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In vitro analysis of susceptibility to *Agrobacterium rhizogenes* in 65 species of Mexican cacti

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Abstract

Susceptibility of Mexican cacti to *Agrobacterium rhizogenes* was evaluated in 65 species of 22 genera. Stem discs taken from *in vitro* cultured plants were inoculated with *Agrobacterium rhizogenes* A4 agropine-type strain that contains the wild RiA4 plasmid and the binary vector pESC4 with the *nptII* and *gus* genes. Hairy roots were produced directly from wounds, or starting from calli generated on the wounded surface, in 34 of the evaluated species. The frequency of hairy roots formation, the number of roots per explant and its growth rates were variable among the tested species. In the 31 remaining species the production of transformed roots was not observed under the conditions used in these experiments. Histochemical detection of β-glucuronidase (GUS) activity demonstrated the expression of this foreign gene in the hairy roots. PCR analyses demonstrated the presence of the *rolB* and *nptII* genes in the DNA of the transformed roots. The patterns of alkaloid-like compounds obtained by thin layer chromatography in some of the tested species were qualitatively similar between the transformed and non-transformed roots.

Additional key words: alkaloids, β-glucuronidase, *Cactaceae*, transformed roots.

Introduction

The *Cactaceae* is a large and diverse family native to America. These plants have been used for human food (fruits and vegetables), forage, materials for diverse constructions, ornamental plants and sources of secondary metabolites like alkaloids, betalains, triterpenes and sterols (Anderson 2001, Bravo-Hollis and Sánchez-Mejorada 1991). Many important secondary metabolites are synthesized in roots, which can then be stored *in situ* or transported to other plant organs (Waller and Nowacki 1978). For this reason, one of the most promising *in vitro* technique to produce plant secondary metabolites is the generation and culture of transformed roots induced as a result of the infection with *Agrobacterium rhizogenes*. Transformed roots have several advantages: *1*) these roots can be removed from the original explant and cultured in hormone-free media where they exhibit a high rate of growth and genetic stability; *2*) transformed roots produce secondary metabolites in higher quantities and in a more stable way that other types of *in vitro* cultures like callus or cell suspension (Bourgaud *et al*. 1997); *3*) sometimes it is possible to regenerate complete transformed plants starting from them, as in *Lotus corniculatus* (Nikolić *et al*. 2003), *Centaurium erythraea* (Subotić *et al*. 2003) and *Blackstonia perfoliata* (Bijelović *et al*. 2004).

Porter (1991) reported the results of *A. rhizogenes*-infection tests in 463 plant species belonging to 109 families. Of these, 264 species (57 %) were susceptible and produced transformed roots. With regard to the *Cactaceae*, Porter reports negative results with all the species that he analyzed (*Echinocactus* sp., *Lophophora williamsii*, *Opuntia hemifusa*, *Pereskia aculeata*, *Pereskiopsis velutina* and *Rebutia* sp.). Because of these results, Porter argued that the *Cactaceae* family is resistant to the infection with *A. rhizogenes* for what is not possible to produce transformed roots of these plants. In this work, the result of a survey made with a higher number of cacti species is reported.

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Abbreviations: EDTA - ethylenediaminetetraacetic acid; GUS - β-glucuronidase; MS - Murashige and Skoog medium; PCR - polymerase chain reaction; CTAB - hexadecyltrimethylammonium bromide.

