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Original Paper

Micropropagation of *Solanum quitoense* var. *quitoense* by apical bud, petiole and hypocotyl culture

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Abstract The development of in vitro propagation methods can improve the current commercial use and conservation of plants like naranjilla (*Solanum quitoense*), a distinctive Andean crop and key emerging agricultural product. In the present study, we report *in vitro* culture protocols for naranjilla apical buds, hypocotyls and petioles. In apical bud culture, MS medium supplemented with 0.10 mg l^{-1} 1-naphtaleneacetic acid (NAA) produced longer plantlets with greater number of leaves. Hypocotyl culture yielded higher number of shoots when using older explants in MS medium supplemented with different combinations of NAA, 6-benzylaminopurine (BAP) and gibberellic acid (GA_3). Petiole culture produced a significantly higher number of shoots per explant, with more abundant and bigger leaves, when using MS medium supplemented with 0.02 mg l^{-1} NAA, 4.50 mg l^{-1} BAP and 1.00 mg l^{-1} GA_3. A factorial analysis reveals that the interaction between GA_3 and NAA/BAP plays an important role in shoot regeneration. These results provide new tools for the in vitro regeneration of naranjilla plants, improving on previously reported protocols for this species by using alternative explant types and regeneration protocols.

Key words: 1-naphtaleneacetic acid (NAA), 6-benzylaminopurine (BAP), Andean crop, gibberellic acid (GA_3), in vitro culture, *Solanum quitoense*.

Introduction

Solanum quitoense var. *quitoense*, known as lulo or naranjilla, is a perennial shrub native to Colombian and Ecuadorian mountainous regions (Heiser 1985). Its fruits are appreciated for their unique flavor and fragrance, as well as for their rich vitamin A and C contents (Gancel et al. 2008). This makes this species an important commercial product with export potential (Revelo et al. 2010). For countries like Ecuador, the naranjilla production exceeded 14 thousand metric tons according to the 2012 National Agronomic Census (SINAGAP 2012), illustrating its economic importance for the central Andean region.

Unfortunately, its susceptibility to fungal infections and nematode attacks causes reduction in yields and fruit quality, and the lack of intraspecific sources of resistance makes the development of breeding programs difficult (Patiño 1986). An alternative is the hybridization of naranjilla with interspecific members of the *Lasiocarpa* section (to which *S. quitoense* belongs), where resistance genes for different pathogens may exist (Heiser 1993). Nevertheless, these hybrids cannot be sexually propagated due to a progressive loss of the genotype of interest caused by gene-segregation in subsequent generations and sexual incompatibilities of genomes (Benítez et al. 1991). One way to preserve and reproduce potentially interesting interspecific hybrids is vegetative propagation through in vitro culture.

In vitro micropropagation studies have been conducted for some members of the *Lasiocarpa* section. Methodologies for shoot induction from hypocotyl segments, stem apices and young leaf explants have been successfully established for *Solanum sessiliflorum* (Schuelter et al. 2009), *Solanum surattense* (Gupta and Handra 1982) and *Solanum melongena* (Mukherjee et al. 1991). Shoot regeneration studies have also been reported from foliar explants in *Solanum candidum*, *S. sessiliflorum* and *S. quitoense*, with the latter showing a lower regeneration frequency (Hendrix et al. 1987; Medina-Rivas et al. 2008).

The current study presents three protocols for the in vitro regeneration of *S. quitoense* using apical buds, hypocotyls and petioles as explants. We also aim to

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identify some of the different plant growth regulators interactions in the petiole protocol reported in this study to better understand their effect in the regeneration of naranjilla plants.

Materials and methods

Plant material

Solanum quitoense fruits were obtained from local markets in Quito, Ecuador. Seeds were extracted, air dried and sterilized in a laminar air-flow hood.

