Biomass accumulation in an ornamental Cactaceae (*Mammillaria elongata* subsp. *echinaria*) in response to a single 6-benzylaminopurine (BAP) spray

Jorge Lozano-Miglioli[1], Ernesto Giardina[2], and Adalberto Di Benedetto[2, 3*]

[1]Scientific Research Committee of the Province of Buenos Aires (C.I.C.), 526 Street between 10 and 11 (1900), La Plata, Province of Buenos Aires, Argentina.

[2]Faculty of Agronomy, University of Buenos Aires, San Martín Av. 4453 (C1417DSE), Buenos Aires, Argentina.

[3]Faculty of Agricultural Sciences, National University of Mar del Plata, Route 226, (B7620ZAA), Balcarce, Province of Buenos Aires, Argentina.

Corresponding Author: dibenede@agro.uba.ar

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ABSTRACT

In ornamental plants growing in pots, root restriction would be presumably related to an endogenous long-distance cytokinin signal from roots. In this context, it has been recently indicated that, in potted ornamental plants, a single 6-benzylaminopurine (BAP) spray can be used to increase biomass accumulation. Although cytokinin sprays and cytokinin-containing substances have been previously used to improve growth in cacti, the physiological mechanism involved has not been elucidated. Thus, this work aimed to evaluate the effect of a single BAP spray on the growth of the vegetative axillary stems of the cactus *Mammillaria elongata* subsp. *echinaria*. To achieve this general objective, *M. elongata* subsp. *echinaria* plants were sprayed with 0, 5, 50, 100 or 200 mg L^{-1} BAP solutions (40 plants per treatment) and grown for 210 days under greenhouse conditions. The results showed that a single BAP spray increased both fresh and dry weights, photosynthetic stem area, root length and a number of axillary stems at 90 days after the beginning of the experiment. The higher biomass accumulation was related to a higher rate of stem area expansion (RSAE), relative growth rate (RGR), net assimilation rate (NAR) and partitioning of photoassimilates into stems. This results indicate that *M. elongata* subsp. *echinaria* biomass accumulation can be increased by a single BAP spray of 5 mg L^{-1}, although the highest response was found with a 200 mg L^{-1} BAP spray.

***Keywords*:** Cactus growth, cytokinin, photoassimilate partitioning.

INTRODUCTION

Mammillaria elongata subsp. *echinaria* is a facultative sun plant (Leirana-Alcocer and Parra-Tabla, 1999) endemic to Mexico, which is usually sold as an ornamental pot plant.

In nature, the growth of most cacti is limited more by the water availability than by the soil space (Bacilio *et al*., 2011). In contrast, under pot culture conditions, with large water and nutrient supply, the plant can suffer from different abiotic stress related to root restriction, such as that observed in other ornamental plants (Di Benedetto, 2011). However, quantitative data regarding this issue are still lacking.

Cramer *et al.* (2011) defined abiotic stress as an environmental condition that reduces growth and yield below optimal levels. Regarding the abiotic stress related to root restriction, Puig *et al.* (2012) and Chen *et al.* (2015) concluded that plants can sense the volume of the rooting space available, and a few studies have shown that plant roots may sense the identity of neighboring roots and respond accordingly. This abiotic stresses may trigger a wide range of local and long-distance signals such as cytokinins (Nishiyama *et al.*, 2011), which must be coordinated and integrated into whole-plant processes (Zwack and Rashotte, 2015).

Previous reports have shown that plant growth-promoting bacteria (Puente *et al*., 2009), vesicular-arbuscular mycorrhizae (Pimienta-Barrios *et al*., 2002) and seaweed extracts (Bashan *et al*., 2009) can increase biomass accumulation in cacti. Products containing these organisms include nutrients, carbohydrates, metabolites and plant growth regulators, such as auxins and cytokinins (Khan *et al*., 2009).

In this sense, it has been claimed that the close coordination between the root and shoot growth is controlled by a signaling pathway which is largely hormonal (Bartoli *et al*., 2013). In this regard, several studies have found an increase in the number of vegetative stems and reproductive buds in cactus plants sprayed with benzyl aminopurine (BAP) (Ho *et al*., 1985; Boyle, 1992; Harkess and Lyons, 1994; Boyle and Marcotrigiano, 1997; Arrellano-Perusquía *et al*., 2013). The effect of BAP on biomass accumulation has been related to a change in photo-assimilate partitioning in favor of shoots (Boyle, 1995). Similarly, exogenous cytokinin sprays have been recently indicated as a tool to improve plant growth in shade and sun ornamental plants (Di Benedetto *et al*., 2010). However, a quantitative analysis of the physiological mechanisms involved is still lacking.

