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SHORT NOTE [NOTA CORTA]

EFFECT OF α-SOLANINE, α-CHACONINE AND SOLANIDINE ON THE GROWTH OF *IN VITRO* CULTURED POTATO (*Solanum tuberosum)* L. SEEDLINGS

[EFECTO DE α-SOLANINA, α-CHACONINA Y SOLANIDINA SOBRE EL CRECIMIENTO DE PLÁNTULAS DE PAPA (*Solanum tuberosum*) CULTIVADAS *IN VITRO*]

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RESUMEN

Los glicoalcaloides naturales de la papa son producidos en tejidos meristemáticos y tienden a acumularse bajo ciertas condiciones adversas como el estrés por calor. Algunas observaciones preliminares sobre la respuesta de la papa al calor, parecen indicar un efecto modulador del crecimiento por parte de los alcaloides que no ha sido estudiado. Aquí reportamos resultados obtenidos al adicionar glicoalcaloides de la papa al medio de enraizamiento de yemas axilares usadas como explantes para el cultivo de plántulas *in vitro*. Los glicoalcaloides α–solanina, α-chaconina y solanidina fueron extraídos de meristemos de papa y purificados usando cromatografía en capa fina. Los alcaloides se encontraron consistentemente en una proporción de 45:45:10, respectivamente. Los alcaloides aislados fueron mezclados y adicionados al medio de cultivo en la misma proporción en que se encontraron o en proporciones equimolares para evaluar su efecto sobre el crecimiento de las plántulas in vitro. A temperatura ambiente, en la proporción endógena de 45:45:10, los GAs provocaron un claro efecto negativo sobre el alargamiento del tallo de manera dependiente con respecto a su concentración en el medio. A una concentración de 10μM los alcaloides causaron 48% de reducción en la longitud del tallo después de 45 días, en comparación con el tratamiento control; a 50 μM provocaron aún mayor reducción de longitud, además de clorosis extensiva de tallos e inhibición del crecimiento de la raíz. Exposición a 100 μM inhibió el crecimiento totalmente. Presentes en proporción equimolar, los alcaloides no provocaron efectos tan severos. A una concentración de 50 μM de alcaloides totales y a 30º C en la sala de incubación se observó una alteración dramática en el patrón de desarrollo, resultando plántulas en forma de roseta. Dada la presencia de los alcaloides preponderantemente en los meristemos de la planta, se propone que los alcaloides en estos tejidos pueden mostrar efectos regulatorios sobre el desarrollo.

Palabras clave: solanina; chaconina; solanidina; papa; plántula; crecimiento.

SUMMARY

Naturally occurring potato glycoalkaloids are produced mainly in meristematic tissues; an accumulation of these compounds may be observed particularly in response to some adverse conditions such as heat; previous preliminary observations in this respect seem to indicate a possible growth modulating effect by glycoalkaloids in the potato plant. Here we report the results from a series of experiments designed to describe the effect of potato glycoalkaloids in the rooting media on the growth of seedlings developed from axillary buds cultivated *in vitro*. Axillary buds were obtained from greenhouse plants to study the effect of alkaloids on their growth at ambient and supra optimal temperature. α-Solanine, α-chaconine and solanidine were extracted from meristems and purified by thin layer chromatography; they were found to occur consistently in a ratio of 45:45:10, respectively. Either in these or in equimolar proportions, the isolated alkaloids were mixed and added to the growth medium in order to evaluate their effect on seedling growth. At ambient temperature, when alkaloids were present in the 45:45:10 ratio, results showed a clear negative effect on stem elongation in an concentration dependent fashion; 10μM caused a 48% reduction in stem length with respect to controls after incubation for 45 days; 50 μM further inhibited elongation, caused chlorosis and inhibition of root growth. 100μM caused complete inhibition of seedling development over the same period. Present in equimolar proportion alkaloids did

not have as severe an effect. The presence of 50μM total alkaloids in equimolar proportions at 30º C caused a dramatic alteration in developmental pattern resulting in rosette seedlings. A regulatory capacity of alkaloids is discussed.

Key words: solanine; chaconine; solanidine; potato; seedling; growth.