The sterilization process consisted in the immersion of the seeds in 70% (v/v) ethanol for 3 min, followed by sodium hypochlorite (2.5%) plus 0.80 ml l^{-1} Tween®-20 (PanReac, Barcelona, Spain) for 20 min and five rinses in sterile distilled water. Sterilized seeds were germinated in Murashige and Skoog (MS) solid basal medium (7.00 g l^{-1} agar) (Murashige and Skoog 1962). Seed germination took 4 weeks at 22±1°C, in a 16 h photoperiod. The resulting naranjilla seedlings and plants were used as the source of the three different explant types used in this study (apical buds, hypocotyls and petioles).

Apical bud culture

Apical buds were obtained from 4-week-old seedlings and cultured in solid MS medium supplemented with 3% sucrose and five different 1-naphtaleneacetic acid (NAA) concentrations: 0.01, 0.05, 0.10, 0.15 and 0.20 mg l^{-1}. These values were chosen based on preliminary results from assays performed with different plant growth regulators in wider concentration ranges (0.01–1.00 mg l^{-1}) (data not shown). For each of the 5 treatments tested, 30 explants were distributed in 6 petri dishes with 5 explants per dish. Explants were cultivated for 4 weeks at 22±1°C, in a 16 h photoperiod. Shoot regeneration efficiency (percentage of buds that developed a shoot) was recorded and plantlet growth was determined based on the following parameters: plantlet height, number of leaves and average leaf length. All length measurements were performed with a ruler, and when multiple leaves were observed in an explant, a single mean length value was recorded per explant and averaged for the total number of explants per petri dish.

Hypocotyl culture

Hypocotyls from in vitro naranjilla seedlings were cultured in MS medium supplemented with NAA (0.01 mg l^{-1}), gibberellic acid (GA_3) (0.10 mg l^{-1}) and 6-benzylaminopurine (BAP) in 0.50 mg l^{-1} intervals in a range between 4.00 mg l^{-1} and 6.00 mg l^{-1}. The effect of the explant age was also tested, as either 1–2 week old hypocotyls (A) or 3–4 week old hypocotyls (B) were used (Figure 1). For each of the 5 treatments, a total of 30 explants, of each age, were distributed in 6 petri dishes with 5 explants per dish. Explants were grown for 4 weeks at 22±1°C, in a 16 h photoperiod. Shoot regeneration efficiency was estimated based on the average number of shoots per hypocotyl. Plantlet growth (shoot length, leaf number and average leaf length) was also assessed for each experiment. All length measurements were performed with a ruler, and when multiple shoots or leaves were observed in an explant, a single mean length value was recorded per explant and averaged for the total number of explants per petri dish.

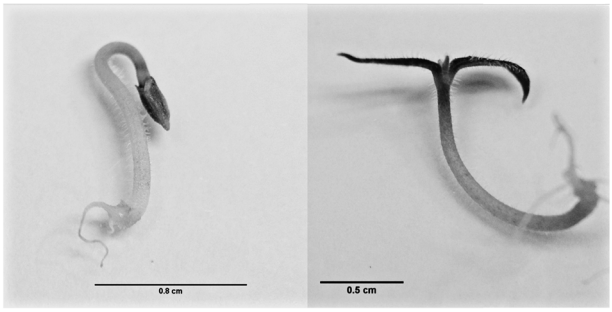

Figure 1. Representative images of, left: 1–2 week old hypocotyls (sprouts without leaves and epicotyl, with presence of cotyledons, and with a complete curved hypocotyl) and right: 3–4 week old hypocotyls (sprouts with presence of folia primordia and with small epicotyl, without cotyledons, and with almost straight hypocotyl).

Petiole culture

Petioles were obtained from 4-week-old plantlets derived from germinated seeds and cultured in solid MS medium supplemented with combinations of BAP (3.00, 3.50, 4.00, 4.50 and 5.00 mg l^{-1}), NAA (0.01, 0.02 and 0.03 mg l^{-1}) and GA_3 (0.50 and 1.00 mg l^{-1}). The location of the petiole in the plantlet was also analyzed by either using petioles from the three uppermost leaves of each plantlet (henceforth called upper petioles) or petioles from the three lowermost leaves of each plantlet (henceforth called lower petioles) and comparing their performance. For each of the 30 treatments tested, 20 explants, from each location, were distributed in 4 petri dishes with 5 explants per dish. Explants were cultivated for 4 weeks at 22±1°C, in a 16 h photoperiod. The parameters measured to determine the efficiency of naranjilla plant regeneration from petioles were the same as in the hypocotyl experiment previously described.

