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Gibberellins in Leaves of *Alstroemeria hybrida:* Identification and Quantification in Relation to Leaf Age

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Abstract. In alstroemeria (*Alstroemeria hybrida*), leaf senescence is retarded effectively by the application of gibberellins (GAs). To study the role of endogenous GAs in leaf senescence, the GA content was analyzed by combined gas chromatography and mass spectrometry. Five 13-hydroxy GAs (GA_{19}, GA_{20}, GA_1, GA_8, and GA_{29}) and three non-13-hydroxy GAs (GA_9 and GA_4) were identified in leaf extracts by comparing Kováts retention indices (KRIs) and full scan mass sprectra with those of reference GAs. In addition, GA_{15}, GA_{44}, GA_{24}, and GA_{34} were tentatively identified by comparing selected ion monitoring results and KRIs with those of reference GAs. A number of GAs were detected in conjugated form as well. Concentrations of GAs in alstroemeria changed with the development of leaves. The proportion of biologically active GA_1 and GA_4 decreased with progressive senescence and the fraction of conjugated GAs increased.

Key Words. Alstroemeria—Chlorophyll—GC-MS—Gibberellins—Leaf age—Senescence

Chlorophyll degradation is the most striking physiological change occurring during leaf senescence and is influenced by various external and internal factors. With respect to growth regulators, the role of cytokinins in this process has been emphasized. However, in a number of species senescence is delayed more effectively by gibberellins (GAs). Whyte and Luckwill (1966) first described the delayed senescence of dark-incubated leaf discs of *Rumex* by gibberellins. Also, *Taraxacum* (Fletcher and Osborne 1966), *Tropaeolum* (Beevers and Guernsey 1967), *Lactuca* (Aharoni and Richmond 1978), and *Lilium* (Han 1995) showed a delayed senescence when GAs were applied. In alstroemeria, leaf senescence is delayed very effectively by the application of GAs, whereas cytokinins exhibited less and auxins and polyamines no effect (Jordi et al. 1995).

It is accepted widely that GAs play a crucial role in the control of internode elongation in plants (e.g. reviewed by Graebe 1987). Other functions include stimulation of seed germination, control of flower development, and fruit growth. In most species studied to date (maize, pea, rice, spinach) the major or only pathway in shoot tissues is the early 13-hydroxylation pathway yielding GA_1. However, these studies were limited to seedlings in which stem elongation is the major feature studied. Only a few reports, however, describe GA patterns in mature and older plant parts. Kurogochi et al. (1979) reported the presence of GA_1, GA_4, and GA_{19} in rice and determined the concentrations of GA_{19}, the most abundant GA, throughout its life cycle. Endo et al. (1989) reported the presence of a number of 13-hydroxylated GAs in *Phaseolus vulgaris* shoots during their development. In tulip it was suggested that the major GA pathway is the non-13-hydroxylation pathway as GA_4, GA_9, GA_{12}, GA_{24}, and GA_{34} were identified in different plant parts (Rebers et al. 1994).

Young leaves are thought to be major sites for GA synthesis (Sponsel 1995, Zeevaart and Gage 1993), whereas senescing leaves contain a high concentration of conjugated GAs as determined by bioassay procedures (Aharoni and Richmond 1978, Zheng and Zhou 1995). Recently, Choi et al. (1995) reported high levels of GA_1,

Abbreviatons: GA(s), gibberellin(s); GC-MS, gas chromatography-mass spectrometry; FW, fresh weight; QAE, quaternary ammonium ether; HPLC, high performance liquid chromatography; BSTFA, *N,O*-bistrimethylsilyltrifluoroacetic acid; TCMS, trimethylchlorosilane; MeTMSi, metyl trimethylsilyl derivative; SIM, selected ion monitoring; KRI, Kováts retention index.

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GA_{19}, and GA_{53} in young leaves of rice compared with older leaves and suggested GA synthesis in the young leaves.

