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A bioassay for brassinosteroid activity based on the *in vitro* fluorimetric detection of nitric oxide production

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ABSTRACT

Recent studies have shown that low concentrations of brassinolide induce a rapid generation of nitric oxide in mesophyll cells of maize leaves, which can be easily detected by fluorimetric methods. In this work we describe a series of natural and synthetic brassinosteroids that are able to trigger *in vitro* NO production in tomato cells that exhibits dose–response behavior. We propose that this effect can be used to develop a new rapid and very sensitive bioassay for brassinosteroid activity that offers several advantages when compared to the current methodologies.

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1. Introduction

The role of steroids as hormones in mammals is known since the 1930s, later their endocrine function in insects and fungi was well stablished. Although plants are able to produce a wide range of steroids, it was only in 1979 when researchers from the United States Department of Agriculture described the structure of brassinolide (**1**), a steroidal lactone isolated from pollen of rapeseed (*Brassica napus*), with a remarkable activity on plant growth [1]. Since then, a large number of structurally related steroids, collectively named as brassinosteroids (BRs), were found in all plant species: higher plants, ferns and algae [2]. BRs proved to have regulatory activity on the growth and development of plants [3,4], such as stimulation of cell division, vegetative growth, reproduction and tolerance to stress and pathogens [5–7]. Due to the low concentration of BRs in plant tissues, their chemical synthesis and that of a wide variety of analogs was mandatory in order to obtain the compounds needed to undertake studies on BRs biosynthesis, metabolism, the underlying molecular mechanisms involved in signal transduction [8–10] and the subsequent genomic responses [11,12]; nowadays, these processes are among the best studied in plant biology.

Once the presence of BRs in plants had been established, the suitability of these new substances for improving the yield of economically important crops was explored. There are many reports, which have been summarized in monographs [13–15], about the beneficial effects of exogenously applied BRs on yield, general vigor, and stress tolerance of commercially important crops [16,5].

Among the major constraints for large-scale use of BRs in crop production are their high cost and limited half-life in the environment. However, progress in the chemical synthesis of long-lasting analogues and non-steroidal mimetics [17] has led to economically feasible approaches that have brought their practical application in agriculture within reach. A key application of BRs that is currently under development is their use as growth factors in techniques involving plant propagation from cuttings, grafting, micropropagation and tissue culture, especially of valuable recalcitrant species [18–20].

In the search for new synthetic BRs with potential use in agriculture, it is vital that hormonal activity is evaluated with a reliable bioassay. Several bioassays have been developed [21], but the most prevalent are the bean second internode elongation assay (BSIE), and the rice leaf lamina inclination test (RLIT).

The bean internode elongation assay uses bean (*Phaseolus vulgaris*) seedlings when the second internode from the root-shoot junction is still growing [22]. BRs characteristically provoke elongation in a dose–response manner, accompanied by curvature, swelling and splitting. The rice leaf lamina inclination test, which is used extensively for the identification of BRs during purification protocols and for determining the potency of natural and synthetic

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BRs, is based on the bending of the lamina of rice leaves away from the sheath when exposed to BRs [23]. Explants consisting of lamina, lamina joint, and leaf sheath from etiolated rice seedlings are floated on test solutions and the bend of the leaf lamina is measured. In a modified protocol, intact rice (*Oryza saliva*) seedlings are used and the test solutions are applied as a microdrop at the junction between the lamina and the sheath [24]. The bending seems to involve the activation of genes that modulate cell division at the lamina joint [25].

These bioassays are quite simple and inexpensive, but their lengthiness (two to four days are needed, not including the one week span required to grow the seedlings) renders them inappropriate for high throughput screening of large collections of BRs.

Recently, it has been shown that low concentrations of brassinolide induce a rapid generation of nitric oxide in mesophyll cells of maize leaves, which can be easily detected by fluorimetric methods [26]. Guided by these results, here we present a preliminary study showing that BRs-induced NO production in cultured plant cells can be developed into a new rapid and sensitive bioassay for measuring brassinosteroid activity.

2. Experimental

2.1. Tested compounds

Brassinolide (**1**) was purchased from Sigma–Aldrich Co. Other natural brassinosteroids and analogs tested in this work were synthesized as previously described [27–29].

2.2. Rice lamina inclination test (RLIT) [25]

Rice seedlings (*Oryza sativa*, cv Chui) were washed with ethanol (1 min) and water and then left in water at 30 °C for two days (with a 16 h photoperiod). Germinated seeds were cultivated in agar under the same growing conditions for four days. Twenty intact seedlings (4–5 cm tall) were inoculated with 0.5 μL of the test compound solution (in ethanol) just under the second leaf joint. Seedlings were kept in the dark (at 30 °C), and after 48 h the magnitude of the induced angle between the leaf and the sheath was measured. Activity for each compound at a given dose is reported as:

(Mean angle of treated plant
– mean angle of control)/mean angle of control

where ethanol was injected as the control.

