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ORIGINAL PAPER

Growth-active gibberellins overcome the very slow shoot growth of *Hancornia speciosa*, an important fruit tree from the Brazilian "Cerrado"

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Abstract Shoot elongation of *Hancornia speciosa*, an endangered tree from the Brazilian savannah "Cerrado", is very slow, thus limiting nursery production of plants. Gibberellins (GAs) A_1, A_3, and A_5, and two inhibitors of GA biosynthesis, trinexapac-ethyl and ancymidol were applied to shoots of *Hancornia* seedlings. GA_1 and GA_3 significantly stimulated shoot elongation, while GA_5 had no significant effect. Trinexapac-ethyl and ancymidol, both at 100 μg per seedling, inhibited shoot elongation up to 45 days after treatment, though the effect was statistically significant only for ancymidol. Somewhat surprisingly, exogenous GA_3 more effectively stimulated shoot elongation in SD-grown plants, than in LD-grown plants. The results from exogenous application of GAs and inhibitors of GA biosynthesis imply that *Hancornia* shoot growth is controlled by GAs, and that level of endogenous growth-active GAs is likely to be the limiting factor for shoot elongation in *Hancornia*. Application of GAs thus offer a practical method for nursery production of *Hancornia* seedlings for outplanting into the field.

Keywords 3β-hydroxylated gibberellin · Ancymidol-Mangabeira · Shoot elongation · Trinexapac-ethyl · Tropical savannah

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Introduction

The "Cerrado" biome, the largest tropical savannah in the world, harbors over 6,000 native species of vascular plants (Mendonça et al. 1998). Forty-four percent of these are endemic, which makes the "Cerrado" flora the most diverse among the world's tropical savannahs (Klink and Machado 2005; Silva et al. 2006). Originally, the "Cerrado" occupied about 2,000,000 km^2 in the center of South America (da Silva and Bates 2002). However, more than half of the "Cerrado's original area has been transformed into pasture, cash crop agriculture, and other uses during the past 35 years (Klink and Machado 2005). The area transformed is in fact about three times the amount of land deforested to date in the Brazilian Amazon and the destruction of the "Cerrado" biome continues at a fast pace (Klink and Machado 2005). Myers et al. (2000) have ranked this ecosystem among the 25 most important terrestrial biomes under threat. Despite its rich biodiversity and its importance as a corridor for species inhabiting neighboring biomes, such as the Amazonian and Atlantic rainforests, the "Cerrado" has received much less attention from the conservation community than the Amazon or Atlantic forests. One approach now being used to promote biodiversity conservation in threatened ecosystems is the identification of products that can be sustainably harvested. *Hancornia speciosa*, popularly known as mangabeira, is one of the most endangered native fruit trees in Brazil (Moura et al. 2005). It is also one of the most promising fruit trees for programs of sustainable harvesting in the "Cerrado". For example, its fruit is a rich source of protein (up to 3% on a fresh weight basis), it has a highly desirable flavor for fresh consumption, and is used in the production of juice and ice cream (Correa 1978; Parente et al. 1985). Incorporation of *Hancornia* into horticultural systems has thus been proposed as a way to contribute to its conservation and also to ameliorate poverty in areas where *Hancornia* is native and grows well. However, the *Hancornia* seedling and mature tree has a very slow growth, as do many "Cerrado" trees, and this slow growth has appreciably reduced interest in propagating *Hancornia* as a fruit tree. In this paper, we demonstrate that seedlings of *Hancornia* can have their inherently slow shoot growth effectively reversed by exogenous applications of two highly growth-active gibberellins (GAs) GA_1, and GA_3 (Fig. 1) and that this GA-induced shoot growth requires the presence of a C-3β hydroxyl group on the GA molecule. We also show, through the use of inhibitors of GA biosynthesis, that *Hancornia* seedlings require GAs for their normal shoot elongation. Our results imply that the inherently slow growth of *Hancornia speciosa* may be due to reduced levels of endogenous growth-active gibberellins.

Methods

Plant material

Mature seeds were pooled from several parent plants and germinated in opaque black polyethylene bags (10 × 60 cm) with perforated bottoms. The bags were filled with washed sand and recently collected "Cerrado" soil (1:4, w/w). The soil, a red oxisoil, was collected from the top 30 cm of the soil profile and all dead root fragments separated out with a mesh and discarded. Germinated seeds (very young seedlings) were then planted in the plastic bags, which were assigned random positions in a glasshouse with natural sunlight. Watering was accomplished twice daily.

