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24-EPIBRASSINOLIDE UPTAKE IN GROWING MAIZE ROOT SEGMENTS EVALUATED BY MULTIPLE-SELECTED ION MONITORING

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Abstract—The evaluation of the uptake of 24-epibrassinolide (BR) in growing maize root segments by means of multiple-selected ion monitoring showed that BR is accumulated into the fresh tissue independently of energy supply, while the experiments performed with frozen–thawed roots show a large adsorption to cell structures. The adsorption is freely reversible in frozen–thawed roots, whereas 30% of BR taken up in fresh roots is irreversibly bound

INTRODUCTION

(2*R*,3*S*,22*R*,23*R*,24*S*)-2,3,22,23-Tetrahydroxy-B-homo-7-oxa-5α-ergostan-6-one (brassinolide), a steroidal lactone isolated from the pollen of rape (*Brassica napus* L.) [1] is the first identified member of a unique class of naturally occurring steroids, brassino steroids, present in several plant materials [for recent review see 2]. Brassinolide and its synthetic 24-epimer brassino sterol (BR) [3], fed at μM concentrations, stimulate growth by cell enlargement in different tissues irrespective of their sensitivity to auxins, gibberellins or cytokinins [4–9] The stimulation of growth by BR is associated with an increase in the electrogenic H^+ extrusion, suggesting that its effect on cell enlargement depends at least in part on an increased cell wall plasticity consequent on the acidification of the wall space, in agreement with the 'acid growth' theory generally accepted for auxin [10].

The whole of the results so far obtained (lack of tissue specificity, additivity or synergism in some cases and opposite effects in some others with auxins, giberellins, cytokinins) [4–9, 11–14] indicates brassinolide and related compounds as a sixth group of phytohormones.

The understanding of the mechanism of action of the brassino steroids requires information about their mode of uptake by plant tissues, possible metabolism, and the existence of specific receptors. The more commonly used methods for this type of study require the use of radioactive compounds In order to avoid the use of radioactive compounds, in this work we investigated the uptake of BR in growing maize root segments by means of multiple-selected ion monitoring (MSIM) This method was applied by using as internal standard deuterated BR, the synthesis of which is first reported in this paper.

RESULTS

In preliminary experiments we incubated maize root segments with 2 μM BR in order to check its recovery after a treatment of 45 or 360 min Table 1 (Expts 1–3) shows that the sum of the BR contents of the tissue and of the medium at the end of the incubation corresponds to the initial BR content of the medium, thus indicating that BR is not substantially destroyed and/or metabolized during the incubation. Moreover the same recoveries are obtained at short (45 min) and long (360 min) times of incubation and with frozen-thawed roots (Table 1, Expt 4).

The time-course of BR uptake by fresh tissue (Fig. 1) shows that the uptake is high within the first 45 min and becomes saturated after 90 min. The reported values were not corrected for the amount of BR present in the free space in equilibrium with the external medium. This amount, however, accounts for no more than 10% of the uptake, even assuming that the free space is 20% of the

Table 1 Recovery of BR after different incubation times in fresh and in frozen-thawed roots*

Experiment no	Incubation (min)	nmol BR in the† medium (18 ml)	nmol BR in the† tissue (300 mg)
1	0	36.0 ± 0.79	0 ± 0.02
	45	34.9 ± 0.75	1.1 ± 0.03
	360	34.4 ± 0.76	1.5 ± 0.04
2	45	34.7 ± 0.76	1.2 ± 0.03
	360	34.4 ± 0.76	1.6 ± 0.04
3	45	34.8 ± 0.76	1.1 ± 0.03
	360	33.7 ± 0.74	1.4 ± 0.04
4	45	32.1 ± 0.70	3.9 ± 0.10
	360	31.8 ± 0.69	4.6 ± 0.12

*Root segments (*ca* 300 mg) were preincubated for 60 min in 0.3 mM $CaSO_4$ and then incubated in a medium (18 ml) containing 0.15 mM Na-MES (pH 6.2), 0.3 mM $CaSO_4$, 5 mM K_2SO_4 and 2 μM BR. Experiments 1,2,3: fresh roots and experiment 4: frozen-thawed roots.

