

Plant growth regulation activity of steviol and derivatives

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Abstract

This work describes the preparation of tetracyclic diterpenoids and determination of their plant growth regulator properties. Stevioside (**2**) was used as starting material and the derivatives 13-hydroxy-*ent*-kaur-16-en-19-oic acid (steviol, **3**), *ent*-7 α ,13-dihydroxy-kaur-16-en-19-oic acid (**4**), 13-hydroxy, *ent*-kaur-16,17-epoxi-19-oic acid (steviol epoxide, **5**), 17-hydroxy-16-ketobayeran-19-oic acid (17-hydroxyisosteviol, **6**), 17-hydroxy-16-hydroxyiminobayeran-19-oic acid (**7**), 16-ketobayeran-19-oic acid (isosteviol, **9**), 16,17-dihydroxybeyeran-19-oic acid (**8**), and 16-hydroxyiminobayeran-19-oic acid (isosteviol oxime, **10**) were obtained by simple chemical procedures. Another derivative, *ent*-7 α ,13-dihydroxycaur-15-en-19-oic acid (**4**), was obtained by biotransformation of steviol (**3**) by *Penicillium citrinum*. In order to determine the plant growth regulator activity the compounds were submitted to the lettuce hypocotyl and barley aleurone bioassays. All compounds showed significant activities in both bioassays. Steviol (**3**) and isosteviol (**9**) were also tested in field-grown grapes resulting in an increase in berry weight and size.

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1. Introduction

Gibberellins are important plant growth hormones with agricultural applications. They influence a variety of physiological properties like germination, stem elongation, flowering and others (Mander, 2003). Because some of them are produced by microorganisms, various fermentative processes have been used for their commercial production. Gibberellic acid (GA₃, Fig. 1, **1**), the most important commercial gibberellin, is produced by fermentation by some fungi, like *Gibberella fujikuroi*. The inherent high costs of fermentation processes limit the applications of these compounds. The search for cheaper alternatives are, therefore, justified.

Some diterpenoids of the kaurene series have gibberellin-like activity. An early work described the weak activity of steviol (**3**) on the elongation of d-5 dwarf mutant of maize seedlings (Ruddat et al., 1963). The activity of steviol on d-5 and an-1 mutants was confirmed and extended it to its relatives (–)-kaur-16-en-19-ol and (–)-kaur-16-en-19-oic acid; modifications at C-17 methylene resulted in loss of activity (Katsumi et al., 1964). Another report described the activities of steviol, (–)-kaur-16-ene and (–)-kauran-19-ol in other systems (Brian et al., 1967). Some kaurane, beyerane and atisane diterpenoids, isolated from *Elaeoselinum* sp., were tested on six bioassays and some compounds were more active than GA₃ (Villalobos et al., 1994). These reports, therefore, indicated the potential of tetracyclic diterpenes other than gibberellins as plant growth regulators.

It has been suggested that, instead of having biological activity itself, steviol acts as a precursor and it is transformed into active gibberellins *in vivo* (Alves and Ruddat,

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1979). The conversion of steviol into gibberellins by gibberellin-producing fungi also support that claim (Bearder et al., 1975; Murofushi et al., 1982). It may be related to the fact that kaurene itself is a key intermediate in the biosynthesis of gibberellins. Therefore, steviol could be a valuable starting material for the preparation of potentially active derivatives.

Steviol (**3**) can be obtained in large scale from the natural sweetener stevioside (**2**), a natural glycoside extracted from *Stevia rebaudiana* which is produced commercially in several countries. Although steviol itself has a weak gibberellin activity the modification of its molecule could provide more active derivatives. In our continuing work for obtaining bioactive derivatives of steviol we now describe the preparation of derivatives by chemical and biotransformation methods. The compounds were then tested for plant growth activity using the lettuce hypocotyl and barley aleurone bioassays. Steviol (**3**) and isosteviol (**9**) were also tested on field-grown grapes for cluster and berry evaluation.

2. Results and discussion

The test compounds were prepared according to known procedures and their structures were determined by spectroscopic methods. They can be grouped according to their carbon skeleton. Steviol and its relatives have the *ent*-kaurene carbon skeleton (compounds **3–5**) whereas isosteviol and derivatives have the beyerene skeleton (compounds **6–10**). The main difference between these two groups is the spatial arrangement of rings C and D. All compounds were submitted to the lettuce hypocotyl and barley aleurone bioassays and GA₃ was used as positive control and for method validation.