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Materials and methods

In vitro cultured plants of 65 Mexican cacti species (Table 1) were used as tissue source for *Agrobacterium rhizogenes*-inoculation. These plants were taken from an *in vitro* conservation and massive propagation program that is carried out in our laboratory. The *Agrobacterium rhizogenes* A4 agropine-type strain contains the wild type plasmid pRiA4, that confers the hairy root phenotype, and the binary vector pESC4 that contains in the T-DNA region the *nptII* gene with the *nos* promoter and terminator, and the *gus* gene with the *cab* promoter and the *ocs* terminator (Jofre-Garfias *et al*. 1997). The bacteria were grown in YMB medium (10 g dm^{-3} D-mannitol, 0.2 g dm^{-3} $MgSO_4 \cdot 7\,H_2O$, 0.5 g dm^{-3} $K_2HPO_4 \cdot 3\,H_2O$, 0.4 g dm^{-3} yeast extract and 0.1 g dm^{-3} NaCl) (Hooykaas *et al*. 1977) with 50 mg dm^{-3} rifampicin and 50 mg dm^{-3} kanamycin at 28 °C. A 72 h liquid culture of the bacteria was diluted to 10^8 cells cm^{-3} with Murashige and Skoog (MS) liquid medium containing 50 μM acetosyringone (4-hydroxy-3´-5´-dimethoxyaceto-phenone), 100 mg dm^{-3} ascorbic acid and 100 mg dm^{-3} citric acid. Stem discs of *in vitro* cultured plants (approximately 4 mm wide) were immersed in the bacterial suspension for 45 min, then blotted dry with sterile muslin to remove the excess bacteria and placed horizontally on MS medium pH 5.7 containing 3 % sucrose and 4 g dm^{-3} *Agargellam* (*Phyto-Technology Laboratories*, Shawnee Mission, USA). Co-cultivation was carried out in the dark at 28 °C for 72 h, and then the infected explants were transferred to MS medium with 3 % sucrose, 4 g dm^{-3} *Agargellam*, 5 cm^3 dm^{-3}, *Plant Preservative Mixture* (PPM, *Phyto-Technology Laboratories*) to eliminate the bacteria, and 50 mg dm^{-3} kanamycin, as selective agent. For this experiment, 15 explants of each species were used, and the complete experiment was carried out twice. Cultures were maintained and multiplied by means of subcultures to fresh selection medium each 30 - 45 d and 90 d after the start of the experiments, the frequency of explants that developed roots, the number of roots per explant and the size of the roots were recorded.

GUS activity was detected histochemically in the presumably transformed roots as described by Stomp (1992). Tissues were stained 12 h at 37 °C in 0.1 M phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 0.1 % (v/v) Triton X-100 and 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). After staining, tissues were washed and conserved in 70 % (v/v) ethanol.

The *rolB*, *nptII* and *virD1* genes were detected in the GUS positive roots by polymerase chain reaction (PCR). DNA was extracted with hexadecyltrimethylammonium bromide (CTAB) as described by Wilkie (1997). The *nptII* gene is in the T-DNA of pESC4 plasmid and the *rolB* gene is in the T-DNA of the pRiA4 plasmid. Both genes are transferred to the plant genome. The *virD1* gene is outside of the T-DNA in the pRiA4 plasmid and was used as control to check the presence of residual *Agrobacterium* in the transformed roots. For the *nptII* gene the 5´ primer was TATTCGGCTATGACTGGGCA and the 3´ primer was GCCAACGCTATGTCCTGAT. These probes amplified a 517 bp fragment. For the *rolB* gene the 5´ primer was ATGGATCCCAAATT GCTATTCCTTCCACGA and the 3´ primer was TTAGGCTTCTTTCTTCAGGTTTACTGCAGC. These probes amplified a 780 bp fragment. For the *virD1* gene the 5´ primer was ATGTCGCAAGGACGTAAGCCCA and the 3´ primer was GGAGTCTTTCAGCAT GGAGCAA. These probes amplified a 450 bp fragment. Cycling conditions were: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. Samples were subjected to 30 cycles. Amplification products were analyzed by electrophoresis on 1.2 % agarose gels and detected by staining with ethidium bromide. *A. rhizogenes* plasmid DNA was used as positive control in the PCR assays. Plasmids were extracted by the small-scale boiling lysis method as described by Holmes and Quigley (1981).

