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Gibberellin A_3 levels in bark and flower buds of peach *(Prunus persica* L.) before leaf fall

María Dolores Soriano and Rubén Bottini

SUMMARY

The endogenous gibberellins of cambial tissue were studied in comparisson with those of flower buds in one year limbs of peach *(Prunus persica* L.) trees cv. Novedad de Córdoba. Sampling was done approximately one month before leaf fall. Gibberellin A_3 was the only gibberellin characterized by gas chromatography-mass spectrometry (full spectrum) in cambial tissue from both, free acid ethyl acetate soluble extract (free gibberellins), and free acid ethyl acetate after enzymatic hydrolisis of the water soluble extract (glucosyl conjugated gibberellins). The quantitations, by gas chromatography-selected ion monitoring using deutero gibberellin A_3 as internal standard, were respectively of 803 and 52 ng g-1. dry weight of tissue for the two different extracts. No gibberellin could be detected in the flower bud extracts.

Key words: peach - floral buds - bark - gibberellins.

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RESUMEN

Se estudió en forma comparativa los niveles endógenos de giberelinas en tejido cambial y yemas florales de ramas de un año de duraznero *(Prunus persica* L.) cv. Novedad de Córdoba. El muestreo se realizó aproximadamente un mes antes de la caída del follaje. La única giberelina caracterizada por cromatografía de gases-espectrometría de masas (espectro completo) tanto en la fracción ácida soluble en acetato de etilo (giberelinas libres) como en la fracción ácida soluble en acetato de etilo luego de hidrólisis enzimática de la fracción soluble en agua (glucosil conjugados de giberelinas), fue A_3. Mediante cromatografía de gases-monitoreo selectivo de iones usando giberelina A_3 deuterada como estándar interno, se cuantificó respectivamente 803 y 52 ng g-1. de peso seco de tejido para los dos extractos. No se pudo detectar ninguna giberelina en los extractos de yemas florales.

Palabras clave: duraznero - yemas florales - corteza - giberelinas.

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INTRODUCTION

In the genus *Prunus* the gibberellins (GAs) A_1, (Bottini *et al.*, 1985; Luna *et al.*, 1990), A_3 (Luna *et al.*, 1990), A_5, (Yamaguchi *et al.*, 1975; Bottini *et al.*, 1985), A_6 (de Bottini *et al.*, 1987), A_8 (Luna *et al.*, 1990), A_{29} (Reed and Martin, 1976; Bottini *et al.*, 1985), and A_{32} (Yamaguchi *et al.*, 1970 and 1975, Coombe, 1971; Bukovac *et al.*, 1979; Bottini *et al.*, 1985), have been characterized by gas chromatography-mass spectrometry selected ion monitoring (GC-MS-SIM).

However, different organs and at different ontogenetic stages have different GAs in variable levels (see the references above cited, and Luna *et al*, 1992). Working with peach *(Prunus Persica* L.) flower buds, we reported in a previous paper (Luna *et al.*, 1990) the characterization of the GAs A_1, A_3, and A_8 as endogenous of this tissue. The levels of those GAs determined by using GC-SIM with stable isotope-labeled internal standards varied sustancially along the dormancy period (Luna *et al.*, 1992), and the maximum amounts of the 3 GAs were found one month before the bud development has been activated.

In regard of the later, we proposed a possible role for those GAs in some developmental events of the flower bud (mainly the fertile verticils) which could allow it to sprout (Luna *et al.*, 1991 and 1992). However, no clues are at hand to assest about the origin of the GAs, whether if they are a metabolic product of the bud itself or if they come from adjacent tissues like phloem. On the other hand, cambial tissue of peach (when both, xylem exudates and phloem extracts were analyzed) showed capable to metabolize $^3HGA_5$ (Bottini R., G.Abdala, M.Koshioka, D.Pearce, R.P.Pharis, unpublished results), although no GA has been identified in this tissue up to now.

Thus, the aim of this paper was to study the endogenous GAs of cambial tissue in comparisson with those of flower buds, in a time in which shoot elongation has stopped but green leaves were still onto the tree as receptors for environmental signals.