2.1. Lettuce hypocotyl bioassay

Solutions of the test compounds, and GA₃, were prepared in the range 10⁻¹¹ to 10⁻⁶ M, applied to lettuce seeds, and after 3 days the hypocotyls were measured. The results, summarized in Fig. 2, show that all compounds promoted an increase on hypocotyls length relative to the control, and that the beyeranes (Fig. 2B) were more active than the kauranes (Fig. 2A).

The kaurane compounds, namely steviol (**3**) and its derivatives 7β-OH (**4**) and epoxide (**5**) (Fig. 2A), were significantly more active than GA₃ (**1**), at concentrations ≤10⁻¹⁰ M (*P* < 0.05) but the means of these compounds, at the same concentration, were not significantly different. At higher concentrations, however, they were less active than GA₃ with the exception of 7β-hydroxysteviol (**4**) which, at 10⁻⁷ M, was comparable do GA₃ at 10⁻⁶ M. The gibberellin-like activity of steviol (**3**) is known (Ruddat et al., 1963) and was confirmed in this experiment. Its conversion into the more polar derivatives 7-hydroxy (**4**) and epoxide (**5**), however, did not result in increased activity at the lowest concentrations.

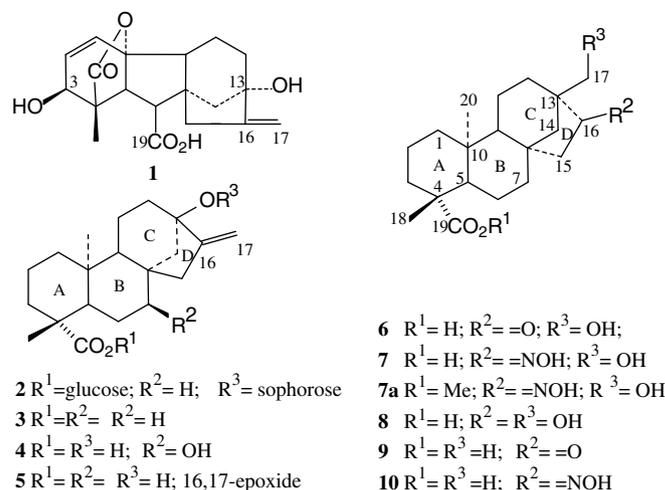


Fig. 1. Chemical structures of gibberellic acid (**1**), stevioside (**2**), steviol (**3**), 7β-hydroxysteviol (**4**), steviol epoxide (**5**), 17-hydroxyisosteviol (**6**), 17-hydroxy-16-hydroxyiminobayeran-19-oic acid (**7**), 16,17-dihydroxybeyeran-19-oic acid (**8**), isosteviol (**9**) and isosteviol oxime (**10**).

All beyerene compounds tested (Fig. 2B) were more active than GA₃ at concentrations <10⁻⁹ M (*P* < 0.05). Isosteviol (**9**) and its oxime (**10**) were the most active but at the highest concentration the activity decreased. Modification of the carbonyl at C-16 of isosteviol (**9**) alone (conversion to -OH or =NOH), therefore, did not result in significant change in activity. The presence of a hydroxyl at C-17 (**6** and **7**), however, decreases the activity of these beyeranes, especially at the lowest concentrations.

2.2. Barley aleurone bioassay

The results of the barley aleurone bioassay (Fig. 3) showed that all compounds significantly induced the formation of α-amylase (*P* < 0.05). Although less active than gibberellic acid (**1**) they were more potent at the lowest concentration. Comparing the compounds with kaurane skeleton (Fig. 3A) the 7β-OH (**4**) and epoxide (**5**) derivatives of steviol (**3**) were significantly more active than the parent compound at concentrations <10⁻⁷ M.

For the compounds with beyerane skeleton there was a significant increase in the response at the lowest concentrations. The modification (Fig. 3B) of the parent compound, isosteviol (**9**), was more effective with its conversion to the corresponding oxime (**10**).