INTRODUCTION

α-Solanine, α-chaconine and solanidine are glycoalkaloids (GAs) which may accumulate to relatively high concentrations in potato (Gregory, 1984; Friedman, 2006). As much as 100 mg of total GAs 100 g^{-1} of tissue have been reported in potato meristematic tissues (Linnemann and Hartman, 1985; Maine *et al*., 1988), while an upper limit of 20 mg 100 g^{-1} of potato is generally accepted for food safety purposes (Cantwell, 1996). Although genetically determined (Kozukue *et al*., 2008), concentration of GAs may be strongly affected by environmental factors; particularly by light and limiting temperature regimes (Dao and Friedman, 1994; Bowles *et al*., 2006).

Glycoalkaloids are believed to owe their evolutionary origins and diversity to their function in deterring predators from overfeeding on the plant (McCue, 2009) and their toxicity to mammals would indicate so; in particular, it has been proposed that these compounds may play a role as part of a mechanism for plant defense against microbes (Friedman, 2006; Bowles *et al*., 2006; Friedman and McDonald, 1992). However, pathogens do not seem to induce synthesis of GAs, but rather, they tend to prevent their accumulation and, in fact, potato plants selected for late blight resistance (*Phytophtora infestans*) consistently showed lower concentrations of GAs over the selection program (Sarquís *et al*., 2000). Thus, present evidence with regard to the role of GAs in plant defense mechanisms remains a subject for debate. Nonetheless, in recent years the genes encoding the enzymatic steps responsible for their synthesis have begun to be elucidated (McCue, 2009).

In potato tuber cells, maximum GA accumulation has been shown to modify membrane permeability (Coria *et al*., 1998), adding to the evidence supporting GAs activity on the basis of their capacity to attach to membrane components and form complexes which cause membrane disruption and leakage (Jenifer, 1987; Roddick and Drysdale, 1984; Rayburn *et al*., 1994; Keukens *et al*., 1995). On the other hand, little is known about the effect of these natural compounds on the growing plant tissues themselves, although they have been reported to accumulate even to relatively large amounts as, for example, in potato tubers cultivated in warm regions, where the crop exhibits a relatively high and sustained expansion rate (CIP, 1994). Indeed, one of the most severe limitations to yield and quality of potato in these areas is GA build up in the tubers (Sowokinos, 1990). Here we report our findings on the effect of exogenous alkaloids on the elongation of *in vitro* potato seedlings at contrasting temperatures in an effort to obtain evidence of a possible role of the endogenous GA balance in early growth at high temperature.

MATERIALS AND METHODS

In vitro seedlings micropropagated from axillary meristems from mature plants (cv. DTO-33) cultured in a greenhouse were used in these experiments. Mother plants were grown from tubers kindly donated by the National Potato Program at the Instituto Nacional de Investigaciones Agrícolas Forestales y Pecuarias, in Metepec, Estado de Mexico, Mexico.

Determination of endogenous GAs

Since the highest GA concentration in plants generally occurs in meristematic tissues (Gregory, 1984) we used meristems as source of GAs. All meristems were removed from at least six healthy mature potato plants grown in a greenhouse; these meristems were then processed to extract, purify and quantify GA content as follows: 10 g tissue samples were homogenized with mortar and pestle in 50 mL methanol at room temperature; homogenates were filtered and centrifuged at 13,000 rpm for 15 min (Sorvall Heraeus, Biofuge). Precipitates were discarded and supernatants carried on to the purification steps. Methanolic extracts were precipitated with concentrated ammonium hydroxide at pH 11.5 (Fitzpatrick *et al*., 1978; Friedman and Dao, 1962); samples were incubated at 70º C for 30 min and then centrifuged at 12,500 rpm for 1 h; supernatants were discarded and precipitates re-suspended in 1 mL absolute methanol. This mixture was used to separate alkaloids on silica gel (Merck) by thin layer chromatography in a chloroform:methanol:ammonium hydroxide (25:25:2.1) solvent system. Alkaloid detection on chromatoplates (100 x 200 mm) was achieved using iodine vapors; once bands were visualized they were identified by drawing their perimeter with a pencil. To retrieve the alkaloids adsorbed onto the plates these were kept at room temperature until all trace of iodine had disappeared. Then a sharp scalpel was used to scrape the silica within pencil lines off the plates. Silica dust from each separate band was placed in a test tube, added with 5 ml methanol, then vortexed vigorously (Thermolyne, Maxi Mix II); this solution was filtered by passing through a 2 mm cotton filter placed at the bottom of a 5 mL syringe barrel; the elution was centrifuged at 8,000 rpm during 30 min. Precipitates were discarded

and supernatants placed in eppendorf vials and stored until used for quantification of GAs. For this purpose, samples were applied onto new silica plates and bands were visualized by spraying serical ammonium sulfate in diluted sulfuric acid. After spraying, plates were incubated at 110º C for 10 min. Upon development, identification and concentration of each alkaloid was determined by densitometry as described previously (Coria *et al*., 1998). Each determination was replicated at least three times.