†The values represent the mean ± s.e. ($n = 3$ for expt 1,2,3, $n = 5$ for expt 4)

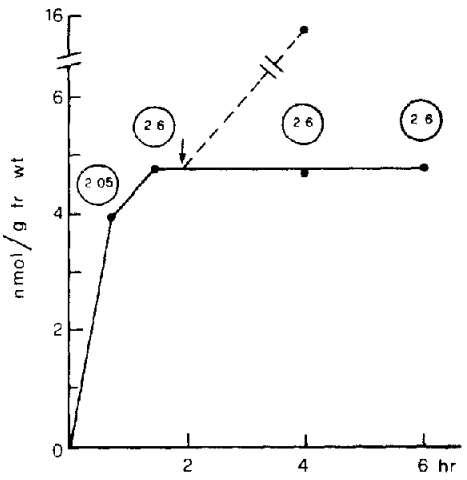


Fig 1 Time-course of 2 μM BR uptake in fresh roots For incubation conditions see Table 1 The figures in the circles are the values of the tissue concentration factor, that is the ratio between BR concentration in the tissue (1 g fr wt being taken as equal to 1 ml) and BR concentration in the medium at the end of the incubation At the arrow, some samples were rapidly frozen and thawed in the same medium and further incubated for 2 hr (dashed line), s e did not exceed $\pm 3\%$

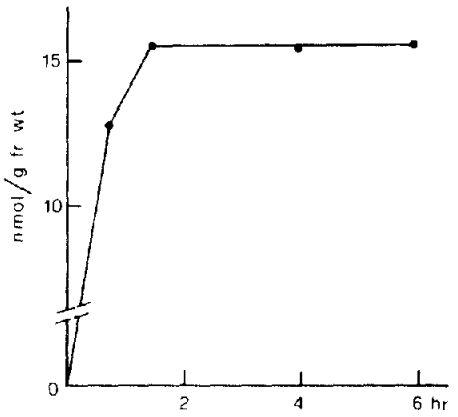


Fig 2 Time-course of 2 μM BR uptake in frozen-thawed roots For incubation conditions see Table 1, s e did not exceed $\pm 3\%$

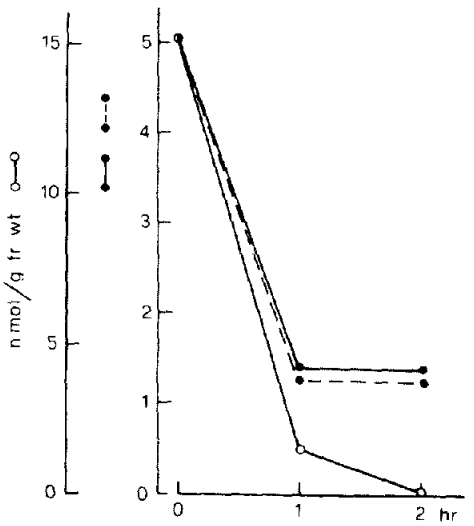


Fig 3 Release of BR by fresh and frozen-thawed roots The fresh or frozen-thawed roots, loaded with BR for 2 hr, were transferred (Zero time) into a medium of equal composition without BR The solution was renewed after the first hour of elution After loading, some samples of fresh roots were rapidly frozen and thawed in the elution medium before the release measurement. ●—— ● loading and release in fresh roots, ●- - -● loading in fresh roots and release in frozen-thawed roots, ○ —○ loading and release in frozen-thawed roots s e did not exceed $\pm 3\%$

tissue volume and that its content is unchanged by the rapid wash with distilled water at the end of the incubation. The values of the tissue concentration factor, reported in the circles, calculated assuming a homogeneous distribution of BR inside the tissue and the cells, show that the concentration of BR in the tissue is larger than that in the medium, showing that BR is accumulated into the tissue. The uptake of BR is unaffected by the presence of cycloheximide (CH), an inhibitor of protein synthesis and of carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation With BR uptake by the control of 4 5 $\pm$0 11 nmol/g fr wt, uptake in 70 μM CH (present during the 60 min preincubation in 0 3 mM $CaSO_4$), was 4.4 $\pm$0 10 and in 2 μM FCCP was 4.6$\pm$0 12 nmol/g fr. wt. The lack of effect of FCCP on the accumulation of BR indicates that this process is not energy dependent and suggests that it may depend on an unspecific adsorption to the cell structures