2.3. Activity of steviol and isosteviol on grapes

The effect of GA₃ on grape development has been known for a long time and its application on vines is now a standard practice (Read and Gu, 2003). Among other effects, its application in the form of potassium salt results in looser, longer and heavier clusters as well as heavier and enlarged berries. The effect of other diterpenoids, like steviol and isosteviol, however, has not yet been described. In view of the positive response of the test

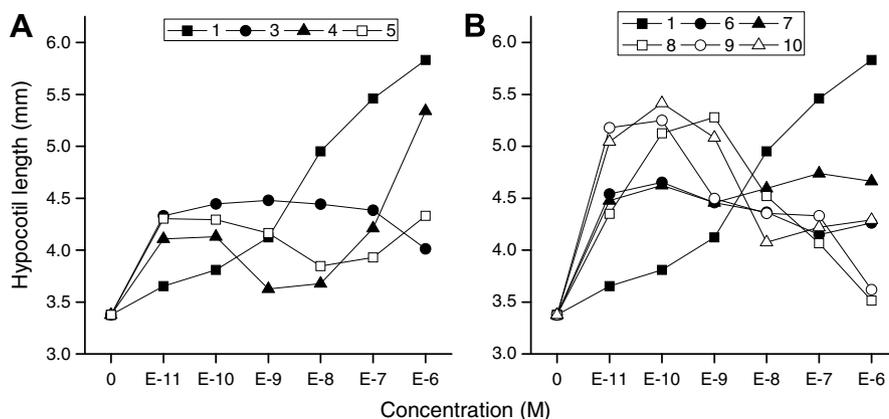


Fig. 2. Activities of the test compounds against water (control) and GA₃ (1) on the lettuce hypocotyl bioassay. (A) Kauranes (3 = steviol; 4 = 7 β -hydroxysteviol, 5 = steviol epoxide). (B) Beyeranes (6 = 17-hydroxyisosteviol, 7 = 17-hydroxy-16-hydroxyiminobayeran-19-oic acid, 8 = 16,17-dihydrox-beyeran-19-oic acid, 9 = isosteviol, 10 = isosteviol oxime).

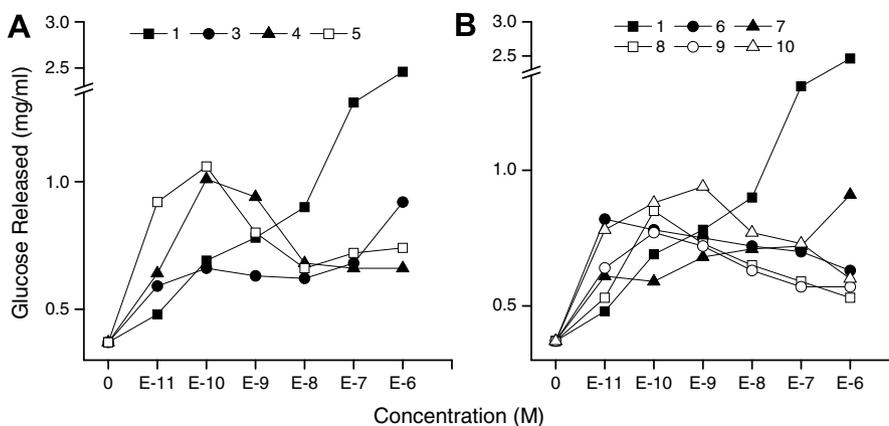


Fig. 3. Activities of the test compounds against water (control) and GA₃ (1) on the barley aleurone bioassay. (A) Kauranes (3 = steviol; 4 = 7 β -hydroxysteviol, 5 = steviol epoxide). (B) Beyeranes (6 = 17-hydroxyisosteviol, 7 = 17-hydroxy-16-hydroxyiminobayeran-19-oic acid, 8 = 16,17-dihydrox-beyeran-19-oic acid, 9 = isosteviol, 10 = isosteviol oxime).

compounds on the previously described bioassays we have decided to test the effects of steviol and isosteviol on field-grown grapes in comparison with GA₃. The compounds were tested at concentrations of 30 and 60 ppm and the results are summarized in Fig. 4.

The first parameter considered was cluster weight which, despite being larger for the test compounds, could not be considered statistically significant ($P < 0.05$) due to a large s.d.. The same happened with cluster size (data not shown). The berry weight (Fig. 4B) and diameter (Fig. 4D), on the other hand, significantly increased after treatment with steviol (3), at 30 ppm when compared to the control. It is interesting to notice that the results with steviol were higher than those of isosteviol at 30 ppm. At 60 ppm, however, isosteviol was more active. The concentration of soluble solids (data not shown), on the other hand, were not significantly different from the control.

