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Fluctuation of Endogenous Levels of Free and Conjugated Gibberellins throughout Seed Maturation in *Leucaena leucocephala* (Lmk) De Wit

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Combined gas chromatography-selected ion monitoring (GC-SIM) analysis of a purified extract from seeds of *Leucaena leucocephala* showed the presence of GA_1, GA_8, GA_{17}, GA_{19}, GA_{20}, GA_{23}, GA_{29} and GA_{53}. GA_1, GA_8 and GA_{29} were also found both as glucosyl ester-like and glucosyl ether-like conjugates, and GA_{20} as a glucosyl ester-like conjugate; these conjugates were analyzed as free GAs after enzyme hydrolysis. GA_{23} was shown to be the main free gibberellin in immature seeds (268 ng/seed), though its level drastically decreased during their maturation. GA_1 was the main free C_{19}-gibberellin and GA_1 glucosyl ester-like and glucosyl ether-like conjugates were found at the highest levels in the seeds prior to maturation. Fluctuation of endogenous levels of gibberellins is discussed in terms of seed development.

Key words: GC-SIM — Gibberellin — Leguminosae — *Leucaena leucocephala* — Seed — Tropical plant.

Immature seeds of Leguminosae are known to contain large amounts of GAs (Bearder 1980), the components and quantities varying with the physiological stage (Yamane et al. 1977). GA sugar conjugate formation during seed maturation has been observed in *Phaseolus vulgaris* (Yamane et al. 1975, 1977) and *Phaseolus coccineus* (Sembdner et al. 1972). Endogenous GAs from ten legumes have been found (Bearder 1980), and quantitative and qualitative changes of free and conjugated GAs during seed maturation have been reported for *Phaseolus multiflorus* and *Phaseolus vulgaris* (Durley et al. 1971, Yamane et al. 1977) and *Pisum sativum* (Frydman et al. 1974). But Leguminosae growing in the tropical area has not been adequately studied yet.

Our previous communication (Arigayo et al. 1983) reported the identification of eight endogenous free GAs in immature seeds of *Leucaena leucocephala* grown in Indonesia. Among them, GA_{23} was the most abundant, suggesting that it might be the characteristic endogenous GA of this plant. These results led us to investigate the quantitative changes of free and conjugated

Abbreviations: AB fraction, butanol soluble-acidic fraction; AE fraction, ethyl acetate soluble-acidic fraction; GA(s), gibberellin(s); GC-SIM, combined gas chromatography-selected ion monitoring; GPC, gel permeation liquid chromatography; HPLC, high performance liquid chromatography; Me/TMS, methyl ester trimethylsilyl ether; TMS/TMS, trimethylsilyl ester trimethylsilyl ether.

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GAs during seed maturation. Seeds of six ages were analyzed by GC-SIM after solvent fractionation and chromatographic purification (Yamaguchi et al. 1982). We report herein the identification of free and conjugated GAs in seeds of *L. leucocephala* (family Leguminosae, subfamily Mimosoideae, tribe Mimoseae) and the fluctuation of their levels during seed maturation.

Materials and Methods

Plant materials—Seeds for analysis of GAs were collected in June 1983 from five trees of *Leucaena leucocephala* (Lmk) De Wit var. K71/K20 in Bandung, Indonesia. Pods were harvested and seeds were divided into several groups by their weights. Six groups with mean seed weights of 9.5, 21.0, 35.0, 55.0, 77.0 and 92.5 mg were subjected to analysis at 33, 37, 40, 43, 49 and 56 days after anthesis, respectively, estimated by the growth curve shown in Fig. 1.

Extraction and solvent fractionation—Each group of seeds (50 g fr wt) was homogenized and extracted three times in 80% aqueous methanol with a Waring blender. After removal of the methanol by evaporation under reduced pressure at 35°C, half of the aqueous concentrate was fractionated as shown in Fig. 2.