Alkaloid-like compounds produced by transformed and non-transformed roots of five of the studied cacti species were investigated using the method of Wagner and Bladt (1996). For this, 100 mg powdered samples of transformed and non-transformed roots were mixed thoroughly with 1 cm^3 10 % ammonia solution and then extracted for 10 min with 0.5 cm^3 methanol under reflux, filtered and 0.03 cm^3 of each extract were applied to the TLC plate (3×0.01 cm^3). The plates were developed in the dark using a solvent system of toluene-ethyl acetate-diethylamine (70:20:10). The plates were analyzed using three detection systems: UV-254 nm, Dragendorff reagent, and Marquis reagent. The first two methods are general and detect a wide range of alkaloids and related compounds. Marquis reagent is more specific and detects mainly opium alkaloids (Wagner and Bladt 1996).

Results and discussion

Five to thirty days after infection with *A. rhizogenes* A4, hairy roots protruded from the wound surfaces of some of the cacti explants cultured in hormone-free medium with 50 mg dm^{-3} kanamycin (Fig. 1*A-D*). The frequency of hairy root formation was variable among the tested species, with values between 0 and 87 % (Table 1). In some species the first response after the infection was callus formation on the wound surface, and the transformed roots arose later starting from the callus tissue (Fig. 1*E*). These results were obtained using

