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On the Role of Opines in Plants Transformed with *Agrobacterium rhizogenes*: Tropane Alkaloid Metabolism, Insect-Toxicity and Allelopathic Properties

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Summary

The opines, mannopine and mikimopine, which are formed in roots after transformation with *Agrobacterium rhizogenes* 15834 and *A. rhizogenes* MAFF03-01724, were synthesized chemically and their influence on the alkaloid production in root cultures of *Hyoscyamus albus* was examined. In adventitious root cultures the addition of opines resulted in a rapid alkaloid degradation. Alkaloid content was restored to a «normal» level within 5 days. Alkaloid accumulation in hairy roots reacted differently: The addition of mikimopine enhanced alkaloid production whereas the addition of mannopine generally reduced the alkaloid content. Most of the different tropane alkaloids produced by the adventitious and the hairy root cultures were affected in the same way by the addition of the opines. Roots were able to take up exogenously supplied opines. The addition of opines to the culture medium replaced carbon or nitrogen sources of the media to some degree.

Mikimopine and mannopine reduced growth of *Manduca sexta* larvae and had deterrent properties at higher concentrations. In addition, mikimopine showed allelopathic properties and retarded the germination of *Lepidium sativum* seeds and growth of seedlings. It is speculated that the opines serve both *Agrobacteria* and the infected plants, supposedly the latter in plant-plant and plant-insect interactions. Thus, *Agrobacteria* – plant relationships might be considered to be of rather symbiotic than pathogenic nature.

Key words: Hyoscyamus albus; Agrobacterium rhizogenes; adventitious root culture; hairy root culture; mannopine; mikimopine; tropane alkaloid production; insect-toxicity; allelochemical property; symbiotic relationship.

Introduction

Agrobacterium rhizogenes causes the so-called «hairy root» disease of plants [White and Sinkar, 1987; Tepfer, 1990]. The ability of the bacterium to promote plant tumors is determined by its virulence encoding genes on Ri (root inducing) plasmids. When *A. rhizogenes* penetrates a wounded site in plants, a part of the plasmid, the so called T-DNA, is transferred and incorporated into the plant's genomic DNA. In addition to genes that encode enzymes of auxin biosynthesis also genes that are connected with the formation of opines are located on the T-DNA [Petit et al., 1983]. The transformed roots produce opines, which may be classified chemically as non-protein amino acids. Opines are secreted into the soil or the culture media even after the elimination of the bacteria [Jung and Tepfer, 1987]. Depending on the strain of *A. rhizogenes* used either agropine, mannopine, cucumopine or mikimopine are formed (see Fig. 1 for structures).

What can be regarded as the function of opines produced by tissues transformed with *A. rhizogenes* or *A. tumefaciens*? It has been suggested that the opines serve as carbon or sometimes nitrogen source for the infecting bacteria and that they stimulate the induction of the *vir* genes by acetosyringone [Tempé and Goldmann, 1982; Tempé and Petit, 1982; Tepfer, 1990; Krishnan et al., 1991]. Another function of opines may be connected with their allelochemical proper-

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ties. Therefore, we tested whether opines can be of some advantage for the infected plants in an ecological context as defence against herbivores or against competiting plants. In addition, we studied the influence of opines on growth and production of tropane alkaloids in adventitious and transformed root cultures of *Hysocyamus albus*, in which we had previously found a high dependency of the alkaloid production on the strain of *Agrobacterium* used for transformation [Sauerwein and Shimomura, 1991; Shimomura et al., 1991; Sauerwein et al., 1992].

Materials and Methods

Plant material

Adventitious root cultures were established from axenic shoot cultures of *H. albus* on Woody Plant (WP) solid medium [Lloyd and McCown, 1980]. Hairy roots were induced by co-culture of leaf-discs with strains of different *Agrobacteria*. The axenic hairy roots thus obtained were subcultured in hormone-free WP liquid medium. Transformation was verified by the isolation and detection of opines (mannopine and mikimopine) as described previously [Sauerwein et al., 1992]. The root cultures were maintained in 50 mL medium in 200 mL Erlenmayer flasks on a rotary shaker at 100 rpm in the dark or under continuous light (4000 lux) at 25°. All media contained 3 % sucrose and the pH was adjusted to 5.7 before autoclaving.