Enzymatic hydrolysis—The NB and AB fractions were dissolved in sodium acetate buffer (0.2 M, pH 4.0) and crude hesperidinase (Tanabe Seiyaku Co.) was added; the ratio of the reaction mixture was 1 mg extract : 1 mg enzyme : 1 ml buffer (Yokota et al. 1971, Sakamoto et al. 1975). The mixture was shaken in an incubator at 40°C. Preliminary experiments were conducted to find the optimal hydrolysis times for the different GA conjugates. The authentic GA_4 glucosyl ester was incubated with the enzyme for 5, 24 and 48 h, and the yield of free GA_4, determined by GC, was optimal at 24 h. The hydrolysis time for the AB fraction was examined using the AB fraction of the matured seeds of *L. leucocephala*. The extract was hydrolyzed by the enzyme, and the yield of free GAs liberated was determined by GC-SIM after 5, 24 and 48 h incubation. The results showed the optimum time for the GA_8 conjugate to be 5 h, but that for GA_1 and GA_{29} conjugates to be 48 h. After washing the hydrolyzate with ethyl acetate at pH 8, the aqueous solution was acidified to pH 3 and extracted three times with ethyl acetate to obtain the AE fraction.

Purification of the AE fraction by GPC—The AE fraction (equivalent to 5 g fr wt of the seeds) was dissolved in tetrahydrofuran (0.5%, w/v) and subjected (0.5 ml/injection) to GPC on a Shodex GPC A-801 column (8×500 mm) with a precolumn of Shodex GPC A-800P (8×50 mm). The columns were eluted with tetrahydrofuran special grade for HPLC at a flow rate of 1.0 ml/min. The effluent was monitored by an RI detector (Shodex SE-11). The effluent of the retention time between 12.4 and 16.4 min was collected to give a fraction containing GAs according to the procedure of Yamaguchi et al. (1982).

Separation by HPLC—The GA fraction obtained by GPC purification was dissolved in 0.5 ml methanol and subjected to HPLC on a column of Nucleosil $5N(Me)_2$ (6×100 mm). The column was eluted with methanol containing 0.05% acetic acid at a flow rate of 2.0 ml/min. The effluent was monitored with a UV detector at 210 nm and separated into four fractions as shown in Table 1 (Yamaguchi et al. 1982).

GC-SIM analysis—The fractions I, II and III from HPLC were analyzed as TMS/TMS derivatives prepared by treatment of TMS reagent [pyridine : *N,O-bis*(trimethylsilyl) acetamide : trimethylchlorosilane (10 : 5 : 1, v/v/v)]. Fraction IV containing C_{20} aldehyde GAs was analyzed as Me/TMS (Fujisawa et al. 1985) after esterification with ethereal diazomethane followed by treatment with the TMS reagent. GC-SIM analysis was conducted using a Hitachi RMU-80A mass spectrometer equipped with an M-003 data processing system under the following conditions: glass column (3×1,000 mm) packed with 2% OV-1 on Chromosorb WAW DCMS (80–100 mesh); oven temperature between 195 and 220°C (isothermal); carrier gas of He

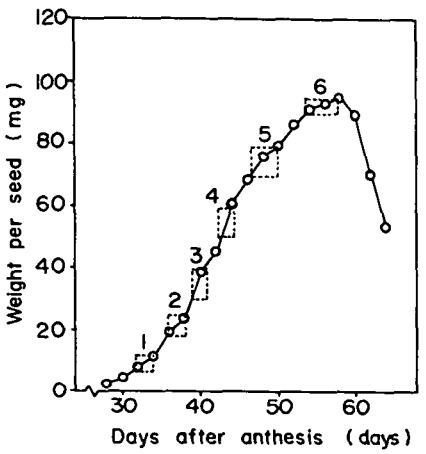


Fig. 1 Changes in fresh weight of *L. leucocephala* seeds during maturation. Pods were marked at anthesis and harvested at 19 different ages from anthesis to maturation for 62 days, and the seeds were weighed. ⬚: weight ranges of the seeds used. Numbers indicate the estimated seed age: 1, day 33; 2, day 37; 3, day 40; 4, day 43; 5, day 49; 6, day 56.

at a flow rate of 50 ml/min; ionization voltage of 22 eV. For the quantitative analysis, calibration curves were made for the peak area of the appropriate monitored ion using a known amount of corresponding authentic GA derivatives.