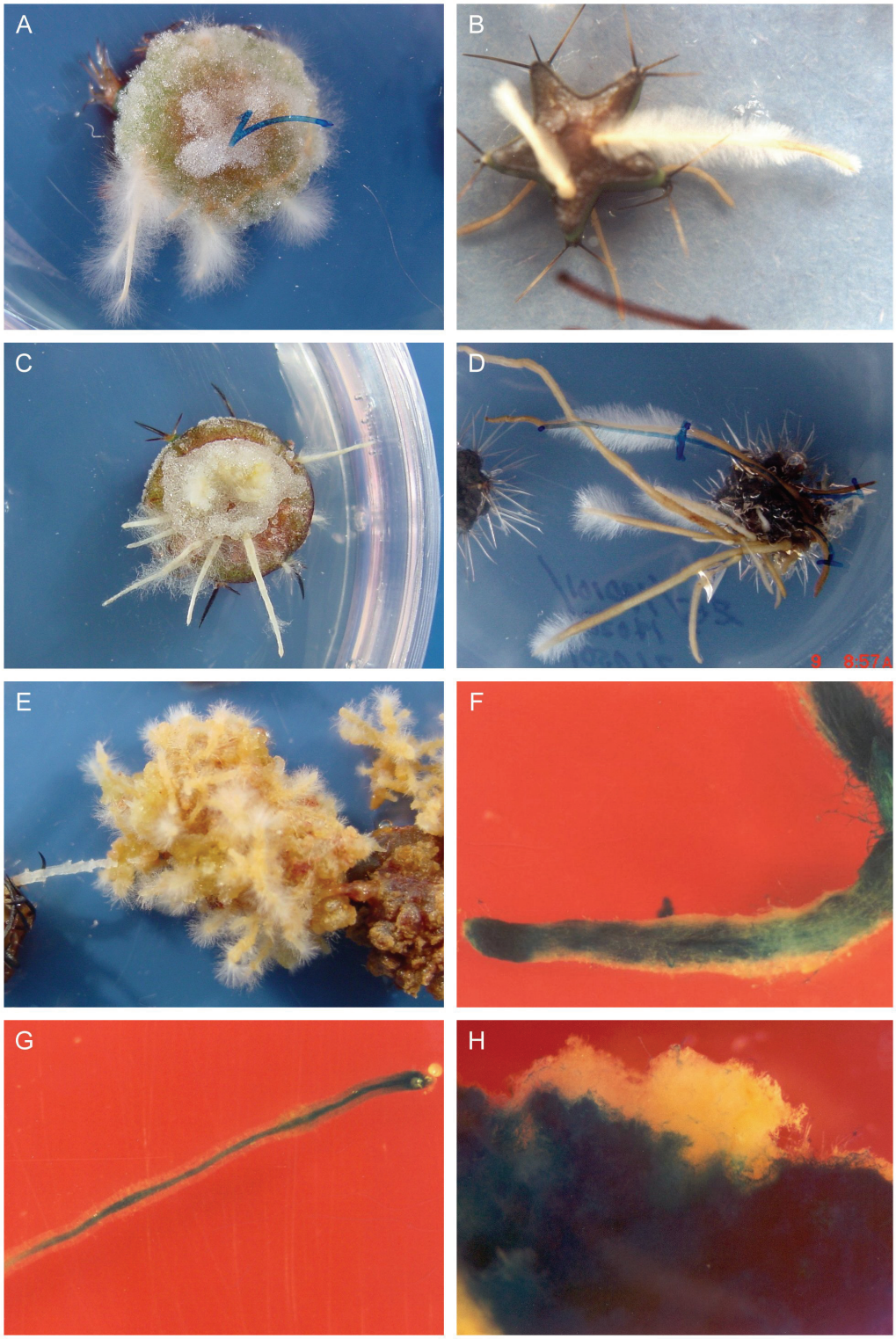

Fig. 1. Induction of hairy roots on shoot segments of *Mammillaria plumosa* (*A*), *Pachycereus schotii* (*B*), *Turbinicarpus schmiedickeanus* var. *schmiedickeanus* (*C*) and *P. pringlei* (*D*), infected with *A. rhizogenes*. *E* - Transformed roots formed from callus on a *T. schmiedickeanus* var. *schwarzii* explant infected with *A. rhizogenes*. *F,G* - Histochemical staining for GUS expression in transformed roots of *T. schmiedickeanus* var. *schmiedickeanus* (*F*) and *M. hutchinsoniana* var. *louisae* (*G*). *H* - Histochemical staining for GUS expression in callus produced in a *Coryphantha radians* explant infected with *A. rhizogenes*.

Table 1. Response of 65 species of Mexican cacti to *Agrobacterium rhizogenes* A4 infection. Means ± SD. All parameters were measured after 90 d in media with 50 mg dm^{-3} kanamycin. Mean number of roots per explant was calculated by dividing the total number of roots by the number of explants that generated roots.