Since the morphogenetic response commonly varies, auxin–cytokinin ratio must be empirically determined for each species. For example, vegetative propagation of cacti includes three plant ideotypes: i) cacti with single stems or monopodial stem with an apical meristem, which produce auxins that maintain areoles in a dormant state and cytokinins from a root meristem, which can activate areoles at the apical area after damage; ii) cacti with proliferous stems in which apical dominance has no effects on areoles at the base, but are activated by cytokinins; and iii) cacti with branched stems, such as *M. elongata* subsp. *echinaria*, where there is no apical dominance and where cytokinins can activate the growth of the areoles of any part of cladodes (Ramírez Serrano and Texeira da Silva, 2008).

Based on all the above, the aim of this work was to evaluate the effect of a single 6-benzylaminopurine (BAP) spray on the growth of the vegetative axillary stems of the cactus *Mammillaria elongata* subsp. *echinaria* to test the hypothesis that this synthetic plant growth

regulator can improve biomass accumulation and plant architecture through both higher photoassimilate production and a change in photoassimilate partitioning to shoots.

MATERIALS AND METHODS

Plant material, treatments, and experiments

The experiment was conducted in a greenhouse facility placed in Mar del Plata city, Argentina (37° 54′ S and 57° 35′ W and altitude of 130 m.a.s.l.) from September 23, 2015 to May 8, 2016.

Stem cuttings (0.697 $\pm$ 0.033 g plant^{-1}) of *Mammillaria elongata* subsp. *echinaria* L. were transplanted into 1.2 liter plastic pots (one cutting per pot) filled with a 2:2:1 (v/v) mix of *Sphagnum maguellanicum* peat, river waste, and perlite. Plants were watered daily to saturation and fertilized weekly with N, P, K and Ca fertilizer added to the irrigation water (50 mg L^{-1} N) (2:1:2:2 N:P:K:Ca).

Seedlings from vegetative stems were sprayed with different BAP (6-benzylaminopurine; SIGMA EC 214-927-5) (Sigma-Aldrich Co., St. Louis, MO, USA) solutions (0, 5, 50, 100 and 200 mg L^{-1}) two weeks after transplant. BAP was previously diluted in alcohol 80%.

Daily maximum and minimum air temperature and global solar radiation were recorded from a meteorological station 500 m from the experimental site. During the experiment, the mean air temperatures ranged between 11.5 and 14.4°C (minimum) and 18.2 and 28.8°C (maximum), whereas the global solar radiation ranged between 18.2 and 24.1 MJ m^{-2} day^{-1}. While greenhouse polyethylene did not change the mean light significantly, the greenhouse ventilation system allowed only a small increase (2-3°C) outside temperature.

Sample and growth evaluations

For destructive measurements, ten plants per treatment and sampling date were randomly chosen at the beginning of the experiment (transplant stage) and 30, 60, 90 and 210 days after transplant. Roots were washed and root and shoot fresh weights (FW) were recorded. Dry weights (DW) were recorded after drying roots and shoots to constant weight at 80°C for 96 hours. The number of axillary stems was recorded as well. To quantify the external stem surface area (ESSA), we used the ellipsoid formula:

$$ESSA = 2\pi \frac{b}{\sqrt{a^2 - b^2}} \left[ä^2 arcsen\left(\sqrt{1 - \left(\frac{b}{a}\right)^2} \right) + \sqrt{1 - \left(\frac{b}{a}\right)^2}\, ab \right]$$

Where, ESSA: external stem surface area (cm^2); a: stem length (cm) and b: stem width.

Axillary stem length and width and root length were measured with a digital caliper.