Experiments with root cultures

Influence on alkaloid metabolism: The opines were added aseptically to cultures grown for 18 days in WP liquid medium to give a final concentration of 0.5 mg/mL. Three cultures were harvested after a certain time of incubation and subjected to HPLC analyses. The experiments were performed in triplicate.

Fate of opines in root cultures: ^{3}H-labeled mikimopine (0.5 mg/mL corresponding 0.25 µCi per flask) was added at day 18 of the culture period and the cultures were harvested 3 and 5 days later. The radioactivity of the medium, the alkaloid fraction, the water soluble fraction and the insoluble residue of the roots was determined in a scintillation counter (Pharmacia). Two cultures were used in each experiment, which was repeated twice.

Opines as a nutrient for hairy roots: To test whether opines might serve as carbon or nitrogen source for transformed roots WP liquid media without sucrose or NH_4NO_3 were prepared. One g/L mikimopine was added aseptically to those media after autoclaving and two root tips were inoculated into the culture media. After 21 days the fresh weight of the roots was determined.

Experiments with insects

Toxicity against insects was determined by feeding the opines to larvae of *Manduca sexta* (Lepidoptera) reared on an artificial diet [Yamamoto, 1969]. This diet was supplied with the opines at concentrations of 0.5 %, 1 %, 2 % and 3 % (w/w) in the case of mikimopine and 0.5 % and 1.5 % in the case of mannopine. Five g diet per animal per day was applied and offered in no-choice experiments. The weight of the insects was determined daily and the non-consumed diet was replaced by a fresh one containing the same concen-

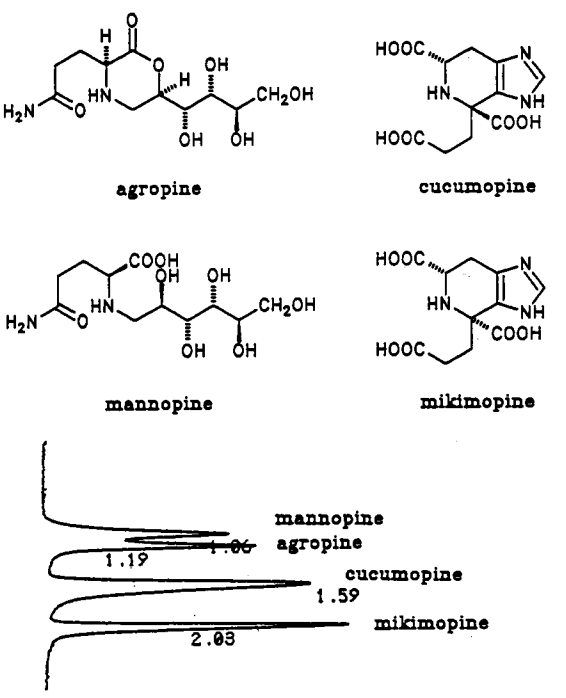

Fig. 1: HPLC analyses of opines induced by *Agrobacterium rhizogenes*. Column: RP-18 100, eluent: 10 mM NH_4OAc, pH 7, flow: 1.2 mL·min^{-1}, RI-detector.