2.3. BRs-induced nitric oxide production

Tomato (*Solanum lycopersicum* 'Money Maker'; line Msk8) suspension-cultured cells were grown at 24 °C in the dark at 125 rpm in Murashige and Skoog medium (Duchefa), supplemented with 5.4 mM naphthylacetic acid, 1 mM 6-benzyladenine, and vitamins (Duchefa). After 5 days of culture cells were transferred to a multiwell plate and pretreated for 30 min with the NO-specific fluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA at 0,5 μM, and subsequently incubated in the presence or absence of the different BRs (previously dissolved in DMSO) in a final volume of 100 μL.

Fluorescence was measured with a Fluoroskan Acsent fluorometer (Thermo Electron Company, Finland), using D480-40 and D525-30 filters for excitation and emission, respectively, every 2 min for 2 h in each well. Cell suspensions were kept at 25 °C and 120 rpm during measurements, which were made in triplicate. Activity for each compound at a given dose is defined as:

Slope of the fluorescence vs. time curve/slope of
the fluorescence vs. time curve of the control.

where DMSO was injected as the control.

2.4. Synthesis

2.4.1. General

All the reagents were purchased from Sigma–Aldrich Chemical Co. Melting points were determined on a Fisher Johns apparatus and are uncorrected. All NMR spectra were recorded on a Bruker AM-500 (500 MHz for ^{1}H, 125.1 MHz for ^{13}C and 470.5 MHz for ^{19}F). Chemical shifts (Δ) are given in ppm downfield from TMS as the internal standard for ^{1}H and ^{13}C, and downfield from CCl_3F for ^{19}F). Coupling constant (J) values are in Hz. For unambiguous assignment of both ^{1}H and ^{13}C signals gHSQC and gHMBC spectra were measured using standard parameters sets and pulse programs. All solvents and reagents were of analytical grade. ESI-HRMS were measured on a Bruker micrOTOF-Q II. Analysis of all new compounds yielded satisfactory combustion data (purity ⩾98%) on an Exeter CE 440 Elemental Analyzer. Optical rotations were measured on a Jasco J-815 spectropolarimeter. IR spectra were recorded on a Nicolet iS50 FT-IR spectrometer and are shown as Supplementary Data.

2.4.2. Synthesis of (22E)-2β-fluoro-5α-stigmasta-22-en-3,6-dione (**15**)

200 mg (0.43 mmol) of (22*E*)-3-acetoxy-5α-stigmasta-2,22-dien-6-one **14** (obtained from stigmasterol, as previously described [28]) were dissolved in 10 mL of dry dichloromethane in a polypropylene round bottom flask under an argon atmosphere. 108 mg (0.56 mmol) of xenon difluoride were added. The reaction was stirred for 3 h at room temperature and poured into ice. The mixture was extracted with dichloromethane (3 × 15 mL) and the organic layer was washed with brine, water, dried with sodium sulfate and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane/EtOAc 95:5), yielding 34% (65 mg, 0.15 mmol) of (22*E*)-2α-fluoro-5α-stigmasta-22-en-3,6-dione [28]. Further elution gave 97 mg (51% yield) of the desired (22*E*)-2β-fluoro-5α-stigmasta-22-en-3,6-dione **15** M.p.: 114–115 °C. $[\alpha]_D^{20}$−44 (c 0.25, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 0.71 (H-18, 3H, *s*); 0.79 (H-26, 3H, *d*, *J* = 6.8); 0.80 (H-29, 3H, *t*, *J* = 7.0); 0.84 (H-27, 3H, *d*, *J* = 5.9); 1.02 (H-21, 3H, *d*, *J* = 8.6); 1,03 (H-19, 3H, *s*); 2,70 (H-5α, 1H, *dd*, *J* = 3.9 and 12.9); 3.03 (H-4β, 1H, *dm*, *J* = 13.3); 4.75 (H-2α, 1H, *dt*, *J* = 4,5 y $^{2}J_{HF}$ = 49.0); 5.03 (H-23, 1H, *dd*, *J* = 8.5 y 15.3); 5.14 (H-22, 1H, *dd*, *J* = 8.5 y 15.3). ^{13}C-NMR ($CDCl_3$): 12.2 and 12.2 (C-18 and C-29); 14.4 (C-19, *d*, $^{4}J_{CF}$ = 4.6); 18.9 (C-26); 21.0 and 21,1 (C-21 and C-27); 21.6 (C-11); 24.0 (C-15); 25.3 (C-28); 28.6 (C-16); 31.8 (C-25); 34.0 (C-4); 37.4 (C-8); 39.2 (C-12); 40.3 (C-20); 41.0 (C-10); 42.8 (C-13); 44.0 (C-1, *d*, $^{2}J_{CF}$ = 20.2); 46.2 (C-7); 51.2 (C-24); 54.0 (C-9); 55.8 (C-17); 56.5 (C-14); 56.9 (C-5); 91.3 (C-2, *d*, $^{1}J_{CF}$ = 180.8); 129.7 (C-23); 137.8 (C-22); 206.0 (C-3, *d*, $^{2}J_{CF}$ = 18.7); 207.7 (C-6). ^{19}F-NMR ($CDCl_3$): −182.0 (*dddd*, $^{2}J_{HF}$ = 49.2; $^{3}J_{HF}$ = 37.1 y 19.5; $^{4}J_{HF}$ = 1.7). MS (EI): *m*/*z* (rel.int.): 444 $[M]^+$ (10); 401 (15); 331 (16); 303 (29); 55 (100).