The growth parameters used in this work were performed according to Di Benedetto and Tognetti (2016). The rate of stem area expansion (RSAE) was calculated as the slope of the regression of the natural logarithm of ESSA versus time (in days), whereas the relative growth rate (RGR) was calculated as the slope of the regression of the natural logarithm of the whole plant on a DW basis versus time (in days). The mean net assimilation rate (NAR), and the leaf area ratio (LAR) was calculated as follows:

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

Where, k_w: RGR (g g^{-1} $days^{-1}$); W_0: extrapolated value of total dry weight at time zero (g); A_0: extrapolated value of external stem area at time zero (cm^2); k_a: RSAE (cm^2 cm^{-2} $days^{-1}$); t: time (in days) at the midpoint of the experimental period and e: base of natural logarithms.

The photosynthetic outward area ratio (POAR), as a stem photosynthetic area estimate, was calculated as:

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

The allometric coefficients between root and stems were calculated as the slope (**β**) of the straight-line regression of the natural logarithm of the root DW versus the natural logarithm of the stem DW.

Statistical analysis

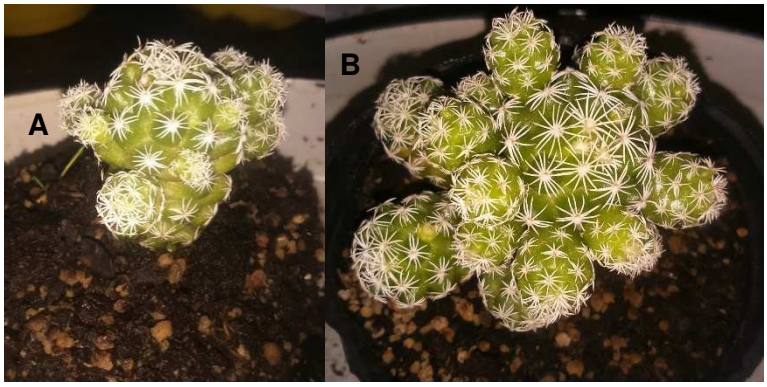
Data were subjected to a one-way ANOVA for a completely randomized design after checking ANOVA assumptions, which include normality of variances (Shapiro-Wilk´s test) and homogeneity of variances (Levene´s test). Means were separated by the Tukey´s test ($P<0.05$). When applicable, Fisher's LSD-test ($P<0.05$) was applied to determine the direction of the differences between treatment mean values. Slopes from straight-line regressions of RSAE, RGR, NAR, POAR, and allometric values were tested using the SMATR package (Warton *et al.,* 2012).

RESULTS

Fresh weight accumulation and photosynthetic stem area

Control and BAP-sprayed plants showed no significant differences in FW during the first 90 days of the experiment. However, at the final harvest, BAP-sprayed plants showed significant higher FW than control ones (Figure 1A, 1B and Figure 2A). The photosynthetic stem area

(Figure 2B), number of axillary stems (Figure 2C) and root length (Figure 2D) showed the same response pattern. Plants sprayed with 200 mg L^{-1} BAP always showed the highest responses.



Control plants **200 mg L^{-1} BAP-sprayed plants**

Figure 1. Images of the control plants (A) and plants sprayed with 200 mg L^{-1} BAP (B) at the end of the experiment.

Dry weight accumulation, photoassimilate partitioning and growth rates

At the end of the experiment (210 days from transplant), all BAP-sprayed plants showed a significant increase in total DW related to control plants (Figure 3A), although the highest response was found in plants sprayed with 200 mg L^{-1} BAP. When the mean stem DW was plotted against the mean root DW (Figure 3B), a positive correlation was found (r^2 = 0.647; $P \leq 0.001$).

A single BAP spray significantly increased RSAE and RGR, with no significant differences between BAP concentrations. The highest NAR response was found in plants sprayed with 100 and 200 mg L^{-1} BAP. An inverse response in POAR was found. The allometric analysis between roots and stems showed higher photoassimilate partitioning to roots in control plants and a change to stems in BAP-sprayed plants (Table 1).