To our knowledge, only limited information is available discussing the role of endogenous GAs in the regulation of leaf senescence using modern analytic techniques such as gas chromatography-mass spectrometry (GC-MS). Because leaf senescence in alstroemeria is retarded very effectively by applied GAs, endogenous GAs are most likely involved in the regulation of leaf senescence in this species. To obtain insight in this regulatory role it is essential to clarify the nature of GAs endogenous in alstroemeria leaves and determine their concentrations during development. The present study revealed 11 GAs endogenous to alstroemeria leaves and reported the amounts of GAs found in three stages of leaf development. The results provide a basis for further studies on the role and mode of action of GAs in the process of leaf senescence.

Materials and Methods

Chemicals

GA_1, GA_4, GA_8, GA_9, GA_{12}, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{34}, GA_{44}, [17-$^2H_2$]GA_1, [17-$^2H_2$]GA_4, [17-$^2H_2$]GA_8, [17-$^2H_2$]GA_9, [17-$^2H_2$]GA_{19}, and [17-$^2H_2$]GA_{20} were obtained from Dr. L. N. Mander (Australian National University, Canberra). [2,3-$^3H_2$]GA_9 was from Dr. A. Crozier, Glasgow, UK. [1,2,3-$^3H_3$]GA_{20} was from Dr. J. MacMillan, Bristol, UK, and [1,2-$^3H_2$]GA_1 and [1,2-$^3H_2$]GA_4 were from Amersham (Buckinghamshire, UK). The specific activity of [^{3}H]GAs was about 50,000 Ci mol^{-1}. Petroleum ether 40–60 was obtained from Lamers and Pleuger ('s Hertogenbosch, The Netherlands).

Plant Material and Experimental Conditions

Alstroemeria hybrida cv. Westland plants were grown at a commerical nursery. The five uppermost leaves, positioned in a whorl directly below the flowers, were used for the experiments. Flower stalks were cut at approximately 60 cm from the top of the inflorescence. Three developmental stages were defined: (1) young leaves 5–7 cm long and flower buds ± 0.5 cm; (2) full grown mature leaves 10–12 cm long with the flower still closed but petals of the first flower bud already colored red; (3) senescent yellow leaves with the flowers open. To induce a homologous leaf senescence, stems were placed in demineralized water and kept in the dark for 8 days at 20°C and 70% relative humidity. Leaves were collected, weighed, plunged into liquid N_2, and stored at −80°C until extraction. A subsample of 100 mg was taken to analyze chlorophyll (*a* + *b*) content by extraction in *N*,*N*-dimethylformamide and measuring the absorbance at 664.5 and 647 nm according to Inskeep and Bloom (1985).

Extraction and Prepurification

The purification method was adapted from Rebers et al. (1994). All glass material was presilanized with dichloromethylsilane and rinsed thoroughly with demineralized water before use to reduce the loss of GAs caused by aspecific absorption. Frozen samples (about 50–100 g FW) were ground to powder with pestle and mortar under liquid N_2. Ice-cold methanol [MeOH:tissue = 4:1 (v/w)] containing 0.01% (w/v) ascorbic acid was added, and the homogenate was stirred for 4 h at 4°C. To quantify endogenous amounts of GAs, [17-$^2H_2$]GAs GA_1, GA_4, GA_8, GA_9, GA_{19}, and GA_{20}, 100 ng of each was added at the start of the extraction period. After centrifugation (10 min, 4°C, 5,000 rpm) the residue was reextracted with 80% methanol overnight at 4°C. Methanol and part of the plant water were removed under reduced pressure. The aqueous residue was frozen at −20°C. After thawing, the volume of the residue was adjusted to 60 mL with H_2O and adjusted to pH 7.5–8.0 with 1 N KOH.