Chemicals

GA_3 was purchased from Sigma Chemical Co., St. Louis, MO, USA). We verified its purity (90%) by full scan gas chromatography–mass spectrometry (GC–MS). GA_1 and

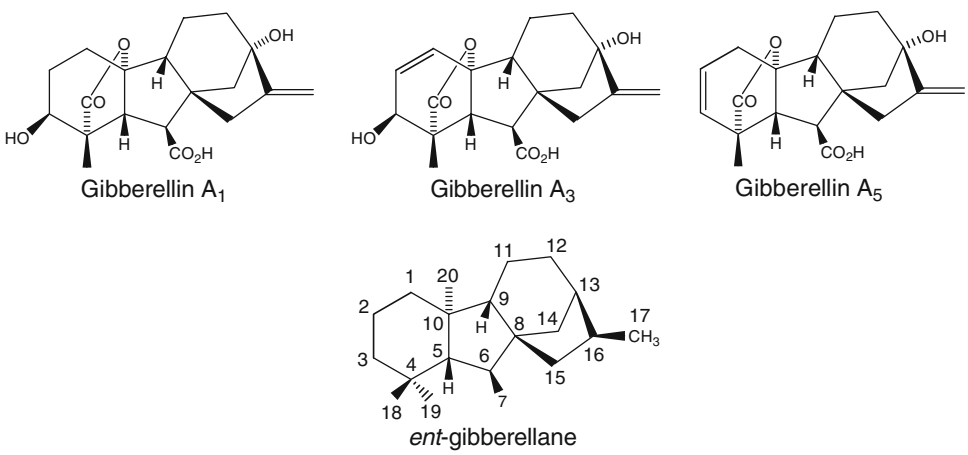

Fig. 1 Chemical structure of GA_1 and GA_3, two gibberellins presenting the growth-activation step, the 3β-hydroxylation, GA_5, a gibberellin with no C-3 hydroxyl group, and the *ent*-gibberellane ring system, the common feature of all of the gibberellins

GA_5 (purity certified to be over 99%) were purchased from Professor L. N. Mander, Research School of Chemistry, Australian National University, Canberra, ACT, 0200, Australia. Ancymidol [α-cyclopropyl-α-(ρ-methoxyphenyl)-5-pyrimidinemethanol] was purchased from Dow AgroSciences, Indianapolis, IN, USA. Trinexapac-ethyl [4-(cyclopropyl-α-hydroxy methylene)-3,5-dioxocyclohexane carboxylic acid ethyl ester], also known as cimectacarb or Primo^{TM}, was purchased from Novartis Inc. (Greensboro, NC).

Application of gibberellins and inhibitors of gibberellin biosynthesis to *Hancornia* seedlings grown under natural day lengths

Seventy per cent aqueous acetone (v/v) microdrops (5 μL) containing 0, 1, 10 or 100 μg of GA_1 and GA_5 or aqueous solutions of ancymidol and trinexapac-ethyl (0, 1, 10 or 100 μg per seedling) were pipetted onto the shoot apex of 6 weeks-old seedlings. Only a single application was used. Control seedlings for the GA treatments received 5 μL of aqueous acetone microdrops, while control seedlings for the ancymidol and trinexapac-ethyl treatments received 5 μL of distilled water microdrops. Elongation, e.g., cumulative increase in shoot length after treatments, and the number of new nodes produced on each tree's shoot were recorded at intervals of 15 days for 60 days following treatment, under naturally lengthening day lengths (spring/summer).

Application of GA_1 and GA_3 to *Hancornia* seedlings grown under natural day lengths

In a separate trial, GA_1 and GA_3 were applied at 10 μg per seedling in 70% (v/v) acetone microdrops under naturally lengthening day lengths (spring/summer) as described above. Seedlings were 6 weeks old at the time of GA application.