MATERIAL AND METHODS

Plant material

One year limbs of peach *(Prunus persica* L.) trees cv. Novedad de Córdoba (10 years old) were collected in an orchard at Río Cuarto, Argentina. The date of collection was April 9, 1990, aproximately one month before the leaf fall, when the shoot elongation has stopped, no more fruits were beared by the limbs, and leaves were begining to senesce. From the collected limbs, flower buds an bark scrappings were inmediately separated, weighed, frozen in liquid nitrogen, freeze dried, and kept at –40° C until processing.

GA procedure

From our experince of working with *Prunus* tissues, special care has to be taken to assure the adequate cleaning-up of the extracts, in order to get mass spectra of reasonable quality. Otherwise, good GC-MS (or even SIM) characterization becomes impossible (Baraldi *et al.*, 1990). In doing so, we adopted for both, flower buds and bark scrappings, the procedure outlined in Figure 1, and described as follows.

One g dry weight of material was grounded in a mortar with liquid nitrogen, and extracted overnight with methanol (MeOH) 80 % aqueous, plus 3.3 10^3 Bq of high specific activity (1.1 10^{11} Bq mM^{-1}) of 1,2 ^3H GA_5, as internal standard in order to check losses during the procedure of purification. After filtration on Wathman N° 1 paper, the residue was re-extracted twice with extra MeOH, and then discarded.

The MeOH was evaporated under vacuo at 35°C, and the remaining water freezed, thawed, and centrifuged for 10 min at 2,500 rpm.

The supernatant was adjusted to pH 7.0, and shaked 30 min with polyvinylpolypirrolidone (20 g L^{-1}, centrifuged 10 min at 2,500 rpm, and the pH adjusted to 2.5. The extract was then partitioned three times with equal volume of ethyl acetate (EtOAc) saturated with 1 % acetic acid (AcOH), and two different fractions were collected: EtOAc1, and aqueous 1.

Free-acid EtOAc soluble GAs

The fraction EtOAc 1 was partitioned three times against half volume of 0.1 M $NaHCO_3$. The $NaHCO_3$ fraction was acidified to pH 2.5, partitioned 3 times with half volume of 1 % AcOH saturated EtOAc, then discarded. The EtOAc fraction obtained (EtOAc 2) was pooled with EtOAc 1.

After evaporation, the extract was re-taken with MeOH 80 % aqueous, passed through a small (1x4 cm) column of reverse phase (RP) C18 (octadecyl silane) at a flow rate of 2-4 mL^{-1}, and evaporated again.

The residue was re-dissolved with a small amount of 10 % MeOH in 1 % AcOH, filtered through a Millipore 0.5 uM filter, and injected onto a RP C18 (u-Bondapack, Waters Associates) high performance liquid chromatography (HPLC) column. The following elution program was performed with a KONIK 500 apparatus at a flow rate of 2 mL min^{-1}: 10 % MeOH in 1 % AcOH for 10 min, 10 to 73 % MeOH in 1 % AcOH from 10 to 40 min, 73 % MeOH in 1 % MeOH from 40 to 50 min, then 100% MeOH from 50 to 60 min. Thirty fractions of 2 min (4 mL) each were collected, and bioassayed.

To locate and approximately quantitate endogenous GA-like substances the dwarf rice *(Oryza sativa* cv. Tan-ginbozu) microdrop (0.5 uL) assay