In the first experiments, [^{3}H]GAs (about 50,000 dpm each) were added to estimate recoveries during purification. The sample was partitioned against petroleum ether 40–60 (three times with an equal volume) and the aqueous phase passed down a polyvinylpyrrolidone column (about 2 g, preequilibrated with H_2O at pH 8.0), which was eluted with 10 mL of H_2O at pH 8.0. The eluate was adjusted to pH 2.5 with 6 N HCl and partitioned against ethyl acetate (four times). The combined organic phases were partitioned against 5% (w/v) sodium bicarbonate (three times). The aqueous phases were acidified to pH 2.5 with 6 N HCl and partitioned against ethyl acetate (four times) and subsequently against diethyl ether (once). The organic phases were combined and reduced to dryness. The sample was dissolved in 10 mL of H_2O and adjusted to pH 8.0, for QAE-anion exchange chromatography.

Prepurification of Conjugated GAs

The aqueous phase remaining after (the first) ethyl acetate partitioning may contain conjugated GAs. This phase was repartitioned with H_2O-saturated *n*-butyl alcohol (three times). For quantification, [17-$^2H_2$]GAs GA_1, GA_4, GA_8, GA_9, GA_{19}, and GA_{20}, 100 ng each, were added. The combined butyl alcohol phases were reduced to dryness with small amounts of H_2O added to remove final traces of butyl alcohol. The residue was dissolved in 10 mL of 0.5 M sodium acetate at pH 4.8. Cellulase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) (Boehringer Mannheim; about 3 units of each) were added, and the sample was incubated for 24 h at 35°C. After hydrolysis, the pH was adjusted to 2.5, and the buffer phase was extracted with ethyl acetate (four times). The ethyl acetate phases were taken to dryness, and the residue was dissolved in 10 mL of H_2O and adjusted to pH 8.0 for QAE-anion exchange chromatography.

Anion Exchange Chromatography

The fractions resulting from the prepurification were each loaded on a column (10 cm × 1 cm, inner diameter) of QAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden), equilibrated with sodium formate [1% (w/v) at pH 8.0–8.5], and subsequently washed with H_2O (40 mL). GAs were eluted with 40 mL of 0.2 M formic acid and loaded directly on a preequilibrated C_{18} Sep-Pak cartridge (Waters, Millipore Corporation, Milford, MA USA). GAs were eluted with 10 mL of 80% methanol and evaporated to dryness.

GAs were purified further by reverse phase HPLC using a Chromspher 5 C_{18} column (Chrompack, Bergen op Zoom, The Netherlands; 250 × 10.0 mm). The column was eluted at a flow rate of 1 mL min^{-1} with 30% methanol for 15 min followed by a linear gradient to 75% methanol over 35 min, and subsequently to 90% methanol over 5 min (solvents contained 0.01% acetic acid). Samples were dissolved in 300 μL of methanol and made up to 1,000 μL with H_2O and injected into the column. Detection occurred with an UV absorbance monitor at 210 nm. For the qualitative analysis, 50 fractions of 1 mL were collected and taken to dryness under reduced pressure to remove all traces

of acetic acid. For quantitative analysis, fractions of 18–21 min (A), 23–26 min (B), 26–29 min (C), and 40–48 min (D) were collected.

Extracts were methylated with excess ethereal diazomethane. The methylated extracts were taken to dryness under a N_2 stream and redissoved in 25 μL of a fresh mixture of BSTFA:TMCS:pyridine [20:1:79 (v/v)] and heated for 20 min at 70°C to produce the trimethylsilyl ethers of the methyl esters (MeTMSi) for GC-MS.

Capillary Column GC-MS

Derivatized samples were analyzed using a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 mass selective detector (Hewlett-Packard Company, Wilmington, DE, USA). Aliquots of 3 μL were injected splitless into a fused silica capillary column (CPSil 5CB; Chrompack, 30 m × 0.25 mm × 0.4 μm) and separated by a temperature gradient: 0–2 min, 25°C min^{-1} to 250°C and 4°C min^{-1} to 280°C; 4-min hold. Helium was used as a carrier gas at 0.94 mL min^{-1}. The injector and interface temperature were 250 and 290°C, respectively. GC-MS with selected ion monitoring (GC-SIM) was used to search HPLC fractions for some GAs not detected by full scan MS. The system was set to monitor ions of *m/z* as follows: GA_{12}: 241, 269, 285, 300, 328, 360; GA_{15}: 239, 284, 312, 344; GA_{24}: 226, 286, 314, 342, 374; GA_{34}: 223, 288, 372, 416, 506; GA_{51}: 284, 328, 386, 418; GA_{53}: 207, 208, 251, 389, 448. Kováts retention indices (KRI) for standards and endogenous GAs were measured using coinjection of a mixture of *n*-alkanes (Gaskin et al. 1971).

