviations: BS – bourse shoot; BL – bourse leaf; CB – cluster base; F – Fruit inner part

Table 4 (cont'd)

[x] % of total injected

abc (see table 3)
* Significantly different from spurs with seedless fruits or from 24 hour sample at $p < 0.05$(*), $p < 0.01$(**) or $p < 0.001$(***) by ANOVA

Figure 3 - Effects of time of treatment and presence of seeds on total radioactivity recovered (DPM/100 mg DW) in inner part of fruit following injection of ^{14}C-GA_{12} into bourse shoots of 'Spencer Seedless' apple in 1992.


Radioactivity (DPM/100 mg DW)
500
400
300
200
100
0
35
40
45
50
55
60
Days after full bloom
Seedless
Seeded

the bourse shoots and more in the cluster base and fruits. Both the total amount and the percentage of radioactivity recovered from bourse shoots, bourse shoot leaves and fruit flesh declined as fruit enlarged, while recovery from the cluster base varied little with time (Table 4).

1993. Radioactivity was again transported from the apex to other parts, although the amounts were much lower than in 1992 (Table 5). Less than 0.2% was found outside of the point of injection; of this about 70% remained in the bourse. None was recovered from fruit or pedicel tissue. Neither the presence of seeds nor sampling time had a significant effect on transport, but the amount of radioactivity in the bourse shoot again fell with time.

1994 Transport was again very low in 1994; approximately 0.13% of tne radioactivity was transported, of which 80-90% remained within the bourse. Bourse shoots on spurs bearing seeded fruits contained less radioactivity than did those on spurs bearing seedless fruits, as was observed for bourse shoot leaves in 1992. Data for bourse shoot leaves paralleled those for bourse shoots, but differences were not significant. No activity was found in the fruit (table 6).

Characterization of transported metabolites by HPLC and TLC. In 1992, one polar metabolite was found in the cluster base following injection of ^{14}C-GA_{12} into the seeds or apices on 37, 45 and 52 DAFB (Fig. 4). Its retention time, 6 minutes,

suggested that it was a conjugate, although acid hydrolysis of this polar metabolite did not change its Rf value (0.12) on TLC (Fig. 4). In 1993 although radioactivity was detectable following oxidation of tissue samples (Table 2), no metabolites were evident using HPLC with the flow-through detector. Two metabolites present in fruit flesh following injection of ^{14}C-GA_{12} into seeds had retention times of 29 and 34 min on HPLC on all sampling dates in 1992 and at 30 DAFB in 1993 (Fig. 5).

When examined by TLC, these metabolites had Rf values of 0.04 and 0.17, respectively (Fig. 6). The elution time of the more polar compound on HPLC suggested that it might be GA_9, but it was much more polar than GA_9 on TLC. The less polar compound was eluted after GA_9 on HPLC, but it, too, had a much lower Rf on TLC.

Table 5. Effects of time of treatment and presence of seeds on total radioactivity recovered in different tissues, and on distribution of radioactivity following injection of ^{14}C-GA_{12} into bourse shoots of 'Spencer Seedless' apple in 1993. All samples (about 100 mg dw,) oxidized and $^{14}CO_2$ collected.

DAFB	Seed	Time(h)	BS[Y]	BL	CB	CL	Total
			Radioactivity recovered (DPM/100 mg DW)				
30	-	24	116	51	30	0	
		48	90	32	35	16	
	+	24	97	32	25	24	
		48	104	45	50	19	
Mean			102a	40a	35a	15a	
36	-	24	66	29	18	0	
		48	81	19	40	12	
	+	24	45	23	13	37	
		48	75	22	39	9	
Mean			67b	23a	28a	15a	
41	-	24	71	21	29	0	
		48	96	32	29	0	
	+	24	53	31	23	0	
		48	76	16	28	0	
Mean			74ab	25a	27a	0b	
48	-	24	42	17	17	14	
		48	38	18	11	0	
	+	24	31	18	17	0	
		48	37	14	22	8	
Mean			37c	17a	17a	6ab	
Mean	-		75	27	26	5	
	+		65	25	27	12*	
		24	65	28	22	10	
		48	74	25	32	8	