2.4.3. Synthesis of (22E)-2β-fluoro-3α-hydroxy-5α-stigmast-22-en-6-one (**16**)

The fluorodiketone **15** (105 mg, 0.21 mmol) was dissolved in 5 ml of dry CH_2Cl_2 under an argon atmosphere and 15 mg of tetrabutylammonium borohydride was added in one portion. The reaction was stirred for 1 h and the organic layer was washed successively with NH_4Cl (s.s.), brine, and water, and then dried over Na_2SO_4. The solvent was evaporated and the resulting products were separated by silica gel column chromatography (hexane/AcOEt gradient) to yield 90% (95 mg, 0.21 mmol) of

(22*E*)-2β-fluoro-3α-hydroxy-5α-stigmast-22-en-6-one **15**. M.p.: 104–105 °C. $[\alpha]_D^{20}$−68 (c 0.22, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 0.69 (H-18, 3H, *s*); 0.79 (H-26, 3H, *d*, *J* = 6.1); 0.80 (H-29, 3H, *t*, *J* = 7.6); 0.84 (H-27, 3H, *d*, *J* = 6.4); 0.89 (H-19, 3H, *s*); 1.02 (H-21, 3H, *d*, *J* = 6.6); 2.33 (H-7α, 1H, *dd*, *J* = 4.5 and 12.2); 3.55 (H-3β, 1H, *dm*, $^3J_{HF}$ = 29.6); 4.82 (H-2α, 1H, *dm*, $^2J_{HF}$ = 50.5); 5.03 (H-23, 1H, *dd*, *J* = 8.7 y 15.1); 5.14 (H-22, 1H, *dd*, *J* = 8.5 and 15.3). ^{13}C-NMR ($CDCl_3$): 12.2 and 12.2 (C-18 and C-29); 14.8 (C-19, *d*, $^4J_{CF}$ = 6.4); 18.9 (C-26); 21.0 and 21.1 (C-21 and C-27); 21.5 (C-11); 24.0 (C-15); 24.8 (C-4); 25.3 (C-28); 28.7 (C-16); 31.8 (C-25); 37.2 (C-8); 39.3 (C-12); 40.2 (C-10, *d*, $^3J_{CF}$ = 1.8); 40.3 (C-20); 41.6 (C-1, *d*, $^2J_{CF}$ = 18.3); 42.8 (C-13); 46.4 (C-7); 51.2 (C-24); 54.4 (C-9); 55.9 (C-17); 56.7 (C-14); 56.7 (C-5); 70.9 (C-3, *d*, $^2J_{CF}$ = 19.8); 91.6 (C-2, *d*, $^1J_{CF}$ = 169.7); 129,6 (C-23); 137.9 (C-22); 209.9 (C-6). ^{19}F-NMR ($CDCl_3$): -197,3 (*dddd*, $^2J_{HF}$ = 48.9; $^3J_{HF}$ = 48.9; 29.1; 11.7). MS (EI): *m*/*z* (rel.int): 446 $[M]^+$ (5); 403 (3); 334 (4); 305 (7); 55 (100). Anal. calc. for $C_{29}H_{47}O_2F$: C, 77,98; H, 10,61. Found: C, 78,00; H, 10,51.