Data analysis

The experiments were performed with a complete randomized block design. Data was subjected to an analysis of variance (ANOVA) to evaluate the contributions of each variable to the response, and the differences between means were identified with Tukey tests. In addition, comparisons between explant types were performed through multiple two-sample T-tests (subject to a Bonferroni correction). Finally, the effect of interactions between hormones was tested in upper petioles through ANOVA tests performed on a linear model that incorporates three factors (NAA, BAP and GA_3) and the interaction terms of each paired combination of growth regulators. A 2×2×5 multifactorial experimental design was implemented to optimize the analysis of the upper petiole data. All of the analyses were assessed through a 95% of confidence interval and performed in Minitab 17 (Minitab 17 Statistical

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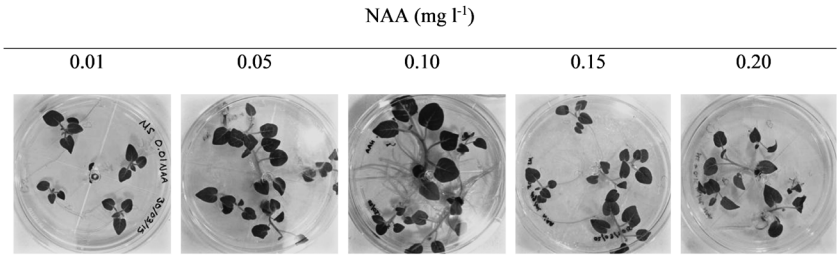

Figure 2. Representative images of plantlets developed from apical buds cultured in MS medium supplemented with different NAA concentrations. The best results were observed with the use of 0.10 mg l^{-1} NAA.

Table 1. Effect of various NAA concentrations on naranjilla plantlet growth from apical bud culture.

	Plantlet height (cm)	Number of leaves	Average leaflength (cm)
NAA (mg l^{-1})	Mean± SE	Mean± SE	Mean± SE
0.01	1.39 ± 0.20^{b}	5.31 ± 0.80^{b}	0.59 ± 0.10^{ab}
0.05	1.46 ± 0.10^{b}	5.84 ± 1.40^{b}	0.59 ± 0.00^{bc}
0.10	2.04 ± 0.80^{a}	6.90 ± 0.40^{a}	0.86 ± 0.00^{a}
0.15	1.46 ± 0.10^{b}	5.38 ± 0.80^{b}	0.70 ± 0.00^{abc}
0.20	1.51 ± 0.20^{b}	5.34 ± 0.30^{b}	0.67 ± 0.00^{c}

Means followed by the same lower case letter in a column are not statistically different from each other (One Way ANOVA, Tuckey, $p<0.05$).

Software 2010).

Results and discussion

The protocols described in this study were efficient for the regeneration of naranjilla plants from different explant types. All apical buds (100%) developed into plants with roots in all NAA concentrations. This regeneration capacity has been previously described in other *Solanum* species such as *S. tuberosum* (Khadiga et al. 2009), suggesting that apical buds in this genus remain viable under the effect of exogenous NAA. In addition, the results of a Tukey test showed that a concentration of 0.10 mg l of NAA supplemented to the media produced significantly higher number of leaves per shoot ($p=0.008$), average leaf length ($p=0.008$) and plantlet height ($p=0.044$) (Table 1). These observations suggest that NAA in low concentrations triggers a positive effect in the growth of naranjilla plantlets from apical buds. However, this response could be species-dependent, as other studies report inhibitory effects of NAA alone or in combination with BAP for shoot development in *Solanum sessiliflorum* (Boufleuher et al. 2008).

