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STEROL COMPOSITION OF DWARF AND TALL *PISUM SATIVUM* SEEDLINGS IN RELATION TO GIBBERELLIC-ACID-ENHANCED SHOOT GROWTH

LYNNE WESTERMAN and JAMES G. RODDICK*

Department of Biological Sciences, University of Exeter, Exeter, EX4 4QG, U.K.

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Key Word Index—*Pisum sativum*; Leguminosae; pea; shoot growth; gibberellic acid; sterols; campesterol; stigmasterol; sitosterol.

Abstract—Gibberellic acid (GA_3) stimulated shoot elongation in both dwarf and tall cultivars of pea, but more so in the dwarf cultivar. The sterol composition of shoots of both cultivars was similar, with sitosterol being the most abundant compound, followed by stigmasterol and campesterol. Cholesterol could not be detected. Following GA_3 application, levels of free sterols in whole shoots increased whereas glycoside levels tended to fall. The magnitudes of the changes in both classes of sterol were similar in both cultivars. Analyses of stems and leaves separately revealed a greater growth response to GA_3 in the former but no effect of the hormone on the sterol composition of either organ. It is concluded that GA_3 enhancement of shoot growth in pea is not mediated through quantitative changes in cell sterols.

INTRODUCTION

The enhancement of stem growth in physiological or genetic dwarfs of a number of plant species (e.g. maize, pea, rice, peach) remains one of the most striking examples of the potent growth-regulatory properties of the gibberellins. This effect was first demonstrated by Brian *et al.* [1] in 1955 but despite considerable advances in our knowledge of gibberellins since then (see reviews [2, 3]), the underlying biochemical events are still not well understood. In pea shoots, it is known that gibberellic acid (GA_3) can stimulate RNA synthesis [4, 5] and α-amylase [6] and β-fructofuranosidase [7] activity, but the possibility that other metabolic events are affected or triggered which could be equally (if not more) important in the initiation of GA_3 action cannot be ruled out. For example, in barley aleurone it appears that GA_3 enhances not only the activity of the protein-synthesizing machinery, particularly the production and aggregation of ribosomes [8, 9] and enzyme synthesis [10–12], but also elaboration of certain membrane systems (e.g. the endoplasmic reticulum) [13, 14], whilst causing changes in the permeability of others [2, 15]. Furthermore, findings such as the stimulation of enzyme synthesis having a lag of 4–20 hr [2] but gibberellin-induced elongation being detectable within 10 min [16] have led to growing speculation that the primary action of gibberellins may involve changes to the properties and transport characteristics of certain cell membranes. Alterations to the fluidity and permeability of synthetic lipid membranes by GA_3 [17, 18], usually within a few minutes of treatment, must be viewed with some caution, but equally rapid effects on the permeability of plant cell membranes have also been demonstrated [19, 20]. In eukaryotic cells the fluidity and permeability of the lipid bilayer is very much influenced by the free sterol component [21, 22] and this, together with reports of gibberellins influencing sterol metabolism in some plant tissues [23, 24], raises the question of the involvement of sterols in gibberellin effects on membranes and development in plants.

In this study, we have attempted to determine whether any relationship exists between GA_3-enhanced stem elongation in dwarf pea seedlings and levels of sterols in seedling shoots. For comparison, experiments have also been conducted using a tall cultivar which shows a much reduced growth response to applied GA_3.

RESULTS

Preliminary analyses of 2-week-old shoots revealed that, in both cultivars, free sterols were the most abundant form and sitosterol the most abundant sterol, followed by stigmasterol and campesterol. According to GLC analysis, cholesterol appeared to be present but the large quantities observed gave rise to doubts and GC/MS failed to confirm the presence of this sterol. Glycosides were present in smaller quantities and their relative abundance was as for free sterols (i.e. sitosterol glycoside was present in largest amounts) but on a number of occasions, inexplicably, they could not be detected. Esters were usually not detectable but, on the few occasions that they were, quantities were very small. Attempts to identify acylated glycosides were not successful.

Application of 100 μg GA_3 to seedling leaves caused the widely observed differential enhancement of shoot growth. Elongation in the dwarf cultivar

*To whom requests for reprints should be sent.