Application of GA_3 to *Hancornia* seedlings grown under controlled day lengths

Plants (128) were organized into four blocks within each of 2 day-length treatments in the glasshouse. Within these four blocks plants were arranged randomly. For the short-day (SD) treatment the plants were covered with opaque black polyethylene boxes (1 × 1 × 1 m) from 1800 to 0700 hours, e.g., they received about 11 h of light, a day length very close to the minimum natural day length of 11.12 hours found for the study site. For the long-day (LD) treatment, supplemental light (30 μmol m^{-2} s^{-1}) was provided by incandescent bulbs from 1730 to 1900 hours, e.g., the LD treatment plants received about 13 h of light, a day length very close to the maximum day length of 12.88 hours found for the study site. Half of the plants in each of the SD and LD treatments were given one application of 100 μg per seedling of GA_3 in aqueous acetone, as described above. Plants were 6 weeks old at the time of GA_3 application.

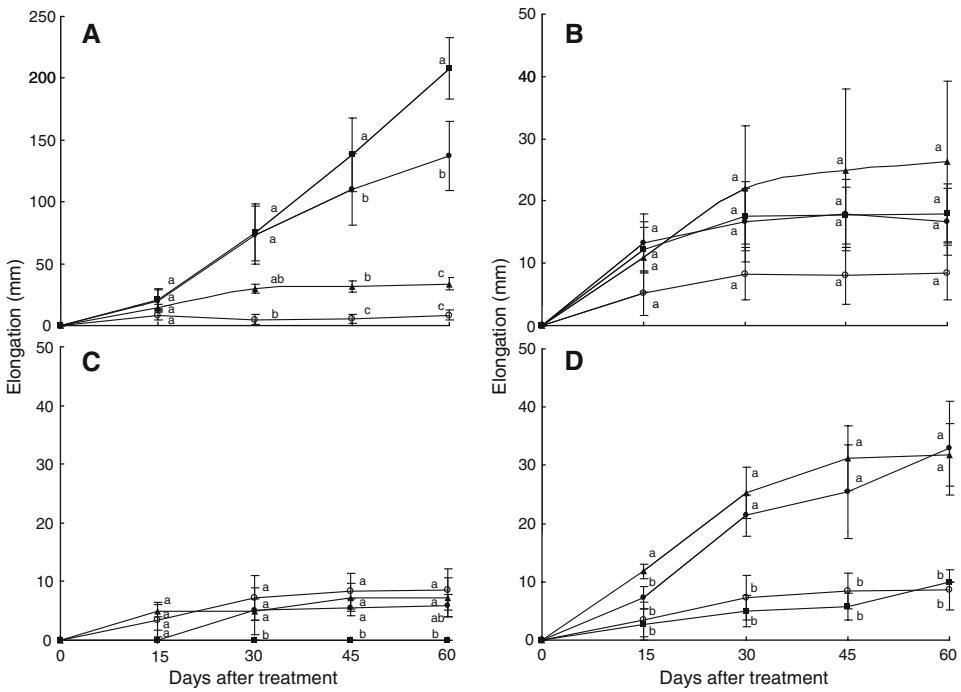

Fig. 2 Time course of shoot elongation for *Hancornia speciosa* in response to one application of GA_1 (**a**), GA_5 (**b**), ancymidol (**c**) or trinexapac-ethyl (TNE, **d**) at 1 (*filled triangle*), 10 (*filled circle*) and 100 (*filled square*) μg per seedling. Control (*open circle*) plants were treated with seventy per cent aqueous acetone (v/v), for the GA_1 and GA_5 experiments, or water, for the ancymidol and trinexapac-ethyl (TNE). Data are means of six replicates ±standard error. Based on Student's *t* test, average elongation for different treatments followed by the same letters do not differ at $P = 0.05$ within each of the days after treatment. *Vertical bars* indicate ±one standard error of the mean

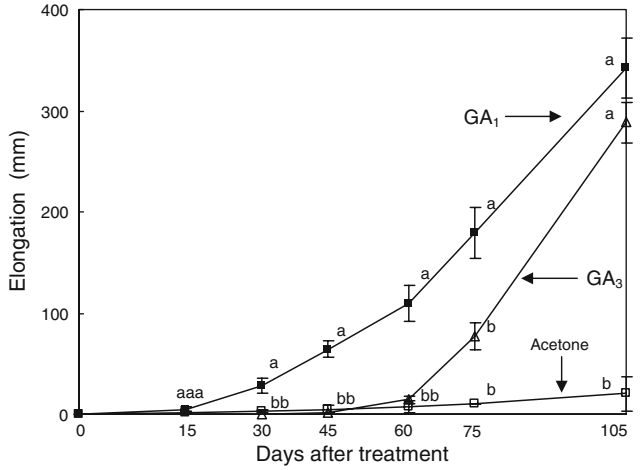

Fig. 3 Time course of the shoot elongation of *Hancornia speciosa* in response to application of GA_1 and GA_3 at 10 μg per seedling. Data are means of six replicates ±standard error. Based on Student's *t* test, average elongation for different treatments followed by the same letters do not differ at $P = 0.05$ within each of the days after treatment. *Vertical bars* indicate ±one standard error of the mean