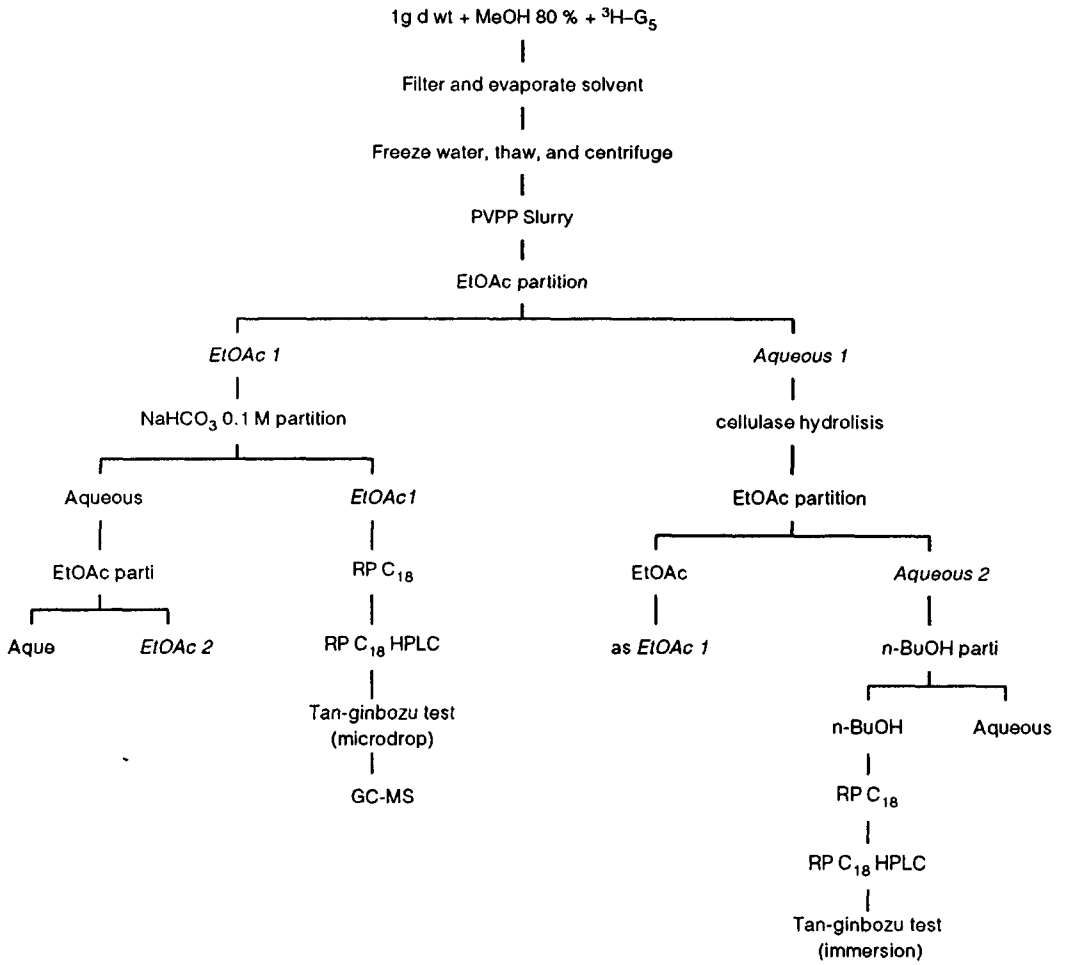

Figure 1. Schematic diagram of the technique used for GA procedure.

(Murakami, 1968) was used in serial dilution (1/50, 1/200) on each fraction from the HPLC. After 48 h the second leaf sheath growth was measured, and the results expressed as ng of GA_3 equivalents per g dry weight of tissue.

Samples showing bioactivity were pooled ac cording HPLC fraction zones, and $^2HGA_1$ and $^2HGA_3$ (100 ng of each GA) were added. Then they were converted to the MeTMSi derivatives with ethereal diazometane and 1:1 pyridine:BST-FA (bis -trimethylsilyl-trifluoroacetamide) plus 1% trimethylchlorosylane (Pierce Chemical Co). After dissolved in hexane, the samples were injected directly onto a Durabond DB-1-15N (15 m lenght x 0.25 um internal diameter) capillary column (J.and W. Scientific Inc) fitted in a Hewlett Packard Series II GC with a capillary direct interphase to a 5970B Mass Selective Detector. The GC tempe rature program was 60° to 195°C at 15°C min^{-1}, then 5° C min^{-1} to 275° C.