Possible adsorption of BR to cell structures was investigated by measuring its uptake by frozen-thawed tissue The time-course is quite similar to that in the fresh tissue (Fig 2), a rapid uptake being observed in the first 45 min and saturation being achieved after 90 min The amount of BR found in the root segments is one order of magnitude larger than that accounted for by the free diffusion, indicating a strong adsorption of BR to cell structures (possibly membranes) The adsorbed BR at each incubation time is about three times that taken up by the fresh tissue. Thus, more binding sites seem accessible to BR in the frozen-thawed tissue than in the fresh one, suggesting that in the fresh roots the penetration and/or the binding of BR are somehow limited This is confirmed by the finding that if the living roots are incubated for 2 hr with BR and then frozen-thawed in the same medium, the amount of BR in the tissue rapidly increases and reaches the values already obtained with the killed tissue (Fig 1, dashed line)

In order to investigate the degree of reversibility of BR uptake in both frozen-thawed and fresh tissue, the roots were transferred, after an incubation time of 2 hr with BR, into a similar BR-free incubation medium and were incubated for 2 hr, the medium being renewed after the first hour The results (Fig. 3) show that the adsorption of BR by frozen-thawed tissue is rapidly and completely reversible In fact, 90% of the adsorbed BR is released during the first hour and the further 10% during the

second hour. On the contrary, only 70% of BR taken up by the fresh tissue is released during the first hour and no further release is observed during the second hour Two possibilities can be envisaged to interpret this result: (i) the adsorption of BR to cell membranes in the fresh roots is at least in part due to irreversible binding, (ii) a fraction of BR taken up is penetrated and kept in the cells. The second possibility is ruled out by the observation that the amount of BR released by the fresh tissue is practically unaffected by a treatment of freezing–thawing in the elution medium before the release measurement (Fig 3).

DISCUSSION

In our previous papers [4, 5] we showed that BR promotes the elongation of maize root segments and increases the electrogenic proton extrusion. In order to investigate the mechanism of action of BR in maize root segments we have considered MSIM for the measurement of BR in experiments aimed to study the BR uptake. Thus we have established a simple synthesis of BR deuterated at position 5,7,7'(BR-d_3) used as internal standard and have determined BR levels after conversion into the corresponding bismethaneboronates. This method was suitable for the evaluation of BR content in plant tissues without the use of the radioactive compound and was sensitive and specific enough to permit the evaluation of the BR uptake at a concentration optimal for biological activity.

Quantitative evaluation of BR in uptake experiments shows that BR seems to be neither destroyed nor metabolized during the incubation, at least at the level of the nucleus moiety of the molecule, thus suggesting that the physiological responses of growth and H^+ extrusion are elicited by BR as such.

In the fresh roots BR is taken up with saturation kinetics and it is accumulated into the tissue independently of energy supply. The results, obtained with frozen–thawed roots, show similar kinetics and indicate the occurrence of a large adsorption of BR to the cell membranes This adsorption in the frozen–thawed roots seems to be rapidly and completely reversible, whereas a certain amount of BR (*ca* 30% of that taken up) is irreversibly bound in the fresh roots and is not released after freezing. This different behaviour of BR binding could be rationalized in two different ways. The possibility exists that the freezing–thawing treatment irreversibly alters the BR binding sites that survive the freezing when BR is already bound. The other possibility is that some cytoplasmic components and/or reactions are necessary for the irreversible binding, found only in the intact tissue.