*2.4.4. Synthesis of (22R,23R)-2β-fluoro-3α,22,23-trihydroxy-5α-stigmastan-6-one (**12**) and (22S,23S)-2β-fluoro-3α,22,23-trihydroxy-5α-stigmastan-6-one (**13**)*

A mixture of **16** (95 mg, 0.21 mmol), *t*-butanol/water (1:1, 10 ml), $(DHQD)_2$-Phal (72 mg, 0.09 mmol), methanesulfonamide (42 mg, 0.42 mmol), potassium ferricyanide (450 mg, 1.32 mmol), potassium carbonate (192 mg, 1.32 mmol), and potassium osmate dihydrate (12 mg, 0.024 mmol) was stirred at room temperature for 5 days. An excess of $NaHSO_3$ was added until the evolution of bubbles was no longer observed. Layers were separated and the aqueous phase was thoroughly extracted with CH_2Cl_2/MeOH (95:5). Combined organic layers were washed with 0.25 M H_2SO_4 (3 × 10 mL) and 2% NaOH (3 × 10 mL). Purification by silica gel column chromatography (CH_2Cl_2/acetonitrile gradient) allowed to obtain 21% (21 mg, 0.04 mmol) of (*22S,23S*)-2β-fluoro-3α,22,23-trihydroxy-5α-stigmastan-6-one **13**. M.p.: 105–107 °C. $[\alpha]_D^{20}$+24 (c 0.26, $CHCl_3$). ^{1}H-NMR ($CDCl_3$/MeOD 9:1): 0.71 (H-18, 3H, *s*); 0.88 (H-19, 3H, *s*); 0.89 (H-26, 3H, *d*, *J* = 6,7); 0.95 (H-27, 3H, *d*, *J* = 6,8); 0.96 (H-29, 3H, *t*, *J* = 7.5); 1.03 (H-21, 3H, *d*, *J* = 6.8); 2.28 (H-5α,, *dd*, *J* = 2.9 and 12.6); 2.33 (H-7α, 1H, *dd*, *J* = 4.5 and 12.6); 3.53 (H-3β, 1H, *m*); 3.61 (H-22 and H-23, 2H, *m*); 4.83 (H-2α, 1H, *dm*, $^2J_{HF}$ = 50.2). ^{13}C-NMR ($CDCl_3$/MeOD 9:1): 11.9 (C-18); 14.2 (C-21); 14.5 (C-29); 14.9 (C-19, *d*, $^4J_{CF}$ = 5.9); 17.7 (C-26); 18.5 (C-28); 21.6 (C-11); 21.7 (C-27); 24.3 (C-15); 24.8 (C-4); 26.9 (C-25); 27.8 (C-16); 37.3 (C-8); 39.2 (C-12); 40.3 (C-10, *d*, $^3J_{CF}$ = 1.9); 41.7 (C-1, *d*, $^2J_{CF}$ = 18.7); 42.2 (C-20); 43.4 (C-13); 46.4 (C-7); 49.5 (C-24); 52.5 (C-17); 54.3 (C-9); 56.7 (C-5); 56.3 (C-14); 70.7 (C-23); 70.9 (C-3, *d*, $^2J_{CF}$ = 18.6); 72.0 (C-22); 91.7 (C-2, *d*, $^1J_{CF}$ = 171.7); 209.9 (C-6). ^{19}F-NMR ($CDCl_3$/MeOD 9:1): -197,5 (*m*). HRMS (ESI): *m*/*z* $[M+Na]^+$ 503,35185 (calc. for $C_{29}H_{49}FO_4$, found: 503.35275; error: 1,8 ppm). Anal. calc. for $C_{29}H_{49}FO_4$: C, 72.46; H, 10.27. Found: C, 72.35; H, 10.35.

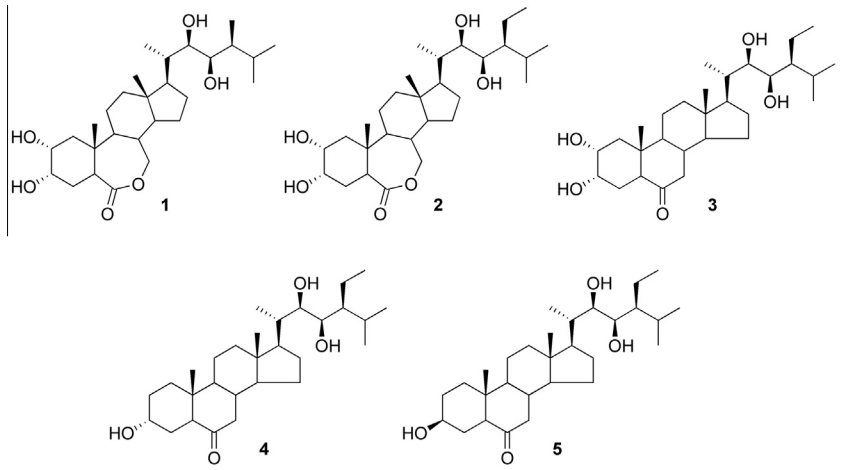


Fig. 1. Structure of brassinolide (**1**) and some active natural brassinosteroids.