In vitro tissue culture

Micropropagation of potato cuttings was done by incubating axillary meristems in MS (Murashige and Skoog, 1962) solid medium without any vitamins or hormones added. Carbon source was 3% sucrose and Phytagel (2.3 $g.l^{-1}$) was used for jellification of the medium, which was adjusted to pH 5.8 (Oakton 2500), prior to sterilization (Chromalox) at 15 psi for 15 min. Plant material used for micropropagation were *in vitro* seedlings with at least six internodes. All operations were conducted in a laminar flow tissue culture chamber (Figursa, CFH-120). Stems were sectioned into two or three portions each containing one axillary meristem. In order to evaluate the effect of exogenous alkaloids on the growth of *in vitro* seedlings various concentrations of α-solanine, α-chaconine y solanidine were added to the tissue culture medium after sterilization. Dose-response curves were obtained for the range of concentrations between 0 and 300 μM. Final alkaloid concentration added to the culture media was kept fixed, but the proportion of each of the three alkaloids employed was made to vary. For tests, either α-Solanine, α-chaconine and solanidine purified in our lab were added to the growth medium in the same proportion in which they were found to be present in the tissues analyzed (45:45:10, respectively) or commercial alkaloids were combined in equimolar concentrations, i.e., 1:1:1. The same procedure was used when evaluating the effect of alkaloid addition to the growth medium prior to subjecting the seedlings to 30º C throughout the duration of the experiment. The effect of exogenous alkaloids on seedling growth was studied by measuring shoot length every other day for 45 days. Data reported are means and standard deviations. Each concentration treatment included 20 individual seedlings grown in 25 x 200 mm test tubes (Kimax). Dry weight (20 seedlings) was recorded (Ohaus, Explorer) separately for roots, stems and leaves. After weight determination, seedlings of each different lot were ground in a mortar to determine chlorophyll concentration by extraction in cold acetone (10 $mL.g^{-1}$); once homogenized in a Polytron (Ultraturrax), samples were centrifuged at 5,000 rpm for 10 min; supernatants were recovered and O.D. was determined at 680 nm (Genesis, 5 Milton Roy). Extinction coefficient (ε□) used to calculate chlorophyll concentration was 2.0. In all tests in vitro seedlings were incubated under 150 μM m^{-2} s^{-1} PAR.

RESULTS AND DISCUSSION

Potato explants micropropagated *in vitro* grow relatively easily and rapidly. Typically, when grown under optimal incubation conditions, the explants used in these studies grew to 10 cm seedlings after a month of incubation; they developed normally, showing a profuse root system, a vigorous shoot and wide leaf blades of a dark green color. Stem elongation rate was determined by measuring stem length every other day and plotting against time. Figure 1 shows the resulting characteristic sigmoidal pattern of early stem elongation (Hunt, 1975) over whose duration stems elongated an average of 0.23 $cm.day^{-1}$.

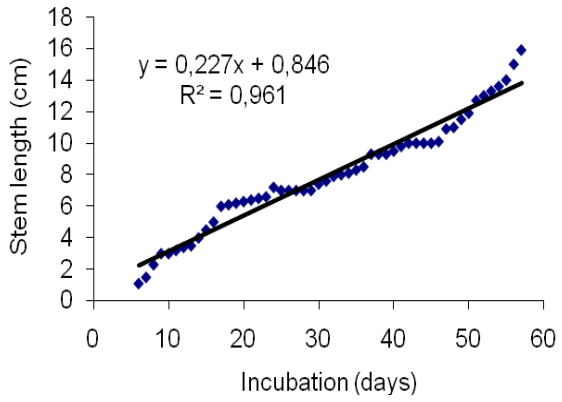

Figure 1. Stem elongation of *in vitro* potato seedlings incubated at 21º C and under 150 μM m^{-2} s^{-1} PAR.