EXPERIMENTAL

Synthesis of the deuterated standard. Mps· uncorr ^{1}H NMR spectra were recorded on a Varian EM-360L or on a Varian XL-200 spectrometer as $CDCl_3$ solns and are reported in δ units relative to TMS. The mass spectra were determined on a Varian MAT 112 S spectrometer using EIMS (70 eV) by direct inlet methods. The progress of all reactions and column chromatography (silica, 230–400 mesh) was monitored by TLC on silica gel (HF_{254}) plates. Hexane–EtOAc mixtures were used as developing solvents and spots were detected by spraying with 70% H_2SO_4 followed by heating. Elemental analysis are all with ± 0.2 from calculated values The unlabelled BR was obtained according to ref. [15].

Synthesis of (2R,3S,22R,23R)-2,3,22,23-*tetrahydroxy B-homo-7-oxa-5α-ergostan-6-one*-5,7,7'-d_3 (**8**) The synthesis was accomplished by a major improvement, according to our more recent results [16, 17] of our previous synthesis of the unlabelled compound [15] (Scheme 1).

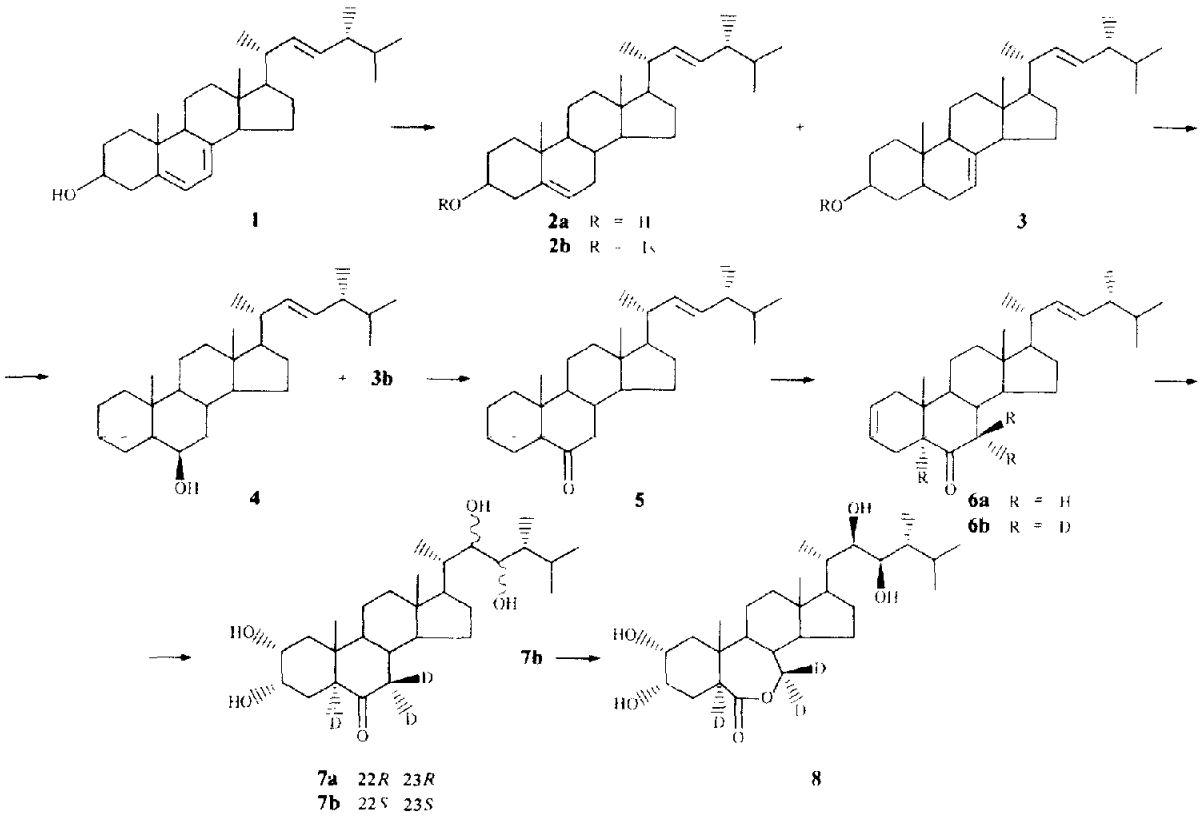
(i) (22E)-3α,5-*Cyclo*-5α-*ergost*-22-*en*-6β-*ol* (**4**) Ergosterol (**1**, 8 g) dissolved in ethylamine (40 ml) was treated with Li (1 6 g), and the mixture was stirred at reflux for 30 min longer than required for the initial appearance of a blue colour Work-up afforded, after extraction with $CHCl_3$, a crude mixture (6 4 g) of (22*E*)-ergosta-5,22-dien-3β-ol (**2a**) and (22*E*)-5α-ergosta-7,22-dien-3β-ol (**3a**) in a 3·2 ratio. The mixture of compounds (**2a**) and (**3a**), dissolved in pyridine (40 ml), was treated with toulene-*p*-sulphonyl chloride (7 g) in pyridine (20 ml) The mixture was kept in the dark for 18 hr at room temp and was poured into cracked ice and H_2O (25 ml) After work-up, the wet tosylates (**2b** and **3b**, 3 g portions) were dissolved in Me_2CO (1 2 l) at 20° and were added to a boiling soln of $KHCO_3$ (7 g) in H_2O (360 ml). The mixture was refluxed for 6 hr, the Me_2CO was evapd under red pres and the residue diluted with H_2O and extracted with Et_2O The ethereal soln was evapd and the residue was chromatographed on silica to afford (22*E*)-3α,5-cyclo-5α-ergost-22-en-6β-ol (**4**, 2.6 g), mp 115–116° (from Me_2CO), $[\alpha]_D^{21}$ +16° ($CHCl_3$; *c* 1) (lit [18] mp 113–115°, $[\alpha]_D$ +15°); λ_{max}^{nujol} cm^{-1}· 3015 and 3060; ^{1}H NMR: δ 0.30–0.60 (3 H, *m*), 0 72 (3 H, *s*), 1 02 (3 H, *s*), 3.24 (1 H, *m*, 6-H), 5 10–5.30 (2 H, *m*, 22- and 23-H), *m/z* 398 $[M]^+$. The tosylate (**3b**) was recovered unchanged by chromatography.

