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Correlation of Endogenous Gibberellic Acid with Initiation of Mango Shoot Growth

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Abstract

Stems of mango (*Mangifera indica* L.) rest in a nongrowing, dormant state for much of the year. Ephemeral flushes of vegetative or reproductive shoot growth are periodically evoked in apical or lateral buds of these resting stems. The initiation of shoot growth is postulated to be primarily regulated by a critical ratio of root-produced cytokinins, which accumulate in buds and by leaf-produced auxin, which decreases in synthesis and transport over time. Exogenously applied gibberellic acid (GA_3) delays initiation of bud break but does not determine whether the resulting flush of growth is vegetative or reproductive. We tested the hypothesis that endogenous GA_3, which influences release of these resting buds, may decrease in stem tips or leaves with increasing age of mango stems. GA_3 and several other GAs in stem tip buds and leaves were identified and quantified in stems of different ages. The major endogenous GAs found in apical buds and leaves of vegetative mango stems were early 13-hydroxylation pathway gibberellins: GA_1, *epi*-GA_1, GA_3, GA_{19}, GA_{20}, and GA_{29}, as identified by gas chromatography-mass spectrometry (GC-MS). A novel but unidentified GA-like compound was also present. The most abundant GAs in apical stem buds were GA_3 and GA_{19}. Contrary to the hypothesis, the concentration of GA_3 increased within buds with increasing age of the stems. The concentrations of other GAs in buds were variable. The concentration of GA_3 did not change significantly with age in leaves, whereas that of most of the other GAs declined. GA_1 levels were greatest in leaves of elongating shoots. These results are consistent with the concept that rapid shoot growth is associated with synthesis of GAs leading to GA_1. The role of GA_3 in delaying bud break in mango is not known, but it is proposed that it may enhance or maintain the synthesis or activity of endogenous auxin. It, thereby, maintains a high auxin/cytokinin ratio similar to responses to GA_3 that maintain apical dominance in other plant species.

Key words: Stem tip growth; Gibberellins; *Mangifera indica*

Introduction

Extension and lateral growth of mango (*Mangifera indica* L.) stems occurs in periodic flushes of elongating shoots forming the terminal intercalary units of branches. Stems are here defined as nongrowing, dormant vegetative structures that remain in rest most of the year, whereas shoots are actively growing vegetative or reproductive structures that are evoked from apical or lateral buds of these stems. Growth of individual shoots lasts only about 2 weeks, forming 10 to 20 leaves before returning to a

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dormant or resting state that lasts 2 months to nearly a year, depending on the age of the tree and environmental conditions. Once elongation is completed, these shoots form the terminal intercalary unit of resting stems as defined by Davenport and Nuñez-Elisea (1997).

Vegetative shoots undergo distinct changes from early shoot development to maturation of leaves. Developing shoots are light green in color during early elongation and enlargement (elongating green leaf [EGL] stage). When nearing full size, approximately 2 weeks after initial bud break, they become red in color and possess little lignification in the cell walls, resulting in limp leaves that hang vertically from stems (limp red leaf [LRL] stage). They are thin and soft to the touch but soon (2–3 days) return to a light green color while strengthening. New leaves continue to strengthen by increased lignification but remain light green in color (immature green leaf [IGL] stage) for 1–2 months after the LRL stage before turning the deep green color typical of mature leaves (mature green leaf [MGL] stage). Depending on the vigor of the tree, one to four episodes of vegetative shoot growth may occur on each stem between flowering flushes, which typically occur in February in the Northern Hemisphere.

Flowering occurs in the subtropics when resting buds initiate growth during cool, inductive temperatures (Batten and McConchie 1995; Davenport and Nuñez-Elisea 1997; Nuñez-Elisea and Davenport 1995; Nuñez-Elisea and others 1996). Flowering responses to floral inductive conditions, however, are moderated by stem age (Nuñez-Elisea and Davenport 1995). Young, resting stems bearing light green or recently matured leaves are more likely to produce vegetative instead of reproductive shoots even if shoot growth is initiated in floral inductive conditions (Nuñez-Elisea and Davenport 1995).