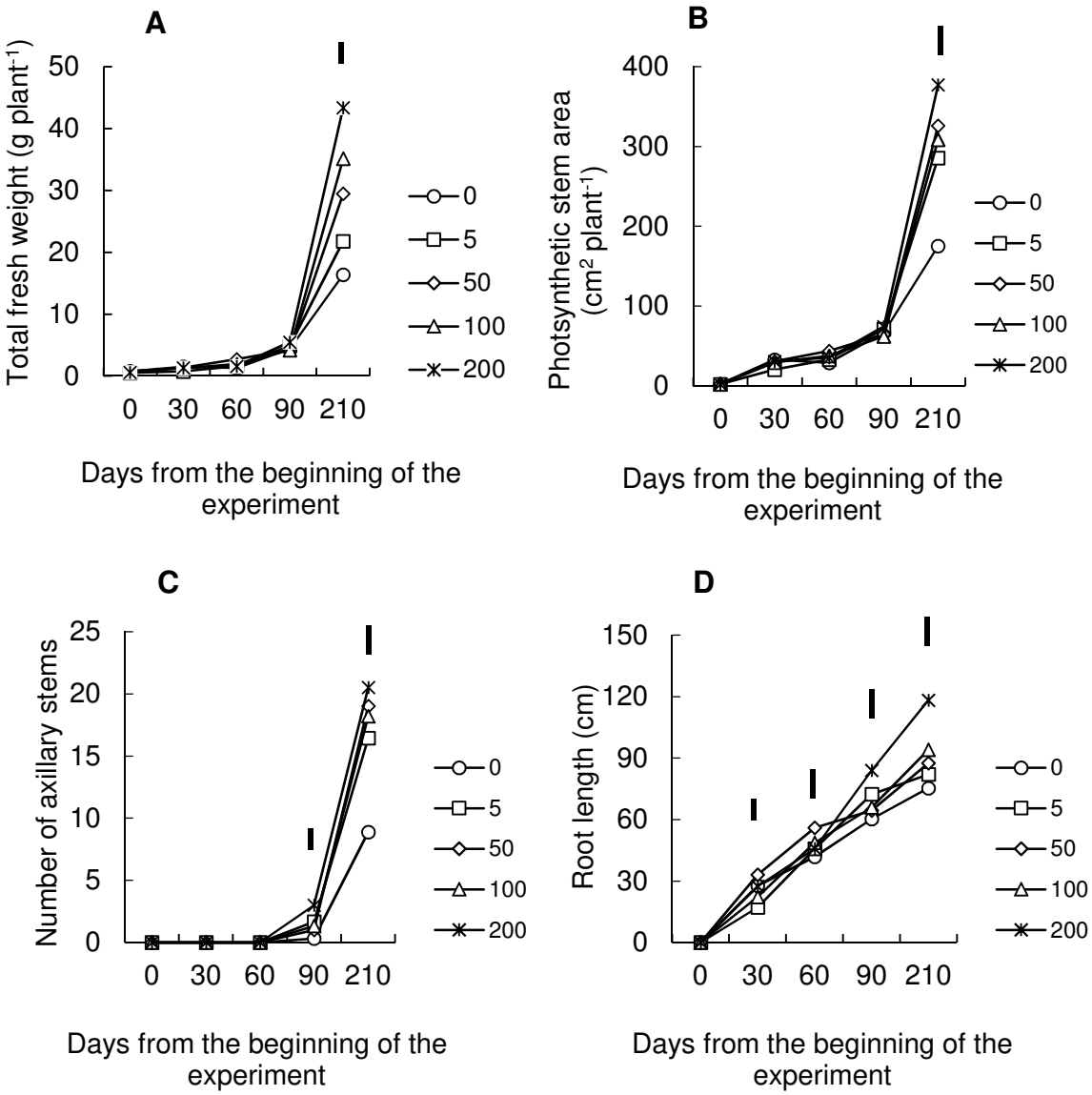


Figure 2. Changes in total fresh weight (A), photosynthetic stem area (B), number of axillary stems (C) and root length (D) during the experiment in plants of *M. elongata* subsp. *echinaria* sprayed with different BAP concentrations (0, 5, 50, 100 or 200 mg L^{-1}). Vertical lines indicate the least significant differences (Fisher's LSD) at each harvest time.