Carrier gas (He) flow rate was 1 mL min^{-1}, the interphase temperature was 280° C, and data adquisition was controlled by a HP 300 Series computer. For full spectra the mass range was 50 to 750 atomic mass units (amu), scan rate 0.66 Hz; for SIM (3 characteristic ions for the deutero isotope, 3 for the endogenous compound), scan rate 1.34 Hz, dwell time 25 m/sec ion^{-1}. A mixture of n-alkanes was co-injected with the sample to allow later calculations of the Kovats retention index (KRI; Kovats, 1958). By comparison of the peak area for the M^+ of endogenous GA versus the M^+ of the correspondent ^2H GA, the amount of free acid GA was calculated.

Free-acid EtOAc soluble GAs after enzymatic hydrolisis of glucosyl-conjugates

Fraction aqueous 1 from above (see Figure 1) was submitted to enzymatyc hydrolisis in order to

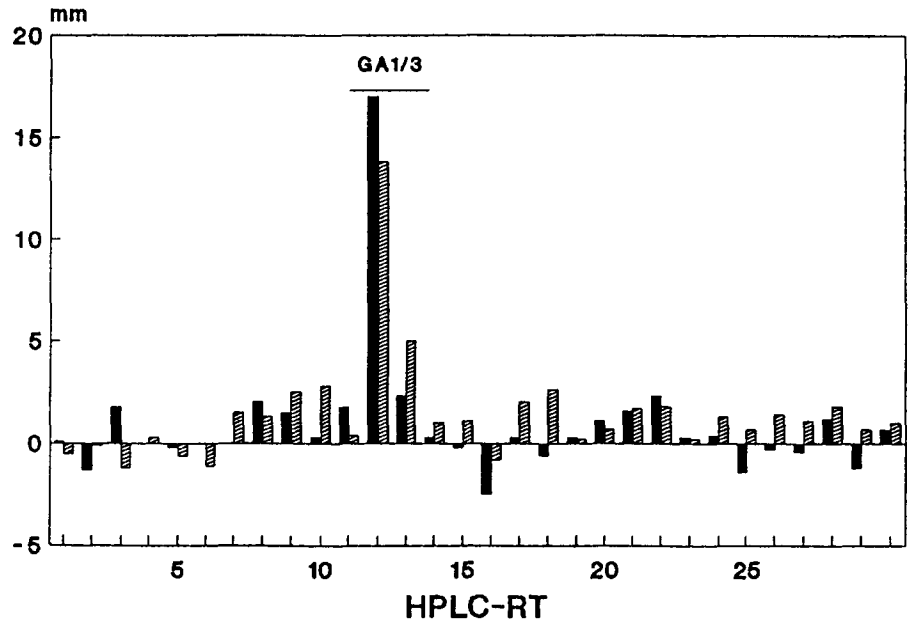

Figure 2. Elution profile from C_{18} HPLC fraction of EtOAc soluble free-acid GA-like extracts of peach bark, bioassayed by the dwarf rice *(Oryza sativa* L.) cv Tan-ginbozu microdrop test. Ordinate, growth of the rice leaf sheath in mm, abscissa, thirty 2 min (4 ml) HPLC fractions