2.4. Structure–activity relationship considerations

Structure–activity studies of gibberellins have shown some general trends. The gibberane carbon skeleton, the

β -hydroxyl at C-3, the free carboxyl at C-19, the C-13 hydroxyl group, the lactone bridge and the 16–17 methylene group are important for higher activity, although not essential (Brian et al., 1967; Hoard et al., 1981; Serebryakov et al., 1984). These features are present in GA₃ (1). For some species small changes in the gibberellin structure may lead to a reversal of its activity. In graminaceous species (barley and wheat), for example, exo-16,17-dihydro-GA₅-13-acetate act as a very potent growth retardant (Rademacher, 2000).

For other diterpenoids like kauranes and beyeranes, however, there are no structure–activity studies available due to the few published data on the plant growth regulation properties of these compounds. Based on our results beyeranes are more active than kauranes on the hypocotyl system, and modification of isosteviol (9) did not improve potency. On the aleurone bioassay, however, kauranes were more active especially the oxidized derivatives of steviol (3). In order to have a broader perspective on the structure–activity relationship of these compounds more data are necessary.

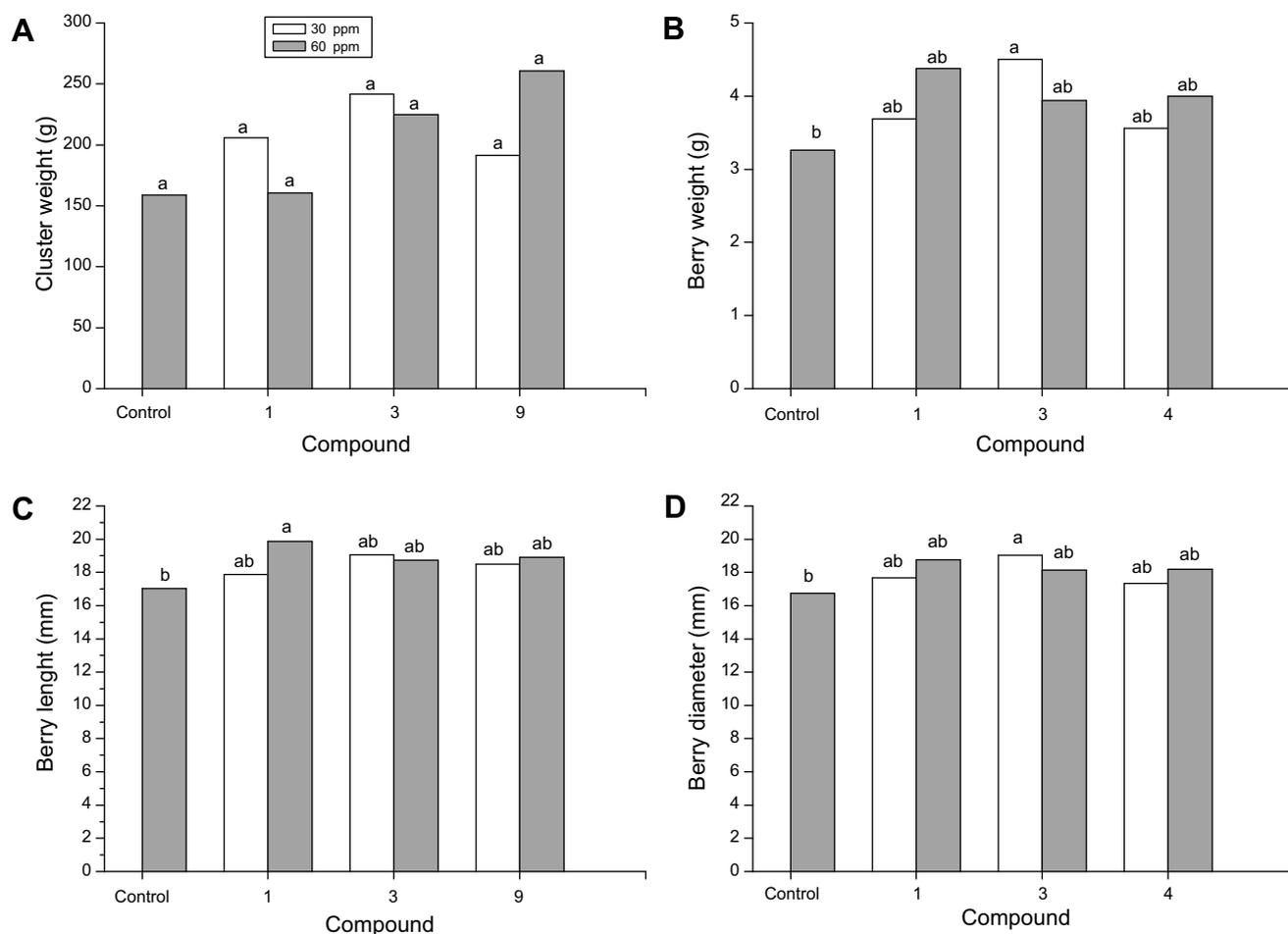


Fig. 4. Activities of GA₃ (1), steviol (3) and isosteviol (9) against control (water) on grape cluster weight (A), berry weight (B), berry length (C) and berry diameter (D). Values of columns with the same letter are not significantly different at $P < 0.05$.

3. Conclusions

The results above show that kaurane and beyerane tetracyclic diterpenoids are promising alternative to gibberellins for agriculture applications. The positive results of steviol and isosteviol on field-grown grapes confirmed the bioassays indications of the potential use of steviol and derivatives as plant growth regulators.

4. Experimental

4.1. General experimental procedures

IR spectra were recorded in KBr discs using a Bio-Rad spectrometer model FTS3500GX. NMR spectra were acquired with Bruker spectrometer models Avance DRX400 and Avance DPX 200 using TMS as internal standard; 1D and 2D experiments (DEPT, 1H–1H COSY, HMQC, HMBC, NOESY) were carried out for structure determinations. Mass spectra were recorded in an API 3000 System from Applied Biosystems/MDS Sciex, whereas high resolution mass spectra employed a Micro-