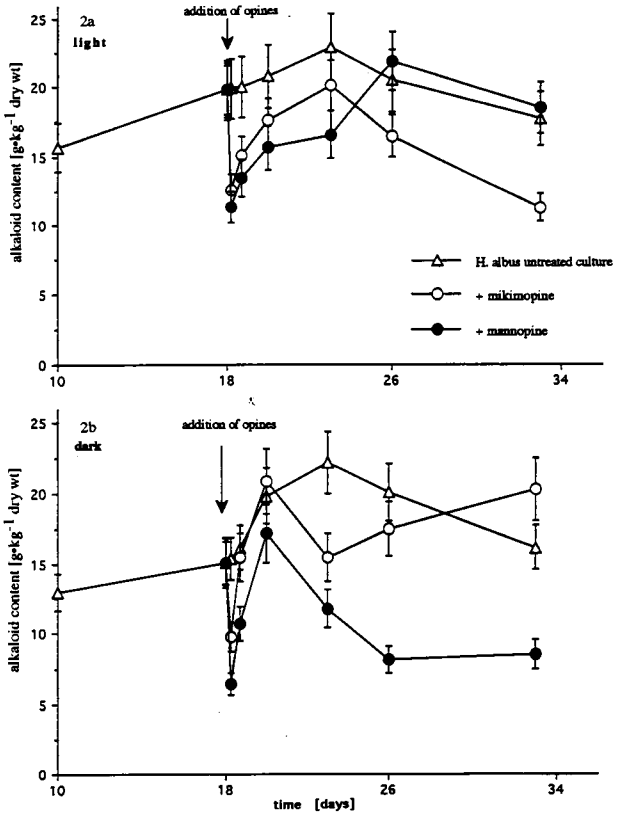

Fig. 2: Influence of opines on alkaloid production in adventitious roots of *Hyoscyamus albus*. Opines (0.5 g·L^{-1}) were added after 18 days of culture, a) in the light, b) in the dark. Bars indicate the standard error.

trations of opines. Twelve animals were used for each concentration tested and each experiment was repeated twice.

Allelopathy experiments

To test the allelopathic properties of the opines fifty seeds of *Lepidium sativum* were placed into a Petri dish (∅ 9 cm) containing two filter paper disks moistened with 10 mL water or aqueous opine solutions (5 or 25 mg mikimopine/10 mL water). The Petri dishes were closed tightly with Parafilm® and incubated at 20 °C under continuous light. After 5 days the length of roots and hypocotyl of the seedlings was measured individually. In addition, the germination rate of the seedlings was determined. For the control it approached 100 %. Comparison between treatments was analyzed using the t-test. Each experiment was carried out in duplicate and repeated twice.

Analysis of tropane alkaloids

Three cultures were harvested and fresh and dry weights (after lyophilization) of tissues were determined individually. Ca. 50 mg of each sample was extracted with 5 mL $CHCl_3$/MeOH/NH_4OH (15 : 5 : 1) using sonication (10 min). After purification the alkaloid extracts were dissolved in MeOH and analyzed by HPLC (Hewlett Packard 1040, Photo-Diode-Array detector) as described previously [Shimomura et al., 1991; Sauerwein and Shimomura, 1991].

Analyses of opines

The opines were extracted from the fresh root by homogenizing ca. 500 mg roots with a glass rod in an Eppendorf tube. After centrifugation an aliquot of the supernatant was directly analyzed by HPLC. A RP-18 100 column (Merck, 4.6 I.D. × 250 mm) was used and eluted with 10 mM ammonium acetate (pH 7). The flow rate was 1.2 mL/min and the effluent was detected by an RI detector (Knauer). For quantification the system was calibrated with authentic samples and the detection limit was at app. 0.05 µg for cucumopine and mikimopine and at 0.02 µg for agropine and mannopine (see Fig. 1).

Synthesis of opines

Mikimopine and ^{3}H-mikimopine were synthesized from α-oxoglutaric acid and L-histidine (ring-labeled ^{3}H-histidine, respectively) according to the method of Isogai [Isogai et al., 1990]. Mannopine was synthesized from monosodium L-glutamate and D-mannose in a similar reaction as described by Petit [Petit et al., 1983] using Raney-Ni and H_2 instead of sodium cyanoborohydride.