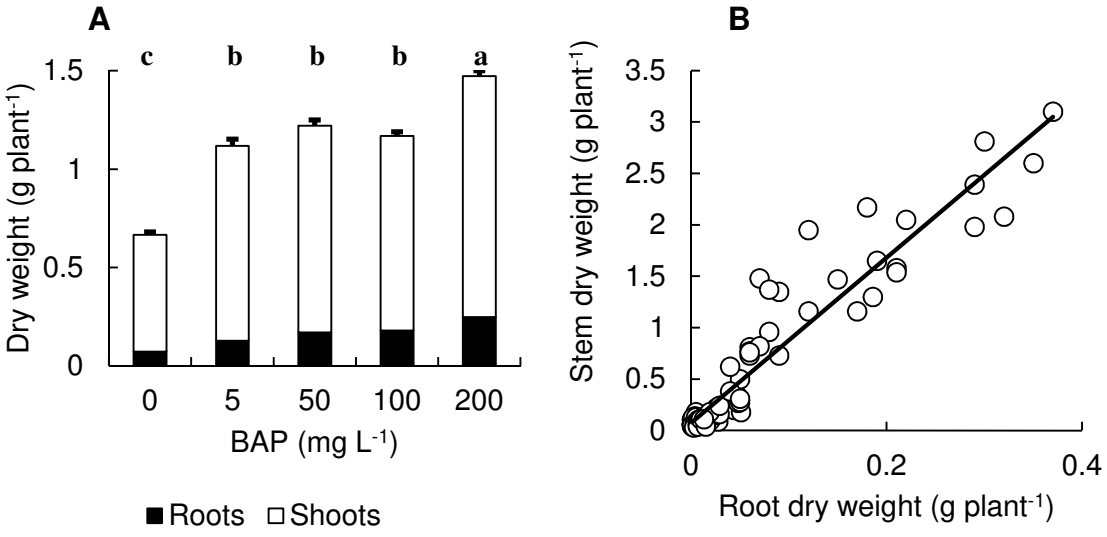


Figure 3. Changes in roots and stems dry weight at the end of the experiment in plants of *M. elongata* subsp. *echinaria* sprayed with different BAP concentrations (0, 5, 50, 100 or 200 mg L^{-1}). Different lower case letters indicate significant differences ($P<0.05$) (A). Stem-root dry weight relationships of *M. elongata* subsp. *echinaria* plants when all experimental data were plotted together (B). The linear regression equation is Stems dry weight = 8.04 and Roots dry weight + 0.072 ($r^2 = 0.907$ $P<0.001$). The probability of the slope being zero was $P<0.001$.

Table 1. Changes in the relative stem expansion rate (RSAE), relative growth rate (RGR), net assimilation rate (NAR), photosynthetic outward area ratio (POAR) and allometric relationships between roots and stems of *M. elongata* subsp. *echinaria* plants sprayed with different BAP concentrations (0, 5, 50, 100 or 200 mg L^{-1}). The slope straight-line (β) are indicated. The probability of the slope being zero was $P < 0.001$ for all growth parameters. Different lower case letters indicate significant differences ($P < 0.05$) between control and BAP-sprayed plants.

BAP (mg L^{-1})	RSAE (cm^2 cm^{-2} day^{-1})	RGR (g g^{-1} day^{-1})	NAR (g cm^{-2} day^{-1}) (x 10^{-5})	POAR (cm^2 g^{-1})	Root:stem allometries β
0	0.0207^{b}	0.0142^{b}	8.67^{c}	163.84^{c}	1.183^{a}
5	0.0225^{ab}	0.0181^{a}	9.34^{b}	211.17^{a}	1.153^{b}
50	0.0246^{a}	0.0219^{a}	9.86^{b}	222.76^{a}	1.162^{b}
100	0.0249^{a}	0.0197^{a}	10.82^{a}	167.32^{c}	0.991^{c}
200	0.0238^{a}	0.0209^{a}	11.41^{a}	183.12^{b}	1.153^{b}

When plotting the data from all treatments, it was found a close direct relationship ($r^2= 0.864$) between NAR and RGR (Figure 4A) and a weak direct relationship between RGR and POAR

(r^2= 0.218) (Figure 4B). Nevertheless, BAP-sprayed plants showed the higher NAR and POAR values.

At the end of the experiment, relationships between RSAE (Figure 5A), RGR (Figure 5B), NAR (Figure 5C) and root DW were positive (r^2 = 0.678, 0.704 and 0.925 respectively). Control plants always showed the lowest values.