mass Q-ToF from Waters. Solvents were distilled before use. TLC was performed on 0.2 mm thick silica prepared plates from Merck. Spot visualizations were made by spraying with H₂SO₄/EtOH (1:1) followed by heating and inspection under UV light. Planar centrifugal chromatography was performed with a Chromatotron (Harrison Research, Palo Alto, CA, USA) model 7924T.

4.2. Test compounds

The known compounds steviol (13-hydroxy-*ent*-kaur-16-en-19-oic acid, 3) (Ruddat et al., 1965; Ogawa et al., 1980), steviol epoxide (13-hydroxy, *ent*-kaur-16,17-epoxy-19-oic acid, 5) (Avent et al., 1990b), 17-hydroxyisosteviol (17-hydroxy-16-ketobayeran-19-oic acid, 6), isosteviol (16-ketobayeran-19-oic acid, 9), (Avent et al., 1990a) and isosteviol oxime (16-hydroxyiminobayeran-19-oic acid, 10) (Al'fonsov et al., 2003) were prepared according to the literature methods. *ent*-7 α ,13-Dihydroxy-kaur-16-en-19-oic acid (4) was prepared by the biotransformation of steviol (3) with *Penicillium citrinum* or *G. fujikuroi* (Shigematsu et al., 1982). The spectroscopic data of those compounds were in accordance with those in the literature.

4.2.1. 17-Hydroxy-16-hydroxyiminobayeran-19-oic acid (7)

17-Hydroxyisosteviol (**6**, 333 mg) was dissolved in MeOH/H₂O (4:1, 50 ml), NaOAc (120 mg) and hydroxylamine hydrochloride (139 mg) were added and the mixture was stirred at room temperature for 24 h. Water was then added and the mixture was extracted with EtOAc (3 × 50 ml). The organic phase was dried with Na₂SO₄, filtered and the solvent evaporated. The residue was purified by planar centrifugal chromatography on silica producing 240 mg of the product (74% yield). A portion of the product was treated with ethereal CH₂N₂ and the ester (**7a**) was obtained as an amorphous solid.

IR ν_{\max} (cm⁻¹): 3450, 2922, 2858, 1708, 1650, 900. ¹H NMR (400 MHz, CDCl₃) δ : 3.62 (*s*, 3H, OMe), 3.64 (*d*, 1H, H-17; *J* = 10.8 Hz), 3.55 (*d*, 1H, H-17; *J* = 10.8 Hz), 1.18 (*s*, 3H, H-18), 0.75 (*s*, 3H, H-20). ¹³C NMR (CDCl₃) δ : 13.1 (C-20), 18.9 (C-2), 19.9 (C-11), 21.7 (C-6), 28.8 (C-18), 34.1 (C-12), 37.0 (C-14), 38.0 (C-3), 38.1 (C-13), 39.9 (C-7), 40.8 (C-1), 41.0 (C-10), 43.8 (C-4), 49.4 (C-8), 51.1 (C-15), 55.6 (C-9), 57.1 (C-5), 67.0 (C-17), 170.4 (C-16), 177.9 (C-19). HRMS *m/z*: 363.2415 [M]⁺ (calcd for C₂₁H₃₃NO₄: 363.2409).