Species	Explants generating roots [%]	Number of roots [explant^{-1}]	Length of the roots [mm]	Roots with GUS activity [%]
Acharagma aguirreana	20	1.33 ± 0.52	3.6 ± 2.3	0
Ariocarpus retusus var. *retusus*	17	1.20 ± 0.45	2.0 ± 1.0	0
Astrophytum capricorne	0	-	-	-
A. myriostigma	0	-	-	-
A. ornatum	3	1.00 ± 0.0	3.0 ± 0.0	100
Carnegiea gigantea	3	1.00 ± 0.0	4.0 ± 0.0	0
Cephalocereus senilis	30	1.67 ± 0.87	4.3 ± 2.2	80
Coryphantha clavata	17	3.80 ± 1.30	0.8 ± 0.1	0
C. durangensis	30	1.56 ± 0.88	5.5 ± 2.8	100
C. elephantidens	30	2.22 ± 1.79	8.9 ± 5.1	50
C. radians	7	27.5 ± 13.4	4.2 ± 2.9	64
Echinocactus platyacanthus	27	1.13 ± 0.35	2.0 ± 0.3	0
Echinocereus leucanthus	23	1.86 ± 0.69	0.5 ± 0.1	0
E. pectinatus var. *pectinatus*	10	2.00 ± 1.00	2.4 ± 0.2	100
Epithelantha bokei	10	1.33 ± 0.58	0.7 ± 0.2	0
Ferocactus flavovirens	40	2.25 ± 2.13	4.1 ± 2.1	0
F. hamatacanthus var. *hamatacanthus*	0	-	-	-
F. histrix	7	2.50 ± 0.71	6.4 ± 3.7	100
F. latispinus var. *latispinus*	7	5.00 ± 4.2	3.8 ± 3.0	100
F. pilosus	20	1.83 ± 0.75	4.8 ± 3.1	100
Leuchtenbergia principis	73	2.10 ± 1.30	3.1 ± 1.3	66
Mammillaria bocasana var. *bocasana*	30	6.22 ± 4.21	5.3 ± 4.2	100
M. bombycina	37	7.36 ± 5.87	0.5 ± 0.1	0
M. carmenae	20	4.00 ± 2.76	8.7 ± 6.9	0
M. crinita	17	8.20 ± 6.97	6.2 ± 4.9	100
M. formosa var. *formosa*	20	2.00 ± 1.09	5.9 ± 3.3	100
M. herrerae	7	3.00 ± 1.41	5.3 ± 3.2	100
M. hutchisoniana var. *louisae*	20	4.00 ± 1.55	8.8 ± 4.7	73
M. oteroi	10	2.67 ± 1.15	7.7 ± 5.6	0
M. pennispinosa	27	2.25 ± 1.03	4.1 ± 2.3	33
M. perbella	0	-	-	-
M. perezdelarosae	0	-	-	-
M. petterssonii	7	26.0 ± 7.07	9.0 ± 4.1	100
M. plumosa	67	1.60 ± 0.88	2.9 ± 1.2	25
M. senilis	40	2.42 ± 1.62	0.5 ± 0.1	0
M. solisioides	0	-	-	-
M. sonorensis	57	1.23 ± 0.56	4.4 ± 3.5	100
M. sphacelata var. *sphacelata*	13	1.75 ± 0.96	8.1 ± 3.3	100
M. theresae	13	3.25 ± 2.22	0.5 ± 0.1	0
M. uncinata	67	1.40 ± 0.82	7.5 ± 3.5	100
Mammilloydia candida	0	-	-	-
Melocactus curvispinus var. *curvispinus*	87	1.85 ± 1.43	2.8 ± 2.1	54
Pachycereus pringlei	20	3.33 ± 1.21	18.6 ± 4.4	0
P. schotii	17	1.60 ± 0.89	9.0 ± 2.2	25
Pelecyphora aselliformis	43	2.31 ± 1.75	0.6 ± 0.1	0
P. strobiliformis	33	1.70 ± 1.06	7.9 ± 2.1	10
Peniocereus serpentinus	13	3.50 ± 1.91	7.6 ± 5.0	0
Pilosocereus chrysacanthus	27	3.75 ± 2.31	7.3 ± 4.5	30
Sclerocactus uncinatus var. *wrightii*	60	2.17 ± 1.34	6.2 ± 3.1	67
Stenocactus coptonogonus	27	1.50 ± 0.76	0.5 ± 0.1	0
Stenocereus stellatus	33	2.40 ± 2.01	3.1 ± 2.4	50

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S. thurberi var. thurberi	7	2.00 ± 1.41	2.0 ± 1.4	0
Thelocactus bicolor var. *schwarzii*	53	2.37 ± 1.71	3.9 ± 2.4	67
T. hexaedrophorus	33	2.20 ± 1.23	4.4 ± 2.5	89
Turbinicarpus hoferi	0	-	-	-
T. laui	20	3.67 ± 2.42	8.7 ± 3.0	55
T. lophophoroides	50	1.33 ± 0.62	7.5 ± 3.8	100
T. pseudomacrochele var. *lausseri*	27	-	-	-
T. pseudopectinatus	30	1.89 ± 1.36	7.2 ± 4.7	100
T. rioverdensis	20	4.00 ± 2.37	6.8 ± 4.5	0
T. schmiedickeanus var. *flaviflorus*	0	-	-	-
T. schmiedickeanus var. *gracilis*	0	-	-	-
T. schmiedickeanus var. *klinkerianus*	0	-	-	-
T. schmiedickeanus var. *schmiedickeanus*	33	2.60 ± 1.26	7.0 ± 4.8	67
T. schmiedickeanus var. *schwarzii*	17	2.20 ± 1.31	6.5 ± 2.8	67