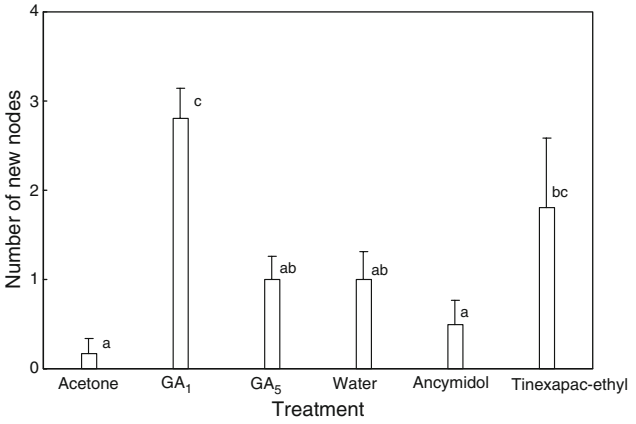

Fig. 4 Comparative effects of applications of 10 μg per seedling of GA_1, GA_5, ancymidol, and trinexapac-ethyl on the formation of new nodes in *Hancornia speciosa*, on day 60 after the treatment. Control plants were treated with acetone, for the GA_1 and GA_5 experiments, or water, for the ancymidol and trinexapac-ethyl (TNE). Data are means of six replicates ±standard error. Based on Student's *t* test, average number of new nodes for different treatments followed by the same letters do not differ at $P = 0.05$. *Vertical bars* indicate ±one standard error of the mean

Statistical analyses

Except for the photoperiod trial, each experiment consisted of six individual seedlings per treatment. Each experiment was repeated at least twice, yielding similar results. An analysis of variance (ANOVA) was performed using the entire data set. Differences between means for treatments were also tested using the Student's *t* test ($P = 0.05$).

Results

There was a progressive increase in shoot elongation with increased doses of GA_1 (Fig. 2a). Sixty days after the treatment, seedlings treated with 100 μg GA_1 showed elongation that was 23.5-fold greater than the acetone-only controls (Fig. 2a). GA_3 at a dose of 10 μg per seedling was also able to significantly ($P = 0.05$) enhance elongation in *Hancornia* shoots (Fig. 3), although there was a (surprising) ca. 40-day lag in response time for GA_3, relative to the shoot's response to GA_1 (Fig. 3). The increased growth in shoot elongation seen for GA_5-treated seedlings, relative to the acetone-treated control seedlings (Fig. 2b) did not reach statistical significance at $P = 0.05$.

By day 30 after the GA biosynthesis inhibitor, ancymidol, was applied at 100 μg per seedling, the *Hancornia* seedlings showed a significantly ($P = 0.05$) reduced elongation (Fig. 2c). Fifteen days after the treatment with the 1 μg dose of the late stage GA biosynthesis inhibitor, trinexapac-ethyl (TNE), the *Hancornia* seedlings had shown a significant ($P = 0.05$) increase in shoot elongation, relative to untreated control seedlings (Fig. 2d).

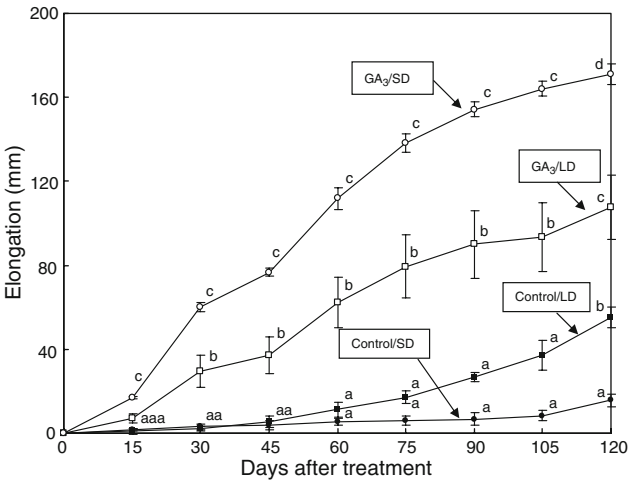

Fig. 5 Time course of the shoot elongation of *Hancornia speciosa* in response to day length (SD or LD) treatments, with and without a single initial application of 100 μg per seedling of GA_3. Data are means of 32 replicates ±standard error. Based on Student's *t* test, average elongation for different treatments followed by the same letters do not differ at $P = 0.05$ within each of the days after treatment. *Vertical bars* indicate ±one standard error of the mean

