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New insight into how thigmomorphogenesis affects *Epipremnum aureum* plant development

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ABSTRACT

Climbing *Epipremnum aureum* plants develop larger leaves than unsupported, hanging plants. This effect may be regarded, in part, as a thigmomorphogenic response, but gravimorphogenetic effect may also be involved, since polar auxin transport is known to be negatively affected in plants with horizontal or hanging stems, which may result in an altered hormone balance at the whole plant level. The present work was aimed at studying how exogenous auxins and cytokinins may influence growth of *E. aureum* rooted cuttings under different training systems. Rooted cuttings of *E. aureum* were cultivated either climbing on an upright wooden board or creeping on the glasshouse bench or hanging from a basket. All leaves of each plant were sprayed to run-off at sunset with four indole-3-acetic acid (IAA) doses 7 days after transplanting and one week later, with four benzylaminopurine (BAP) concentrations, rendering 16 hormone combination treatments. The application of IAA or BAP at 50 mg L⁻¹ to creeping and hanging plants significantly promoted growth but, in climbing plants, a negative effect was generally observed. Changes in net assimilation and photosynthetic rates, together with modified allometric coefficients, accounted for these responses. The higher growth promotion by exogenous growth regulators observed in creeping or hanging plants compared to climbing plants, may be interpreted mostly as a gravimorphogenetic response.

Keywords: auxin, citokinin, foliage plants, gravitropism, leaf growth, leaf anatomy, photosynthesis.

RESUMO

Nova compreensão sobre como a thigmomorfogênese afeta o desenvolvimento de plantas de *Epipremnum aureum*

Plantas trepadeiras (*Epipremnum aureum*) conduzidas suspensas, desenvolvem folhas maiores em comparação com plantas conduzidas sem suporte (rasteiras). Esse efeito pode ser visto, em parte, como uma resposta thigmomorfogênica; mas o efeito gravimorfogênico também pode estar envolvido, uma vez que o transporte polar de auxinas é afetado negativamente em plantas com hastes horizontais ou pendentes, o que pode resultar em um balanço hormonal alterado, a nível da planta como um todo. O presente trabalho teve como objetivo estudar como as auxinas e citocininas exógenas podem influenciar o crescimento de estacas enraizadas de *E. aureum* sob diferentes sistemas de condução. Estacas enraizadas de *E. aureum* foram cultivadas como trepadeiras em uma tábua de madeira vertical ou rasteiras sobre bancada em casa de vegetação ou suspensas em uma cesta. Todas as folhas de cada planta foram pulverizadas até escoamento à tarde, com quatro doses de ácido indol-3-acético (AIA), 7 dias após o transplante e, após uma semana, com quatro concentrações de benzilaminopurina (BAP), resultando em 16 tratamentos combinados com hormônios. A aplicação de IAA ou BAP a 50 mg L⁻¹ em plantas rasteiras e suspensas, promoveu crescimento significativo mas, em plantas trepadeiras, observou-se, em geral, efeito negativo. Mudanças na assimilação líquida e taxas fotossintéticas, juntamente com coeficientes alométricos modificados, foram responsáveis por essas respostas. A maior promoção do crescimento por reguladores de crescimento exógenos, observada em plantas rasteiras ou suspensas em comparação com plantas trepadeiras, pode ser interpretada principalmente como uma resposta gravimorfogênica.

Palavras-chave: auxina, citoquinina, plantas de folhagem, gravitropismo,

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The Golden Pothos (*Epipremnum aureum*), a naturally variegated plant, is a tree-climbing vine native to the Solomon Islands. Golden Pothos varieties are among the most popular tropical ornamental hanging basket crops for interior landscape. In natural

tropical settings, 'pothos' can grow to be large plants, producing leaves up to 90 cm in length. However, plants used for interior landscape purposes usually have heart-shaped leaves that rarely exceed 15 cm in length. Leaf size depends, among other factors, on how

vines are trained. When plants grown in pots under commercial facilities are vertically supported, leaf size significantly increases in comparison to leaves from basket hanging plants (Di Benedetto *et al.*, 2010).

Native shade plants environments

are characterized by a low light intensity, and for this reason, carbon gain is an important aspect of plant performance. A strategy for maximizing light capture in several shade-adapted plants consists of exploring the vertical environment through the anchorage to an adjacent surface such as host plants, rocks or walls. These changes in plant growth habit modify, in turn, both shoot and root growth (Di Benedetto *et al.*, 2010).

Steinitz *et al.* (1992) showed that young *E. aureum* plants with soft stems grow in a vertical downwards hanging manner unless they encounter a surface on which they can climb and grow in a direction that is different from the one given by the gravitational vector. The climbing vine finds anchorage with adventitious aerial clasping roots appraised to an adjacent surface of host plants, rocks or walls.

Steinitz & Hagiladi (1987) and Steinitz *et al.* (1992) found that climbing *E. aureum* plants had not only larger leaves, but also shorter internodes and a better-developed aerial root system than unsupported plants that were kept stretched in an upright position by hanging them on a hook at the petiole of the youngest leaf. They suggested that the morphological differences between climbing and free hanging plants represent thigmomorphogenic responses of climbing plants to a mechanical stimulus generated by the contact of the stem with the support surface.

It is well known that many species, including other ornamental shade plants such as *Monstera obliqua* and *Philodendron scandens*, have developed sensory mechanisms to detect mechanical stimulation (Steinitz & Hagiladi, 1987). This stimulation often results in a suite of responses of anatomical, physiological, biochemical, biophysical and molecular nature, termed “thigmo-morphogenesis” (Li & Gong, 2011), a syndrome that is mediated by auxin, other hormones and signalling molecules as well (Chehab *et al.*, 2009).

However, when comparing climbing plant with plants hanging freely from a pot, an alternative or complementary explanation of the morphologic responses of Golden

Pothos to vine orientation may be given. It is known that stem orientation with respect to the gravity vector determines changes in plant morphology and physiology, a kind of response that has been named ‘gravimorphism’. Gravimorphic responses are associated with modified auxin flux, which is known to be inhibited by placing stems in horizontal or downward, instead of upward position. Auxins normally move in a polar manner from shoot to root apices, but in downward-pointing shoots, auxins accumulate near the shoot tip (Lovisolo *et al.*, 2002).

Auxins play key roles in organ development. Organ initiation at the shoot apical meristem is thought to result from localized accumulation of auxin at the site of future primordium development (Monshausen & Haswell, 2013). Auxins are known to promote the differentiation of lateral roots as well (Pacurar *et al.*, 2014). The latter is a very important control point of plant growth, since root apices are the main source of cytokinins. In turn, cytokinins, which are transported via xylem to the shoot apex, exert a multiple-faced growth promoting action in the aerial part of the plant. Thus, inhibition of polar auxin transport in horizontally or downwardly oriented stems may ultimately result in a decreased whole-plant growth (Keller, 2015). Gravimorphism and thigmomorphism may influence growth at the same time; for example, Steinitz *et al.* (1992) reported an interaction between *E. aureum* vine orientation and touch response, being only upwards growing vines those sensitive to touch stimuli.