Davenport and Nuñez-Elisea (1997) proposed that there exist two physiologic switches that regulate growth and developmental events in mango. The first switch regulates initiation of bud break and has no regulatory role in determining its reproductive or vegetative fate. If present, the apical bud is always the one that initiates a new shoot. It is proposed that the ratio of promotive and inhibitory initiation components determines when shoot initiation occurs. A root-generated initiation promoter, perhaps a cytokinin, may accumulate in stem tips over time as has been found in citrus (Hendry and others 1982), which displays a phenology similar to mango (Davenport 1990). An inhibitor formed in leaves that decreases with age, such as auxin, may antagonize it. Decreases in availability of auxin to stem tips as leaves age have been reported in species other than mango (Davenport and others 1980; Veen and Jacobs 1969). Davenport and Nuñez-Elisea (1997) suggested that a rising ratio of cytokinin to auxin acts to stimulate initiation of bud break in resting stems of mango once a critical ratio is reached. It is analogous to the relationships described for correlative inhibition of lateral branching (apical dominance) in growing plants (Cline 1997; Cline and others 1997).

Several lines of evidence also support the notion that a gibberellin (GA), possibly GA_3, may also inhibit initiation. Numerous articles have reported the concentration-dependent inhibitory and delaying effects of exogenously applied GA_3 on flowering of mango (Nuñez-Elisea and Davenport 1998; Oosthuyse 1995). The most notable response of mango trees is inhibition of inflorescence shoot initiation, not prevention of floral induction. The delay in inflorescence initiation is always followed by inflorescence development as long as initiation takes place during cool, floral-inductive conditions. If GA_3 inhibited floral induction, one would expect initiation of a high proportion of vegetative shoots instead of reproductive shoots during cool, floral-inductive conditions, especially in response to high GA_3 concentrations. Moreover, triazole plant growth retardants, which are known to inhibit gibberellin biosynthesis (Rademacher 1991), stimulate early flowering of mango (Sergent and others 1996; Tongumpai and Chantakulchan 1996).

The delaying effect of GA_3 on shoot initiation without influencing flowering in inductive conditions and the accelerating effect of paclobutrazol on reproductive shoot initiation implicates GA_3 in the initiation events of mango, perhaps as an inhibitor augmenting the auxin/cytokinin ratio described earlier. Endogenous gibberellins have been reported in mango leaves and shoots under a variety of conditions using various analytical protocols (Chen 1987; Pongsomboon and others 1997). Most are not useful comparisons to this study. Chen (1987) reported levels of gibberellins $A_{1/3}$, $A_{4/7}$, A_5, A_{17}, A_{20}, and A_{29} (identified by gas chromatography-mass spectrometry [GC-MS]) in xylem sap extracted from stumps during the fall vegetative flush, during the mature green leaf dormant period, and during the early and full flowering flush in spring. Highest levels of $GA_{1/3}$ (2.9 ± 0.9 ng · mg^{-1} (fresh mass [fm] xylem sap) were observed during early vegetative flush in the fall. Lower amounts ($1.0–1.4 \pm 0.2$ ng · mg^{-1} xylem sap) were noted during the dormant and flowering flush periods. Although none of the preceding estimates of gibberellins in stem tips or leaves of mango provide unambiguous estimates of individual gibberellins as related to stem age, they suggest that

gibberellin levels may be relatively high during vegetative shoot growth and gradually decrease over time during the subsequent rest period.

As part of a study of the role of gibberellins in flowering in mango, the primary purpose of this investigation was to test the hypothesis that apical stem tips and leaves from older stems would contain less GA_3 than those from young stems or shoots. Thus, we identified the typical complement of endogenous gibberellins contained in 'Tommy Atkins'; mango leaves and buds by GC-MS. We then quantified those GAs in leaves and stem tips from young shoots in the limp red leaf stage, from two age groups of mature stems bearing dark green leaves, and from stem tips that were either resting or were initiating new vegetative growth at the time of sampling.