(ii) (22E) -3α,5-*Cyclo*-5α-*ergost*-22-*en*-6-*one* (**5**) Jones reagent [19] was added at −15° to a soln of (**4**, 1 g) in Me_2CO (50 ml) until present in an excess which was discharged with *iso*-PrOH After work-up the crude product was crystallized from MeOH to yield (22*E*)-3α,5-cyclo-5α-ergost-22-en-6-one (**5**, 900 mg), mp 110–111° (from moist Me_2CO), $[\alpha]_D^{21}$ +6° ($CHCl_3$, *c* 1) (lit. [3] mp 108–110°, $[\alpha]_D$ 5°); $\lambda_{max}^{CHCl_3}$ cm^{-1} 1695, ^{1}H NMR δ 0.30–0.60 (3 H, *m*), 0.72 (3 H, *s*), 1.00 (3 H, *s*), 5.10–5 30 (2 H, *m*, 22- and 23-H); *m/z* 396 $[M]^+$.

(iii) (22E)-5α-*Ergosta*-2,22-*dien*-6-*one*-5,7,7'-d_3 (**6b**) The ketone (**5**, 700 mg) was dissolved in DMF (10 ml) and pyridinum hydrobromide (450 mg) was added. The mixture was refluxed for 3 hr and then poured into H_2O and extracted with Et_2O. Work-up followed by chromatography afforded (22*E*)-5α-ergosta-2,22-dien-6-one (**6a**, 600 mg), mp 123–124° (from MeOH), $[\alpha]_D^{21}$ +2° ($CHCl_3$, *c* 1) (lit. [15] for non deuterated compound mp 123–124°, $[\alpha]_D^{25}$ +3°); ^{1}H NMR (200 MHz). δ 0.677 (3 H, *s*), 0 702 (3 H, *s*), 1.006 (3 H. *d*, *J* = 6.7 Hz, H-21), *m/z* 396 $[M]^+$

The ketone (**6a**, 150 mg) was added to a soln of Na (80 mg) in MeOD (30 ml), and the soln was refluxed for 3 hr under a N_2 atmosphere Addition of D_2O (10 ml) and removal of most of the MeOD under red pres gave a suspension which was extracted with Et_2O The combined extracts were washed with a satd NaCl soln in D_2O, dried over Na_2SO_4, and evapd under red press to give crystalline **6b**, (150 mg) which showed mp 123–124° (from MeOD), *m/z* 399 $[M]^+$, 98% isotopically pure d_3, with other physicochemical properties similar to those of (**6a**).