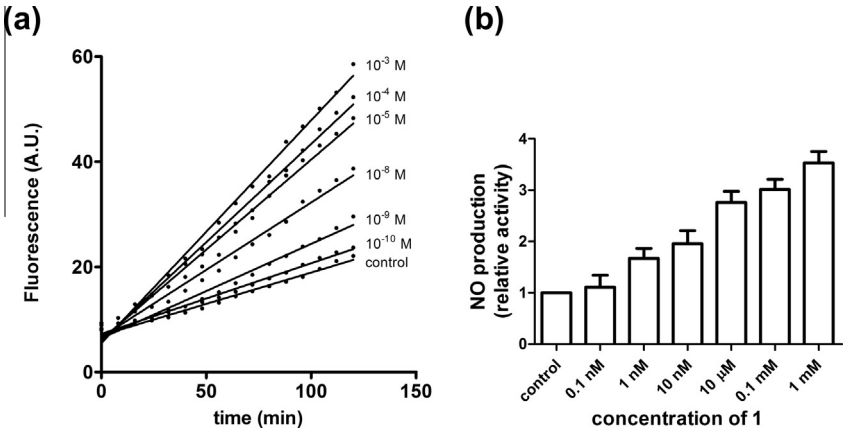


Fig. 2. (a) Time-dependent production of NO in tomato cells induced by brassinolide. A.U.: arbitrary units of fluorescence. (b) Dose–response production of NO induced by brassinolide.

Further elution yielded 67% (67 mg, 0.14 mmol) of (22*R*,23*R*)-2β-fluoro-3α,22,23-trihydroxy-5α-stigmastan-6-one **12**. M.p.: 101–102 °C. $[\alpha]_D^{20}$+17 (c 0.25, $CHCl_3$). ^{1}H-NMR ($CDCl_3$/MeOD 9:1): 0.69 (H-18, 3H, *s*); 0.89 (H-19, 3H, *s*); 0.91 (H-21, 3H, *d*, *J* = 6.5); 0.95 (H-29, 3H, *t*, *J* = 7.4); 0.96 (H-26, 3H, *d*, *J* = 7.2); 0.97 (H-27, 3H, *d*, *J* = 6.3); 2.27 (H-5α, 1H, *dd*, *J* = 2.9 and 12.6); 2.34 (H-7α, 1H, *dd*, *J* = 4.6 and 12.6); 3.53 (H-3β, 1H, *m*); 3.59 (H-22, 1H, *dd*, *J* = 1.2 and 8.6); 3.72 (H-23, 1H, *dd*, *J* = 1.2 y 8.6); 4.83 (H-2α, 1H, *dm*, $^2J_{HF}$ = 50.3). ^{13}C-NMR ($CDCl_3$/MeOD 9:1): 11.9 (C-18); 11.9 (C-21); 13.4 (C-29); 14.9 (C-19, *d*, $^4J_{CF}$ = 6.0); 18.8 (C-28); 19.4 (C-26); 21.2 (C-27); 21.6 (C-11); 23.8 (C-15); 24.8 (C-4); 27.6 (C-16); 28.8 (C-25); 36.9 (C-20); 37.3 (C-8); 39.4 (C-12); 40.3 (C-10, *d*, $^3J_{CF}$ = 1.9); 41.6 (C-1, *d*, $^2J_{CF}$ = 18.7); 42.8 (C-13); 46.3 (C-24); 46.3 (C-7); 52.5 (C-17); 54.3 (C-9); 56.5 (C-14); 56.7 (C-5); 70.9 (C-3, *d*, $^2J_{CF}$ = 19.5); 72,8 (C-23); 74.5 (C-22); 91.6 (C-2, *d*, $^1J_{CF}$ = 171.9); 209,9 (C-6). ^{19}F-NMR ($CDCl_3$/MeOD 9:1): −197,4 (*m*). HRMS (ESI): *m*/*z* $[M+Na]^+$ 503,35185 (calc. for $C_{29}H_{49}FO_4$, found: 503.35126; error: 1,2 ppm). Anal. calc. for $C_{29}H_{49}FO_4$: C, 72,46; H, 10,27. Found: C, 72,51; H, 10,25.