Results

The growth curve of *Leucaena leucocephala* seeds is shown in Fig. 1. Seeds were harvested simultaneously and grouped into six ages by their weights. Each seed extract was subjected to solvent fractionation as shown in Fig. 2 to give the AE, NB and AB fractions.

Free GAs—The AE fraction was purified successively by GPC and HPLC, giving four fractions. A portion of each fraction was subjected to GC-SIM as TMS/TMS or Me/TMS derivatives.

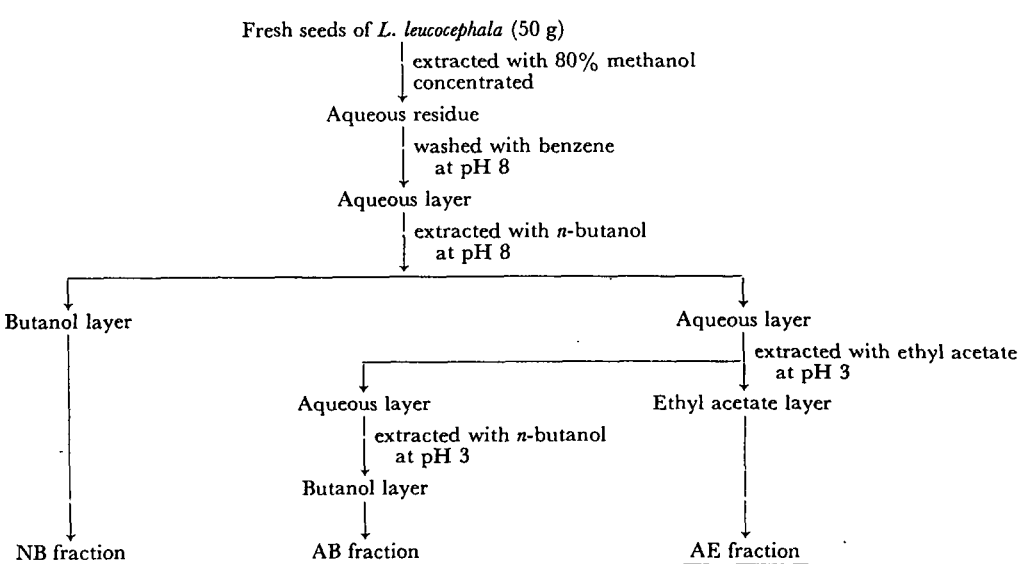


Fig. 2 General procedure of extraction and fractionation of free and conjugated GAs from *L. leucocephala* seeds at various growth stages.

The retention time and intensity ratio of the monitored ion peaks were compared with those of authentic GA derivatives. Eight free GAs, GA_1, GA_8, GA_{17}, GA_{19}, GA_{20}, GA_{23}, GA_{29} and GA_{53}, were identified as shown in Table 1. Their structures are shown in Fig. 7. Internal standards cannot be used to quantify GAs, but semiquantitative analyses can be useful for studying the fluctuations of endogenous GA levels.

Fig. 3 shows the fluctuations in endogenous GA levels for C_{20}-GAs and Fig. 4 those for C_{19}-GAs. GA_{23} was the most abundant of the endogenous GAs in *L. leucocephala*. It was present in a low level in the youngest seeds, but reached its maximum level on day 37 (268 ng/seed), and then drastically decreased to a very low level at maturity. As for the other C_{20}-GAs, GA_{17} reached maximum at day 43 (19 ng/seed), GA_{19} at day 37 (18 ng/seed) and GA_{53} at day 40 (47 ng/seed). The major GA among C_{19}-GAs was GA_1 which showed the maximum level at day 43 (83 ng/seed). GA_8 and GA_{20} also had maxima at day 43 (53 and 21 ng/seed, respectively)