Previous reports have shown that leaf size, leaf number (Di Benedetto *et al.*, 2013, 2015a) and leaf shape (Di Benedetto *et al.*, 2010; De Lojo & Di Benedetto, 2014) were significantly modified when *E. aureum* or *Monstera deliciosa* creeping plants were sprayed with a single 5, 50 or 100 mg L⁻¹ 6-benzylaminopurine (BAP) dose. Leaf area and whole-plant biomass accumulation increased with relatively low BAP application (5-50 mg L⁻¹), while 100 mg L⁻¹ BAP generally appeared as a supra optimal concentration. In these works, BAP-driven growth promotion

was associated with increased net assimilation rate (NAR) and net photosynthetic rate.

Exogenous sprays of indole acetic acid on *E. aureum* plants resulted in morpho-physiological responses that, largely resembled those obtained by BAP sprays, suggesting that growth promotion by exogenous application of both growth regulators may occur via the same signalling cascade (Di Benedetto *et al.*, 2015b).

Examining *E. aureum* growth patterns under different training systems and in response to exogenously supplied growth regulators may help to understand the contribution of mechanical stimuli and gravity to plant development. Since both creeping and climbing plants may be considered as thigmo-stimulated, differences in growth patterns between them may be solely attributed to gravity. If departure from upward position results in decreased growth due to an impaired auxin transport to the root system, then exogenous auxin and cytokinin application to the foliage should be a straightforward manner to overcome such restriction. The aim of this work was to study *E. aureum* growth under three different training systems (creeping, climbing or hanging downwards) and to evaluate how exogenously supplied auxin and cytokinin may modify it and, in this context, to gain insight on the hormonal regulation of thigmo-/gravimorphogenetic responses of *E. aureum* to vine orientation.

MATERIAL AND METHODS

Rooted cuttings of *E. aureum* were obtained from a commercial propagator (Vivero Kogiso, Buenos Aires, Argentina), and transplanted into rigid 1,200 cm³ plastic pots (one plant per pot). At the transplant stage, cuttings had on average 3.3±0.15 leaves, with 145.49±24.45 cm² leaf area and 7.57±0.83 g fresh weight per cutting. Pots were filled with a 40-40-20 (v/v/v) mix of *Sphagnum maguellanicum* peat: river waste: perlite. Plants were watered daily to saturation with high quality tap water (pH= 6.64; electrical conductivity = 0.486 dS m⁻¹) and were fertilized each

week with 50 mg L⁻¹ N as 1.0: 0.5: 1.0: 0.5 (v/v/v/v) N: P: K: Ca (nitric acid, phosphorus acid, potassium nitrate and calcium nitrate) via overhead irrigation water.

The experiment was carried out in a greenhouse at the Faculty of Agronomy, University of Buenos Aires, Argentina (34°28'S), from September 8th, 2007 to March 12th, 2008. Plants were cultivated either climbing (supported through an upright wooden board 5 cm in width and 100 cm in length, coated with black polyethylene), creeping on the glasshouse bench or hanging from a basket, to obtain three different growth habits. Both climbing and creeping plants were assumed thigmo-stimulated, being their aerial roots in contact with the respective surfaces (either wooden board or bench surface). Care was taken to ensure similar light exposure to the plants regarding the training system employed. The greenhouse was covered with a black shade light cloth for 50% full sun, divided into three blocks, and within each block, 160 plants were grown under each training system. The red:far-red ratio (R:FR) was not significantly modified by the shade-cloth, as shown by a 660/730 sensor (Skye Instruments, Wales, UK). Daily mean temperatures ranged between 21.80 to 37.50°C and daily photosynthetic active radiation between 14.80 to 25.64 molphotons m⁻² day⁻¹ during the experiment. Temperature and light intensity were recorded with three HOBO sensors (H08-004-02) (Onset Computer Corporation, MA, USA) connected to a HOBO H8 data logger.

Seven days after transplantation, all leaves of each plant were sprayed to runoff at sunset with IAA solutions (0, 5, 50, or 100 mg L⁻¹), followed 7 days later by sprays with BAP solutions (0, 5, 50, or 100 mg L⁻¹), rendering 16 hormone combination treatments. Chemicals were of highest purity (Sigma-Aldrich Co., St. Louis, MO, USA). To prepare growth regulators stock solutions, both IAA and BAP were dissolved in 80% (v/v) ethanol. No surfactants were used. Ten plants, either climbing, creeping or hanging, randomly distributed within each greenhouse block, were sprayed with each of the 16 growth regulators

combinations tested.

At 0, 60, 90, and 120 days after transplanting, two plants per block treated with each combination of growth regulators under each training system were destructively sampled. The number of leaves was recorded, and individual leaf area was determined using a LICOR 3000A automatic leaf area meter (LI-COR, Inc., Lincoln, NE, USA). Fresh weights (FW) of the different aerial parts i.e. leaf blades, petioles, and stems on each plant were determined. Dry weights (DW) were obtained after drying aerial parts and roots to constant weight at 80°C for 96 hours.

The relative rate of leaf area expansion (RLAE) was calculated as the slope of the regression of the natural logarithm (ln) of total leaf area versus time (in days). The rate of leaf appearance (RLA) was calculated as the slope of the number of visible leaves (including unrolled ones >1cm) versus time (in weeks). Relative growth rate (RGR) was calculated as the slope of the regression of the ln DW (whole plant) versus time (in days). Mean net assimilation rate (NAR) was calculated as:

$$NAR = \frac{k_w W_0 e^{k_a t}}{A_0 e^{k_a t}}$$

where W_0 : extrapolated value of total DW (g) at time zero; k_w : RGR (days⁻¹); A_0 : extrapolated value of leaf area (cm²) at time zero; k_a : RLAE (d⁻¹); t : time (days) at the midpoint of the experimental period and e : base of natural logarithm.

Mean leaf area ratio (LAR) was calculated as:

$$LAR = \frac{k_w}{NAR}$$

Since in most treatments plant biomass accumulation approached a plateau towards the end of the experiment, the last sampling (120 days after transplant) was not included for performing this analysis, which is best suited for plants growing exponentially.

Specific leaf area (SLA) was calculated as the ratio of individual leaf area to leaf DW (average of all plant leaves).

The allometric coefficients between root and shoot were calculated as the slope (β) of the straight-line regression

of natural logarithm root DW versus natural logarithm shoot DW.

The net photosynthetic rate was measured at ambient O₂ and CO₂ concentrations at a saturating photon flux density (>1,700 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) between 11.30-13.00 h on a sunny day just before final harvest. The youngest fully expanded leaf on three plants from each hormone treatment was selected for measurements, which were performed using a portable LICOR LI-6200 photosynthetic system (LI-COR Inc., Lincoln, NE, USA).