Materials and Methods

Plant Material

Shoot or stem samples were randomly harvested from branches on ten 15-year-old 'Tommy Atkins' mango trees located in a commercial orchard near Homestead, Florida USA. The trees were in excellent condition, planted in trenches dug into the calcareous Rockdale gravelly soil, and were fertilized and irrigated regularly. Stems were sampled on July 22, 1994 by excising the terminal intercalary unit from stems approximately 1 month after fruit harvest. Stem tip and leaf samples were categorized into one of four distinct types representing different ages of shoots or stems:

1. Early developing green-leaf shoots (EGL) 3–32 mm long (≤1 week since shoot initiation), borne on MGL stems of the previous vegetative flush (approximately 6- to 7-months-old).

2. LRL shoots (approximately 2-weeks-old).

3. MGL stems with resting apical buds, which evoked soon after February, 1994 flowering (approximately 3-months-old).

4. MGL stems that had not flushed since flowering about 7 months earlier and bearing no apical bud, having developed into a panicle that subsequently aborted.

Harvested shoots or stems were placed with leaves intact in plastic bags on ice in an insulated chest and quickly transferred to the laboratory. Shoot or stem tips (1 cm in length), consisted of the apical bud (except for category type 4, which lacked an apical bud) and about 10 lateral buds. The leaves were excised from the stems in each category. The stem tips and leaves were each pooled into three replicate samples. Each shoot or stem and each leaf sample consisted of 10 tips and all leaves, respectively, on stems in category 2 and five tips and all leaves on stems in categories 1, 3, and 4. Each replicate pooled stem tip or leaf sample was frozen in liquid nitrogen and stored at −80°C until lyophilized. All of the buds on each dried stem tip were pared from the tips and pooled to form the final samples. Three replicate samples of each were analyzed for GAs.

Large samples (>1 kg fm) of LRL and MGL were also harvested from the same mango trees and similarly prepared for preliminary identification of gibberellins. After removal of midribs and petioles, 100 g of dried leaf lamina was used for analyses of each sample.

GA Analysis

Extraction, purification, and analytical procedures outlined in the following were used for quantitative analyses of all samples with minor modifications in some cases. The same steps were used on a larger scale to purify large quantities of leaves for identification of the major gibberellin components.

The three replicate samples of emerging apical shoots (category 1) had a dry mass (dm) of 530–740 mg. Buds pared from the three replicate stem tip samples harvested at the LRL stage (category 2) yielded 86 to 111 mg (dm). Buds excised from stem tip samples taken from the MGL samples with or without apical buds (categories 3 and 4) yielded samples ranging in mass from 79–125 mg. Samples were ground in a mortar immediately before extraction.

About 50 g dm of leaves per replicate was harvested for green-leaf samples, and about 20–30 g dm per replicate for red-leaf samples. Subsamples were taken from each of the triplicate pooled leaf samples, the midribs and petioles were removed, the remaining leaf blades were ground in a Wiley mill equipped with a 40-mesh screen, and a 2-g subsample was used for GA analysis.

Samples were extracted overnight in 10–40 mL 80% methanol (MeOH) at 4°C, centrifuged at 2,000 × *g* for 10 min (or filtered) and briefly re-extracted twice in 100% MeOH. Supernatants were pooled before addition of internal standards. For quantitative analysis, 10 to 50 ng of [$^2H_2$]-labeled internal standards of GA_1, GA_3, GA_4, GA_6, GA_8, GA_9, GA_{19}, GA_{20}, GA_{29}, GA_{44}, and GA_{53} were each added to the extracts. The methanol concentration of extracts was then adjusted to 80% with water followed by adjustment to pH 7 with NH_4OH. Chlorophyll and other nonpolar components were removed on a reversed-phase column (1–5 g C_{18}; Waters Corp., Mil-