Table 5 (con't)

			Per cent of total radioactivity recovered[x]				
30	-	24	0.04	0.08	0.03	0	0.14
		48	0.03	0.05	0.03	0.02	0.14
	+	24	0.03	0.05	0.03	0.03	0.14
		48	0.03	0.07	0.06	0.03	0.19
Mean			0.03	0.06	0.04	0.02	
36	-	24	0.02	0.04	0.02	0	0.08
		48	0.03	0.03	0.04	0.02	0.12
	+	24	0.01	0.04	0.02	0.02	0.09
		48	0.03	0.04	0.04	0.02	0.12
Mean			0.02	0.04	0.03	0.01	
41	-	24	0.03	0.04	0.03	0	0.09
		48	0.04	0.06	0.04	0	0.11
		48	0.03	0.03	0.04	0	0.09
Mean			0.03	0.05	0.03	0	
48	-	24	0.01	0.03	0.02	0.02	0.09
		48	0.02	0.04	0.01	0	0.07
	+	24	0.01	0.03	0.02	0	0.07
		48	0.01	0.03	0.03	0.01	0.08
Mean			0.02	0.03	0.02	0.01	
Mean	-		0.02	0.05	0.03	0.01	
	+		0.02	0.04	0.03	0.01	
		24	0.02	0.04	0.03	0.01	
		48	0.03	0.04	0.04	0.01	
Interaction			ns	ns	ns	ns	

Abbreviations: **BS** – bourse shoot **BL** – bourse leaf
CB – cluster base **CL** – cluster leaf

Table 5 (cont'd)

[Y] Excluding point of injection

[X] % of total injected

abc (see table 3)

* Significantly different from seedless fruits at $p < 0.05$ by ANOVA

Table 6. Effects of seeds and time of treatment on total radioactivity and distribution among tissues 72 hr after injection of ^{14}C-GA_{12} into bourse shoots of 'Spencer Seedless' apple in vitro in 1994

DAFB [Z]	Seed	BS[Y]	BL	CB
		Radioactivity recovered (DPM/100 mg DW)		
40	-	130	84	17
	+	114	47	18
Mean		122	65	18
45	-	190	66	31
	+	87	25	28
Mean		138	46	30
Mean	-	160	74	24
	+	101	36	23
		Per cent of total radioactivity recovered[X]		
40	-	0.04	0.15	0.02
	+	0.03	0.08	0.03
Mean		0.04	0.12	0.03
45	-	0.07	0.12	0.04
	+	0.03	0.05	0.04
Mean	-	0.06	0.13	0.03
	+	0.03**	0.07	0.03

[Z] Abbreviations **DAFB**– Days after full bloom

BS – Bourse shoot

BL – Bourse shoot leaves

CB – Cluster base

[Y] Excluding point of injection

Table 6 (cont'd)

[x] % of total injected

** Significantly different from seedless fruits at $p < 0.01$ by ANOVA

Figure 4 - Chromatographic characteristics of a polar metabolite in cluster base following injection of ^{14}C-GA_{12} into the seeds or apices of 'Spencer Seedless' apple. A: HPLC profile, using methanol/water gradient as described in the text. B: Radioautogram of this metabolite following TLC in ethyl acetate : chloroform : acetic acid (15:5:1). 1- before hydrolysis; 2-after acid hydrolysis.