Samples of young fully expanded leaves were collected to examine leaf anatomy i.e. overall leaf thickness, thickness of the mesophyll and the epidermal layers and volume of intercellular spaces, on the final harvest (120 days from transplanting). Tissue from the middle region of the lamina was fixed in a mixture of 70% ethanol, 5% formalin, 5% glacial acetic acid, and 20% distilled water prior to dehydration in an ethanol and tert-butyl alcohol series. Samples were sectioned at 10-20 μm thick on a rotary microtome and stained with safranin-crystal violet-fast green. Data presented are the means of three leaves per treatment using ten leaf cross-sections per leaf. Quantitative anatomical data were obtained using Image Pro Express version 6.0 (Media Cybernetics, MD, USA).

The experiment was arranged in a three-way factorial design with four concentrations of IAA, four concentrations of BAP and three plant training systems. Data were subjected to three-way analysis of variance (ANOVA) using STATISTICA 8 software, after checking ANOVA assumptions and means were separated using Tukey's test. Least significant differences (LSD) values were calculated.

RESULTS AND DISCUSSION

At the end of the experiment, climbing plants (Figure 1A) had higher FW than creeping or hanging ones (Figure 1B and C, respectively). The application of IAA or BAP at any concentration, either in single or combined manner, led to a significant increase in final FW

in creeping and hanging plants, but not in climbing ones. However, in creeping plants untreated with IAA, the higher BAP doses appeared to be supra-optimal (Figure 1B).

There were no significant differences in dry matter content among training systems or hormone treatments (data not shown). Average values (%) were 7.828 ± 0.116 , 7.157 ± 0.190 , 6.643 ± 0.152 and 8.443 ± 0.170 for roots, stems, petioles and leaves respectively.

In untreated controls, climbing plants had significantly higher DW-based RGR than either creeping or hanging ones. RGR for climbing plants was about 20% higher than for creeping plants, which in turn had about 15% higher RGR than hanging plants (Table 1). Single IAA or BAP sprays at either 5 or 50 mg L⁻¹ significantly increased RGR of creeping plants, while 100 mg L⁻¹ appeared to be a supra-optimal concentration for both hormones. In hanging plants, single hormone applications increased RGR at all concentrations tested, but maximum promotion was found when either IAA or BAP were applied at 50

mg L⁻¹. Combined hormone application in all combinations promoted RGR of creeping plants. On the other hand, in hanging plants, the most effective hormone combinations were the 50-100 and 100-100 mg L⁻¹ IAA-BAP treatments, although several other treatments also had an effect on RGR. No growth promotion was observed in the 5-50, 5-100 and 50-5 mg L⁻¹ IAA-BAP treatments. On the other hand, growth regulators applications on climbing plants resulted in a significantly decreased RGR in almost every IAA-BAP combination tested (of about -20% in most cases), with the sole exception of 100-50 mg L⁻¹ IAA-BAP treatment in which no significant RGR decrease was observed (Table 1).

In untreated controls, differences in RGR among training systems could be attributed more due to differences in NAR than to variation in LAR, although differences in NAR between climbing and creeping plants were not significant. Similarly, growth regulators-driven decrease in RGR of climbing plants was generally associated with a

decrease in NAR, and conversely, the increase in RGR of growth regulators-treated creeping or hanging plants was associated with a significant increase in NAR. Variation in LAR was in general low, climbing plants tend to present higher values than either creeping or hanging ones. LAR was rather unaffected by growth regulator treatments but in the few treatments in which significant growth regulators effects were found (i.e., 50-100 and 100-100 mg L⁻¹ IAA-BAP treatments in hanging plants) a decrease response was observed (Table 1).

The root versus shoot allometric analysis of untreated controls showed a trend for higher root vs. shoot β coefficient (which indicates lower DW partitioning to shoots) when departure from the upward growing position, being β coefficient values of hanging plants about 12.2% higher than that of climbing ones (Table 2). Single IAA or BAP application resulted in decreased β coefficient values under all training systems, but especially in hanging plants, in which values