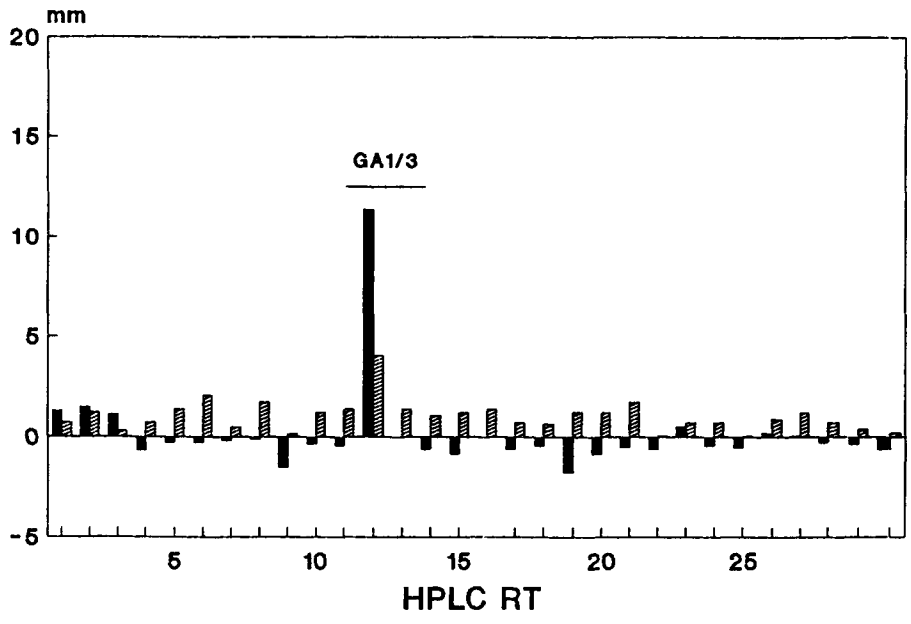

Figure 3. Elution profile from C_{18} HPLC fraction of EtOAc soluble after enzymatic hydrolisis of glucosyl-conjugated GA-like extracts of peach bark, bioassayed by the dwarf rice *(Oryza sativa* L.) cv. Tan-ginbozu microdrop test. Ordinate, growth of the rice leaf sheath in mm; abscissa, thirty 2 min (4 ml) HPLC fractions.

obtain the free GA moieties (Rood *et al.*, 1983). The residue was dissolved in 0.4 mL of 0.2 M acetate buffer (pH 3.0), and 0 4 mL of a 1 % cellulase (Sigma Chemical Co) solution was added. The mixture was incubated at 37°C for 16 h, centrifuged, and then partitioned against 1 % AcOH saturated EtOAc. The dried EtOAc soluble residue was then processed exactly as the EtOAc 1 fraction, according it has been described above (see Figure 1), and finally the amount of endogenous free-acid GA liberated by enzymatic hydrolisis of glucosyl conjugated forms was estimated.

Glucosyl-conjugated GAs.

The aqueous, namely aqueous 2 (see Figure 1), was processed as follows Aqueous 2 was partitioned three times with half volume of 1 % AcOH saturated n-BuOH After solvent evaporation, the n-BuOH fraction was re-taken in MeOH 80 % aqueous, passed through a RP C18 column as noted above, submitted to RP C18 HPLC, and the 20 fractions collected bioassayed (1/5 and 1/20 dilutions) by the dwarf rice test in the inmersion variant (Murakami, 1973). The purported activity would correspond to glucosyl ethers and/or glucosyl esters of endogenous GAs.

RESULTS AND DISCUSSION

Figures 2 and 3 show the bioactivity (expressed as growth of the second leaf sheath of the dwarf rice cv. Tan-ginbozu seedlings) of the different HPLC fractions from extracts of bark scrappings of peach. The bioactivity found (1/50 and 1/200 dilutions) of both, free-acid EtOAc soluble GAs and free-acid EtOAc soluble GAs after hydrolisis (Figures 2 and 3, respectively) concentrated in the HPLC-Rt zone in which authentic ^3H $GA_{1/3}$ run. The amounts of endogenous $GA_{1/3}$-like substances were estimated in 850 ng g^{-1}dry weight of tissue for the free acid GAs, and 50 ng g^{-1} for the GA liberated by enzymatic hydrolysis. No significant peaks of bioactivity measured by the dwarf rice cv. Tan-ginbozu (inmersion version) were found for glucosyl conjugated extracts of bark scrappings (data not showed). In flower buds, significative GA bioactivity was not found for either, free-acid EtOAc soluble GAs, or free-acid EtOAc soluble GAs after hydrolisis, or glu cosyl conjugates (data not showed).