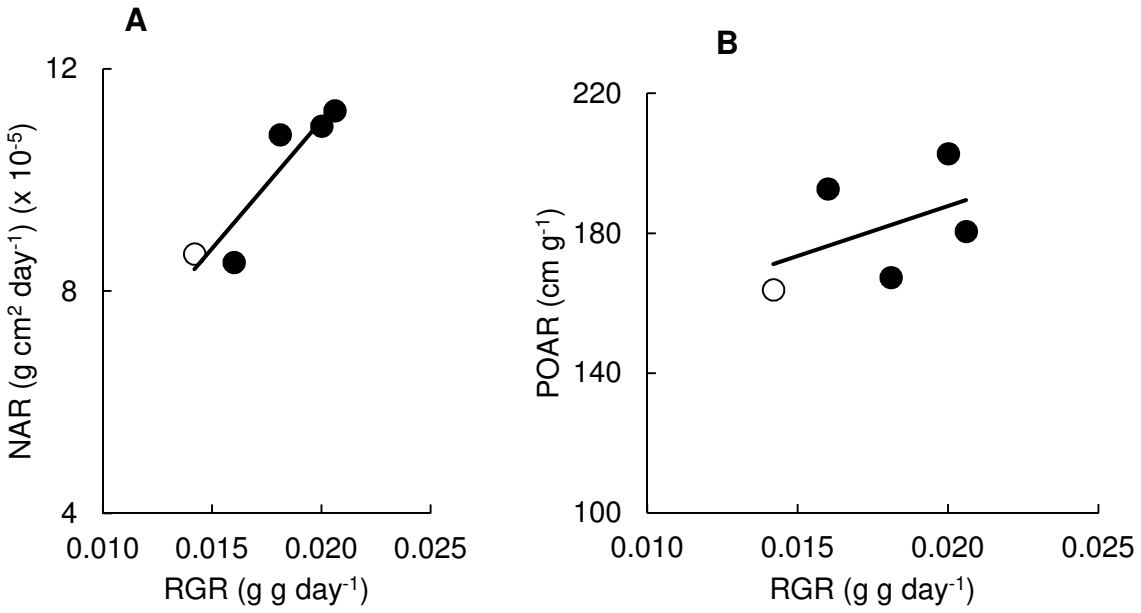


Figure 4. Net assimilation rate (NAR) (A), photosynthetic outward area ratio (POAR) (B) related to relative growth rate (RGR). The straight-line regressions were NAR = 461.1 RGR + 1.85 (r^2= 0.864; P < 0.05) and POAR = 2866.60 RGR + 130.45 (r^2 = 0.218; P < no significant). The empty and full symbols indicate controls and BAP-sprayed plants respectively.