(iv) (2R, 3S, 22R, 23R)-2,3,22,23-*Tetrahydroxy*-5α-*ergostan*-6-*one*-5,7,7'-d_3 (**7a**) and (2R, 3S, 22S, 23S)-2,3,22,23-*Tetrahydroxy*-5α-*ergostan*-6-*one*-5,7,7'-d_3 (**7b**). The ketone (**6b**, 200 mg) dissolved in THF (3 ml) was added to a mixture of *N*-methylmorpholine *N*-oxide hydrate (1.350 g) dissolved in D_2O (2 ml) and THF (3 ml) and OsO_4 (10 mg in 5 ml of *t*-BuOH) The mixture was then stirred at room temp. for 76 hr, CH_2Cl_2 was added and the base was eliminated by washing with dilute HCl.



Scheme 1

After evapn of the solvent under red pres., the residue was dissolved in a mixture of EtOH–CH_2Cl_2 (1:1, 20 ml) and H_2S was bubbled through the soln for 5 hr. The black ppt was filtered off on a pad of celite and washed with EtOH–CH_2Cl_2 (1:1). Removal of the solvent and rapid chromatography of the residue afforded (2*R*, 3*S*, 22*S*, 23*S*)-2,3,22,23-tetrahydroxy-5α-ergostan-6-one-5,7,7′-d_3 (**7b**, 75 mg), mp 184–185° (from EtOAc), $[\alpha]_D^{23} - 3°$ ($CHCl_3$, *c* 1) (lit. [3] for non deuterated compound mp 182–183°, $[\alpha]_D^{25} - 2°$), $\lambda_{max}^{CHCl_3}$ cm^{-1} 1695, ^{1}H NMR δ 0.69 (3 H, s), 0.75 (3 H, s), 3.56 (1 H, *m*), 3.72 (1 H, *m*), 3.79 (1 H, *m*), and 4.06 (1 H, *m*), *m/z* 367 $[M-100]^+$, and then (2*R*, 3*S*, 22*R*, 23*R*)-2,3,22,23-tetrahydroxy-5α-ergostan-6-one-5,7,7′-d_3 (**7a**, 78 mg), mp 241–242° (from EtOAc), $[\alpha]_D^{23}$ 1° ($CHCl_3$, *c* 1) (lit., [18] mp 241–242°, $[\alpha]_D^{23}$ 0°), ^{1}H NMR (pyridine-d_5) δ 0.69 (3 H, s), 0.75 (3 H, s), 3.56 (1 H, *m*), 3.72 (1 H, *m*), 3.79 (1 H, *m*), and 4.06 (1 H, *m*), *m/z* 367 $[M-100]^+$, 98% isotopically pure d_3.

(v) (2R, 3S, 22R, 23R)-2.3,22,23-*tetrahydroxy-B-homo-7-oxa-5α-ergostan-6-one*-5,7,7′-d_3 (**8**). The crude tetraacetate of (**7a**, 140 mg) (prepared by acetylation of the tetrahydroxy-ketone (**7a**) with Ac_2O and pyridine) was added to a soln of $(CF_3CO)_2O$ (150 mg) and H_2O_2 (0.02 ml, 50%) in CH_2Cl_2 (4 ml). The mixture was allowed to react at room temp for 2 hr, worked-up and chromatographed. Recrystallization of the combined chromatographically pure fractions gave 90 mg of a product which was saponified in 3 ml of 2% K_2CO_3 in 70% aq. MeOH. The soln was then acidified with 6 M HCl and heated at reflux for 10 min. Work-up afforded (2*R*, 3*S*, 22*R*, 23*R*)-2,3,22,23-tetrahydroxy-B-α-homo-7-oxa-5α-ergostan-6-one-5,7,7′-d_3 (**8**, 95 mg), mp 256–258° (from EtOAc), $[\alpha]_D^{21} + 32°$ ($CHCl_3$, *c* 1) (lit., [3] for non deuterated compound mp 256–258°, $[\alpha]_D^{25} + 30°$, $\lambda_{max}^{CHCl_3}$ cm^{-1} 3425, 1705, and 1670. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.70 (3 H, s), 1.02 (3 H, s), 3.54 (1 H, *d*, *J* = 9 Hz), 3.72 (2 H, overlapping), 4.03 (1 H, *br* s), *m/z* 465 $[M-H_2O]^+$, 98% isotopically pure d_3.