A similar response occurred for seedlings treated with the 10 μg dose of TNE. However, at the 100-μg dose of TNE there was only a statistically non-significant trend for decreased elongation (day 45), relative to elongation of seedlings treated with water (control).

Seedlings treated with 10 μg of GA_1 also showed a significant increase in the number of new nodes formed, 60 days after the treatment (Fig. 4). Similarly, seedlings treated with 10 μg of TNE also tended to have increased

numbers of new nodes, though this increase was not statistically significant.

For plants not treated with growth regulators, the SD treatment (11 h of light) led to significantly ($P = 0.05$) reduced shoot elongation (Fig. 5) in comparison with plants maintained under LD (13 h of light), both being assessed at day 120 after the initiation of the experiment. GA_3 application increased shoot elongation under both SD and LD treatments, relative to controls which received no GA_3, and this response was seen quite soon after GA_3 application (Fig. 5). Surprisingly, SD-grown plants that were treated with 100 μg of GA_3 per seedling showed an increase in shoot elongation that was 1.7-fold greater than that seen for GA_3-treated seedlings grown under LD.

Discussion

Shoot growth for many native woody perennials found in the "Cerrado" is extremely slow (Hoffmann 2000; Franco 2002). This slow growth has been attributed to inherent low growth rates and also other factors such as light irradiance limitation by canopy shading (Franco 2002). There are two cellular processes controlling shoot elongation and thus final plant height, cell proliferation, and cell elongation. At the cellular level, gibberellins (GAs) have been demonstrated to promote both cell division and cell elongation (Kende and Zeevaart 1997). The major shoot growth-active GAs, including GA_1, GA_3, GA_4, and GA_7, have several common structural attributes, namely a hydroxyl group on C-3β, a carboxyl group on C-6, and a lactone between C-4 and C-10 (Yamaguchi 2008).

We have used a physiological approach to investigate the putative role of GAs in the process of shoot elongation in seedlings of the very slow-growing "Cerrado" fruit tree, *Hancornia speciosa*. Our approach has utilized applications of several well-characterized GA biosynthesis inhibitors (growth retardants) as well as application of GAs of three structural types to glasshouse-grown seedlings of *Hancornia*.

Application of GAs

GA_1 and GA_3, two well-known shoot growth-active GAs (Hedden and Croker 1992; Jones 1973; Zeevaart et al. 1993), significantly stimulated shoot growth (both elongation and number of new nodes) of *Hancornia* seedlings (Figs. 2a, 3). In contrast, GA_5, a 2,3 deoxy GA without a 3β-hydroxyl group, showed no statistically significant effect on either shoot elongation (Fig. 2b) or formation of new nodes (Fig. 4).

Recent physiology research with trees has shown that endogenous GAs, and specifically GA_1, are causal for stem growth, including internode elongation (Eriksson et al. 2000). The exceptional elongation response of the *Hancornia* seedlings to application of GA_1 (Fig. 2a) strongly implies that the *Hancornia* seedling's natural internode elongation may be limited by exceptionally low levels of GA_1 or other per se growth-active GA. A similar exceptional increase in the number of nodes seen from a 10-μg dose of GA_1 (Fig. 4) leads to the same conclusion. Effects obtained from application of GA_3 (Fig. 3 and data not shown) confirm the GA_1 results. In contrast, there was only a non-significant trend toward increased stem elongation and increased numbers of new nodes in response to GA_5 application. This implies that *Hancornia* seedlings may have a very reduced level of GA_3-oxidase activity, which otherwise might convert GA_5 into the growth-active GA_3 (Durley et al. 1973; Fujioka et al. 1990; Moritz and Monteiro 1994; Poole et al. 1995; Wolbang et al. 2004) and/or that GA_5 has very low per se growth-activity. Finally, the fact that *Hancornia* responds strongly to exogenous application of the per se growth-active GAs, GA_1, and GA_3, indicates that a defect in the GA signal transduction pathway is not likely to be responsible for the inherently slow shoot elongation rate of the young *Hancornia* control seedlings. Nor, is *Hancornia* likely to have exceptionally high levels of GA2-oxidase (which rapidly de-activates GA_1 and GA_4), given the sustained growth elongation effects of even low doses of GA_1 (Figs. 2a, 3). That said, TNE at low doses did promote shoot elongation—see discussion below.