Furthermore, a declining tendency of growth was observed for NAA concentrations below and above 0.10 mg l^{-1} (Table 1, Figure 2). Similar patterns caused by this growth regulator are common across different plant families, as it has been previously reported in genera such as *Citrus* (Moreira-Dias et al. 2000).

In order to explore the potential for plant regeneration from naranjilla hypocotyls, two different hypocotyl ages (1–2 weeks old and 3–4 weeks old) were cultured in media supplemented with NAA (0.01 mg l^{-1}), GA_3 (0.10 mg l^{-1}) and five concentrations of BAP (4.00 to 6.00 mg l^{-1}). Older hypocotyls (3–4 weeks) developed a significantly higher number of shoots ($p=0.001$) and leaves ($p=0.01$) (Table 2, Figure 3) compared to the younger explants (1–2 weeks). Age related differences in the response to growth regulators have been previously described in other species, but the tendencies are not congruent between reports. For example, a trend towards decreasing bud and shoot formation as the age of seedlings increased was noted for chili peppers (Ramírez-Malagón and Ochoa-Alejo 1996), a phenomenon which is opposite to our findings. The exact mechanisms for these trends in hypocotyls have not been previously described, but it has been reported that endogenous hormone production and its accumulation in hypocotyls play a direct role during seed germination and early plant development under specific conditions (Zheng et al. 2016).

Regarding the effect of the growth regulators, 3–4 week-old hypocotyls regenerated a larger number of shoots in the media supplemented with BAP 6.00 mg l^{-1}, while the best results for shoot length, number of leaves and average leaf length were observed in the media supplemented with BAP 5.50 mg l^{-1}. However, these results were not significantly different from the other BAP concentrations tested (Table 3). Combinations of auxins and cytokinins have been previously used for the successful hypocotyl regeneration of other Solanum species from the Lasiocarpa section (Schuelter et al. 2009; Te-chato 1988). For *S. sessiliflorum*, high rates of shoot induction have been reported using hypocotyls in media supplemented with 0.01 mg l^{-1} indole acetic acid (IAA) and 20.00 mg l^{-1} kinetin (KIN) (Schuelter

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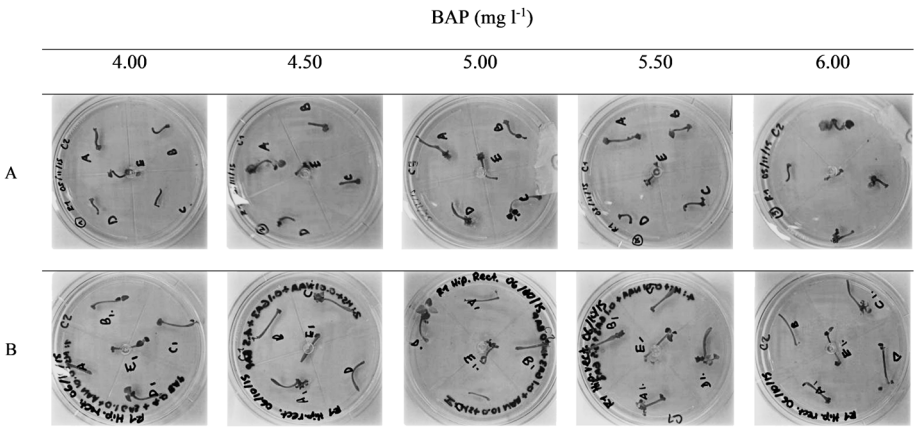

Figure 3. Representative images of the hypocotyl regeneration assays from (A) 1–2 week old hypocotyls and (B) 3–4 week old hypocotyls in MS medium supplemented with 0.01 mg l^{-1} NAA, 0.10 mg l^{-1} GA_3 and different BAP concentrations. Older hypocotyls produced longer shoots with higher number of leaves.

Table 2. Effect of the age of the explant in the regeneration efficiency and naranjilla plantlet growth from hypocotyl culture across all treatments.