Preparation of plant material. Maize (*Zea mays* L. cv. Dekalb XL 72) seeds, surface sterilized with 1% NaClO, were germinated in the dark at 28° for 3 days on filter paper wetted with H_2O. The seedlings were then transferred to germination trays fitted with a perforated plate and the roots were dipped into aerated 0.5 mM $CaSO_4$ for 20 hr.

Subapical root segments, 5 mm long, were excised from the main root 2 mm below the apex. The segments (90 per sample, *ca* 300 mg) were kept for 30 min in 0.3 mM $CaSO_4$, preincubated for 60 min in 0.3 mM $CaSO_4$ and then transferred to the incubation medium (5 segments/ml) 0.15 mM MES (2-*N*-morpholmethansulfonic acid)–NaOH buffer (pH 6.2), containing 0.3 mM $CaSO_4$, 5 mM K_2SO_4 and 2 μM BR, optimal concn for the effects on growth and H^+ extrusion in maize root segments [4]. Frozen-thawed tissue was obtained by freezing the segments twice with liquid N_2 at the end of the preincubation period. All the experiments were run in the dark in a thermoregulated (26°) H_2O bath with shaking (100 rpm), and were performed at least twice in triplicate.

Detection of BR. At the end of the incubation period an aliquot of the medium (5 ml) was spiked with BR-d_3 as int. standard. The aq. medium was satd. with NaCl and the mixture of BR and BR-d_3 was obtained by EtOAc extraction and evapn. The segments, rapidly rinsed with H_2O, spiked with BR-d_3 were homogenized in MeOH–EtOAc (1:1, 5 ml × 3) using a potter homogenizer with a motor driven Teflon pestle, and the mixture was filtered over a 3 mm diameter column containing 100 mg of silica gel and evapd to afford a residue containing BR and BR-d_3. Both residues were converted to the corresponding bismeth-

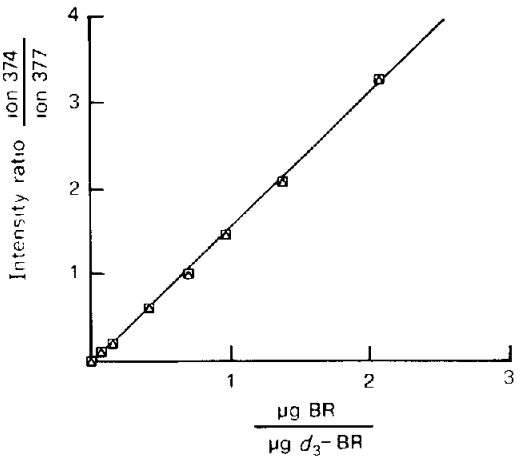


Fig 4 Standard curves (SC) showing the ratio of signal intensities of the bismethaneboronate of BR (*m/z* 374) and those of the same derivative of d_3-BR (*m/z* 377) plotted against the ratio of g of BR to g of BR-d_3 ⊠ indicate the superimposable datum points obtained for segments (□) and buffer (△)