Results and Discussion

Opines and alkaloid production

In a first set of experiments the influence of opines on alkaloid metabolism of normal and hairy roots of *Hyoscyamus albus* was studied. The addition of mannopine and mikimopine to adventitious root cultures resulted in a rapid drop of the alkaloid content (Fig. 2 a, b). Within 5 hours after opine addition alkaloid contents decreased by almost 50 % in both roots cultured in the dark or in the light. On the other hand, the culture media did not contain more alkaloids than before, so that it must be concluded that alkaloids were degra-

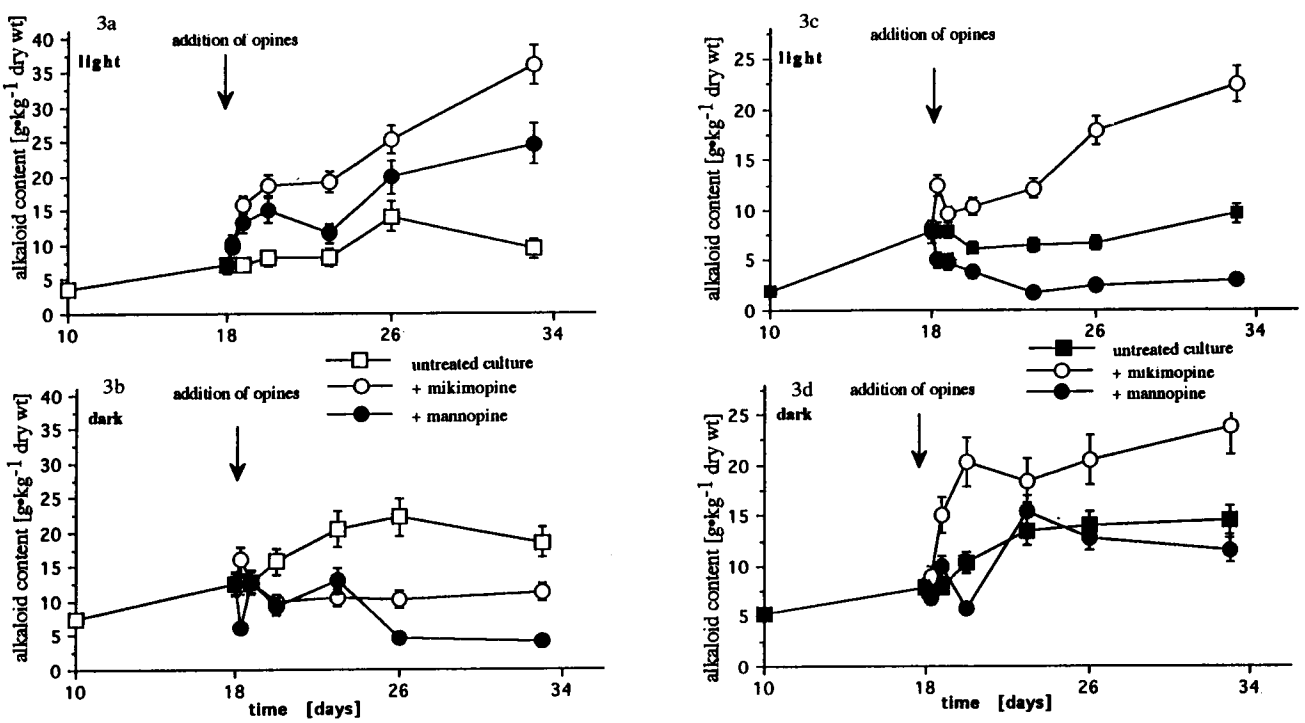

Fig. 3: Influence of opines on alkaloid production in transformed roots of *Hyoscyamus albus*. Opines (0.5 g·L^{-1}) were added after 18 days of culture, a) *H. albus* MAFF03-01724 in the light, b) in the dark, c) *H. albus* 15834 in the light, d) in the dark. Bars indicate the standard error.