	Number of shoots	Shoot length (cm)	Number of leaves	Average leaf length (cm)
Age of the explant	Mean±SE	Mean±SE	Mean±SE	Mean±SE
1–2 weeks	0.57 ± 0.30^{b}	0.23 ± 0.20^{a}	1.32 ± 0.80^{b}	0.18 ± 0.10^{a}
3–4 weeks	1.01 ± 0.50^{a}	0.25 ± 0.10^{a}	2.22 ± 0.90^{a}	0.20 ± 0.10^{a}

Means followed by the same lower case letter in a column are not statistically different from each other (One Way ANOVA, Tuckey, $p<0.05$).

Table 3. Regeneration efficiency and naranjilla plantlet growth from the culture of 3 to 4-week-old hypocotyls in different BAP concentrations.

	Number of shoots	Shoot length (cm)	Number of leaves	Average leaf length (cm)
BAP (mg l^{-1})	Mean±SE	Mean±SE	Mean±SE	Mean±SE
4.00	0.90 ± 0.30^{a}	0.23 ± 0.10^{ab}	2.35 ± 0.90^{ab}	0.22 ± 0.10^{a}
4.50	0.55 ± 0.30^{a}	0.12 ± 0.10^{b}	1.03 ± 0.50^{b}	0.12 ± 0.10^{a}
5.00	1.10 ± 0.60^{a}	0.23 ± 0.10^{ab}	2.15 ± 0.70^{ab}	0.22 ± 0.10^{a}
5.50	1.20 ± 0.70^{a}	0.39 ± 0.10^{a}	2.83 ± 0.70^{a}	0.23 ± 0.10^{a}
6.00	1.30 ± 0.50^{a}	0.28 ± 0.10^{ab}	2.73 ± 1.10^{a}	0.22 ± 0.10^{a}

Means followed by the same lower case letter in a column are not statistically different from each other (One Way ANOVA, Tuckey, $p<0.05$).

et al. 2009). The size of these adventitious shoots from hypocotyl segments were small (<5 mm) (Schuelter et al. 2009), similar to our results (size mean=3.9 mm) with *S. quitoense* hypocotyls.

Cytokinins play an important role in the natural physiology of plant cells, but identifying the optimal concentration is key for their use in in vitro culture, as an excess of these growth regulators may cause a phytotoxic effect (Schuelter et al. 2009). Our results show a favorable response to BAP concentrations of up to 6.00 mg l^{-1}, which agrees with previous reports that suggest that a concentration of 10.00 mg l^{-1} (in combination with NAA 0.01 mg l^{-1}) has an inhibitory effect in shoot formation from hypocotyls in *Solanum quitoense* (Te-chato 1988).

Our results on naranjilla regeneration from petioles show that upper petioles yielded the highest number of shoots per explant ($p=0.004$), longer shoot lengths ($p=0.001$), higher number of leaves ($p=0.037$) and larger leaves per plantlet ($p=0.001$) compared to lower petioles (Table 4). These differences could be explained by the fact that petioles found in the upper stem are younger than the ones found on the lower stem; therefore, younger petioles show higher regeneration rates. Furthermore, a range of NAA, BAP and GA_3 concentrations were tested for their effect on petiole regeneration (Figure 4). Explants in all the treatments successfully regenerated shoots. However, MS medium supplemented with 0.02 mg l^{-1} NAA, 4.50 mg l^{-1} BAP and 1.00 mg l^{-1} GA_3 (Figure 4, red box) provided the best results in three of the traits measured: shoot length (mean=9.20 mm, $p=0.036$), number of leaves per explant (mean=11.00 leaves, $p=0.0079$) and average leaf length (mean=4.40 mm, $p=0.037$) (Supplementary Material S1). These results suggest that the use of a cytokinin in combination with low auxin concentrations and gibberellin induce efficient shoot formation from petioles in *S. quitoense*. In contrast, previous studies report favorable responses in petiole explants by using