Quantification of GAs was achieved using calibration curves constructed by mixing protonated and deuterated GAs in various amounts including corrections made for naturally occurring isotopes according to Hedden (1987) and Croker et al. (1994). The ion pairs monitored by GC-SIM were 506/508 (GA_1), 284/286 (GA_4), 594/596 (GA_8), 270/272 (GA_9), 434/436 (GA_{19}), and 418/420 (GA_{20}). GA_{29} was quantified against the [17-$^2H_2$]GA_8 standard in the same injection, so the values should be taken as approximate although valid for comparison. Corrections for different abundances of GA_{29}- and [17-$^2H_2$]GA_8 standards were estimated by coinjection of both standards. GA_{24} and GA_{15} (against [17-$^2H_2$]GA_9 standard) and GA_{34} and GA_{44} (against [17-$^2H_2$]GA_4 standard) were estimated in the same way.

Results

Identification of GAs in Mature Leaf Tissue

After fractionation of extracts by reverse phase HPLC, six 13-hydroxylated GAs and five non-13-hydroxylated GAs were identified (Table 1). Based on a comparison of full scan mass spectra and KRIs with those obtained for standard GAs, HPLC fraction A was found to contain GA_8; fraction B, GA_1; fraction C, GA_4, GA_{19}, GA_{20}, and GA_{44}; and fraction D, GA_9. For GA_{29} in fraction A no standard was available, and therefore we used reference spectra published by Gaskin and MacMillan (1991). GA_{34} in fraction C and GA_{15} and GA_{24} in fraction D were identified by comparing GC-SIM results and KRIs with standards. No evidence for the presence of GA_{12}, GA_{51}, or GA_{53} was found in any leaf extract as analyzed by both full scan and SIM methods.

The aqueous phase left after the first ethyl acetate partitioning and successive enzymatic hydrolysis was found to contain GA_8 and GA_{29} (see Table 3). In addition, trace of GA_1, GA_4, GA_9, and GA_{20} were found. When the aqueous phase was repartitioned against ethyl acetate instead of being hydrolyzed, no (free) GAs could be detected, indicating that measuring free GAs as spillover instead of conjugated GAs is not likely.

Variation in GA Content

Scintillation counting of samples after HPLC fractionation revealed that the recovery of individual GAs was approximately 60%. Conversion of [^{3}H]GA_1 and [^{3}H]GA_9 to their methyl esters yielded 98% efficiency as analyzed by HPLC. To estimate the variation in the purification and analysis method used, we separately purified and analyzed four samples originating from one batch of frozen leaves and determined concentrations of GA_8 and GA_9, representing a polar and an apolar GA. For both GAs the variation was less than 8% of the mean value obtained (data not shown).

Concentrations of identified GAs varied from 0.04 pmol (g FW)$^{-1}$ for GA_{34} to 18.80 pmol (g FW)$^{-1}$ for GA_8. Mature leaves, harvested at different periods in time, showed a considerable variation in GA concentrations (e.g. GA_9 varied from 0.54 to 3.57 pmol (g FW)$^{-1}$; Table 2). However, the amount of active GAs is significantly less compared with both precursor and inactivated GAs (Table 2).

Quantification of GAs in Three-Leaf Developmental Stages

To relate leaf development to endogenous GA content we determined GA concentrations in three stages of leaf development. Because the dry matter content of the leaves did not change during development (data not shown), GA concentrations can be expressed as pmol per FW. The time from stage 1 (young leaves) to stage 2 (mature leaves) was approximately 2 weeks. Total GA concentration increased strongly as the leaves became older, mainly due to increases in (total) GA_8 and GA_{29} (Table 3). Concentrations of GA_8, GA_{15}, GA_{19}, GA_{20}, GA_{24}, and GA_{29} increased during maturation. In contrast, concentrations of GA_1, GA_4, GA_9, GA_{34}, and GA_{44} decreased.