In general, α-solanine and α-chaconine combined averaged 89% of the total alkaloid content found in the meristems of mother plants used in this study, while solanidine averaged 11%. Total alkaloid content extracted from meristems averaged 69.7 ± 7.3 mg.100 g^{-1} fresh weight. This range of alkaloid concentrations in potato meristematic tissues is within reported values for other potato cultivars (Friedman and Dao, 1962) and substantiates previous reports of more abundant glycosidic forms of alkaloids than aglycans present in potato meristematic tissues (Fitzpatrick *et al*., 1978).

Effect of exogenous alkaloids on seedling growth

Purified alkaloids were added to the growth medium in a range from 0 to 100 μM total alkaloid concentration. Relative proportions of α-chaconine, α-solanine and solanidine in the total mixture prepared were 45, 45 and 10, respectively, as found in potato meristematic tissues normally. Over the six week incubation period

stem elongation was reduced in a concentration dependent pattern (Figure 2). Total alkaloids at 10 μM in the rooting medium was enough to reduce seedling elongation rate by 50%; while at 50 μM very little growth was recorded.

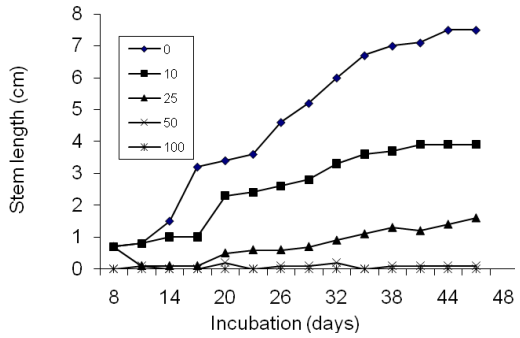

Figure 2. Effect of alkaloids on stem elongation of *in vitro* potato seedlings. Alkaloids were added to the incubation medium in a 45:45:10 ratio for α-solanine, α-chaconine and solanidine, respectively, to attain a total alkaloid concentration of 0, 10, 25, 50 or 100 μM. Seedlings were kept at 21º C and under 150 μM m^{-2} s^{-1} PAR.

Figure 3. *In vitro* potato seedlings after 45 days of incubation in a medium added with GAs. From left to right: 0, 10, 25, 50 or 100 μM total alkaloid concentration in a 45:45:10 ratio for α-solanine, α-chaconine and solanidine, respectively. Seedlings were kept at 21º C and under under 150 μM m^{-2} s^{-1} PAR.

Exposure to increasing concentrations of total alkaloids caused stem dry weight to decrease with decreasing elongation and leaf and root dry weights decreased accordingly (Table 1). Concomitantly, the higher the alkaloid concentration in the growth medium, the less chlorophyll was extracted per gram of tissue in close association with decreased extension growth (Table 2); Jadhav and Salunkhe (1975) reported an inverse relation between GAs and chlorophyll content in potato tubers. Figure 3 shows a photograph of seedlings exposed to increasing concentrations of total alkaloids in the rooting medium.

Typically, even the lowest GA concentration added to the rooting medium caused a significant decrease in stem length (48% inhibition), in comparison to controls, although leaf blade expansion seemed unaffected. Exposure to 50 μM further inhibited stem elongation and additionally caused reduced root growth and leaf expansion. Explants exposed to 100 μM total alkaloids did not show any sign of either root or shoot development; two days after initiation of the treatment these explants began to show a slight chlorosis, and after five days they were completely chlorotic and remained so for the duration of the incubation period.

Table 1. Dry weight of *in vitro* grown potato seedlings exposed to various concentrations of exogenous alkaloids in the rooting medium in a 45:45:10 ratio.

	Dry weight (mg.20 seedlings^{-1})			
Sample	Seedling	Stem	Leaf	Root
Control	530 ± 40	180 ± 30	110 ± 30	280 ± 90
10μM	490 ± 28	110 ± 26	47 ± 10	380 ± 50
25μM	410 ± 30	80 ± 29	50 ± 20	200 ± 10
50μM	22 ± 22	10 ± 5	15 ± 5	0

Table 2. Chlorophyll content and percent inhibition of stem elongation of *in vitro* grown potato seedlings in the presence of exogenous alkaloids in the rooting medium at a 45:45:10 ratio, after 45 days of incubation.