ford, MA) and washed with 10 to 50 mL 80% MeOH. The eluate was reduced to aqueous phase at reduced pressure at 35°C, adjusted to pH 8 with NH_4OH, and centrifuged for 10 min at 2,000 × *g*. The supernatant was slurried with polyvinylpolypyrrolidone (Sigma Chemical Co., St. Louis, MO; 1–10 g total, depending on the sample), and then filtered. The filtrate was adjusted to pH 3 with HCl and partitioned three times against equal volumes of ethyl acetate EtOAc). The acidic EtOAc fraction was dried at reduced pressure at 35°C. The residue was dissolved in 5 mL of (99:1) ethyl acetate:acetic acid)EtOAc:HOAc) and loaded onto a 14 × 20 mm id deactivated silica gel (2 g) column (Koshioka and others 1983b). GAs were eluted with 20 mL of EtOAc:HOAc (99:1) and subsequently dried. Neutral impurities in the residue were removed by chromatography on DEAE-Sephadex A-25 (Turnbull 1985) or QAE-Sephadex A-25 (Lenton and others 1994). Gibberellins in the acidic fraction from ion exchange chromatography were subsequently separated by reversed-phase HPLC (C_{18} μBondapak, 3.9 mm id × 30 cm; Waters Corp., Milford, MA) with [^{3}H]-GA_1 and -GA_4 (0.5 kBq each; Amersham Pharmacia Biotech, Piscataway, NJ) added as internal standards. The eluting solvent program was 10% MeOH 0 to 11.5 min, linear gradient to 73% MeOH at 47.5 min; flow 1.67 mL min^{-1}; 1.2 min (2 mL) fractions were collected (Koshioka and others 1983a).

Fractions from HPLC were first analyzed for GA-like biological activity in preliminary analyses to determine the major GAs present in LRL and MGL extracts using a modified dwarf rice (*Oryza sativa* cv Tan-ginbozu) bioassay (Nishijima and Katsura 1989). Subsequently, groups of fractions were combined on the basis of biologic activity and/or the elution volumes of the [^{3}H]- internal standards and expected elution volumes of the [^{2}H]- internal standards. These samples were methylated with ethereal diazomethane, silylated with N,O,-bis(trimethylsilyl)trifluoroacetamide with 1% TMCS (Pierce Chem. Co., Rockford, IL), and analyzed by GC-MS or GC-MS-selected ion monitoring (SIM) (Zanewich and Rood 1995). Quantities of GA_1, GA_3, GA_8, GA_{20}, and GA_{29} were estimated by isotope dilution analysis from the abundance of their molecular ions and those of their respective [^{2}H]-labeled standards, and for ions at m/z 434 and 436 for GA_{19} and [^{2}H]GA_{19}. *Epi*-GA_1 was estimated by comparison of the abundance of its molecular ion with that of [$^2H_2$]GA_1, present in the same sample in GC-MS-SIM analysis.

For each GA, results were assessed by ANOVA and comparisons of individual treatments (leaf or bud age) were then made by Fisher's protected least significant difference (PLSD) procedure. The results were also compared using the Kruskal-Wallis test.

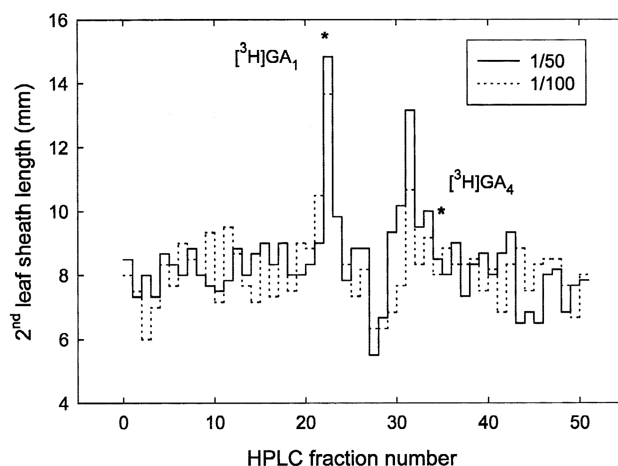

Figure 1. Distribution of biological activity in fractions from C_{18} HPLC of an extract of mature green mango leaves. Dilutions (1/50 or 1/100) of each fraction were assayed on dwarf rice (Tan-ginbozu). [^{3}H]-internal standards were located as shown.