(Meteor) increased by 280% (cf. the control) in a 7-day period compared with 110% in the tall cultivar (Alaska). Sterol analyses of whole shoots of GA_3-treated Meteor seedlings revealed significant increases in the levels of sitosterol and campesterol, but not stigmasterol (Fig. 1). The proportion of sterols was not markedly altered (Fig. 1) but the stigmasterol : sitosterol ratio fell from 0.57 (in controls) to 0.42 (in GA_3 treatments). In contrast, glycosides all showed large decreases (Fig. 1) although their sporadic appearance meant it was not possible to assess these changes statistically. In Alaska seedlings, a similar pattern of changes occurred in both free and glycosylated sterols following GA_3 application. All three free sterols increased significantly although relative amounts were unaffected (Fig. 2). The stigmasterol : sitosterol ratio (0.53) was similar to that of the dwarf cultivar but showed a smaller reduction (to 0.48) in GA_3 treatments. Once again, glycosides appeared sporadically but, where quantifiable, the trend following GA_3 application was decidedly downwards (Fig. 2).

Although applied to leaves, GA_3 exerts its most dramatic growth effects on the internodes. Since analyses of whole shoots could have masked any changes in the sterol composition of these organs, further experiments were carried out to examine growth and sterols in stems and leaves separately.

In both cultivars, the pattern of free sterols in both stem and leaf was essentially as found in whole shoots except that glycosides and esters could not be detected at all. GA_3 caused increases in shoot length similar to those observed previously (Table 1). As expected, stem weight increased in both cultivars, but

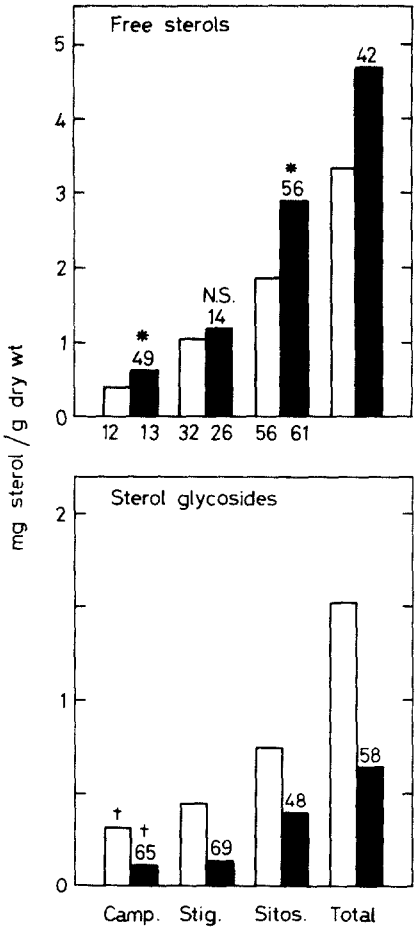

Fig. 1. Effect of GA_3 on the levels of free and glycosylated sterols in whole shoots of the dwarf pea cultivar, Meteor. Plants were treated with 100 μg GA_3 when 10 days old and harvested after a further 7 days. Open columns are controls; filled columns GA_3 treatments. Values at base of columns represent per cent sterol; those above filled colums are the per cent increase (free sterols) or decrease (glycosides) following GA_3 treatment. Significance: $*P = 0.05$; NS, not significant. †Sterols detectable in only one of three extractions. Camp.—campesterol, Stig.—stigmasterol, Sitos.—sitosterol.

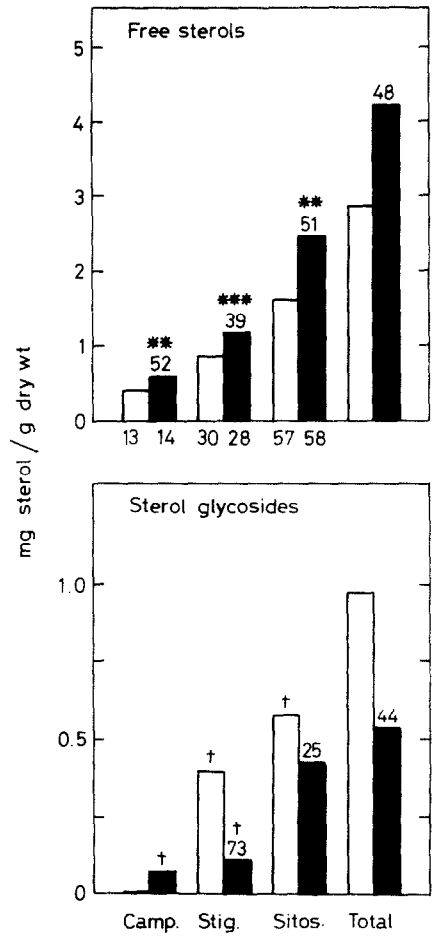

Fig. 2. Effect of GA_3 on the levels of free and glycosylated sterols in whole shoots of the tall pea cultivar, Alaska. Plants were treated with 100 μg GA_3 when 10 days old and harvested after a further 7 days. Open columns are controls; filled columns GA_3 treatments. Values at base of columns represent per cent sterol; those above filled columns are the per cent increase (free sterols) or decrease (glycosides) following GA_3 treatment. Significance: $**P = 0.01$; $***P = 0.001$. †Sterols detectable in only one of three extractions. Camp.—campesterol, Stig.—stigmasterol, Sitos.—sitosterol.