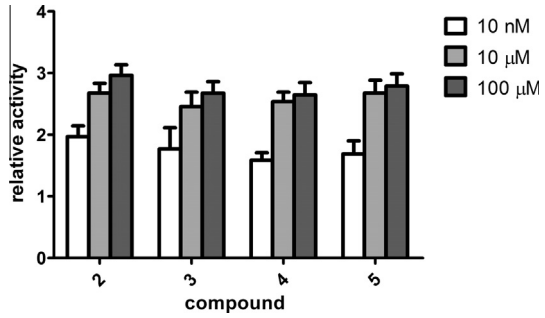


Fig. 3. Dose–response production of NO induced by some natural BRs.

3. Results and discussion

To determine the kinetics of NO production we chose brassinolide (**1**, Fig. 1), the most active natural BRs, for the preliminary assays. Fig. 2a shows that **1** triggers measurable production of NO when tomato cells (*S. lycopersicum*) are treated in the presence of 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA), a specific fluorescent probe for NO. The NO production follows a time-dependent response that is almost linear for each of the tested concentrations ($r^2 \geqslant 0.99$). The activity of **1** is defined as the ratio between the slope of the corresponding curve for each concentration relative to the slope of the line of the blank.

Responses were dose dependent and significant at doses as low as 1 nM (Fig. 2b). The effective dose 50 (EC_{50}) was estimated to be about 5 nM, which, taking into account the final volume of the well, corresponds to 0.5 pmol of compound. For comparison, the EC_{50} of **1** in the RLIT is typically 100 pmol (data not shown), i.e. two orders of magnitude higher. Seeking to extend these results, we repeated the same experiments with other natural BRs, such as homobrassinolide, homocastasterone, homothyphasterol and homoteasterone (compounds **2**, **3**, **4** and **5** respectively, Fig. 1). Fig. 3 shows that a similar dose – response behavior is observed.

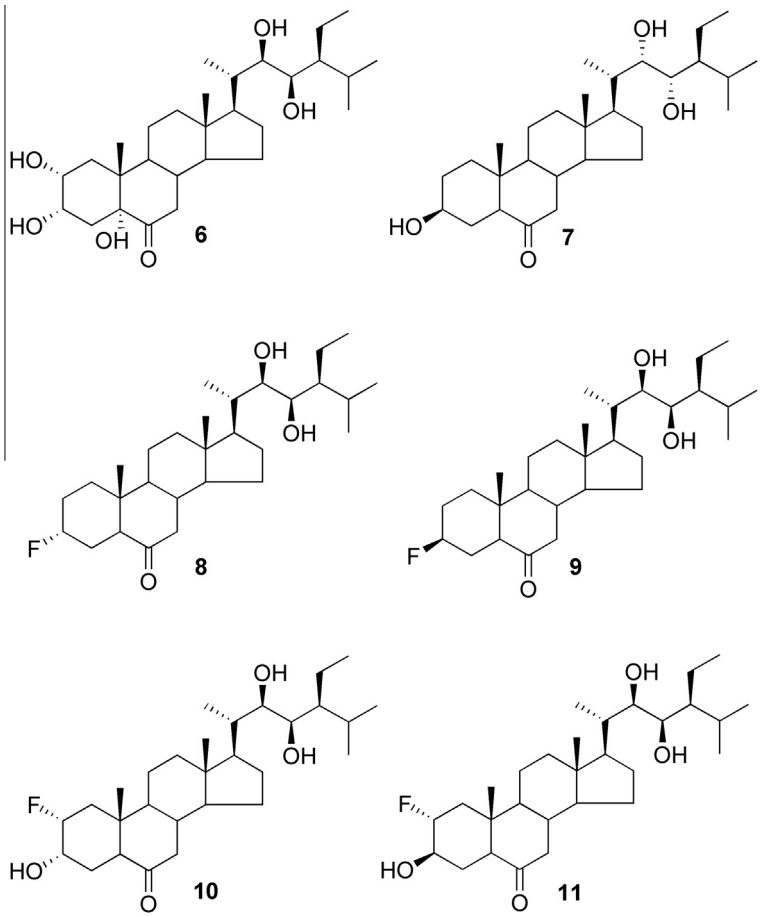


Fig. 4. Structure of the BRs analogs tested in this work.

Table 1
Activity of BRs at a 1 nmol dosage in both bioassays. Activities are reported as described in the Section 2. NO production is reported as the average of five independent experiments performed in triplicates. RLIT experiments were performed using 20–25 seedlings in triplicates.