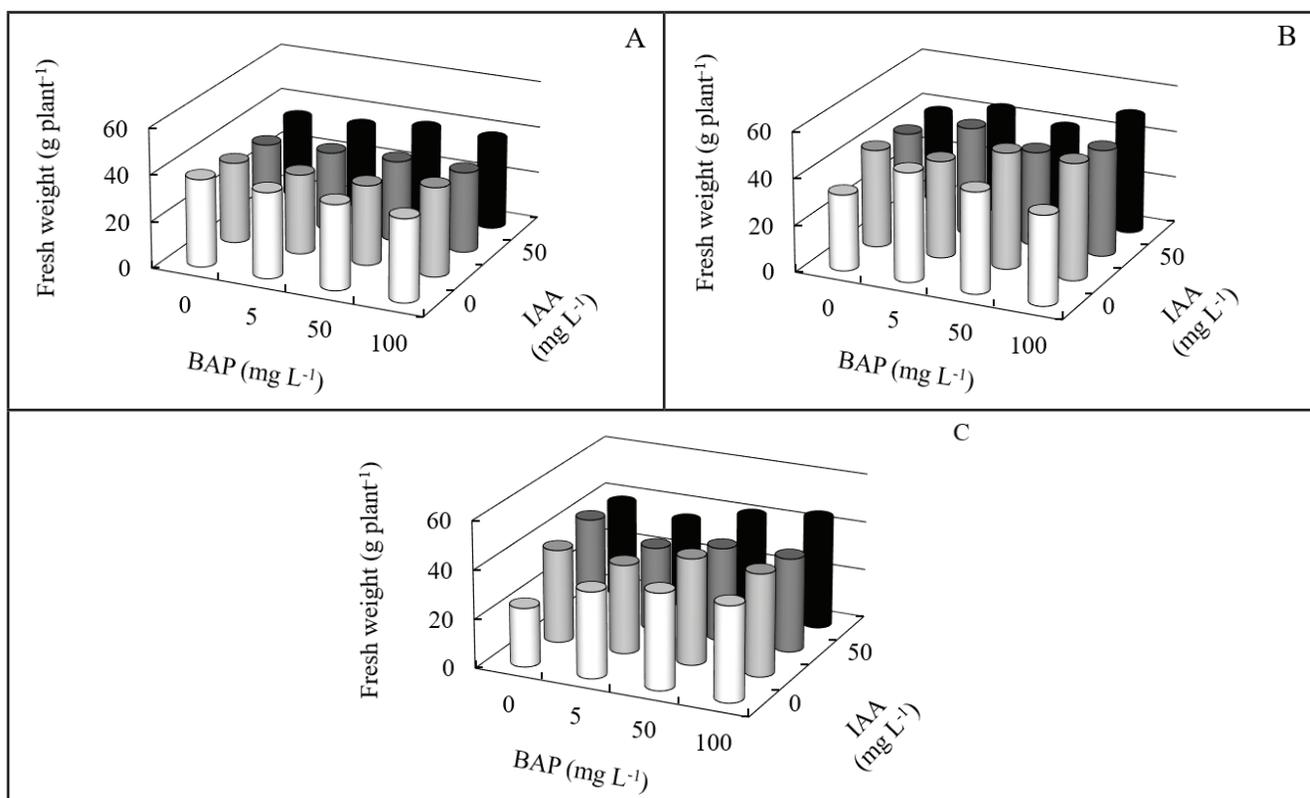


Figure 1. Fresh weight at the end of experiments (120 days from transplanting) of *E. aureum* plants sprayed with BAP at either 0, 5, 50 or 100 mg L⁻¹ on plants previously sprayed with IAA at 0, 5, 50 or 100 mg L⁻¹. Plants were grown under climbing (A), creeping (B) or hanging (C) training systems. Buenos Aires, UBA, 2008.

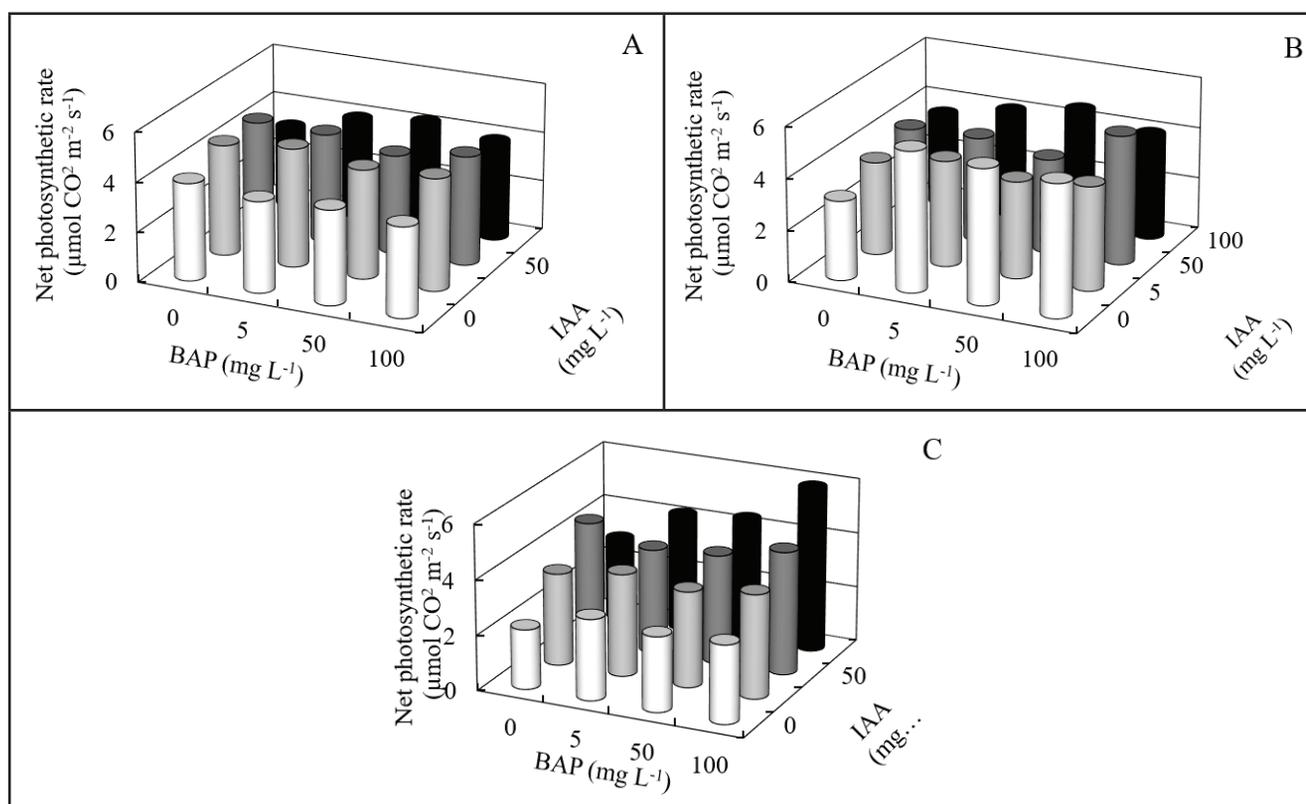


Figure 2. Net photosynthetic rate at the end of the experiments (120 days from transplanting) of *E. aureum* plants sprayed with BAP at either 0, 5, 50 or 100 mg L⁻¹ on plants previously sprayed with IAA at 0, 5, 50 or 100 mg L⁻¹. Plants were grown under climbing (A), creeping (B) and hanging (C) training systems. Buenos Aires, UBA, 2008.

decreased by more than 25%. Under any training system, combined growth regulators treatments produced low β coefficient values, in comparison with untreated controls (Table 2).

The net photosynthetic rate of young, fully expanded leaves of untreated plants showed a similar pattern to that of biomass allocation, this is, higher values in climbing plants than in creeping ones, and higher values in creeping plants than in hanging ones (Figure 2A-C).

In climbing plants, IAA or BAP in either single or combined application did not result in a promotion of photosynthesis (Figure 2A). Conversely, in creeping and hanging plants, a significant increase of net photosynthetic rate was observed when exogenous IAA and BAP, either in single or combined manner, were applied. The promotive effect of growth regulators were maximum in hanging plants, particularly at the highest IAA+BAP doses, which resulted in a two-fold increase of the net photosynthetic rate over untreated controls (Figure 2C).

Trends for plant total leaf area of untreated controls at the end of the experiment, as well as for the relative rate of leaf expansion (RLAE), were similar to those found for FW and DW accumulation, this is, significantly higher values for climbing plants than for creeping or hanging ones (Table 3). Both larger individual leaf area and higher rate of leaf appearance in climbing plants than in either creeping or hanging ones accounted for such differences. Conversely, SLA values of climbing plants were lower than in creeping ones, and they were also lower in creeping than in hanging ones.

In creeping plants, single BAP sprays at 5 or 50 mg L⁻¹ resulted in increased values, being the concentration of 100 mg L⁻¹ supra-optimal, while in hanging plants all concentrations were promotive (particularly the highest one). On the other hand, IAA sprays on creeping plants significantly promoted total leaf area only at the lowest concentration tested (5 mg L⁻¹); while on hanging plants, all IAA concentrations were

similarly effective. Combined IAA+BAP treatments varied in effectiveness in creeping plants, being either promotive or neutral, while in hanging plants all growth regulators combinations were promotive (particularly the highest dose tested, i.e. 100-100 mg L⁻¹ IAA-BAP treatment).