aneboronates as described in ref. [20], by heating at 60° with a soln (10 μl) of methaneboronic acid (10 mg) in pyridine (7 ml), for 30 min The derivatives were analysed by MSIM on a column (0 5 m × 2 mm) packed with 1% OV-17 at 290°. The carrier gas (He) flow-rate was 30 ml/min, and electron energy was 20 eV The ions focussed for the analysis were at *m/z* 374 (C_{21}–C_{22} fission of the bismethane-boronate of the BR [20]) and at *m/z* 377 for the deuterated derivative (BR-d_3-bismethane-boronate) To check the linearity of the method, MSIM analysis was carried out on bismethane-boronates of extracts of frozen subapical root segments (*ca* 300 mg) spiked with BR-d_3 (2 964 μg) and with increasing amounts of BR (O, 0.2070, 0.4140, 1,2427, 2.0712; 2 8997, 4 1425; 6 2137 μg) Similarly the linearity was checked in 5 ml of the buffer Points shown in the plot (Fig 4) represent the results of the MSIM of these samples A linear correlation ($r = 0.999$) was obtained when the ratio of the peak intensities, at the retention time of bismethane-boronates of BR, in the traces of ions at *m/z* 374 and 377 were plotted against the ratio of the amount of BR and BR-d_3 in each sample The linearity observed refers not only to the MSIM itself but also to the derivatization and extraction procedures, due to the fact that the deuterated internal standard (BR-d_3) was added before any manipulation of the incubated materials. Repetitive MSIM analysis of the BR content in a single sample gave ±1 5% relative s d. In addition a good reproducibility was observed for the complete procedure as the s e did not exceed ±3% in the measurement of BR.

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REFERENCES

1. Grove, M D., Spencer, G F, Rohwedder, W K., Mandava, N B, Worley, J F, Warthen, Jr, J. D, Steffens, G L, Flippen-Anderson, J. L and Cook, Jr, J C (1979) *Nature* **281**, 216
2 Adam, G. and Marquardt, V (1986) *Phytochemistry* **25**, 1787
3 Thompson, M J, Mandava, N B., Flippen-Anderson, J L, Worley, J F, Dutky, S R, Robbins, W E and Lusby, W. (1979) *J. Org. Chem* **44**, 5002
4 Romani, G, Marrè, M T., Bonetti, A, Cerana, R, Lado, P and Marrè, E (1983) *Physiol Plant.* **59**, 528
5 Cerana, R, Lado, P, Anastasia, M, Ciuffreda, P and Allevi, P (1984) *Z Pflanzenphysiol Bd* **114**, 211
6 Cerana, R, Bonetti, A, Marrè, M T, Romani, G, Lado, P and Marrè, E (1983) *Physiol Plant* **59**, 23
7 Yopp, J H., Mandava, N B and Sasse, J M (1981) *Physiol Plant* **53**, 445.
8 Mandava, N B, Sasse, J M and Yopp, J H (1981) *Physiol. Plant.* **53**, 453
9 De Michelis, M. I and Lado, P (1986) *Physiol Plant.* **64**, 603
10. Rayle, D L and Cleland, R. C (1977) *Curr Top Dev Biol* **11**, 187
11. Arteca, R N., Tsai, D S., Schlagnhaufer, C and Mandava, N B (1983) *Physiol Plant* **59**, 539
12 Schlagnhaufer, C, Arteca, R N. and Yopp, J H (1984) *Physiol Plant* **60**, 347
13. Schlagnhaufer, C, Arteca, R. N and Yopp, J H (1984) *Physiol. Plant.* **61**, 555
14 Arteca, R. N, Bachmann, J M., Yopp, J H and Mandava, N. B. (1985) *Physiol. Plant.* **64**, 13
15 Anastasia, M, Ciuffreda, P and Fiecchi, A (1983) *J Chem Soc Perkin Trans I* 379
16 Anastasia, M, Allevi, P, Ciuffreda, P and Oleotti, A (1985) *Steroids* **45**, 561.
17 Anastasia, M, Allevi, P, Ciuffreda, P and Fiecchi, A (1983) *Ital Patent Appl* 21524/83
18. Thompson, M J, Cohen, C. F. and Lancaster, S M (1965) *Steroids* **7**, 745
19 Bowden, K, Heilbron, I M, Jones, E. R H and Weedon, B C L (1946) *J Chem. Soc* 39
20 Takatsuto, S., Ying, B, Marisaki, M and Ikekawa, N (1982) *J Chromatog* **239**, 233