Results

Identification of GAs

Bioassays of extracts of young (limp red) and mature (dark green) leaves after reversed-phase HPLC showed two major groups of biologically active fractions. The results for the two leaf types were the same. Figure 1 displays the results on mature green leaves. From these, GA_1, *epi*-GA_1, GA_3, and GA_{20} were identified by GC-MS or GC-MS-SIM (Table 1). GA_{29} and GA_{19} were identified from other fractions (Table 1). GA_8 was indicated (by ions at m/z 594 and 448 in quantitative analyses), and trace amounts of GA_{44} and GA_{53} were indicated in some quantitative analyses (by molecular ion only). Extracts of both red and green leaves contained an unidentified compound with characteristics of a dihydroxylated GA_9 (Gaskin and MacMillan 1991). It was found in HPLC fractions 17 and 20 mL, which showed no biologic activity in the dwarf rice bioassay.

Quantification of GAs

Of those GAs surveyed in the buds of resting stem tips at both the LRL (2-weeks-old) and MGL stages (3-months-old), GA_3 and GA_{19} were the most abundant when normalized on a per unit mass basis (Figure 2). The trends in concentration of the various gibberellins varied with age of the sampled shoots or

Table 1. Inhibition of long day-induced flowering of *Fuchsia* cv. Lord Byron by natural and synthetic gibberellins in three experiments.

Treatment	Dose	Flowers per plant	
		Exp. I	Exp. II
Control (4 LD)		4.3 ± 0.4	3.6 ± 0.2
GA_3	30 ng/plant	0.7 ± 0.4	2.0 ± 0.8
2,2-dimethyl GA_4	30 ng/plant	0	0.3 ± 0.3
2,2-dimethyl-3-*epi* GA_4	30 ng/plant	3.5 ± 0.5	3.0 ± 0.3
16,17-dihydro-2,2-dimethyl-3-*epi* GA_4	30 ng/plant	—	3.0 ± 0.4
GA_1	30 ng/plant	5.2 ± 0.7	—
		Exp. III	
Control (4 LD)		4.9 ± 0.4	
GA_3	100 ng/plant	0.8 ± 0.4	
GA_3 methyl ester	100 ng/plant	4.5 ± 0.5	

*All plants were exposed to 4 LD beginning at the time of a single application of 10 μL of various gibberellins to the shoot tip. The control was treated with the same aqueous:ethanol (90:10, v/v) solution. Values are means ± SEM (*n *= 10–14)*

stems. GA_3, the gibberellin of primary concern to the hypothesis being tested, displayed a clear trend of increasing in buds of stem tips as emerging shoots made the transition to resting stems and increased maturity (Figure 2). Higher levels of GA_1 found in leaves during early shoot development were consistent with the role of GA_1 in shoot elongation. GA_8, GA_{20}, and GA_{29} tended to decrease with advancing stem tip age. The absolute content of all observed gibberellins was greatest in the emerging shoots (≤1 week) (Table 2) and thereafter displayed substantially lower levels with no particular trends evident in any of the gibberellins.

In general the overall levels of gibberellins found in leaves were lower than those found in the stem tip buds (Figure 2). Except for GA_3 and GA_{19}, the GA amounts were the greatest in the youngest (LRL) stage. A nonsignificant trend of increasing GA_3 levels was noted in leaves of increasing age.

Discussion

All GAs identified in mango stem tips and leaves are members of the early 13-hydroxylation pathway, common in elongating tissue of many species (Sponsel 1995). The relationship between GA_1, GA_8, GA_{19}, GA_{20}, and GA_{29} is well established (Hedden and Kamiya 1997). GA_3 might arise from GA_{20} by way of GA_5 (Albone and others 1990; Fujioka and others 1990); however, neither this nor GA_{95} (1,2-didehydro GA_{20}), another possible immediate precursor of GA_3 (Nakayama and others 1996), was found in this study.