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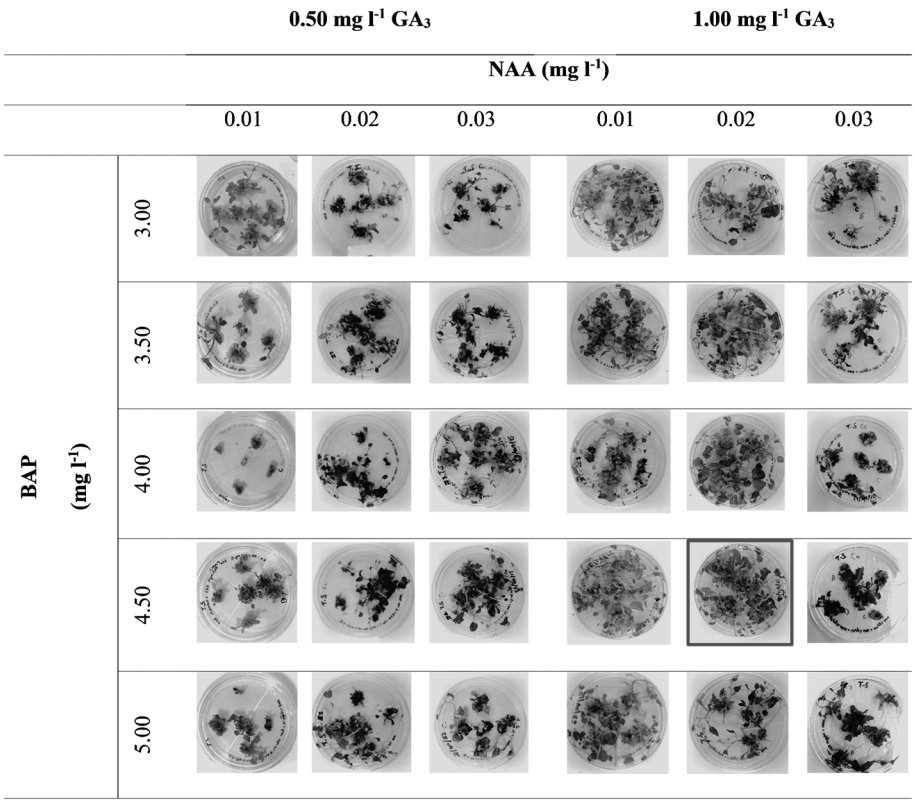

Figure 4. Representative images of the upper petiole regeneration assays with the use of different combinations of NAA, BAP and GA_3. Significant differences were observed for the number of leaves, leaf length and shoot length in upper petioles cultured in MS +4.50 mg l^{-1} BAP, 0.02 mg l^{-1} NAA and 1.00 mg l^{-1} GA_3 (red box).

Table 4. Regeneration efficiency and naranjilla plantlet growth from the culture of petioles from upper and lower plantlet sections.

	Number of shoots	Shoot length (cm)	Number of leaves	Average leaf length (cm)
Position of petiole	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Upper section	2.24±0.50[a]	0.42±0.10[a]	5.03±0.70[a]	0.22±0.10[a]
Lower section	1.77±0.30[b]	0.32±0.10[b]	3.73±0.40[b]	0.18±0.10[b]

Means followed by the same lower case letter in a column are not statistically different from each other (One Way ANOVA, Tuckey, $p<0.05$).

high concentrations of cytokinins in combination with auxins (Hendrix et al. 1987; Schuelter et al. 2009). The incorporation of GA_3 in the growth medium could play an important role by interacting with both auxins and cytokinins, explaining the improved response in our experiments in terms of number of shoots (5.05 shoots per explant, compared to 1.50 shoots per explant reported by Schuelter et al. 2009).