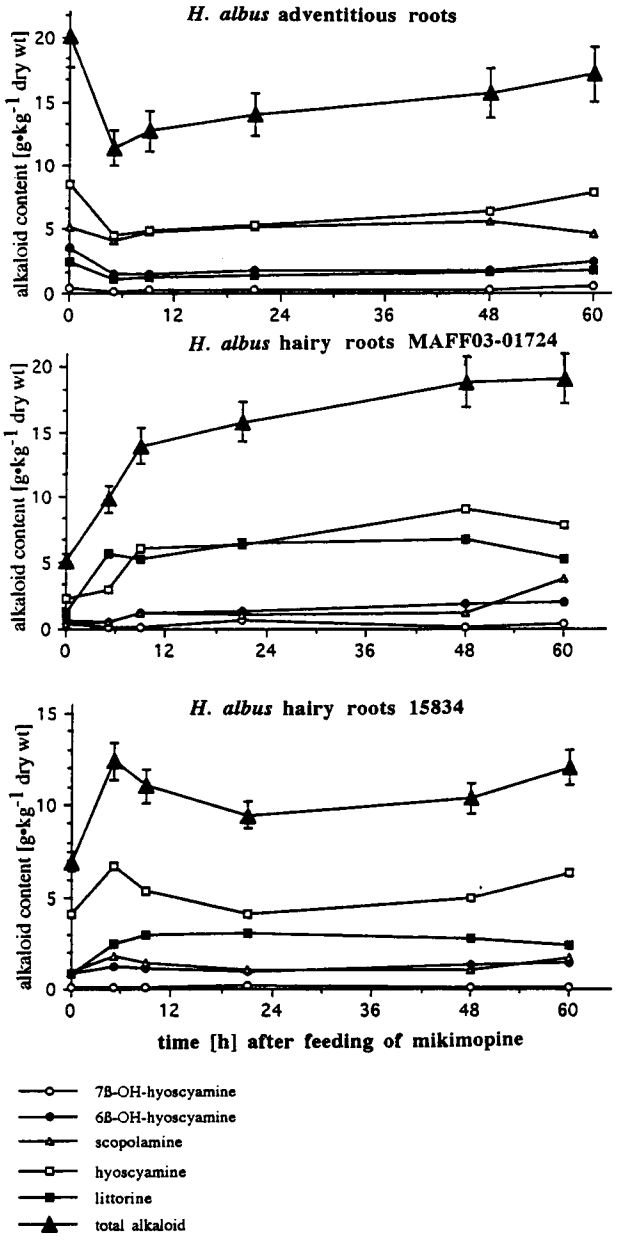

Fig. 4: Influence of mikimopine on the alkaloid pattern in adventitious and hairy roots of *Hyoscyamus albus*. Opines (0.5 g·L^{-1}) were added after 18 days of culture. Bars indicate the standard error.

daded. During the next 5 days of culture alkaloid production recovered and reached approximately the same level as that of untreated cultures. About 10 days later, mikimopine treated light and mannopine treated dark-cultures showed substantially decreased alkaloid levels. The rapid reduction of the alkaloid content in the adventitious roots at the early stage of the opine addition might be advantageous for the infecting *Agrobacteria* and promote the infection and penetration of *A. rhizogenes* into the tissues, especially because tropane alkaloids, namely hyoscyamine, show a certain toxicity against *Agrobacteria* (data not shown).

Transformed root culture were analyzed in a similar fashion. The reaction of transformed root cultures to the addition of opines to the culture medium differed substantially (Fig. 3 a–d). Mikimopine enhanced the alkaloid production in all cultures except for the transformant MAFF03-01724 cultured in the dark, which did not respond. Mannopine had no significant influence or reduced the alkaloid production. Only in the hairy roots transformed with *A. rhizogenes* MAFF03-01724 cultured under light conditions did mannopine enhance the alkaloid production equally to mikimopine, but to a smaller degree.

The addition of opines did not change the alkaloid pattern in root cultures significantly (Fig. 4). Hyoscyamine was the main alkaloid produced in all cultures. Alkaloids that are derived from hyoscyamine such as 7β-hydroxyhyoscyamine, 6β-hydroxyhyoscyamine and scopolamine (Fig. 5) changed in a similar way as hyoscyamine. Only littorine did not follow this pattern.