Compound	NO production	RLIT
1	2.76 ± 0.22	4.3 ± 0.2
2	2.69 ± 0.16	4.1 ± 0.2
3	2.67 ± 0.21	4.0 ± 0.3
4	2.53 ± 0.16	3.1 ± 0.4
5	2.46 ± 0.23	2.9 ± 0.6
6	2.12 ± 0.18	2.5 ± 0.4
7	1.53 ± 0.16	0.4 ± 0.6
8	2.31 ± 0.33	2.6 ± 0.3
9	2.28 ± 0.12	2.4 ± 0.4
10	3.08 ± 0.30	3.4 ± 0.3
11	1.86 ± 0.23	1.0 ± 0.4
12	2.00 ± 0.23	1.5 ± 0.6
13	1.64 ± 0.19	0.6 ± 0.2

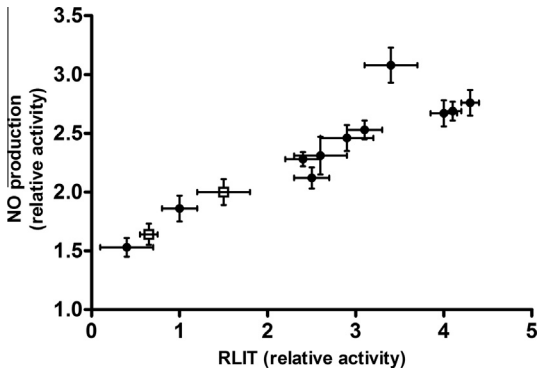


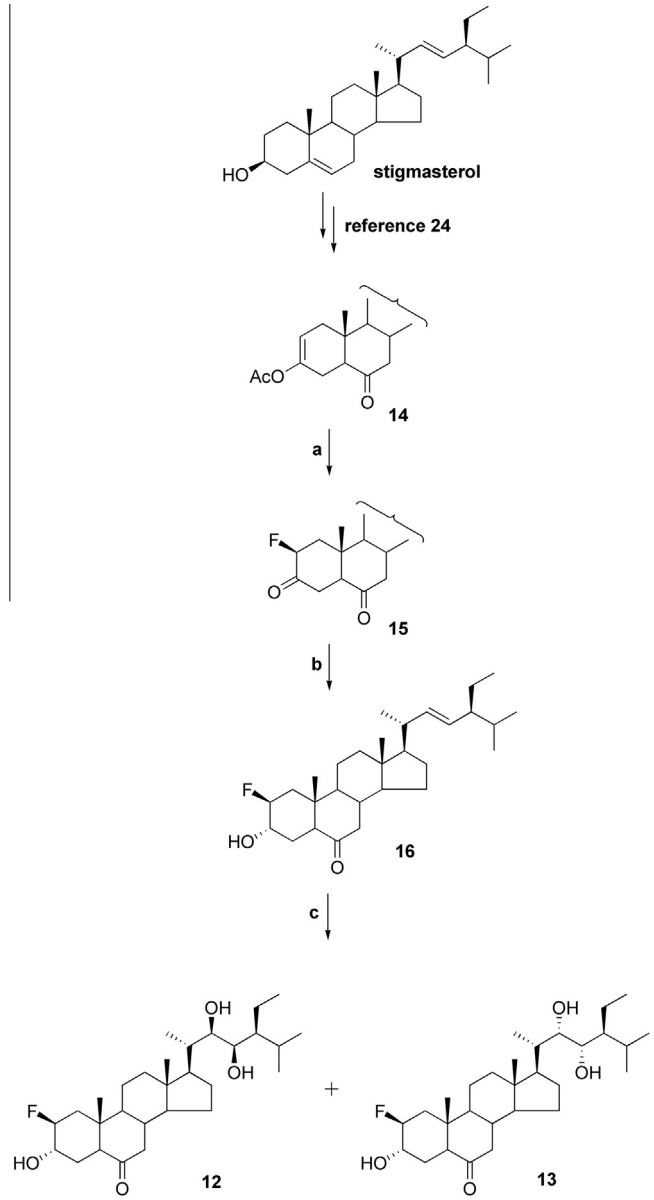
Fig. 5. Correlation plot comparing the activity of each compound at 1 nmol in both assays. Activities in both axis are relative to the blank as described in the Section 2.

With the purpose of comparing our proposed method with well-established BRs bioassays, we tested both the production of NO in tomato cells and the bending of the leaf lamina in rice using a set of synthetic BRs (compounds **6**–**11**), whose structures are shown in Fig. 4. The election of these compounds was made based on previous studies from our laboratory that showed their wide range of growth-promoting activities in the RLIT assay [27,28,30,29].