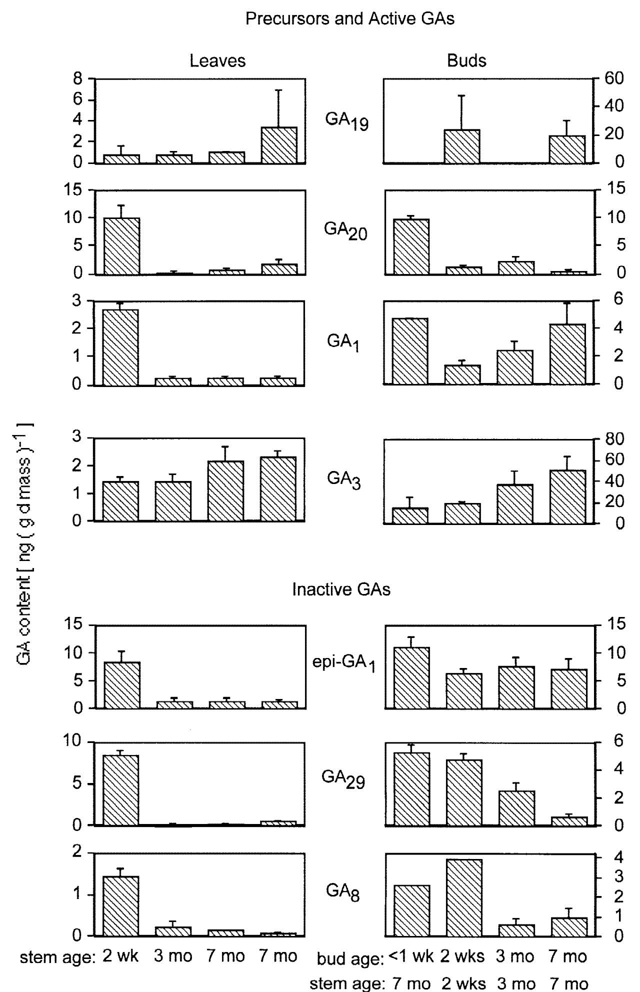

Figure 2. *Left,* Mean (± SD) gibberellin content [ng (g dm)$^{-1}$] of mango leaves borne on limp red leaf shoots (2 weeks), 3-month and 7-month-old mature green leaf stems. Mature green leaves from the ~7 month column of figures were borne on stems from which the newly emerging shoots (≤1-week-old) were forming. *Right,* Mean (± SD) gibberellin content [ng (g dm)$^{-1}$]^z found in emerging shoots (≤1 week) and in buds from apical stem tips of 2-week-old limp red leaf mango shoots or 3- and 7-month-old mature green leaf resting stems.

Other GAs previously reported in xylem sap of mango, that is, GA_4, GA_5, GA_7, and GA_{17} (Chen 1987) were not found in either leaves or stems of mango in our study. There was no evidence of significant quantities of endogenous GAs accompanying the internal standards of [$^2H_2$]- GA_4, -GA_6, or -GA_9 in this study.

Epi-GA_1 could be a natural GA of mango or may

Table 1. Gibberellins[a] Identified from Limp Red or Mature Green Mango Leaves

HPLC Fraction	Gibberellin	KRI	Principal Ions m/z (relative intensity)
20–24	GA_1	2660	506(100), 491(9), 448(21), 377(16), 376(24), 375(31), 313(38), 207(92)
20–24	*epi*-GA_1	2772	506(100), 491(9), 448(34), 377(56), 375(28), 313(10), 238(16), 207(115)
20–24	GA_3	2687	504(100), 489(8), 445(6), 370(11), 347(14), 297(17), 208(76)
33–34	GA_{19}	2619	462(5), 434(100), 402(35), 375(52), 374(47), 345(50)
30–32	GA_{20}	2512	418(100), 403(14), 359(17), 375(49), 301(23), 207(113)
17–19	GA_{29}	2667	506(100), 491(13), 447(10), 375(11), 303(48)
17–19[b];20–24	di-OH GA_9	2625	506(15), 491(12), 475(8), 474(4), 447(6), 446(3), 433(10), 419(3), 416(9), 401(3), 384(6), 357(76), 343(43), 328(11), 311(11), 267(39), 250(10), 241(8), 223(10), 179(100), 135(39)