Uptake of opines by the root cultures

All hairy roots contained ca. 0.5 mg g^{-1} fresh weight of endogenous produced opine prior to the addition of exogenous opines. Twenty one h after the addition of opines (0.5 mg/mL) to the culture medium, roots contained 1 mg mikimopine g^{-1} fw (*A. rhizogenes* MAFF03-01724) and 2.3 mg mannopine g^{-1} (*A. rhizogenes* 15834), respectively. The addition of the «foreign» opine did not enhance the content of endogenous opine in the transformed roots. In the non-transformed roots opines were detected in traces only after their addition to the culture medium (HPLC analyses). Thus, opine uptake by normal roots must have been rather low or the opines were degradated rapidly.

To follow the fate of exogenous opines, ^{3}H-labeled mikimopine was added. Three days after addition of ^{3}H-labeled mikimopine most of the radioactivity (ca. 80 %) was detected in the culture medium (Table 1). The alkaloid fraction only contained traces of radioactivity, whereas ca. 6 % of the ^{3}H-label was detected in the insoluble residue of the roots and ca. 11 % was in the aqueous extract. There were only minor differences in the results obtained with the three root cultures, which indicates that basically the non-transformed roots metabolize the opine in a similar way as the transformed roots.

Table 1: Radioactivity in extracts, tissues and culture media after feeding of ^{3}H-mikimopine to root cultures of *H. albus*.

	Radioactivity (%)		
	adventitious root	hairy root MAFF	hairy root 15834
3 days of culture			
root (solid residue)	6.3	5.3	7.0
alkaloid fraction	3.8	2.1	4.1
aqueous root extract	11.3	13.0	9.9
culture medium	78.4	79.7	79.2
5 days of culture			
root (solid residue)	2.1	1.7	1.9
alkaloid fraction	2.1	2.7	2.3
aqueous root extract	2.9	6.8	4.8
culture medium	92.8	88.5	90.6

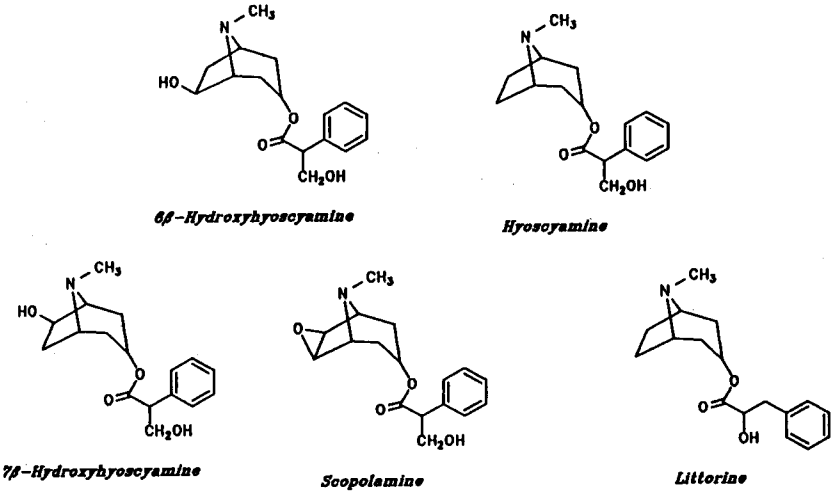

Fig. 5: Tropane alkaloids produced in adventitious and hairy roots of *H. albus*.