Table 1. Effect of GA_3 on growth of dwarf (Meteor) and tall (Alaska) pea cultivars

Cultivar	% increase in height	% increase in stem wt		% increase in leaf wt	
		Fr. wt	Dry wt	Fr. wt	Dry wt
Meteor	320	130	140	29	39
Alaska	150	38	50	3	13

10-Day-old plants were treated with 100 μg GA_3 and harvested after a further 7 days.

proportionately more so in the dwarf cultivar (Table 1), whereas leaf weight increased significantly only in the dwarf cultivar (Table 1). In the dwarf cultivar, free sterol levels (especially sitosterol) were much higher in stems than leaves (Table 2) whereas in the tall cultivar differences were less marked (Table 3). In both cultivars, absolute amounts of sterols were greater in stems and leaves (and particularly stems) of GA_3-treated plants (Tables 2 and 3). However, increases were of a similar magnitude to those in weight (Table 1) so that sterol concentrations were not significantly altered in either organ or cultivar by GA_3 treatment (Tables 2 and 3). Stigmasterol: sitosterol ratios were generally higher in leaves (0.50 in Meteor; 0.61 in Alaska) than stems (0.32 in Meteor; 0.28 in Alaska) but were little affected by GA_3 treatment (0.44 and 0.29 respectively in Meteor; 0.65 and 0.27 respectively in Alaska). Nor did the hormone have any marked effect on the relative amounts of sterols in stems and leaves of either cultivar (Tables 2 and 3).

DISCUSSION

Studies involving analyses of whole shoots suggest GA_3 to be capable of influencing sterol levels in both dwarf and tall pea cultivars. Although the glycoside data are less reliable, their downward trend following GA_3 treatment and the concomitant increase in free sterols could indicate some conversion from glycoside to free form. Wojciechowski *et al.* [25] found high turnover rates of sterol glycosides in *Sinapsis alba* seedlings with much of the newly synthesized glycoside being rapidly deglycosylated. Such a conversion could be associated with the synthesis of new membranes during cell growth, and stimulation of growth by GA_3 could therefore also accelerate this conversion. However, the absolute increases in free sitosterol are much greater than the decreases in its glycoside which may point to some direct effect of GA_3 on the biosynthesis of sitosterol. Of interest in this connection is the report by Shewry and Stobart [24] of GA_3 increasing sterol biosynthesis in germinating hazel seed.

This finding contrasts with a brief report by Grunwald [26] indicating no effect of GA_3 on the free sterols of whole shoots of pea seedlings. Differences in the age of seedlings (7 day cf. 10 day) and cultivars (Progress cf. Meteor) used are unlikely to explain this disparity since growth responses and levels and proportions of sterols were otherwise quite similar. However, the fact that (in our study) sterol changes were very similar in both cultivars while growth responses were markedly different suggests that enhancement of internode elongation by GA_3 does not result from quantitative changes in cell sterols. This conclusion is further supported by data obtained from separate stems and leaves. Thus, although our results differ somewhat from those of Grunwald [26], we concur with his opinion regarding the absence of a causal relationship between GA_3-induced elongation and sterol levels in pea. A similar conclusion has also been arrived at recently by Jusaitis *et al.* [27] who, working with stem segments of *Avena sativa*, did not observe any correlation between levels of free sterols and GA_3-enhanced elongation. Geuns [28] has implicated changes in the stigmasterol: sitosterol ratio with changes in membrane permeability and elongation of mung bean hypocotyl, but there is no evidence from our study of GA_3 exerting its effects on pea internodes in this manner.

The apparent absence of glycosides and of any effects of GA_3 on sterols in leaves and stems is, at first glance, difficult to reconcile with the results obtained from whole shoots. A possible explanation is that these compounds and effects may be located mainly in the shoot apex, which was not analysed in the second series of experiments. Geuns [29], in fact, has pointed out that the apex not only is a major site of sterol biosynthesis but makes a significant contribution to sterol levels and metabolism in the shoot as a whole.