The difference in the sensitivity of the two assays was problematic. We decided, as a compromise, to test the compounds applying a 1 nmol dose in both assays: we showed above (Figs. 2b and 3) that this amount (which corresponds to a final concentration of 10 μM) did not saturate the NO production of the most active compounds. Furthermore, we knew from our previous studies that this level of exogenous BRs is appropriate to give a significant response in the RLIT.

The results in both bioassays are presented in Table 1; in addition Fig. 5 shows the correlation between them, where a close relationship can be observed.

In order to validate the method, we decided to evaluate two new, previously undescribed BR analogs, in both assays. For this purpose, we synthesized the 2β-fluoro brassinosteroids **12** and **13**, whose synthesis is shown in Scheme 1. We previously reported that when enol acetate **14** is reacted with Selectfluor, 2α-fluoro enone is obtained almost exclusively [28]. After some experimentation we found that the use of a more reactive, less selective, electrophilic fluorinating agent such as xenon difluoride leads to a mixture of both 2β- and 2α-fluorosteroids in a 1.5/1 ratio. The configuration of the fluorine at C-2 was established from analysis of the pattern of the coupling constants in both the ^{1}H-NMR and ^{19}F-NMR spectra.



Scheme 1. Synthesis of compounds **12** and **13**. *Reagents and conditions:* (a) XeF_2/CH_2Cl_2, r.t., 3 h. (b) $(Bu_4N)BH_4$/CH_2Cl_2, r.t., 1 h. (c) K_2OsO_4/$K_3Fe(CN)_6$/$(DHQD)_2$Phal/K_2CO_3/t-BuOH/H_2O/$CH_3SO_3NH_2$, r.t., 5 days.

Reduction of the carbonyl of compound **15** with tetrabutylammonium borohydride gave 2β-fluoro-3α-hydroxy steroid **16** as the main product. The observed regioselectivity for the reduction resulted from the greater reactivity of the 3-oxo moiety compared to the 6-oxo group [31,32]. Moreover, the high diasteroselective output of this reaction could be explained, according to Ramachandran et al., by the chelating effect of the fluorine on the Lewis acidic borane [33], which favors attack on the β-face of the steroid.

Finally, compound **16** was stereoselectively dihydroxylated at the Δ^{22} double bond under Sharpless conditions, using $(DHQD)_2$Phal as a catalyst, yielding a mixture of the desired fluorinated brassinosteroids **12** and **13**. The configuration of the vicinal diol for each compound was established by comparing the chemical shifts and coupling constants of known BRs with closely related structures [34]. The bioactivity of **12** and **13** was also evaluated in both the RLIT and in the newly proposed assay using a 1 nmol dosage.

The first point that could be emphasized is that the new bioassays reveal the same relative structure–activity trends that were established for brassinosteroids. [35]. For example, compound **13**, with a non natural 22*S*,23*S* diol configuration in the side chain has a lower activity than that of its 22*R*,23*R* diastereomer, as expected (Table 1 and open squares in Fig. 4). The same effect can be observed when comparing compounds **5** and **7**. On the other hand, it is known that BRs having 7-oxalactones are more active than the corresponding 6-oxa counterparts (compare **2** vs. **3**), whereas substituents in the A ring prefer a 2α- and 3α-configurations. In addition, the bioactivity displayed by natural biosynthetic intermediates increases according to their position on the biosynthetic pathway (**5** ≈ **4** < **3** < **2**) [36], the closer the intermediate in the pathway to homobrassinolide the greater the NO production. These results clearly show that the methodology described in our work is an attractive alternative to the well-established RLIT, mainly because of the higher sensibility. Moreover, two other differences between the assays could be exploited. Firstly, the NO-based assay only requires standard plant cell culture methodologies and fluorimetric equipment, and thus may be more easily adapted to a high throughput screening format, a useful tool in the search of new BRs analogues with practical applications, which is not feasible currently for RLIT.

On the other hand, and as previously mentioned, the bending of the lamina in the RLIT involves the activation of several genes that not only modulate cell division at the lamina joint, but cell growth and division themselves, complex events whose development spans several hours [25]. Conversely, the NO production triggered by BRs is a fast process, involving fewer signaling mediators. Thus, the response observed likely reflects the early steps after BRs perception by their receptor [37].

In conclusion, we have demonstrated that the *in vitro* BRs-induced NO production in tomato cells follows a dose–response behavior, and that this effect can be used as a new rapid and very sensitive bioassay for BRs activity. Most important, the relative activities measured with the new proposed method closely follow that of the widely validated RLIT assay. Finally, NO is known to be involved in the pathway signaling of other phytohormones such as auxins and gibberelins [38]. Thus, it would be interesting to explore the application of the proposed assay to other plant growth promoters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2015.07.003.

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