Up to a large extent, differences in RLAE among training systems for each growth regulators combinations treatment accompanied those found for total leaf area at the end of the experiment. In climbing plants, the growth regulators-induced decrease in both variables measuring total leaf area accumulation, could be ascribed to a reduced RLA rather than to a decrease in individual leaf area, since growth regulators sprays at any combination in comparison with untreated controls, did not significantly affect the latter. In contrast, in creeping and hanging plants, both increased individual leaf area and RLA contributed, in general, to the promotion of leaf area accumulation by IAA and/or BAP. In particular, both

Table 1. Relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) values in climbing, creeping or hanging *E. aureum* plants sprayed with IAA (0, 5, 50 or 100 mg L⁻¹) followed one week later by BAP (0, 5, 50 and or 100 mg L⁻¹). Buenos Aires, UBA, 2008.

IAA-BAP (mg L ⁻¹)	RGR (g g ⁻¹ day ⁻¹)	NAR [g cm ⁻² day ⁻¹ (x 10 ⁻⁵)]	LAR (cm ² g ⁻¹)	IAA-BAP (mg L ⁻¹)	RGR (g g ⁻¹ day ⁻¹)	NAR [g cm ⁻² day ⁻¹ (x 10 ⁻⁵)]	LAR (cm ² g ⁻¹)
0-0				50-0			
Climbing	0.0211 aA	9.74 aA	216.39 aA	Climbing	0.0161 bB	7.31 bB	220.21 aA
Creeping	0.0177 bB	9.20 bA	192.02 aA	Creeping	0.0202 aA	11.87 aA	170.20 aB
Hanging	0.0153 cC	7.62 bB	200.78 aA	Hanging	0.0199 aA	11.12 aA	179.00 bB
0-5				50-5			
Climbing	0.0172 b	7.71 bB	223.14 aA	Climbing	0.0174 bB	7.48 bB	232.48 aA
Creeping	0.0241 aA	13.03 aA	185.03 aB	Creeping	0.0206 aA	11.68 aA	176.44 aB
Hanging	0.0174 bB	8.93 bB	194.82 aB	Hanging	0.0145 cC	7.30 cB	198.74 aB
0-50				50-50			
Climbing	0.0177 bB	7.63 bB	231.90 aA	Climbing	0.0169 bB	7.29 bB	231.76 aA
Creeping	0.0194 aA	10.22 bA	189.84 aB	Creeping	0.0198 aA	11.14 aA	177.75 aB
Hanging	0.0192 aA	10.20 aA	188.31 aB	Hanging	0.0163 bB	7.98 bB	204.30 aB
0-100				50-100			
Climbing	0.0176 bA	7.80 bB	225.74 aA	Climbing	0.0178 bB	7.61 bB	233.95 aA
Creeping	0.0180 bA	9.13 bA	197.16 aB	Creeping	0.0200 aA	10.69 bA	187.17 aB
Hanging	0.0185 bA	9.65 aA	191.77 aB	Hanging	0.0169 bB	8.16 bB	207.08 aB
5-0				100-0			
Climbing	0.0173 bA	7.73 bB	223.83 aA	Climbing	0.0165 bA	7.13 bB	231.53 aA
Creeping	0.0198 aA	10.26 bA	193.04 aB	Creeping	0.0183 bA	9.37 bA	195.35 aB
Hanging	0.0176 bA	9.03 bA	194.98 aB	Hanging	0.0169 bA	8.39 bA	201.36 aB
5-5				100-5			
Climbing	0.0159 bB	7.01 bB	226.72 aA	Climbing	0.0181 bB	7.95 bB	227.44 aA
Creeping	0.0194 aA	10.82 bA	179,25 aB	Creeping	0.0206 aA	12.04 aA	171.07 aB
Hanging	0.0170 bB	8.43 bB	201.76 aA	Hanging	0.0175 bB	8.45 bB	207.20 aA
5-50				100-50			
Climbing	0.0160 bB	6.75 bB	236.91 aA	Climbing	0.0194 aA	9.28 aB	209.03 aA
Creeping	0.0205 aA	11.20 aA	182.97 aB	Creeping	0.0202 aA	10.72 bA	188.52 aB
Hanging	0.0137 cC	6.21 cB	220.48 aA	Hanging	0.0198 aA	11.12 aA	178.06 bB
5-100				100-100			
Climbing	0.0181 bB	8.15 bB	222.16 aA	Climbing	0.0170 bB	7.21 bB	235.90 aA
Creeping	0.0210 aA	12.28 aA	170.97 aB	Creeping	0.0224 aA	13.10 aA	170.97 aB
Hanging	0.0136 cC	6.42 cC	211.94 aA	Hanging	0.0225 aA	13.06 aA	172.33 bB

Different lowercase letters indicate significant differences for each growth parameter between IAA-BAP concentrations for the same training system while different capital letters indicate significant differences among training systems for each IAA-BAP concentration ($p < 0.05$).

growth regulators promoted individual leaf area of hanging plants over 100% of untreated controls in most hormone combinations.

On the other hand, untreated creeping and hanging plants showed higher SLA values (about 15% and 25% respectively) than climbing ones. Growth regulators treatments generally

had no significant effect on SLA values of climbing plants. Conversely, most growth regulators combinations (i.e., except 5-50, 5-100 mg L⁻¹ and 50-5 IAA-BAP mg L⁻¹ treatments) significantly decreased SLA of hanging plants. In creeping plants, results were more variable: while in general no significant differences were observed between

growth regulators-sprayed plants and untreated controls, one growth regulator combination increased SLA while another combination decreased it (50-50 and 5-5 mg L⁻¹ IAA-BAP, respectively) (Table 3).

Control, untreated climbing plants had thicker leaves than creeping and hanging ones. Single or combined IAA-

BAP sprays increased leaf thickness particularly in hanging plants sprayed with BAP at 5 or 50 mg L⁻¹, or with IAA at 5 mg L⁻¹, although a promotive effect was also observed in creeping ones. On the other hand, no significant effect could be observed in climbing plants. As a consequence of the differential effect of growth regulators among training systems, little differences in leaf thickness could be observed in leaves from sprayed plants, especially under IAA+BAP combinations at the higher doses (Figure 3A).

In untreated climbing plants, the epidermal cell layer represented a larger fraction of leaf cross-section than in either creeping or hanging ones. Growth regulators applications tended to reduce this contribution in climbing plants and to increase it in hanging ones, while no clear trend was observed in creeping plants (Figure 3B-D). The intercellular space fraction occupied a relatively low proportion of leaf cross-sections (i.e., 8% to 18%) in untreated controls, irrespective of the training system. In most cases, growth regulators supply led to an increase in this fraction,

particularly in climbing and creeping plants. On the other hand, no clear trends regarding the parenchymatous layer were found.