	Chlorophyll (mg.g fresh weight^{-1})	Stem elongation (% inhibition)
0 μM	2.53 ± 0.20	0.0
10 μM	1.00 ± 0.18	48.0
25 μM	1.03 ± 0.25	78.7
50 μM	0.37 ± 0.15	98.3
100 μM	0.27 ± 0.20	100.0

In another set of trials, commercial alkaloids were added to the rooting medium in equimolar proportions in a range from 0 to 300 μM, instead of the commonly found proportions of each alkaloid (45:45:10), as used in the previous tests. Our objective was to find out whether the observed effects on seedling growth caused by GAs can be altered by varying their relative concentration from what we normally found in tissues; the rational being that present in equimolar proportions

GAs would not exhibit a synergic effect to the same extent as when present in a 45:45:10 ratio, as more of the aglycan form and less of the more active glycosidic forms are present (Rayburn *et al*., 1994). As before, increasing equimolar concentrations of alkaloids in the growth medium caused an increasing reduction of seedling growth. However, as shown in photographs in Figures 4 and 5, at 50 as well as at 100 μM exogenous total alkaloids in equimolar proportions (panels B in both Figures), the negative effect of alkaloids was by far less severe than at the endogenous 45:45:10 ratio (panels A in both Figures).

These observations suggest that over a threshold value, the particular proportion of each component in the endogenous alkaloid balance may trigger a synergy which causes the observed alteration of growth, thus overriding the opposing effect of the aglycan alkaloid on the extent of interaction between glycosidic alkaloids and membrane sterols (Roddick and Drysdale, 1984; Keukens, *et al*., 1995). Recent evidence which supports this interpretation was reported by Mandamika *et al*. (2007) while studying the toxic effects of single and mixtures of potato glycoalkaloids on gene expression in human intestinal epithelial cells. In this case, differences in the responses to the various glycoalkaloid treatments were mainly due to the differing degrees of potency of the glycoalkaloids; α-solanine (10 μM) was the least potent of the glycoalkaloid treatments, as observed in both lactate dehydrogenase (LDH) leakage test and gene profiling experiments; α-solanine administered alone caused less LDH leakage compared to equimolar amounts of α-chaconine and the glycoalkaloid mixtures.

Effect of exogenous alkaloids at supra optimal temperature

The typical bitter flavor developed by potato tubers grown at supraoptimal temperature is due to GA buildup and is well documented (Cantwell, 1996; McCue, 2009). Presuming a possible relation between such accumulation and a protective effect by GAs against heat damage, we tested the application of a GA mixture at equimolar concentrations of each intervening compound to evaluate the effect of incubation of seedlings at high temperature.
Seedlings incubated at 30º C, a supraoptimal temperature for the species, are shown in Figure 6. At 50 μM total alkaloids in the rooting medium, the pattern of seedling growth was visibly altered; in this case, the length of the internodes was drastically reduced and secondary shoots were initiated; as a consequence, seedlings developed into rosettes.

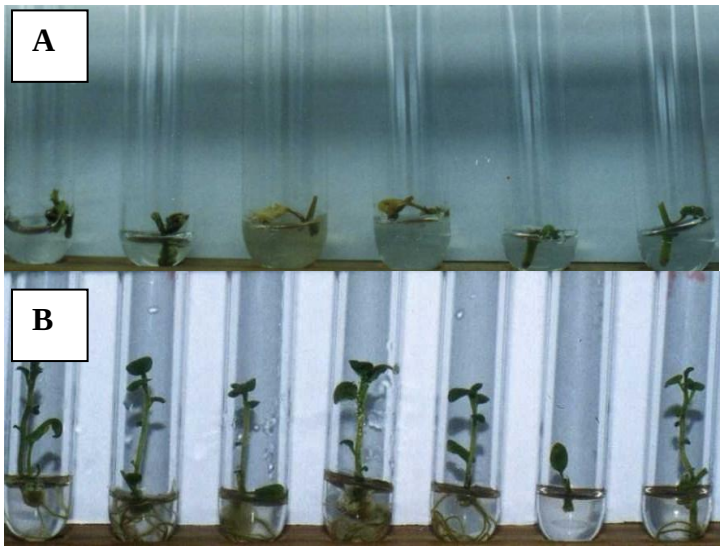

Figure 4. Development of *in vitro* potato seedlings after 30 days incubation in the presence of 50 μM total alkaloids in either: A) 45:45:10 ratio or B) equimolar ratio. Seedlings were kept at 21º C and under 150 μM m^{-2} s^{-1} PAR.