To find the best explant with the potential of generating the largest number of plantlets, we compared the results from the best media for each type of explant. Upper petioles produced significantly higher numbers of shoots and leaves ($p<0.001$) compared to lower petioles, hypocotyls and apical buds. On the other hand, longer shoots and bigger leaves were obtained when using apical buds as starting material ($p<0.001$) (Figure 5). We suggest that petiole explants are an adequate source material to maximize shoot regeneration, whereas apical buds are better suited to deliver plantlets with longer shoots.

The higher regeneration efficiency from petioles compared to other tissues prompted a deeper investigation of the role of hormone interactions in the in vitro regeneration of upper petioles through a multifactorial analysis under a linear model (see Data analysis). This type of analysis is commonly used when an experiment requires a reduction in the number of samples, an optimization of parameters, or if there is an interest in screening the most influential factors of an *in vitro* culture protocol (Gundogdu et al. 2014; Nuri et al. 2004). Our results show that GA_3 could be a key regulator for shoot regeneration, since it interacts directly with NAA ($p=0.017$), and BAP ($p=0.018$) independently. It has been reported that gibberellins regulate the action of other phytohormones through direct interactions (Hamayun et al. 2010; Khalloufi et al. 2017). On the other hand, the auxin and cytokinin interaction was not significant. Furthermore, the three

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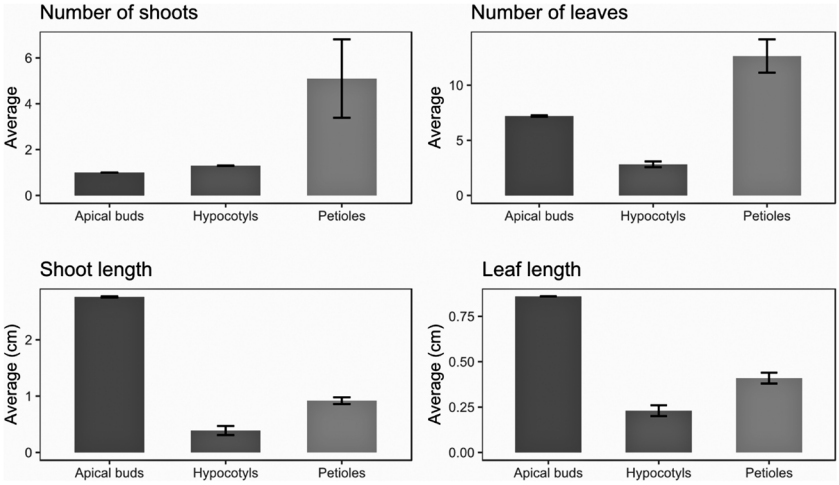

Figure 5. Performance of different explant types regarding regeneration efficiency and plantlet growth rates. Higher numbers of shoots ($p<0.001$) and leaves ($p<0.001$) per explant were obtained with upper petioles cultivated in MS medium supplemented with 0.02 mg l^{-1} NAA + 1.00 mg l^{-1} GA_3 + 4.50 mg l^{-1} BAP (upper panels). At the same time, bigger shoots ($p=0.01$) and leaves ($p=0.02$) were obtained from apical buds cultured in MS medium supplemented with 0.10 mg l^{-1} NAA (lower panels). For all the outcomes, 3–4 week old hypocotyls were the least efficient explant type.

regulators used in this study show significant effects on the number of leaves per regenerated plantlet by themselves, suggesting that, to a certain extent, they act independently. This phenomenon might reflect the action of each hormone in different stages of leaf formation, where cytokinin signaling promotes stem cell differentiation while auxins and gibberellins have the highest activity during cell division and elongation. (Fambrini and Pugliesi 2013).

This report for the successful regeneration of apical buds, hypocotyls and petioles in *S. quitoense* provides different tools for the regeneration of naranjilla plants, while giving an insight into the role of different hormones in the regeneration process. The regeneration protocols themselves could facilitate the ex situ conservation and establishment of crop improvement programs for *S. quitoense*, a species where interspecific hybridization can be an important source for traits of interest. As with other crops, the combined use of efficient propagation methods and modern breeding techniques could maximize their value for the local economy and agrobiodiversity.

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