[a]*MeTMS derivatives.*
[b]*Most in these fractions.*

be an artifact generated from GA_1 during sample processing (Gaskin and others 1995). The latter possibility, however, is unlikely in our samples, because no epimerization of the [^{2}H]-GA_1 internal standard was found. In addition to the 13-hydroxylation pathway, the existence of another route of GA biosynthesis is possible given the presence of the unidentified dihydroxy GA_9-like compound.

The finding that GA_3 was abundant among those GAs surveyed is unusual in higher plants but not unique (Abdala and others 1995; Rood and others 1987). Its accumulation in the buds of stem tips in particular would reflect the relative rates of its synthesis, catabolism, conjugation, and movement into and out of the stem tips. None of these are known for mango; however, some evidence suggests that GA_3 might accumulate, because it is more slowly metabolized than its counterparts such as GA_1 (Goldschmidt and Galili 1981).

The previously described evidence of (1) buds remaining in rest for several months before initiation of new shoots, (2) GA_3 delays bud break in a concentration-dependent manner, and (3) earlier than normal bud break of inflorescences after triazole plant growth retardant treatment supports the hypothesis that GA_3 in buds or leaves or both is reduced with the age of stems and, while at elevated levels in young stems, contributes directly to the delay in resumption of growth between flushes. The evidence presented here not only does not support this hypothesis, it demonstrates trends of increasing levels of GA_3 in buds over time. The mean GA_3 levels were highest in buds from 7-month-old tips (Figure 2).

The concentration of GA_3 was relatively high in buds from resting stem tips compared with leaves (Figure 2). The GA_3 may have been imported into the stem tips from subtending leaves, because GA_3 was found in low concentrations in the leaves (Figure 2), or from roots as implied from the results of $GA_{1/3}$-like biologic activity found in xylem sap vacuum extruded from mango shoots (Chen 1987). Quantification was made difficult in buds from stem tips by the variable bulk of stem tissue that was likely included with the buds when they were excised from the tips. If expressed on a per stem tip basis, the highest levels during bud break and early shoot development were 3- to 10-fold greater than at any other time (Table 2). Despite the variability, there is no evidence that the levels of GA_3 substantially lowered with increasing age.

Endogenous GA_3 alone, thus, does not appear to directly inhibit shoot initiation. It could, however, participate indirectly when GA_3 is exogenously applied by affecting the auxin/cytokinin ratio.

Exogenously applied gibberellins have been reported to intensify apical dominance in conifers (Pharis and others 1972; Ross and others 1983) and other plants (Jacobs and Case 1965; Scott and others 1967). Such elevated levels could either enhance the impact of existing levels of auxin, as has been suggested by Jacobs and co-workers (Jacobs and Case 1965; Scott and others 1967), or possibly increase auxin biosynthesis in leaves (Law 1987; Law and Hamilton 1989). It is possible that similar effects, exhibited by delayed bud break in mango, are mediated through similar auxin synthesis or sensitivity mechanisms (Phillips 1969).

The proposed normal attrition of auxin with age of the resting terminal intercalary unit is possibly influenced by the apparent increase in GA_3 with stem age, which may amplify the impact of auxin

and delay initiation of bud break until the auxin levels are further reduced. Thus, even if the auxin levels decrease with age, thus lowering the auxin/cytokinin ratio, the impact of this loss would be lessened by the increasing levels of endogenous GA_3 with increasing age of the stems. Evidence to support this hypothesis is provided by the consistent observations of early bud break stimulated by triazole plant growth retardants (Sergent and others 1996; Tongumpai and Chantakulchan 1996). Maintenance of apical dominance by endogenous GA_3 has been demonstrated in other species (Brooks 1964; Pharis and others 1965; Ruddat and Pharis 1966) and, thus, parallels the initiation responses to GA_3 and plant growth retardants observed in mango.

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