In darkness, it took 8 days for the leaves to become completely senescent (stage 3). Chlorophyll content was less than 20% of the amount of mature leaves (Table 3). During senescence GA_1, GA_4, GA_{15}, and GA_{19} concentrations decreased, whereas GA_8, GA_{29}, and GA_{34} concentrations increased.

After enzymatic hydrolysis of the aqueous fraction left after the first ethyl acetate partitioning, no GAs could be detected in extracts from young leaves. In similar ex-

Table 1. GC retention times, KRIs, and characteristic ions for identification based on full scan monitoring of Me and MeTMSi derivatives of GAs from alstroemeria leaf tissue.

Gibberellin	R_t (min)	KRI	Characteristic ions *m/z* and relative abundance (% base peak)
GA_1	13.7	2689	506 (M^+, 100), 491 (12), 448 (22), 376 (24)
GA_1 standard	13.7	2689	506 (M^+, 100), 491 (12), 448 (20), 376 (25)
GA_4	12.4	2522	418 (M^+, 27), 328 (43), 289 (54), 284 (100, 225 (63)
GA_4 standard	12.4	2522	418 (M^+, 29), 328 (35), 289 (48), 284 (100), 225 (65)
GA_8	15.0	2832	594 (M^+, 100), 579 (3), 535 (6), 448 (19), 379 (7)
GA_8 standard	15.1	2837	594 (M^+, 100), 579 (7), 535 (7), 448 (15), 379 (9)
GA_9	11.1	2319	330 (M^+, absent), 298 (100), 270 (59), 243 (51), 226 (53)
GA_9 standard	11.1	2319	330 (M^+, 9), 298 (100), 270 (61), 243 (46), 226 (51)
GA_{12}	N.D.		
GA_{12} standard	11.4	2359	360 (M^+, 2), 328 (30), 300 (100), 285 (19), 269 (7), 241 (25)
GA_{15}	13.2	2622	344 (M^+, 21), 312 (44), 284 (73), 239 (100)
GA_{15} standard	13.2	2624	344 (M^+, 25), 312 (27), 284 (71), 239 (100)
GA_{19}	13.1	2611	462 (M^+, 7), 434 (100), 402 (29), 374 (64), 345 (31)
GA_{19} standard	13.1	2610	462 (M^+, 7), 434 (100), 402 (31), 374 (60), 345 (22)
GA_{20}	12.3	2501	418 (M^+, 100), 403 (17), 390 (7), 375 (46), 359 (14)
GA_{20} standard	12.3	2501	418 (M^+, 100), 403 (15), 390 (3), 375 (52), 359 (15)
GA_{24}	12.0	2465	374 (M^+, 4), 342 (43), 314 (100), 286 (96), 226 (72)
GA_{24} standard	12.0	2465	374 (M^+, 4), 342 (31), 314 (100), 286 (96), 226 (97)
GA_{29}	13.7	2691	506 (M^+, 100), 491 (18), 465 (absent), 447 (5), 375 (27), 303 (24)
GA_{29} standard*	13.7	2691	506 (M^+, 100), 491 (14), 465 (2), 447 (7), 375 (17), 303 (20)
GA_{34}	13.6	2675	506 (M^+, 100), 416 (8), 372 (10), 288 (19), 223 (31)
GA_{34} standard	13.6	2675	506 (M^+, 100), 416 (8), 372 (10), 288 (21), 223 (39)
GA_{44}**	14.6	2788	432 (M^+, 61), 417 (7), 373 (9), 238 (47), 207 (100)
GA_{44} standard	14.6	2786	432 (M^+, 49), 417 (7), 373 (15), 238 (34), 207 (100)
GA_{51}	N.D.		
GA_{51} standard	12.5	2532	418 (M^+, 2), 386 (32), 328 (37), 284 (100)
GA_{53}	N.D.		
GA_{53} standard	12.4	2516	448 (M^+, 64), 389 (35), 251 (24), 208 (95), 207 (100)

Note. M^+, molecular ion; N.D., not detected in alstroemeria, i.e. less than 0.05 pmol $(g\ FW)^{-1}$. *, obtained by interpolation of data of Gaskin and MacMillan (1991). **, detected in young leaves only. Identification of $GA_{15,24,34}$ was conducted by GC-SIM, whereas other GAs were identified by full scan GC-MS.