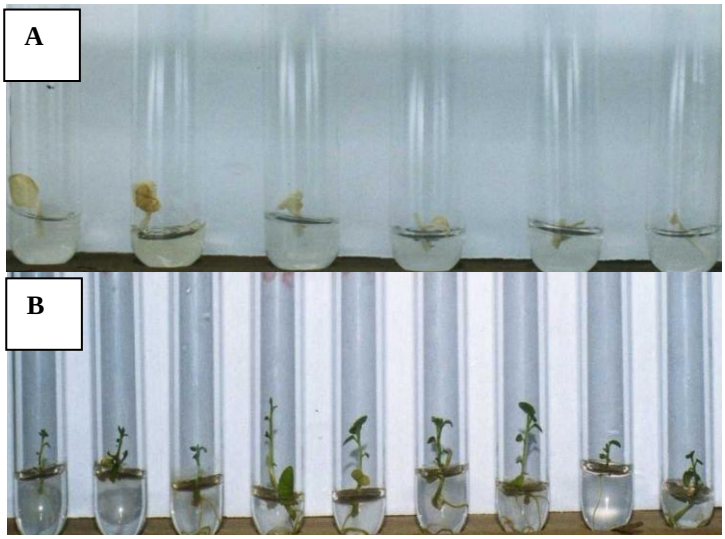

Figure 5. Development of *in vitro* potato seedlings after 30 days incubation in the presence of 100 μM total alkaloids in either: A) 45:45:10 ratio or B) equimolar ratio. Seedlings were kept at 21º C and under 150 μM m^{-2} s^{-1} PAR.

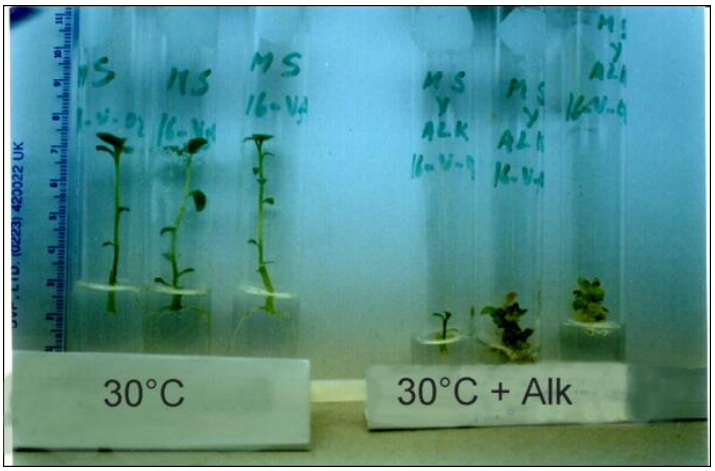

Figure 6. I*n vitro* potato seedlings incubated in MS medium at 30º C either in the absence (left) or in the presence (right) of 50 μM total alkaloids.

This may result from a similar effect by alkaloids as has been reported for brassinosteroids, which are well known plant hormones capable of regulating plant growth and development by controlling gene expression involved in cell division and cell elongation (Vert *et al*., 2005). Results at 35º C were not consistent, but generally seedlings grown at this temperature did not grow a root and their stems did not elongate; they gradually lost their normal green color and became chlorotic regardless of the presence or absence of alkaloids in the media.

The combined effect of high temperature and alkaloids in the growth media did not show a clearly distinct pattern; in some experiments the presence of alkaloids at any equimolar concentration seemed to attenuate heat damage, but in other tests no differences could be recorded between treated seedlings and controls. At any rate, to our knowledge, this is the first report of a probable involvement of regulatory effects of potato GAs in the plant response to supraoptimal ambient temperature.

CONCLUSION

Given that alkaloids occur naturally in meristematic tissues mostly, we tested adding alkaloids extracted from such tissues to the rooting media in which seedlings were grown *in vitro* from axillary buds. The results seem to indicate a possible relation between the presence of alkaloids and a dramatic change in growth pattern from elongation to rosette formation. We

suggest this may be due to impairment of the regulation of elongation growth by glicoalkaloids, under the experimental conditions tested.

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