Compounds such as polyene antibiotics [30] and saponins [31] can alter the permeability of membranes by chemically interacting with membrane sterols rather than by causing changes in their levels, and Wood and Paleg [17] have also demonstrated that the type of sterol can influence the subsequent effect of GA_3 on the permeability of liposomes. The possibility therefore that GA stimulation of extension growth might involve interactions with, or qualitative changes in, membrane sterols cannot yet be excluded. Also, the inability so far to establish any correlation between GA_3 enhancement of elongation and sterol composition should not be taken to imply that sterols are not involved in other developmental processes controlled or influenced by gibberellins.

EXPERIMENTAL

Plant material. Seeds of the dwarf pea cultivar Meteoi and of the tall cultivar Alaska were obtained locally and soaked for 3 hr in distilled H_2O prior to planting in soil-less compost in 7.5 cm plastic pots. Seedlings were raised in a glasshouse at 20°. Where necessary, extended Hg vapour illumination was supplied to supplement natural winter day-

Table 2. Effect of GA_3 on the free sterol composition of stems and leaves of the dwarf pea cultivator, Meteor

Organ	Sterol	Control			+GA_3			% change	
		Sterol/plant (μg)	Sterol concn (μg/g dry wt)	Sterol (%)	Sterol/plant (μg)	Sterol concn (μg/g dry wt)	Sterol (%)	Sterol/plant	Sterol concn
Stem	Campesterol	9	402	14	22	389	15	+144	−3
	Stigmasterol	12	592	21	23	480	19	+92	−19
	Sitosterol	37	1823	65	84	1676	66	+127	−8
	Total (4-demethyl)	58	2817	100	129	2545	100	+122	−10
Leaf	Campesterol	26	236	12	32	209	9	+23	−11
	Stigmasterol	62	558	29	93	640	28	+50	+15
	Sitosterol	126	1122	59	237	1463	63	+88	+30
	Total (4-demethyl)	214	1916	100	362	2312	100	+69	+21

10-Day-old plants were treated with 100 μg GA_3 and harvested after a further 7 days. Leaf consisted of the leaf laminae proper, petioles and stipules; stem consisted, of the remaining main axis but excluding the apical 10 mm.

Table 3. Effect of GA_3 on the free sterol composition of stems and leaves of the tall pea cultivar, Alaska

Organ	Sterol	Control			+GA_3			% change	
		Sterol/plant (μg)	Sterol concn (μg/g dry wt)	Sterol (%)	Sterol/plant (μg)	Sterol concn (μg/g dry wt)	Sterol (%)	Sterol/plant	Sterol concn
Stem	Campesterol	22	371	17	36	409	19	+64	+10
	Stigmasterol	24	401	18	33	384	18	+38	−4
	Sitosterol	85	1429	65	122	1416	64	+44	−0.9
	Total (4-demethyl)	131	2201	100	191	2209	100	+46	+0.4
Leaf	Campesterol	29	228	14	32	328	15	+10	+14
	Stigmasterol	70	668	33	73	715	33	+4	+7
	Sitosterol	110	1095	53	117	1106	52	+6	+1
	Total (4-demethyl)	209	2051	100	222	2149	100	+6	+5

10-Day-old plants were treated with 100 μg GA_3 and harvested after a further 7 days. Leaf consisted of the leaf laminae proper, petioles and stipules; stem consisted of the remaining main axis but excluding the apical 10 mm.

light and/or to ensure a 16 hr day. Plants were treated 10 days after sowing.

Treatment and harvest of plants. Prior to treatment, shoot height was measured from soil level to the point of attachment of the smallest stipules visible without dissection. Gibberellic acid (Sigma) was prepared as a 10 mg/ml soln in MeOH and a 10 μl aliquot applied to the adaxial surface of the central pinna of the first tripinnate leaf. Control plants were similarly treated with 10 μl MeOH and this had no adverse effects on the leaf or plant. After 7 days, plants were re-measured and harvested. For analyses of whole shoots, sufficient plants to give *ca* 10 g tissue were excised at soil level and accurately weighed; for analyses of leaves and stem, sufficient plants to give *ca* 10 g leaf and 10 g stem were excised as above and weighed. Leaves consisted of the leaf lamina proper, petioles and stipules; stems of the remaining main axis but minus the apical 10 mm. After fr. wt determination, tissues were immersed in liquid N_2 and then lyophilized to constant dry wt.