Table 2. Seasonal variation in GA concentrations in pmol (g FW)$^{-1}$ in mature leaves of alstroemeria.

Time of harvest	Gibberellins [pmol (g FW)$^{-1}$]					No. of analysis
	GA_{20}	GA_9	GA_1	GA_4	GA_8	
September 1994	3.64	1.20	0.66	0.72	6.58	3
January 1995	7.56	0.54	N.D.	0.36	4.56	4
September 1995	3.63	2.05	0.74	0.79	7.58	2
October 1995	5.32	3.57	1.22	1.69	18.80	2

Note. N.D., not detected, i.e. less than 0.05 pmol (g FW)$^{-1}$.

Table 3. GA concentration in pmol (g FW)$^{-1}$ in leaves of alstroemeria at three developmental stages. The data are means of three samples ± S.E. Sampling was done in September.

Leaf stage	Young	Mature	Senescent
chlorophyll [mg (g FW)$^{-1}$]	0.77 ± 0.15	1.32 ± 0.09	0.24 ± 0.08
Total gibberellin [pmol (g FW)$^{-1}$]			
Non-13-OH GAs			
GA_{15}	0.36 ± 0.01	0.94 ± 0.05	N.D.[a]
GA_{24}	0.12 ± 0.01	0.49 ± 0.04	0.69 ± 0.16
GA_9	2.56 ± 0.11	1.20 ± 0.04	1.86 ± 0.45
GA_4	1.27 ± 0.03	0.72 ± 0.02	N.D.
GA_{34}	0.66 ± 0.03	0.04 ± 0.02	2.27 ± 0.23
13-OH GAs			
GA_{44}	3.96 ± 0.29	N.D.	N.D.
GA_{19}	1.02 ± 0.03	1.27 ± 0.07	0.55 ± 0.29
GA_{20}	N.D.	3.64 ± 0.19	4.82 ± 0.59
GA_1	0.83 ± 0.02	0.66 ± 0.05	0.20 ± 0.03
GA_8	1.15 ± 0.09	6.85 ± 1.47	15.23 ± 0.69
GA_{29}	N.D.	2.96 ± 0.13	12.11 ± 1.12
Conjugated gibberellin [pmol (g FW)$^{-1}$]			
GA_8	N.D.	1.22 ± 0.36	6.57 ± 0.45
GA_{29}	N.D.	0.15 ± 0.09	7.21 ± 0.88

[a]N.D., not detected, i.e. less than 0.05 pmol (g FW)$^{-1}$.

tracts from mature leaves 1.22 pmol (g FW)$^{-1}$ GA_8 and 0.15 pmol (g FW)$^{-1}$ GA_{29} were detected, making up 16% of total GA_8 and 5% of total GA_{29} analyzed in these extracts. Also, traces of GA_1, GA_4, GA_9, and GA_{20} could be demonstrated in these extracts. Senescent leaves contain traces of GA_1, GA_9, and GA_{20} and 6.57 pmol (g FW)$^{-1}$ GA_8 and 7.21 pmol (g FW)$^{-1}$ GA_{29} (43% and 59%, respectively, of total GA_8 and GA_{29}) in the hydrolyzed aqueous fraction.