Steinitz & Hagiladi (1987) and Steinitz *et al.* (1992) reported the climbing plants of 'Golden pothos' and other ornamental Araceae plants develop larger leaves than hanging plants. In the present work we show that leaves from climbing *E. aureum* plants are not only larger but also thicker, and with a lower SLA, than those from hanging ones, and that similar differences can be found when creeping plants are compared to hanging ones, the latter displaying the smallest leaves with the largest SLA values (Table 3). These differences ultimately lead to a decreasing foliage production (i.e., RLAE) with departure from upward growth position. These effects were also accompanied with an increasing dry mass partitioning towards the root system, from climbing to hanging plants (Table 2). However, an even more important effect of training system on plant performance was that of carbon assimilation, being both the calculated NAR and measured

photosynthetic rates higher in climbing plants than in creeping ones, and higher in creeping plants than in hanging ones (Table 3, Figure 2). This effect led to important differences among training systems in RGR, which decreased together with departure from upward growth position (Table 1).

Auxin and/or cytokinin-sprays on either creeping or hanging plants led to larger leaf size and total leaf area production (Table 3) in parallel with a decreased dry mass partitioning to roots (Table 2). Growth regulators-treated plants also showed higher RGR values, which could be explained mainly because of a strong promotion in NAR by both auxin and cytokinin. In general, single-growth regulators effects were maximum at lower growth regulators concentrations, with higher doses resulting supra optimal in many cases. These results are in good agreement with previous work on creeping *E. aureum* plants (Di Benedetto *et al.*, 2015a, b). Growth regulators-driven promotion of carbon fixation per unit leaf area may be, in part, explained by changes in the leaf anatomy, including thicker leaves

Table 2. Allometric analysis ($\ln \text{Root dry weight} = \alpha + \beta \times \ln \text{Shoot dry weight}$) for climbing, creeping or hanging *E. aureum* plants sprayed with IAA at 0, 5, 50 or 100 mg L⁻¹ followed one week later by BAP at 0, 5, 50 and or 100 mg L⁻¹. Buenos Aires, UBA, 2008.

IAA-BAP (mg L ⁻¹)	Roots vs. shoots					
	Climbing		Creeping		Hanging	
	β	r ²	β	r ²	β	r ²
0-0	1.110 aB	0.932	1.188 aA	0.865	1.246 aA	0.905
0-5	1.013 bA	0.914	1.145 aA	0.927	0.876 cB	0.732
0-50	0.976 bA	0.891	1.056 bA	0.922	0.936 cB	0.892
0-100	0.963 bA	0.854	0.992 bA	0.681	0.992 bA	0.917
5-0	1.086 cA	0.898	1.007 bA	0.941	1.076 bA	0.865
5-5	0.860 cB	0.954	0.965 bA	0.875	1.067 bA	0.871
5-50	0.887 cB	0.862	1.083 bA	0.928	0.929 cB	0.841
5-100	0.998 bA	0.927	1.049 bA	0.939	1.015 bA	0.831
50-0	0.995 bA	0.901	1.093 bA	0.892	0.993 bA	0.905
50-5	0.948 bA	0.870	1.018 bA	0.925	1.022 bA	0.823
50-50	0.860 cA	0.837	1.000 Ab	0.899	0.764 dA	0.821
50-100	0.847 cB	0.821	1.028 bA	0.920	0.972 bA	0.888
100-0	1.041 bA	0.897	1.066 bA	0.925	0.935 cB	0.886
100-5	1.002 bA	0.904	1.071 bA	0.914	0.877 cB	0.882
100-50	0.952 bA	0.857	0.792 cB	0.651	0.942 cA	0.909
100-100	0.883 cB	0.873	1.044 bA	0.947	0.997 bA	0.951

The straight-line regression α coefficient are not shown. All slopes were statistically significant ($P < 0.001$).

Table 3. Plant total leaf area, mean individual leaf area, relative leaf area rate (RLAE), rate of leaf appearance (RLA) and specific leaf area (SLA) in climbing, creeping or hanging *E. aureum* plants sprayed with IAA at 0, 5, 50 or 100 mg L⁻¹ followed one week later by BAP at 0, 5, 50 and or 100 mg L⁻¹. Buenos Aires, UBA, 2008.

IAA-BAP (mg L ⁻¹)	Total leaf area (cm ² plant ⁻¹)	Individual leaf area (cm ² leaf ⁻¹)	RLAE (cm ² cm ⁻² day ⁻¹)	RLA (leaves week ⁻¹)	SLA (cm ² g ⁻¹)
0-0					
Climbing	539.47 aA	77.17 aA	0.0146 aA	1.074 aA	460.58 aC
Creeping	357.81 bB	45.83 bB	0.0116 cB	0.870 cB	521.13 bB
Hanging	262.08 cC	40.80 cB	0.0088 dC	0.894 cB	581.18 aA
0-5					
Climbing	423.67 bA	73.18 aB	0.0123 bB	0.981 bB	492.42 aA
Creeping	488.58 aA	67.33 aB	0.0165 aA	1.284 aA	479.99 bA
Hanging	347.19 bB	83.33 aA	0.0105 bC	1.051 bB	515.54 bA
0-50					
Climbing	436.21 bA	76.41 aB	0.0133 aA	0.970 bB	471.29 aB
Creeping	408.87 bA	71.70 aB	0.0122 cB	1.081 bA	524.83 bA
Hanging	370.66 bB	90.46 aA	0.0118 bB	1.148 aA	494.90 cB
0-100					
Climbing	412.15 bA	68.48 aB	0.0128 bA	0.933 bB	459.68 aB
Creeping	389.62 bB	58.50 aB	0.0113 cB	1.078 bA	554.34 bA
Hanging	406.01 aA	92.91 aA	0.0114 bB	1.047 bA	530.76 bA
5-0					
Climbing	393.10 bA	66.72 aA	0.0123 bA	0.926 bB	483.64 aA
Creeping	459.94 aA	70.83 aA	0.0128 cA	1.133 bA	528.07 bA
Hanging	325.25 bB	78.64 aA	0.0106 bB	1.022 bB	507.17 bA
5-5					
Climbing	388.20 bA	81.38 aA	0.0112 cA	0.898 cB	491.29 aA
Creeping	357.36 bA	71.28 aA	0.0113 dA	1.101 bA	407.43 cB
Hanging	329.43 bA	84.02 aA	0.0106 bA	1.050 bA	510.22 bA
5-50					
Climbing	404.69 bA	71.74 aA	0.0119 bA	0.953 bB	488.74 aB
Creeping	421.26 aA	75.34 aA	0.0128 cA	1.144 bA	498.56 bB
Hanging	378.62 bB	79.48 aA	0.0086 dB	0.978 bB	582.57 aA
5-100					
Climbing	431.86 bA	70.95 aA	0.0132 aA	1.067 aB	455.39 aC
Creeping	449.83 aA	68.19 aA	0.0123 cA	1.241 aA	517.95 bB
Hanging	348.85 bB	75.59 aA	0.0079 dB	0.889 cC	587.72 aA
50-0					
Climbing	374.08 cA	62.82 aB	0.0110 cA	0.906 bB	481.18 aA
Creeping	360.82 bA	68.38 aB	0.0114 dA	1.069 bA	510.12 bA
Hanging	372.87 bA	84.28 aA	0.0117 bA	0.926 cB	486.08 cA
50-5					
Climbing	424.68 bA	72.02 aB	0.0130 aA	1.030 aB	462.30 aB
Creeping	423.55 aA	69.60 aB	0.0121 cA	1.160 bA	529.69 bA
Hanging	325.71 bB	87.00 aA	0.0077 dB	0.944 bB	563.81 aA
50-50					
Climbing	402.07 bA	66.62 aB	0.0125 bA	1.000 aA	476.16 aC
Creeping	385.64 bA	66.20 aB	0.0115 dA	1.056 bA	628.78 aA
Hanging	321.45 bB	90.15 aA	0.0099 cB	0.944 bA	531.64 bB