Extraction of sterols. The procedure used was adapted from refs. [32,33]. Freeze-dried tissue was crumbled into a Whatman parchment thimble which was then closed with muslin. The tissue was extracted for 16 hr with EtOH in a Soxhlet extractor. The EtOH extract was taken to dryness under vacuum at 40°. Flask contents were dissolved in 150 ml 33% EtOH in petrol (40–60°) and partitioned with 30 ml distilled H_2O. The organic fraction (containing free and esterified sterols) was reduced to a few ml and subjected to CC (see below) while the aq. fraction (containing sterol glycosides) was re-extracted with 10 ml petrol, concentrated to *ca* one-third under vacuum at 30° and refluxed in 2 M HCl for 3 hr. The hydrolysate was neutralized with 2 M NaOH and partitioned with Et_2O. The Et_2O extract was partitioned with half its vol. of 30% KOH in MeOH to remove pigments, washed 2× with distilled H_2O and reduced to a few ml under vacuum at 30°.

CC. The organic fraction (containing free sterols and steryl esters) was applied to a 10 mm diam. glass column packed with 10 g of neutral Al_2O_3 activity grade III and eluted with 100 ml vols of 1%, 6% and 20% Et_2O in petrol (40–60°) to yield 4-dimethyl steryl esters, free 4,4-dimethyl sterols and free 4-demethyl sterols respectively [33]. The 6% fraction was discarded and the 1% and 20% fractions evaporated to dryness at 30°. The 20% fraction was dissolved in a small vol. of Et_2O. The 1% fraction was refluxed in 6% KOH in 90% EtOH for 1.5 hr, neutralized with 2 M HCl and partitioned with Et_2O, the vol. of which was reduced under vacuum at 30° to a few ml.

TLC. Et_2O extracts of free, esterified and glycosylated sterols were applied as a band to separate 20 × 30 mm TLC plates (Si gel G, 0.25 mm) along with a marker spot containing authentic sitosterol, stigmasterol, campesterol and cholesterol. Chromatograms were developed in $CHCl_3$, dried, sprayed with 0.5% aq. Rhodamine 6G and viewed under UV light. Authentic sterols ran as a single band ($R_f \approx 0.5$) and the corresponding extract band was scraped off and eluted with Et_2O. Extracts were filtered through Whatman No. 1 paper, evaporated to dryness in an air stream and dissolved in 0.3 ml EtOH.

GLC. To each 0.3 ml of extract was added 0.1 ml of 0.4% 5α-cholestane in EtOH as int. standard. A 1 μl aliquot was injected into the gas chromatograph and sterols separated in a glass column (2.1 m × 3 mm i.d.) packed with 3% OV-101 (80–100 mesh) on Gas Chrom Q. The column was isothermal at 250° and the FID temp. 300°. The carrier gas was N_2 flowing at 40 ml/min. Peaks were identified by R_t values and by co-chromatography with authentic sterols. Where necessary, identities of sterols (as TMSi derivatives) were checked by GLC (as above, but using 3% OV-17 as column packing) and GC MS.

GC/MS. TMSi ethers of both extracts and authentic sterols were prepared by injecting *N,O*-bis-(trimethylsilyl)-acetamide (BSA) on to dried samples. Sealed mixtures were incubated at 50° for 1 hr and 1 μl aliquots withdrawn and injected into a GC interfaced with a single-focusing magnetic deflection MS with a 16-cm radius magnetic sector. The column was as above but at 240° and the carrier gas was He.

Quantitation of sterols. In quantitative (but not preliminary qualitative) analyses, extraction efficiency was determined by measuring recovery of 0.1 μCi of [4-^{14}C] cholesterol (sp. act. 149 μCi/mg) added to the initial extract. A 0.1 ml aliquot of the final extract was counted by scintillation spectrometry in 10 ml of dioxan-based scintillation fluid. Counting efficiency was *ca* 90% and background 28 cpm. Recovery was usually 40–50% and occasionally as high as 65%. Although strictly applicable only to free sterols, this correction was used with all classes of sterols measured. Sterols were quantified by referring peak areas (determined by weighing peak cut-outs on pre-calibrated paper) to the appropriate calibration graph prepared with authentic sterol. Graphs were replotted whenever the column was re-packed or a new syringe used.

Analyses of results. Between 10 and 30 plants were used to provide material for each extraction. Each experiment was carried out 3× and, except where otherwise indicated, data are means of the three runs. Data were statistically analysed using a *t*-test.

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