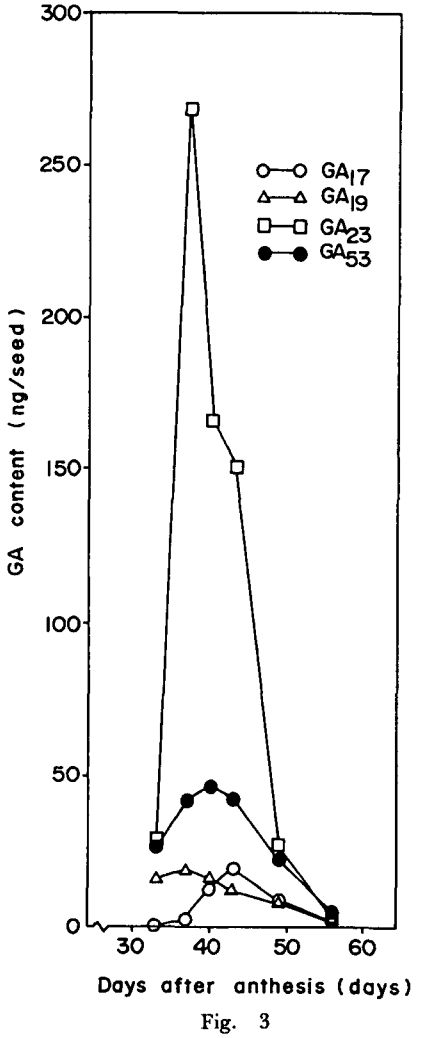


Fig. 3

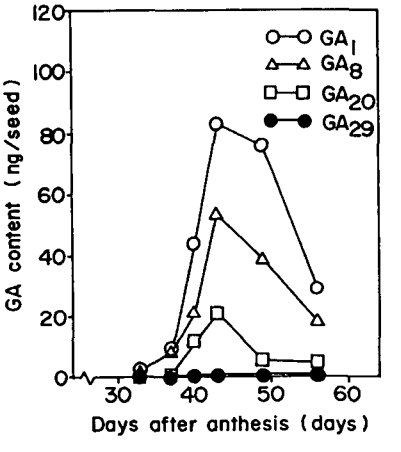


Fig. 4

Fig. 3 Fluctuation of endogenous levels of free C_{20}-GAs throughout maturation of *L. leucocephala* seeds.

Fig. 4 Fluctuation of endogenous levels of free C_{19}-GAs throughout maturation of *L. leucocephala* seeds.

Table 1 GC-SIM data obtained with samples from *L. leucocephala* seeds

Fraction number[a]	t_R of HPLC,[b] min	Monitored ions, m/z (Relative intensity)				t_R of GC,[c] min (Oven temperature, °C)	Identified GAs[d]
I	4.0–12.0	564 (40)	549 (28)	474 (30)	447 (100)[e]	3.7 (205)	GA_{53}
II	12.0–22.0	564 (100)	549 (27)	474 (10)	447 (65)	5.0 (210)	GA_1
		652 (100)	637 (13)	535 (51)		5.7 (220)	GA_8
		666 (66)	651 (96)	576 (100)		5.7 (210)	GA_{17}
		476 (100)	461 (25)	433 (30)	359 (78)	5.4 (195)	GA_{20}
III	22.0–24.0	564 (100)	549 (23)	474 (5)	447 (54)	5.0 (210)	GA_{29}
IV	24.0–38.0	462 (10)	447 (7)	434 (100)		4.8 (200)	GA_{19}
		550 (18)	535 (11)	522 (100)	519 (12)	7.5 (200)	GA_{23}

[a] Fractions from HPLC on Nucleosil 5N(Me)$_2$ column.
[b] Retention time of HPLC.
[c] Retention time of gas chromatography in the analysis of GC-SIM.
[d] Obtained relative intensities and retention times of GC showed good coincidence with those of the corresponding authentic derivatives of GAs.
[e] Intensities of the underlined ions were used for quantification of GAs.

and decreased with further maturation. The level of GA_{29} was very low throughout seed maturation with the maximum at day 56 (0.6 ng/seed).