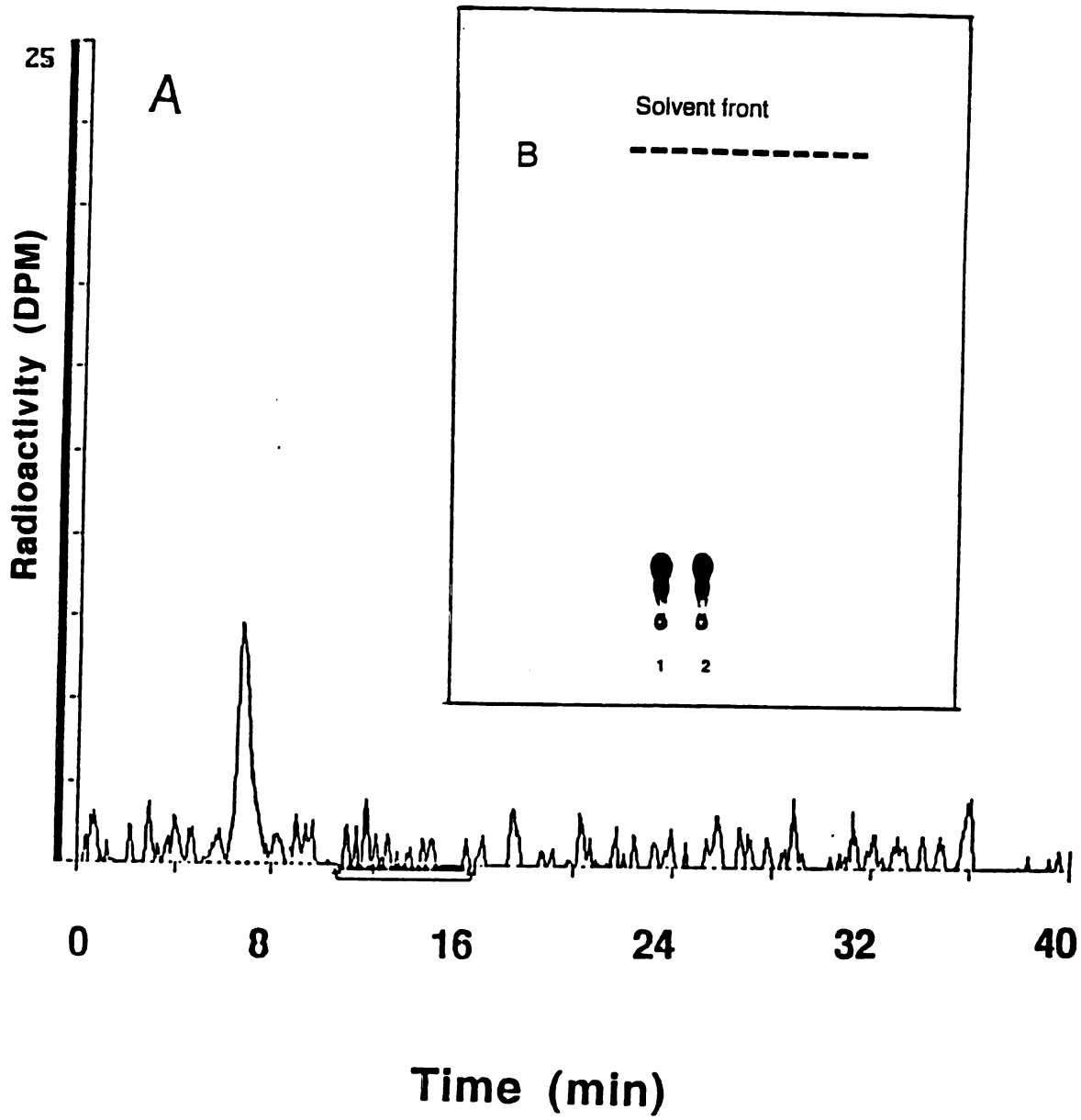

25
A
Radioactivity (DPM)
Solvent front
B
1
2
0
8
16
24
32
40
Time (min)

Figure 5 - HPLC traces (see text for conditions) of metabolites extracted from fruit flesh following injection of ^{14}C-GA_{12} into seeds in 1992 (A) and 1993 (B).