Discussion

Surveys of GAs in mature tissues are rarely reported; most studies are restricted to GAs determined in seedlings or developing buds, all young and rapidly growing tissues. Rebers et al. (1994) analyzed endogenous GAs in sprouts and bulbscales of tulip during storage and subsequental growth. Kurogochi et al. (1979) determined the GA pattern in rice plants and found that GA_{19}, the most abundant GA, varied by a factor 15 throughout the growth season. However, growth and development could not be correlated with the GA_{19} pattern, and the authors concluded that seasonal variation seemed a general feature in rice plants. In alstroemeria, large variations in GA concentrations were found in leaves of comparable developmental stage harvested at different moments in time, suggesting that the history of the plants influenced GA concentrations. Although seasonal variation in GA content was not the emphasis of this study, it is clear that statements correlating absolute GA concentrations to distinctive processes (in this case senescence) based on a single time point analysis must thus be interpreted with care.

The presence of (free) GA_1, GA_4, GA_8, GA_9, GA_{19}, GA_{20}, GA_{29}, and GA_{44} in mature alstroemeria leaves was demonstrated by comparing full scan mass spectra and KRI with available standards. GA_{15}, GA_{24}, and GA_{34} were detected by GC-SIM. The GAs identified in alstroemeria leaf extracts suggest that two major GA pathways, the early non-13-hydroxylation pathway to GA_4 and the early 13-hydroxylation pathway to GA_1, exist in alstroemeria. Both pathways are known to be operative in vegetative tissues of many plants (Sponsel 1995). Comparable results have been obtained in a related plant genus *Lilium elegans*, where GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, GA_{34}, and GA_{51} were identified in addition to GA_{44}, GA_{19}, GA_{20}, GA_1, and 3-epi-GA_{34} (Takayama et al. 1993). No evidence was found for the presence of the early GA_{12} and GA_{53}. It is likely that these GAs are metabolized quickly and therefore present at concentrations below detection limits and require much larger scale extraction procedures. Neither GA_{51}, for which GA_9 is thought to be the precursor, nor the conjugated form of GA_{51} was found in any alstroemeria leaf extract.

We arranged the GAs detected in alstroemeria in three groups according to their supposed function in the metabolic pathway (Sponsel 1995). The ratio of precursor to active to inactivated GAs can be calculated from Table 3 and changed from 0.67:0.17:0.16 in young leaves to 0.21:0.005:0.78 in senescent leaves (Fig. 1). In young leaves of alstroemeria, the contribution of GAs assumed to be precursors was about 67% of the total GA pool, indicating that these leaves have the possibility to synthesize rapidly the active GA_1 and GA_4. In rice seedlings it was also found that concentrations of precursor type GAs were higher in younger leaves when compared with older leaves (Choi et al. 1995). Also in pea, cell-free extracts from young tissues have the highest activity for *ent*-kaurene synthesis (Coolbaugh 1985). It is generally accepted that young leaves are sites of GA biosynthesis (Sponsel 1995, Zeevaart and Gage 1993). In senescent leaves the concentrations of GA_9, GA_{20}, and GA_{24} increased, although when related to the total amount of

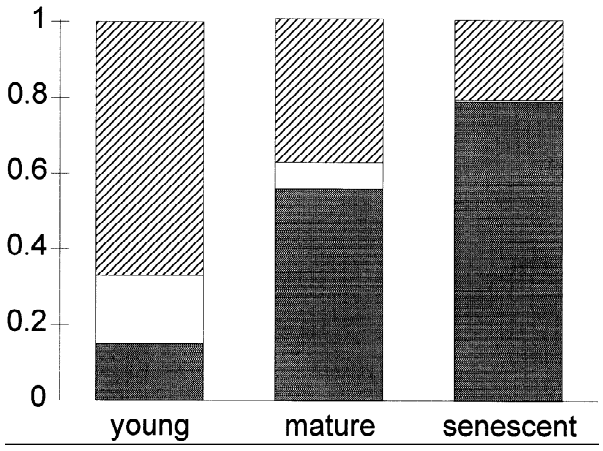

Fig. 1. Ratios of different classes of GAs in leaves of alstroemeria in three stages of development. Classification is as follows: precursor type gibberellins (*hatched*), GA_{15} + GA_{24} + GA_9 + GA_{44} + GA_{19} + GA_{20}; active gibberellins (*white*), GA_1 + GA_4; inactivated type gibberellins (*gray*), GA_8 + GA_{29} + GA_{34}. Data are calculated from Table 3.