Conjugated GAs—The NB and AB fractions from the extracts at each seed age were hydrolyzed by hesperidinase, a crude enzyme mixture produced by *Aspergillus niger* (Sakamoto et al. 1975).

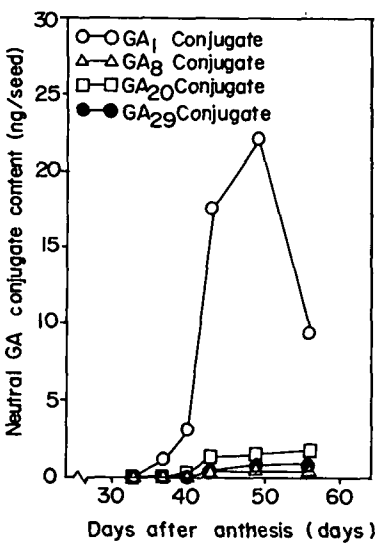


Fig. 5

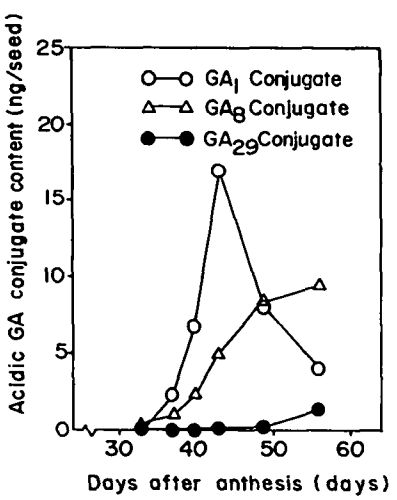
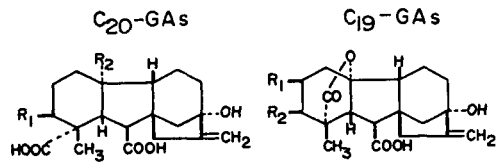


Fig. 6

Fig. 5 Fluctuation of endogenous levels of GA glucosyl ester-like conjugates throughout maturation of *L. leucocephala* seeds.

Fig. 6 Fluctuation of endogenous levels of GA glucosyl ether-like conjugates throughout maturation of *L. leucocephala* seeds.



GA_{17} : R_1=H , R_2=COOH
GA_{19} : R_1=H , R_2=CHO
GA_{23} : R_1=OH , R_2=CHO
GA_{53} : R_1=H , R_2=CH

GA_1 : R_1=H , R_2=OH
GA_8 : R_1=OH, R_2=OH
GA_{20} : R_1=H , R_2=H
GA_{29} : R_1=OH , R_2=H

Fig. 7 Structures of free GAs found in *L. leucocephala* seeds.

The AE fractions obtained from the enzymatic hydrolyzate were purified successively by GPC and HPLC and analyzed by GC-SIM as in case of free GAs.

The NB fractions gave GA_1, GA_8, GA_{20} and GA_{29} as hydrolysis products of their respective glucosyl ester-like conjugates.

Three GAs, GA_1, GA_8 and GA_{29}, were identified from the purified hydrolyzate of the AB fractions. They can be regarded as having been derived by the hydrolysis from GA_1, GA_8 and GA_{29} glucosyl ether-like conjugates, judging from their properties on fractionation and enzymatic treatment using hesperidinase. The acidic and neutral GA conjugates are hereafter referred to as the GA glucosyl ester and the GA glucosyl ether.

The contents of conjugated GAs in developing seeds were estimated by GC-SIM, and the fluctuations of their levels are shown in Fig. 5 for GA glucosyl esters and in Fig. 6 for GA glucosyl ethers. GA_8 and GA_{29} glucosyl ethers increased during maturation of the seeds and reached maximum at the last stage of maturation (9.5 and 1.4 ng/seed, respectively), whereas GA_1 glucosyl ether showed maximum levels at day 43, prior to maturation (17 ng/seed). GA_1 glucosyl ester is the main conjugated GA in this plant with the peak level at day 49 (22 ng/seed). The other glucosyl ester of GA_8, GA_{20} and GA_{29} each increased during maturation and reached its maximum amount at the last stage of maturation (0.6, 1.9 and 1.0 ng/seed, respectively).