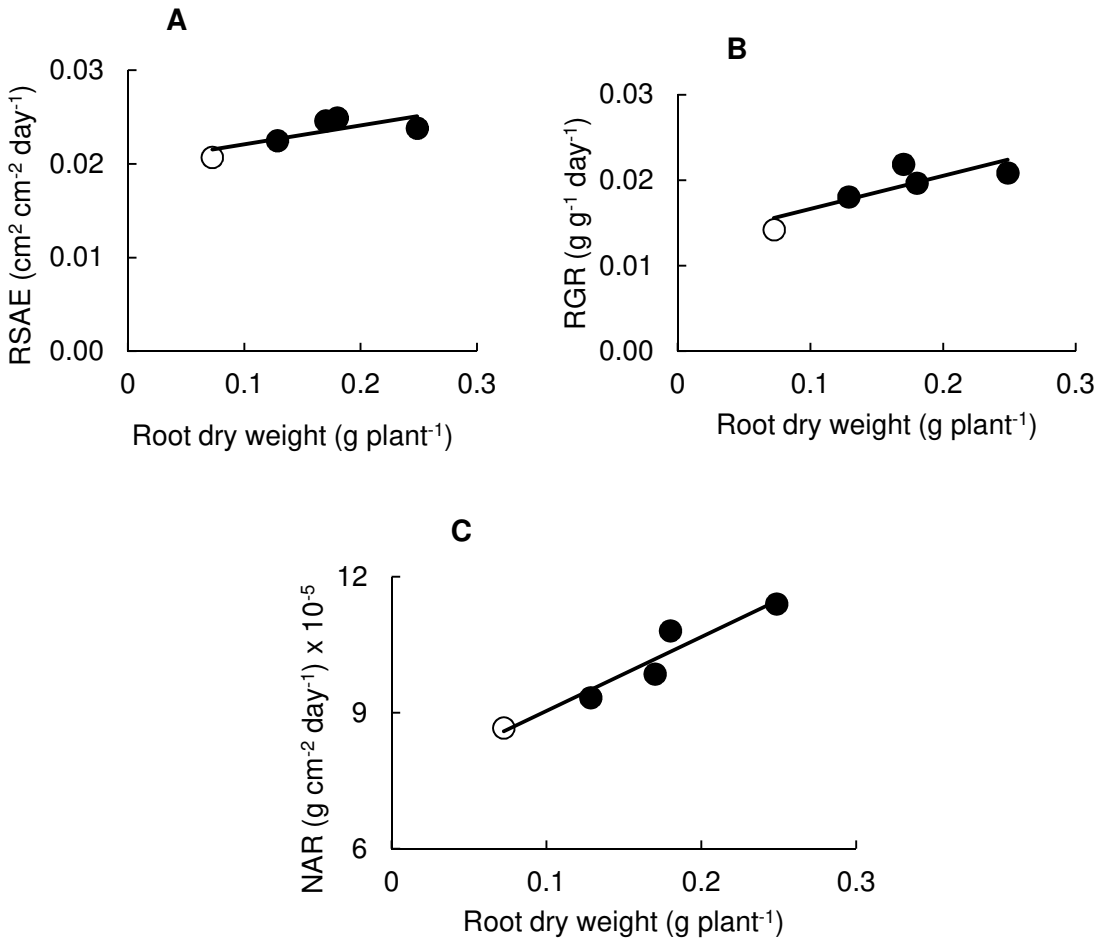


Figure 5. Relationships between the relative stem area expansion rate (RSAE) (A), relative growth rate (RGR) (B), net assimilation rate (NAR) (C) and root dry weight (RDW). Linear regression equations are RSAE = 0.020 RDW + 0.020 (r^2 = 0.678; P<0.001); RGR = 0.039 RDW + 0.01 (r^2 = 0.704; P<0.001); NAR = 16.32 RDW + 7.41 (r^2 = 0.925; P<0.001). The empty and full symbols indicate controls and BAP-sprayed plants respectively.

DISCUSSION

Biomass accumulation in Cactaceae plants is the result of temperature-dependent development processes (Nobel and Castañeda, 1998) and photosynthetic active radiation (PAR)-dependent growth processes (Martínez-Berdeja and Valverde, 2008). The propagation strategies of *M. elongata* subsp. *echinaria* (a facultative sun plant) include shoot nipple-like tubercles, which are important both for propagators and for growers.

The RGR of most cactus species in their native environments is relatively low (Singh and Singh, 2003) due to drought (Bacilio *et al*., 2011) although quantitative data for *M. elongata* subsp. *echinaria* are lacking. However, under greenhouse cropping, *M. elongata* subsp. *echinaria* RGR can be significantly higher in control plants (Table 1). Although there are no data regarding the FW of cacti in their native environments, our results showed a significant increase in *M. elongata* subsp. *echinaria* FW (Figures 1A, 1B and 2A) when plants were sprayed with a single BAP spray. In the same way, a higher DW accumulation significantly increased RGR in BAP-sprayed plants (Table 1).

These results are in agreement with previous reports where, although the responses were associated with the presence of concentrations of auxins or cytokinins similar to those used in this experiment (Khan *et al*., 2009), significant responses on vegetative stems and flower bud appearance were found only in response to a BAP spray (Boyle, 1992; Boyle and Marcotrigiano, 1997; Arellano-Perusquía *et al*., 2013). On the other hand, although Kotov and Kotova (2015) indicated that the higher the root system, the higher the cytokinin-ribosides synthesized, not all the numerous zeatin riboside isomers show the same biological activity (Van Staden *et al*., 2008), because the biological activity of all cytokinin-like compounds is not uniform, and normally depends on several structural aspects (Cassán *et al*., 2014). It was found both an increase in length (Figure 2D) and root DW biomass (Figure 3A) in *M. elongata* subsp. *echinaria* BAP-sprayed plants.