4.2.2. 16,17-Dihydroxybayeran-19-oic acid (8)

17-Hydroxyisosteviol (**6**, 333 mg) was dissolved in EtOH (100 ml), NaBH₄ (500 mg) was added, and the mixture was stirred at room temperature for 5 h. The reaction was then quenched with 10% HCl (50 ml), and the organic solvent was evaporated. The aqueous mixture was then extracted with EtOAc (3 × 50 ml), the organic phase was dried, filtered and the solvent evaporated. The solid residue was purified by planar centrifugal chromatography on silica producing 226 mg of a solid (68% yield).

IR ν_{\max} (cm⁻¹): 3550, 3450, 2982, 2923, 1715. ¹H NMR (400 MHz, CDCl₃ + (CD₃)₂SO) δ : 4.15 (*dd*, 1H, H-16, *J* = 10.9 and 4.6 Hz), 3.36 (*d*, 1H, H-17; *J* = 10.4 Hz), 3.44 (*d*, 1H, H-17; *J* = 10.4 Hz), 1.18 ppm (*s*, 3H, H-18), 0.87 ppm (*s*, 3H, H-20). ¹³C NMR (400 MHz, CDCl₃) δ : 13.1 (C-20), 18.6 (C-2), 19.6 (C-11), 21.5 (C-6), 28.8 (C-18), 29.1 (C-12), 37.8 (C-3), 37.9 (C-10), 39.7 (C-1), 41.7 (C-7), 41.6 (C-8), 41.9 (C-13), 43.0 (C-4), 46.3 (C-14), 49.9 (C-15), 56.3 (C-9), 56.7 (C-5), 69.8 (C-17), 77.2 (C-16), 179.7 (C-19). HRMS *m/z*: 336.2285 [M]⁺ (calcd for C₂₀H₃₂O₄: 336.2300).

4.3. Hypocotyl bioassay

Stock solutions (1000 μ g ml⁻¹) of the test compounds as well as GA₃ (**1**, positive control) were prepared in acetone, and dilutions were made in H₂O in order to make 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M. Lettuce seeds (60) were soaked in the solutions for 12 h and then distributed among Petri dishes (20 seeds/dish), containing a disk of filter paper previously moistened with 2 ml of water, and the dishes were then left in a propagation room with controlled

temperature, humidity and illumination. After 3 days the hypocotyls and main roots were measured with a digital caliper (Hoad et al., 1981).

4.4. Barley aleurone bioassay

Seeds of barley (*Hordeum vulgare*) were treated with H₂SO₄ 50% for 2 h, washed and dried. They were then cut in half, de-embryonated and irradiated with UV light (254 nm) for 15 min. The half seeds (10) were then transferred to sterilized 10 ml glass vials containing 1.0 ml of acetate buffer (0.2 mM, pH 4.8) and 20 mM of CaCl₂. Solutions of the test compounds (10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M, 1 ml) were then added and the incubation was carried out in a shaker at 28 °C, 140 rpm and in the dark for 36 h. The hydrolysates were then centrifuged at 2000 rpm for 10 min and the supernatant was analyzed for their content in reducing sugar using the DNS procedure. All experiments were made in triplicate (Jones and Varner, 1967).

4.5. Activity of steviol and isosteviol on grapes

The experiment was carried out in a commercial vine (*Vitis vinifera* cv. Venus), in Ponta Grossa (Paraná state, Brazil) which has been in production for 13 years. Solutions of steviol (**3**), isosteviol (**9**) and GA₃ (**1**) at 30 and 60 ppm in H₂O were prepared and tested. The application was 15 days after flowering by dipping clusters for 30 s in the test solutions. The weights and sizes of clusters and berries, as well as the soluble solids concentration (°Brix), were evaluated 120 days later. The experiment was conducted in a fully randomized trial with four repetitions of 10 clusters per parcel.

4.6. Statistical analysis

Data were analyzed using OriginPro 7.5 software (OriginLab Corporation). The means were considered significantly different when *P* < 0.05 (Tukey's ANOVA).

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