GAs, their proportion decreased. This indicates that even in senescent leaves GA metabolism occurs. For alstroemeria we consider both GA_1 and GA_4 as biologically active GAs as both GAs were effective in delaying leaf senescence (Jordi et al. 1995). Although we cannot exclude that GA_4 has to be converted to GA_1 before showing activity (Graebe 1987, Sponsel 1995), GA_4 was 2–3 orders of magnitude more effective in retarding chlorophyll in alstroemeria leaves (Jordi et al. 1995). A number of other studies also strongly suggest that GA_4 has intrinsic biological activity in e.g. *Arabidopsis* (Zeevaart and Talon 1992), *Cucumis* (Nakayama et al. 1991), and *Tulipa* (Rebers et al. 1994).

The total GA content of the leaf increased with maturation and subsequent senescence. This seems to be in conflict with the finding that GA concentrations decline rather than increase in senescent leaves (Chin and Beevers 1970, Fletcher et al. 1969). However, in those studies bioassay procedures were used, and as a consequence only GAs active in the bioassay were determined, including some catabolites that show activity but excluding, for instance, inactive GA conjugates. In alstroemeria, concentrations of the biologically active free GA_1 and GA_4 declined after 8 days of darkness. This is in accordance with the study of Aharoni and Richmond (1978) which reported a decrease of GA_3-like substances in senescing lettuce leaves. During maturation and subsequent senescence of alstroemeria leaves the contribution of precursor type GAs decreased to 21% of the total amount. The contribution of free inactivated GAs increased to more than 78% in senescent leaves, which supports the view that these compounds are inactive forms of GAs. Previous studies using bioassay procedures have revealed the increase of GA-like substances in conjugated form during senescence of leaves of *Lactuca* (Aharoni and Richmond 1978) and *Rumex* (Zheng and Zhou 1995). Mature and senescent alstroemeria leaves were found to contain traces of a number of GAs in the hydrolyzed aqueous fraction. The amounts of GA_8 and GA_{29} analyzed in the aqueous fraction of mature and senescent leaves were substantial and suggest the occurrence of conjugated, glycosylated forms. However, in this study GA conjugates could not be characterized individually, and therefore the total amounts of free and originally conjugated GA_8 and GA_{29} were considered as degradation products of the 13-hydroxylation pathway. The function of GA conjugates has been suggested by Sembdner et al. (1994) to be (temporarily) inactive, storage or transport forms. The large increase of GA_8- and GA_{29}-conjugated GAs from trace amounts in young leaves to more than 25% of total GAs in senescent alstroemeria leaves suggests storage of inactive products and is apparently the result of coversion of free GAs to their conjugated forms. In senescent leaves, the conversion of precursors of GA_1 into GA_8 conjugate and GA_{29} conjugate via GA_8 and GA_{29} seems to be more active than in mature leaves. Although GA_8 and GA_{29} increased twofold and fourfold, respectively, during senescence, the concentration of GA_{34} increased 57-fold compared with mature leaves. Since GA_4 is also far more effective than GA_1 in delaying senescence of alstroemeria leaves (Jordi et al. 1995), the results suggest that non-13-hydroxylated GAs are involved in regulation of leaf senescence.

The present study established the identity of 11 GAs in alstroemeria leaves, which represent the occurrence of two major GA pathways in higher plants. The relative distribution of the different types of GA varied throughout the development of the leaves. A shift of so-called precursor type GAs to inactivated GAs was found during maturation and subsequent senescence of the leaves. In previous work using exogenous application of GAs it was suggested that endogenous GAs are important in the regulation of senescence of alstroemeria leaves (Jordi et al. 1995, Van Doorn and Van Lieburg 1993). This study demonstrated that in alstroemeria leaves, the pattern of endogenous GAs correlates with leaf developmental stage. The possible causal relation between endogenous GAs and leaf senescence will be the basis for future studies.

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