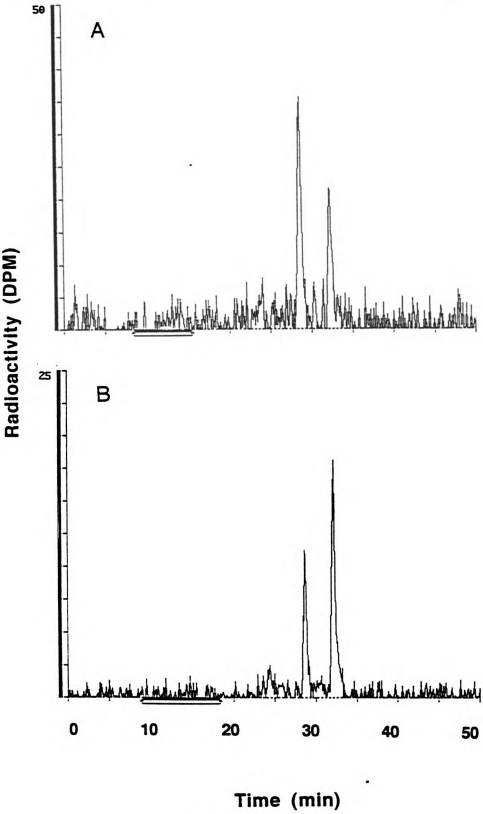

50
A
25
B
Radioactivity (DPM)
0
10
20
30
40
50
Time (min)

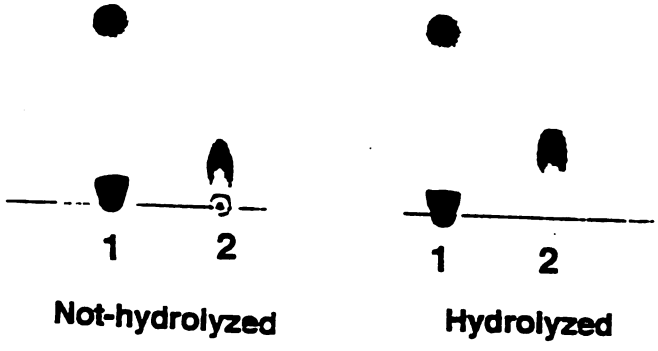
Figure 6 - TLC of metabolites of ^{14}C-GA_{12} extracted from fruit flesh following injection of ^{14}C-GA_{12} into seeds. 1- metabolite eluted at 33 min on HPLC (see Fig. 2); 2- metabolite eluted at 29 min. Left - before acid hydrolysis; right - after acid hydrolysis.

d from

eeds. 1-

2-

hydrolysis



DISCUSSION

Following injection of ^{14}C-GA_{12} into seeds, only low amounts of radioactivity were detected in other tissues. In only one sample was radioactivity found in the apex, where flower initiation occurs, when ^{14}C-GA_{12} was injected into the seeds. Green (1987), working with 2 biennial cider apple cultivars, obtained similar results. She observed very low amounts of ^{14}C-GA_{12}-aldehyde (0.005 to 0.006% of total injected) in diffusates from fruit pedicels following injection of ^{14}C-GA_{12}-ald into seeds, and similar results were obtained with ^{3}H-GA_4. However, radioactive compounds in diffusates from fruits injected with ^{3}H-GA_4 co-chromatographed with standard GA_4. Green (1987) also analyzed endogenous gibberellins by GC-MS, but no qualitative or quantitative differences were found in bearing vs. non-bearing trees of either cultivar. Thus she obtained no evidence that seed GAs are transported to the apex and there inhibit flower initiation.

Several reasons could be suggested for my failure to detect transport from seeds to apices and vice-versa: 1) transport does not occur; 2) the ^{14}C-GA_{12} injected did not enter the proper pool, and was conjugated to give inactive compounds or artifacts; 3) incubation time (24 to 72 hr) was too brief

for measurable transport to occur. Although all ^{14}C-GA_{12} was metabolized by the seeds within the first 24 hours (Ban, 1996, Part II), transport of metabolites may require a longer period of time. Recovery of radioactivity was low (15 to 25%); had recovery been better, greater transport might have been evident.

Transport from apices to fruits was observed only in 1992. One may argue that incubation time was not long enough. Endogenous gibberellins might accumulate in apices after several days, even weeks.