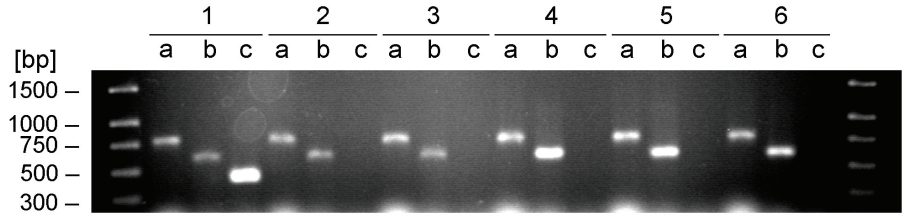

Fig. 2. PCR detection of the *rolB, nptII* and *virD1* genes in *A. rhizogenes* and in transformed roots of several cacti species. 1: Total nucleic acids from *Agrobacterium rhizogenes* A4 pESC4; 2 - 6: DNA from transformed roots of *Turbinicarpus schmiedickeanus* var. *schwarzii* (2), *Pachycereus pringlei* (3), *Mammillaria bocasana* var. *bocasana* (4), *Thelocactus hexaedrophorus* (5), and *Leuchtenbergia principis* (6). *Lanes a*: primers for detection of *rolB*; *lanes b*: primers for detection of *nptII*; *lanes c*: primers for detection of *virD1*.

acetosyringone, ascorbic acid, and citric acid in the bacterial suspension medium. In previous experiments, where these compounds were not used, the frequency of appearance of transformed roots was significantly lower (data not shown). It has already been demonstrated in other plant species that the addition of acetosyringone, a *vir* gene inducer, to co-cultivation medium increases the frequency of explants with transformed roots (Henzi *et al*. 2000). Citric and ascorbic acids impede the oxidation of phenolic compounds excreted by the wounded explants, a process that probably inhibits transformation. Another factor that favored the appearance and growth of transformed roots was the use of PPM to eliminate *Agrobacterium*. In previous experiments, cefotaxime (200 - 400 mg dm^{-3}) was used for this purpose with unsatisfactory results. Cefotaxime was apparently toxic for cacti cells and caused a high mortality among explants.

Growth of the transformed roots was also variable among the species analyzed, showing increments in length from 0.5 to 18.6 mm after 90 days of cultivation. In most species, the higher growth rate was observed in the first 30 d. This agrees with the biphasic growth (rapid during the first weeks and slower thereafter) observed in transformed roots of other plant species (Inomata *et al.* 1993).

The transgenic nature of the roots generated by *A. rhizogenes* infection was confirmed by root induction on an auxin-free medium with 50 mg dm^{-3} kanamycin, histochemical GUS assays and PCR analysis. GUS activity was demonstrated in transformed roots of 34 out of the 65 cacti species tested. Frequencies of GUS positive roots varied from 10 to 100% in the susceptible species (Table 1, Fig. 1*F-G*). GUS activity was also detected in calli produced after the *A. rhizogenes* infection (Fig. 1*H*).

In the species positive for the formation of roots and for GUS analysis, PCR results were also positive, since 780 and 517 bp fragments were amplified in DNA samples, matching the *rol*B and *npt*II genes. In 83 % of the analyzed root samples, the 450 bp fragment corresponding to the *virD1* gene was not found, so the possibility that the results had been caused by contamination with *A. rhizogenes* is discarded (Fig. 2). Additionally, 19 other species generated roots in the selection medium; however, none of these roots showed GUS activity and the *rolB* and *nptII* genes were not detected by PCR analysis of its DNA. Therefore, these roots were considered escapes. This, as well as the presence of a significant proportion of escapes in some of the susceptible species, could be due to the fact that the sensitivity to kanamycin among tested cacti species is different. In these experiments only one concentration of kanamycin was used for all the analyzed species.