Table 3. Continuation

IAA-BAP (mg L ⁻¹)	Total leaf area (cm ² plant ⁻¹)	Individual leaf area (cm ² leaf ⁻¹)	RLAE (cm ² cm ⁻² day ⁻¹)	RLA (leaves week ⁻¹)	SLA (cm ² g ⁻¹)
50-100					
Climbing	473.79 bA	67.73 aB	0.0135 bA	1.009 aA	468.84 aB
Creeping	437.87 aA	67.98 aB	0.0126 cA	1.074 bA	542.35 bA
Hanging	331.88 bB	88.63 aA	0.0109 bB	0.978 bA	538.28 bA
100-0					
Climbing	394.67 bA	66.02 aB	0.0121 bA	0.956 bA	506.13 aA
Creeping	380.86 bA	66.84 aB	0.0113 cA	0.929 cA	522.55 bA
Hanging	347.80 bA	87.81 aA	0.0105 bA	1.020 bA	547.55 bA
100-5					
Climbing	434.65 bA	67.31 aB	0.0134 bA	0.972 bC	466.57 aB
Creeping	377.41 bB	68.62 aB	0.0117 cB	1.203 aA	496.30 bA
Hanging	360.86 bB	91.24 aA	0.0115 bB	1.120 aB	517.20 bA
100-50					
Climbing	411.28 bA	61.99 aB	0.0136 bA	1.121 aA	458.01 aB
Creeping	406.88 bA	69.94 aB	0.0128 cB	1.044 bB	536.04 bA
Hanging	373.25 bB	89.31 aA	0.0116 bB	1.153 aA	504.99 bA
100-100					
Climbing	430.10 bA	71.47 aB	0.0128 bA	0.990 bB	485.80 aA
Creeping	481.00 aA	76.21 aB	0.0137 bA	1.232 aA	481.99 bA
Hanging	442.16 aA	99.79 aA	0.0139 aA	1.308 aA	485.02 cA

Different lowercase letters indicate significant differences for each growth parameter between IAA-BAP concentrations for the same training system while different capital letters indicate significant differences among training systems for each IAA-BAP concentration ($P < 0.05$).

(Figure 3A) with lower SLA (Table 1) and increased proportion of intercellular spaces, which may help decrease resistance to CO₂ diffusion from the sub stomatal cavity to the chloroplast stroma (Figure 3B-D) (Tholen *et al.*, 2012).

As reported before (Di Benedetto *et al.*, 2015a, b), when *E. aureum* plants are grown in pots, roots are impeded to develop normally and this restriction may lead to a limited production of cytokinins by root apices. In turn, this affects shoot development because of the cytokinin promotive role on cell division in meristems (Skylar & Wu, 2011). Then, exogenous cytokinin supply has been found to be an effective means of overcoming root restriction. The reason why exogenous IAA promoted growth in a similar manner than BAP may be less straight forward. A first possibility is a direct effect of auxin on both leaf expansion (Keller, 2007) and leaf initiation (Scarpella *et al.*, 2010), because auxins move from the

shoot meristems to the root apex. A more likely possibility, discussed by Di Benedetto *et al.* (2015b), is that auxin mediates cytokinin production due to its role on lateral root development, being root apices the sites of synthesis of cytokinin (Aloni *et al.*, 2005).

In the present work, we also found that growth of hanging plants was effectively promoted in the same way than creeping ones. At high doses of both growth regulators in combined supply (e.g. 100-50 and 100-100 mg L⁻¹ IAA-BAP treatments), this promotion was enough to make RGR and NAR values of hanging plants similar to those of creeping ones.

On the other hand, growth regulators-sprayed climbing plants showed a general decrease in RLAE, mainly driven by a lower RLA (Table 3). They also showed lower RGR and NAR values than untreated controls in almost every growth regulators combination treatment (Table 1) while

no clear trends in LAR were observed. These results, taken together, may be a response to a possible growth regulator (both auxin and cytokinin) deficiency in creeping and, especially, in hanging plants. Conversely, in hanging plants, endogenous auxin and cytokinin concentrations would not limit growth, and further increase in these concentrations through exogenous sprays could lead to supraoptimal levels with a negative impact on plant development.

Steinitz & Hagiladi (1987) and Steinitz *et al.* (1992) described the promotive effect of thigmo-stimulation on foliage development in several Araceae including *E. aureum*. It is well known that plants respond to external (and internal) mechanical loads through mechano-sensing and transducing pathways that alter the expression of specific sets of transcription factors, ultimately responding by modifying growth rate and direction (Moulija *et al.*,

2016). In the case of *E. aureum* vines, soft stems grow in a hanging manner until they are mechano-stimulated by a surface on which they can climb. Nowadays, significant progress has been made in identifying stimulus-responsive elements and genes, as well as proteins, hormones and inorganic signalling molecules that are involved in plant response to mechanical and gravity stimuli (Coutand & Mitchell, 2016). It is well known that thigmomorphogenetic responses are modulated by hormone homeostasis and especially by distribution patterns of auxins (Chehab *et al.*, 2009). However, in our work, both thigmo- and gravimorphogenic effects may explain differences in plant development among training systems. Vine departure from upward growing position is expected to inhibit polar auxin transport from shoot apex to roots, which depends on stem position respect to the gravity vector (Lovisolo *et al.*, 2002). Lower auxin levels in roots could in turn led to decreased root branching (Pacurar *et al.*, 2014) and thus, lower cytokinin synthesis and export to shoots, being root apices the main site of synthesis of cytokinin synthesis (Pacifci *et al.*, 2015). Exogenous

growth regulators supply could then restore decreased endogenous hormonal levels in plants due to vine departure from vertical upward position.