When the bioactive fraction from figures 2 and 3 were submitted to GC-MS analysis, only GA_3 was characterized by full spectrum (Figure 4) from both, free-acid EtOAc soluble extracts after hydro-

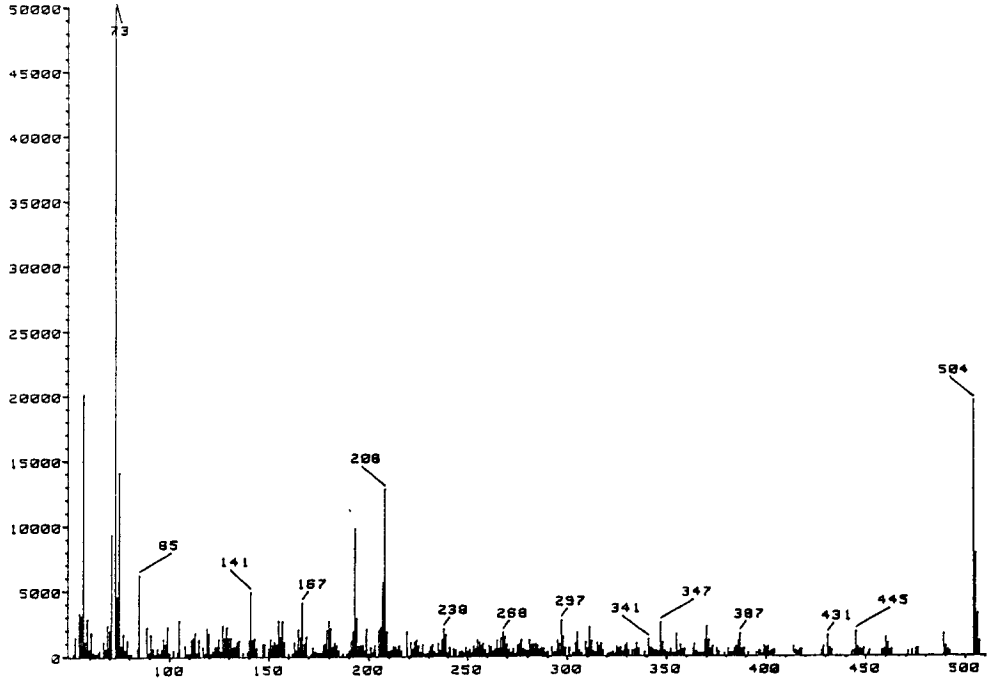

Figure 4. Full spectrum of the compound found at KRI = 2,691 on GC (co-chromatographing with authentic [^2H] – GA_3), after injecting the bioactive C_{18} HPLC fractions 12 and 13 from free-acid EtOAc soluble extracts of peach bark Ordinate, absolute intensity; abscissa, ion mass.

lisis. After GC-SIM quantitation by comparisson of the M + 504 (the parent ion of $^1HGA_3$ area at a KR1 = 2,691 with the M+506 (the parent ion of ^2H GA_3) area at the same KRI, the amount of endogenus free-acid EtOAc soluble GA_3 was estimated in 802 ng g^{-1} dry weight of tissue. By the same procedure, the amount of GA_3 liberated by enzimatic hydrolisis was estimated in 52 ng g^{-1} dry weight of tissue. This quantitations with the aid of stable-isotope-labeled internal standard, correlated fairly well with the estimations by bioassay.

From the results presented, GA_3 was the only GA characterized from bark tissue of peach trees one month before leaf fall. Quite surprinsingly, the amount found largerly exceeded those found in flower buds during dormancy (Luna *et al.*, 1992), wich were of 12.5, 10, and 2,5 ng. g-1 dry weight of tissue respectively for GA_8, GA_3, and GA_1. Most of the GA_3 was present in the free form.

Of course, no correlation can be established with the fact that no GA was detected in flower buds at the same time.

It comes clear from our results, that the states of dormancy of flower buds is not a consequence of lack of promoters which are not coming from another part of the plant, since the amount of GA_3 in adjacent tissues is so high; and from previous results (Luna *et al*, 1990 and 1991) is possible to assest that the buds were truly dormant.

The fact that no GA_3 was found in flower buds says that some kind of impediment is blocking GA flow towards the bud. This correlates fairly well with our former findings (Luna *et al.*,1990), which showed that mature vascular elements are noticeable in buds just a few weeks before blooming.

Perhaps, the overall phenomena at that time implies a source/sink relationship which determines an influx of free as well as glucosyl conjugated GAs either, from the aerial part (leaves) to the roots, or from leaves to the bark.

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