Transformed roots induced by *A. rhizogenes* are widely used for the study and production of secondary metabolites from many plant species. With the goal of verifying if the cacti transformed roots conserve the biosynthetic capacities found in the normal roots, alkaloid-like compound production in some of the studied cacti species was investigated using thin layer chromatography. In all five species analyzed, and with the three detection systems used, it was observed that the patterns obtained in transformed and non-transformed roots were qualitatively equal (Fig. 3). Also, some of the spots appear more intense in the extracts obtained from transformed roots. These results should be confirmed using more sensitive techniques. However, as far as the authors know, this is the first evidence that the transformed roots can be used to obtain and study secondary metabolites in cacti.

In conclusion, this work demonstrated that 34 cacti species, belonging to 15 genera, were susceptible to the infection with *A. rhizogenes* and able to generate transformed roots. Besides, our preliminary study of alkaloid-like compounds production found that cacti transformed roots apparently conserved the same biosynthetic capacity as normal roots.

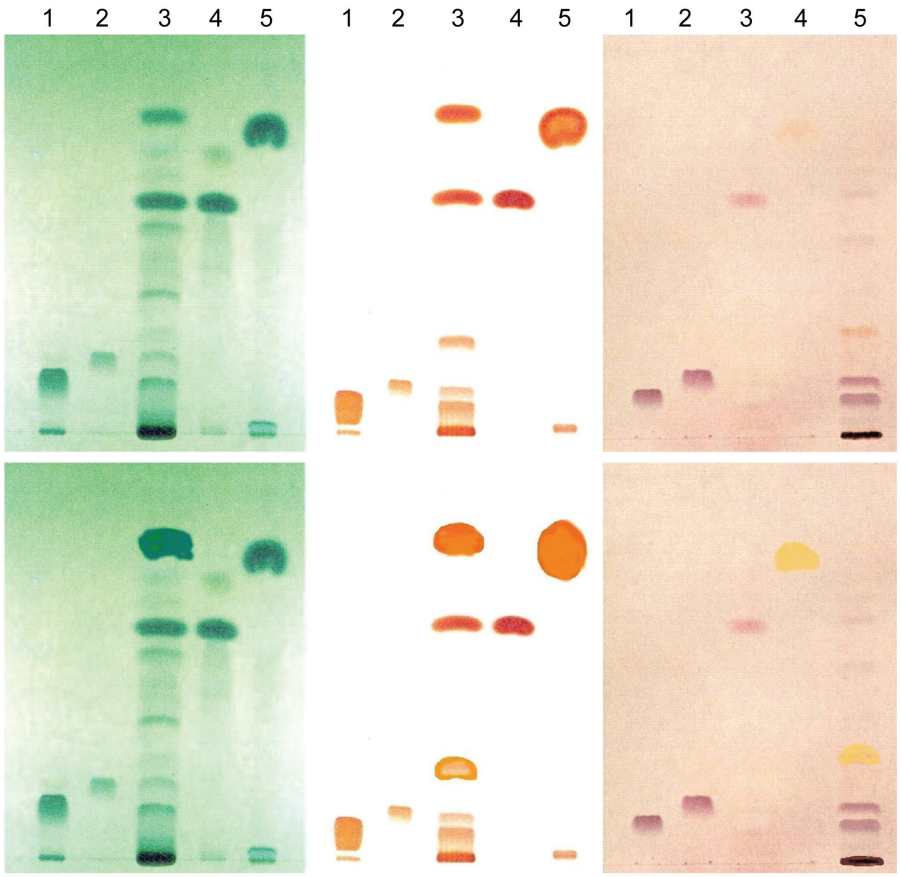

Fig. 3. Thin layer chromatography plates of alkaloid-like compounds extracts from non-transformed (*upper panel*) and *A. rhizogenes* transformed roots (*lower panel*) of five cacti species. *Lanes 1*: *Mammillaria pennispinosa*; *lanes 2*: *M. hutchissoniana* var. *louisae*; *lanes 3*: *Pachycereus pringlei*; *lanes 4*: *Thelocactus hexaedrophorus; lanes 5*: *Turbinicarpus schmiedickeanus* var. *schmiedickeanus*. The plates from left to right were revealed with UV light (264 nm), Dragendorff reagent, and Marquis reagent, respectively.

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