Lopez *et al.* (2014) discuss models describing the gravitropic response following the tilting of plants, highlighted that gravitropic curvature involves both gravi-sensing and mechano-sensing, but noted that most experimental designs do not allow to discriminate between them. As was demonstrated through mathematical models of stem gravitropic movements (Bastien *et al.*, 2013) that both gravi-sensing and mechano-sensing lead to the reorientation of the plant.

While Steinitz & Hagiladi (1987) and Steinitz *et al.* (1992) attempted to separate touch and gravity stimulus by holding upper leaf petioles without touching sensitive stems, this procedure might have modified shoot apex position respect of climbing plants, which in turn could affect downward auxin movement and plant gravity perception. In our experiment, both climbing and creeping plants may be considered as thigmo-stimulated; therefore, differences in plant responses between them may be attributed to gravity effects, yet it must

be taken into account that both stimuli appear not to act in an additive manner, but rather interact (Steinitz *et al.*, 1992).

As Lopez *et al.* (2014) state, it remains a challenge to find an experimental design, which could allow discriminating between gravi- and mechano-sensing mechanisms. Furthermore, it is still not clear whether gravi-sensing and mechano-sensing act through the same mechanisms and to what extent one can differentiate these stimuli, since that gravi-sensing is derived from an ancestral touch perception apparatus.

Several conclusions can be drawn from the results of the present work. First, decreased leaf area development in hanging *E. aureum* plants, as compared to climbing ones, could be attributed to differences in both lower carbon assimilation (i.e., lower NAR and net photosynthetic rate) (Table 1 and Figure 2 respectively) and to a lower carbon assignment to leaf area development (larger root:shoot β allometric coefficient, Table 2).

Second, creeping plants exhibited generally intermediate values, but regarding some variables (e.g., individual leaf area, Table 3); results

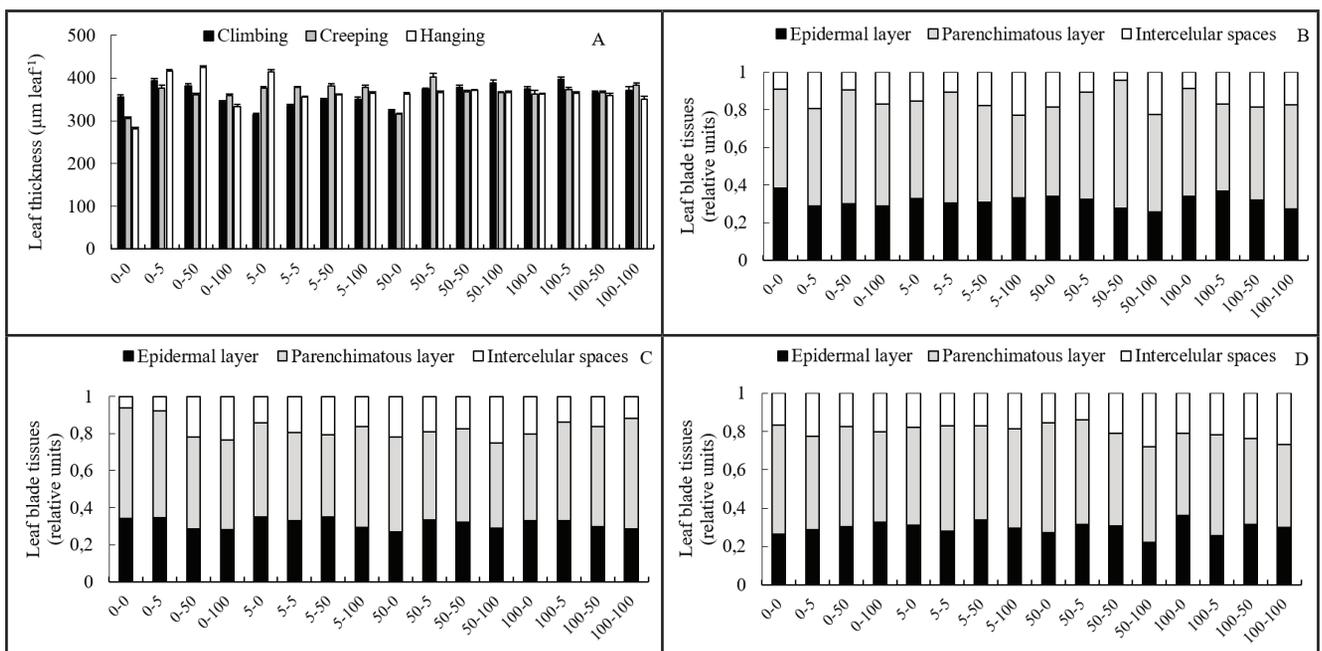


Figure 3. Changes in leaf thickness (A) and in leaf tissue distribution (%) of *E. aureum* plants sprayed with four BAP concentrations (0, 5, 50 and 100 mg L⁻¹) but previously sprayed with IAA at 0, 5, 50 or 100 mg L⁻¹. Plants were grown under climbing (B), creeping (C) and hanging (D) training systems. Bar indicates least significant difference (LSD). Buenos Aires, UBA, 2008.

were much closer to those of hanging plants than to those climbing, suggesting that any departure from upward growing position may severely affect *E. aureum* development.

Third, the effect of exogenous growth regulators applications widely differed among training systems: while in creeping and hanging plants growth was strongly enhanced by both IAA and BAP sprays, no promoting effect was observed in climbing ones. This is consistent with hormone deficiency in creeping and hanging plants, possibly due to impaired IAA transport to roots, and with possible supraoptimal hormone levels in sprayed climbing plants, although direct evidence to check these possibilities is needed.

Fourth, up to a good extent, growth promotion by exogenous growth regulators in creeping and hanging plants was attributable to the same factors that explained differences in growth parameters between training systems in the absence of exogenous growth regulators sprays (i.e. enhanced carbon assimilation per unit leaf area and enhanced accumulation of leaf area in plants treated with either IAA or BAP than in untreated controls) (Figure 2 and Table 3 respectively).

Fifth, the fact that both creeping (thigmo-stimulated) and hanging (non thigmo-stimulated) plants exhibited similarities in their behaviours, either in the absence or in the presence of exogenous growth regulators, in contrast with climbing (thigmo-stimulated) plants stresses the role of gravity on *E. aureum* responses to growth habit. While the regulation of plant form by mechanical stimuli has been described more than two centuries ago by Darwin, the subject has regained interest during the past four decades, partly because of its potential as an innovative means of controlling plant growth (Coutand & Mitchell, 2016).

In the present work we show that not only *E. aureum* development is strongly affected by plant training system, but also that the response to exogenous AIA and BAP treatment varies drastically, either promoting or inhibiting growth, depending on vine orientation. This may in turn be of considerable applied value

for the commercial grower, for who, 'pothos' price is mainly related to leaf and plant size.

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