Discussion

Analyses of endogenous GA levels during maturation of *L. leucocephala* seeds indicate that GA_{23} is a characteristic endogenous GA in this plant. As shown in Fig. 3, the maximum level of GA_{23} (12.8 mg/kg fr wt) appeared on day 37, in an early stage of seed development. GA_{23} has been isolated from immature seeds of *Wisteria floribunda* (Koshimizu et al. 1972) and from immature fruits of *Lupinus luteus* (Fukui et al. 1972) at 1 and 7 mg/kg fr wt, respectively. *L. leucocephala* is the third known leguminous plant which contains GA_{23} as a main GA.

The level of GA_{23} decreased drastically during seed development and the content of GA_1, the main C_{19}-GA in this plant, increased until the middle stage of seed maturation as shown in Fig. 4. This suggests that GA_1 is a main active GA which may be transformed from GA_{23} in this plant. Accumulation of GA_{23} at a young stage of seed maturation as C_{20}-GA suggests that 3β-hydroxylation at an early step of GA biosynthesis is the main pathway in the seed. Immature seeds of *W. floribunda* and *Lupinus luteus* contain GA_{18} (Koshimizu et al. 1972, Fukui et al. 1972), which could be a precursor of GA_{23}. However, GA_{18} could not be detected at any stage of seed development of *L. leucocephala*. This may be due to rapid metabolism of GA_{18} to GA_{23} in the seeds. The presence of GA_{19} and GA_{20}, non-3-hydroxylated GAs in *L. leucocephala*, suggests that the seeds have another biosynthetic route without 3β-hydroxylation as a minor pathway.

The main conjugated GAs in this plant were GA_1 glucosyl ester and ether which showed maximum levels at almost the same age as that of free GA_1, prior to the maturation. On the other hand, GA_8 glucosyl ether increased towards the last stage of maturation. GA_1 was shown to be metabolized to GA_8 glucosyl ether, probably by the inactivation process in *Phaseolus vulgaris* (Yamane et al. 1975). The difference in fluctuation patterns between GA_1 and GA_8 glucosyl ethers may be interpreted as indicating that glucosylation of the two GAs have different physiological functions.

An *L. leucocephala* tree growing in a tropical area, where the day length and temperature vary little throughout the year, has flowers, immature seeds and matured seeds at the same time, while the same tree growing in a temperate zone shows rather definite growth stages depending on the seasonal changes. We showed that even under the tropical conditions, the endogenous level of GAs in seeds of this plant fluctuate during maturation, corresponding to those observed in seeds grown in the temperate zone. This suggests that the fluctuation pattern may be characteristic of growth and development of seeds and may not be related to the seasonal changes.

Chemotaxonomic considerations with respect to endogenous GAs in Leguminosae seeds have been reported by Sponsel et al. (1979). They proposed that there are two groups with to 3β-hydroxylation of GAs: the tribe Phaseoleae (*Phaseolus vulgaris, Phaseolus coccineus* and *Vigna unguiculata*) contains 3β-hydroxylated GAs and conjugated GAs, while the tribe Vicieae (*Pisum sativum* and *Vicia faba*) contains non-3-hydroxylated GAs and scarcely any GA conjugates. In this sense, *L. leucocephala* may belong to the Phaseoleae type containing 3β-hydroxylated GAs and GA conjugates. However, *L. leucocephala* seeds differed from the Phaseoleae type as they accumulated GA_{23} at the early stage of maturation. This resembles the characteristics of *Wisteria floribunda* and *Lupinus luteus*, though they belong to different tribes; *L. leucocephala* to the tribe Mimoseae, *Wisteria floribunda* to Galegae, and *Lupinus luteus* to Lupineae (Heywood 1971).

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