Application of inhibitors of GA biosynthesis

The use of ancymidol, an inhibitor of monoxygenases that catalyze early steps of GA biosynthesis (Rademacher 2000) significantly reduced growth at 100 μg per seedling. Furthermore, the use of TNE, a "late stage" GA biosynthesis inhibitor, did tend to decrease shoot elongation at the relative high dose of 100 μg per plant. These results indicate that *Hancornia* seedlings do utilize endogenous GAs as growth effectors. Alternatively (or additionally), *Hancornia* seedlings could also have a relatively high level of GA inactivation, via C-2β hydroxylation, e.g., see the significant increase in elongation that results from use of the 1 and 10 μg doses of TNE (Fig. 2d). The TNE-induced elongation increases seen at 1 and 10 μg per seedling were, however, not related to significant changes in the number of nodes formed by the *Hancornia* shoots. Thus, the TNE-driven elongation increase is due primarily to increased internode length. TNE is an acylcyclohexanedione with close structural similarities to 2-oxoglutarate, the essential co-substrate of soluble dioxygenases (2-ODDs) involved in the late stages of GA biosynthesis. Thus, TNE is assumed to competitively block late steps in the GAs biosynthesis

(Adams et al. 1992; Griggs et al. 1991; Rademacher 2000; Tan and Qian 2003). These late steps will not only include 3β-hydroxylation, the "activation" step, they will also include the "inactivation" step, e.g., inhibition of GA 2β-hydroxylation (Griggs et al. 1991; Nakayama et al. 1991). Thus, application of TNE can result in inhibition, a nil effect, or even promotion of elongation, depending on "balance" between the relative activity of the plant's inherent "activation" versus the "inactivation" steps. For example, treatment of stock (*Matthiola incana*) with 1 and 10 µg per plant of TNE resulted in enhanced shoot elongation (Hisamatsu et al. 2000a), while treatment with 60 µg of TNE inhibited shoot elongation (Hisamatsu et al. 2000b). This is analogous to what we see for *Hancornia* (Fig. 2d). Given the TNE results, *Hancornia* seedlings could very well have a naturally occurring high rate of C-2β hydroxylation (GA inactivation) in addition to low levels of C-3β hydroxylation (the "activation" step in GA biosynthesis). Such a combination, reduced synthesis of the growth-active "effector" GA_1, together with a rapid deactivation of GA_1 via C-2β hydroxylation, could very well explain *Hancornia*'s inherently slow rate of growth in the 'Cerrado'.

Application of GA_3 under controlled day lengths

In many woody species SD's induce cessation of shoot elongation, while LD's maintain shoot elongation. Current evidence indicates that changes in GA concentrations are, at least in part, responsible for day length regulation of shoot elongation (Yamaguchi 2008). Earlier experiments using *Brassica napus* var. *annua* (Dahanayake and Galwey 1999) plants grown under extended LD conditions (via supplemental incandescent light) and then treated with GA_3, showed that the GA_3 treatment yielded much longer shoots under LD, than comparable applications of GA_3 to plants grown under SD. We thus found it surprising that for *Hancornia* exogenous GA_3 more effectively stimulated shoot elongation in SD-grown plants, than in LD-grown plants (Fig. 5). The reason(s) for such an unusual differential response by the *Hancornia* seedlings remain to be elucidated.

Finally, the significant enhancement of *Hancornia* shoot elongation by the application of growth-active GAs, GA_1, and GA_3, described in this study is expected to result in the incorporation of *Hancornia* into horticultural systems, thus strengthening conservation efforts of this endangered native tree. If this happens, then the increased commercial use and sale of the fruit by local resident should also help to ameliorate poverty in areas where *Hancornia* is native. In addition, there is a possibility that GA-induced enhancement of shoot elongation might be a widespread response by slow-growing "Cerrado" trees. We expect to investigate this possibility in the near future on a range of species whose status is "threatened or endangered".

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