Cactus biomass accumulation is the result of both PAR available and light interception (Nobel 1982; Geller and Nobel 1987; Martínez-Berdeja and Valverde, 2008). Because cacti have changed their anatomy as a response to drought (*i.e*. leaves have been replaced by stems) (Mauseth, 2006), quantifying their photosynthetic capacity through the growth analysis approach is methodologically difficult (Hernández-González and Villarreal, 2007). However, *M. elongata* subsp. *echinaria* stems, which can photosynthesize, are almost cylindrical and the ellipsoid equation can be used to estimate photosynthetic stem area. Based on this approach, a single BAP spray significantly increases photosynthetic stem area as from 90 days after the beginning of the experiment (Figure 2B), which is an expected result that agrees with that previously reported by Ramírez-Serrano and Texeira da Silva (2008). In the same way, Boonman and Pons (2007) found a positive relationship between PAR and endogenous cytokinin concentrations.

RGR can be disaggregated as the product of the net assimilation rate (NAR) (physiological component) and photosynthetic outward area ratio (POAR) (morphological component) (adapted from Poorter and Van der Werf, 1998). On the other hand, the anatomical tissues of cacti with relatively high water content (Soffiatti and Angyalossy, 2009) make cacti with a Crassulacean Acid Metabolism to be more inefficient than photosynthetic C_3 ones (Lerdau *et al*., 1992). However, the results shown in Table 1 indicate NAR and POAR increased in BAP-sprayed plants. These results together with the strong positive relationship between NAR and RGR (Figure 4A) and with the higher values from BAP-sprayed plants and the weak relationship between POAR and RGR (Figure 4B) indicate that RGR is mainly associated with the 'physiological component' NAR. These results agree with Boonman *et al.* (2007), who showed

that cytokinin stimulated the expression of photosynthetic enzymes like Rubisco. On the other hand, Shipley (2006) indicated that, in general, NAR is the best general predictor of variation in RGR.

At the end of the experiment (210 days since pot cropping), it was found both a higher DW accumulation (Figure 3A) and a change in the photoassimilates partitioning to stems (Table 1) in agreement with the results of Boyle (1995) who worked with the cactus *Rhipsalidopsis gaertneri*. *M. elongata* subsp. *echinaria* plants sprayed with a single BAP spray increased their root DW from 77 to 242% and their stem DW from 66 to 105%. Tissues and organs with high endogenous cytokinins such as the shoot apical meristem are sinks of photoassimilates (Francis and Halford, 2006), and cytokinins have major roles in source nutrient remobilization and sink development (Yu *et al.*, 2015). On the other hand, it was found a positive and close (r^2 = 0.907) relationship between stem DW and root DW (Figure 3B), which suggests that the main influence on shoot biomass accumulation is that of roots, and presumably, root-synthesized cytokinins.

It was also found that the higher plant DW accumulation in *M. elongata* subsp. *echinaria* changed the plant architecture with an increase in the number of axillary stems or nipple-like tubercles, a morphological process strongly stimulated by BAP (Figures 1A, 1B and 2C), this is in agreement with the response observed in other cactus species sprayed with the cytokinin benzyl adenine (Boyle, 1992; Harkess and Lyons, 1994).

Cactus root growth has been mentioned as an adaptive strategy in drought environments (Dubrovsky *et al*., 1998). However, under a plentiful water supply, like those of this experiment, the root DW (Figure 3A) and root length (Figure 2D) of control plants were significantly lower than those of BAP-sprayed plants. Root restriction is physical stress imposed on a root system when plants are grown in small pots, which leads to a pronounced decrease in both root and shoot growth. In our experiment, when the root system increased, positive relationships between RSAE (Figure 5A), RGR (Figure 5B) and NAR (Figure 5C) were found. Cytokinins have been shown to be useful in preventing root restriction in annual and perennial ornamental plants (Di Benedetto *et al*., 2010; Di Benedetto, 2011) because endogenous cytokinins synthesized in roots are transported into shoots via the xylem (Kieber and Schaller, 2014).

CONCLUSION

In conclusion, a single BAP spray increased the FW, photosynthetic stem area, root length and a number of axillary stems of *M. elongata* subsp. *echinaria* plants as from 90 days after the beginning of the experiment. The higher biomass accumulation was related to higher RSAE, RGR, NAR, and photoassimilate partitioning into stems. From a grower's point of view, these results clearly show that a single exogenous BAP spray is a tool to improve both cactus propagation capacity and potted cactus yield.

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