The Rf values (0.12 from cluster base, 0.04 and 0.16 from flesh) of transported metabolites indicated that they were not GA_4 or GA_7, which are thought to be the GAs affecting flower initiation in apple. The Rf value of the transported metabolites did not match those of known gibberellins under the conditions I used. These metabolites could be conjugates, given their polarity, but acid hydrolysis did not change their Rf values. More work is needed to identify and investigate the function of these metabolites.

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SUMMARY

Defruiting experiments indicated that seeds inhibit flowering of 'Spencer Seedless' apple. There are two main hypotheses to explain this phenomenon. 1) GAs synthesized in seeds move to the apex, and there inhibit flowering. 2)The presence of seeds controls the metabolism of GAs in the apex, then and this affects flower initiation there. Following injection of ^{14}C-GA_{12} into seeds, only a very small amount of radioactivity was transported, and most of this remained in the fruit flesh. With but one exception no radioactivity was found in the apex. These observations provide little support for the first hypothesis. However, several reasons may be offered for the failure to detect transport from seeds to apices. 1) the ^{14}C-GA_{12} injected did not enter the proper pool or overloaded the system, and was conjugated to give inactive compounds or artifacts. Most of the metabolites in seeds were conjugated, possibly because the amount of ^{14}C-GA_{12} injected was excessive and/or the GA_{12} did not enter the proper pool. 2) The incubation time (24 to 72 hr) was too short for measurable transport to occur. Although, most of the ^{14}C-GA_{12} was metabolized within 24 hr, more time may be required for transport. However, th amount of transport increaseed only slightly when incubation time was extended to 48 hr. 3) The recovery of radioactivity was

low (15 to 25%)of the total injected. Had recovery been better, greater transport might have been evident. Most of the radioactivity was lost (about 70 to 80%) between the time of injection and the time of sampling. The GA may have been metabolized and converted to gaseous ^{14}C-CO_2.

One polar metabolites was found in the cluster base after injecting ^{14}C-GA_{12} into seeds or apices; two were found in the flesh after injecting ^{14}C-GA_{12} into seeds. Rf value of the metabolite found in the cluster base was 0.12, those of metabolites in the flesh were 0.04 and 0.16. Neither acid nor enzymatic (glycosidase, cellulase) hydrolysis changed their Rf values.

The metabolism of ^{14}C-GA_{12} was slower in apices bearing seeded fruits than in those bearing seedless ones, and the rate of metabolism declined during the period of treatment (June - July). There were seven major metabolites found in the apices, six in the seeds. Unfortunately, the quantities of metabolites available were insufficient for identification by GC-MS. However, these results support the second hypothesis proposed by Looney, et al. which is that the presence of seeds reduce the rate of metabolism of GAs in the apices, thereby reduce the flower initiation.

The rate of metabolism of ^{14}C-GA_{12} was much more rapid in seeds than in apices, none remaining after 24 hr. in experiments performed <u>in vivo</u>; however, a significant quantity remained even after 72 hr when excised branches

were used. The number of metabolites formed was also reduced *in vitro*.

Four of the six major metabolites found in apple seeds were strongly polar on TLC, even though they were eluted from the HPLC column at retention times similar to those of 'free' GAs. In contrast, 6 of the 8 metabolites in apices behaved as 'free' GAs on TLC. However, none matched the standard GAs available in both retention time on HPLC and Rf value on TLC. Some seed and apex samples contained a polar metabolite which also occurred in the cluster base, although the amount of this metabolite was very low.

Suggestions for further research

1. Obtain sufficient quantities of metabolites for identification by GC-MS.
2. Select spurs with bourse shoots less than 2 cm in length for comparison the metabolism of ^{14}C-GA_{12} in apices of bearing vs. non-bearing spurs. A length of 2 cm is critical based on J. Neilsen's results (personal communication), longer shoots tending to flower whether seeds are present or not.
3. Apply an auxin such as NAA or 4-Cl-IAA to spurs bearing seedless fruits to determine if it stimulates GA metabolism, as has been reported for pea fruits. Or apply an auxin inhibitor antagonist such as TIBA to spurs bearing seeded fruits to determine the rate of metabolism of ^{14}C-GA_{12} is reduced.

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