Table 2: Effect of mikimopine (0.1 %) on growth of *Hyoscyamus albus* roots cultured for 21 days in media without sucrose or nitrogen.

culture conditions media	mikimopine	growth (g fresh wt) dark		light	
adventitious root					
WP medium (control)	+	5.00	(100 %)	4.30	(100 %)
WP medium	–	5.20	(102 %)	4.16	(97 %)
WP minus sucrose	+	0.95	(19 %)	1.14	(26 %)
WP minus sucrose	–	0.32	(7 %)	0.29	(7 %)
WP minus NH_4NO_3	+	2.46	(49 %)	2.46	(57 %)
WP minus NH_4NO_3	–	0.52	(11 %)	0.40	(9 %)
hairy root MAFF03-01724					
WP medium (control)	+	10.99	(100 %)	10.99	(100 %)
WP medium	–	10.20	(93 %)	10.16	(92 %)
WP minus sucrose	+	3.26	(29 %)	2.59	(23 %)
WP minus sucrose	–	0.29	(3 %)	0.25	(2 %)
WP minus NH_4NO_3	+	6.55	(59 %)	4.23	(38 %)
WP minus NH_4NO_3	–	0.41	(4 %)	0.40	(4 %)
hairy root 15834					
WP medium (control)	+	11.81	(100 %)	5.50	(100 %)
WP medium	–	11.71	(99 %)	5.70	(104 %)
WP minus sucrose	+	1.88	(11 %)	1.56	(28 %)
WP minus sucrose	–	0.31	(3 %)	0.28	(5 %)
WP minus NH_4NO_3	+	3.56	(30 %)	2.32	(42 %)
WP minus NH_4NO_3	–	0.39	(3 %)	0.32	(6 %)

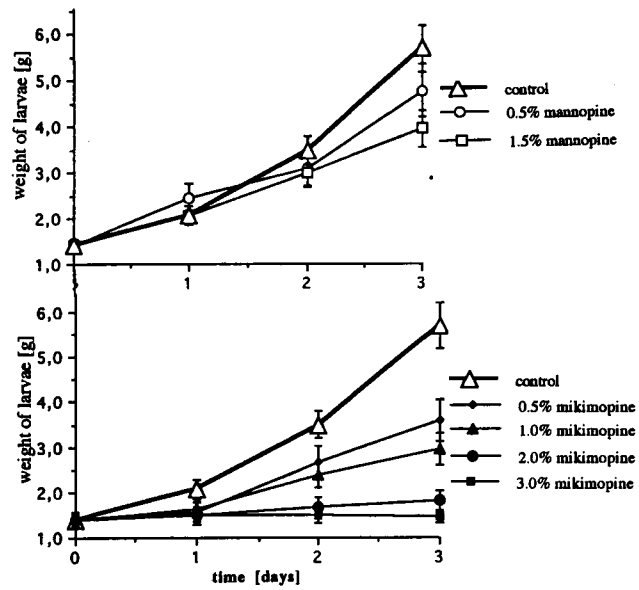

Fig. 6: Allelochemical properties of opines 6a Influence on growth of *Manduca sexta* larvae.

In another set of experiments we examined whether opines can be a N- or C-source for roots. Table 2 shows the growth characteristics of the root cultures on media in which sucrose or nitrogen were replaced by mikimopine. In either case the growth of the cultures was substantially reduced. All of the cultured roots were affected in a comparable manner. The lack of nitrogen was compensated far easier by mikimopine than that of sucrose, but in all experiments a certain limited growth of the cultures took place. Thus, it is likely that the root cultures are able to catabolize exogenous opines to some degree.

Allelochemical properties of opines

The growth of larvae of *Manduca sexta* (Lepidoptera) was retarded substantially by rearing them on an opine-containing diet (Fig. 6 a, b). No growth at all was observed when the diet had 3 % mikimopine and the weight of the larvae only increased slowly with 2 %, 1 % or 0.5 % of mikimopine. Mannopine was less toxic, but still affected the development of the larvae. Thus, the opines reduce larval growth. Since opine-treated larvae ingested less food at higher opine concentrations than control animals, a deterrent effect seems to exist. Whether the opines interfere with the protein biosynthesis as other non-protein amino acids remains to be elucidated.

The germination of seeds of *Lepidium sativum* (Brassicaceae) and growth of seedlings was almost completely inhibited at 0.25 % mikimopine (Fig. 6 c). Already a concentration of 0.05 % mikimopine was effective in retarding the growth of the seedlings significantly. In both cases mainly the elongation of roots was affected.

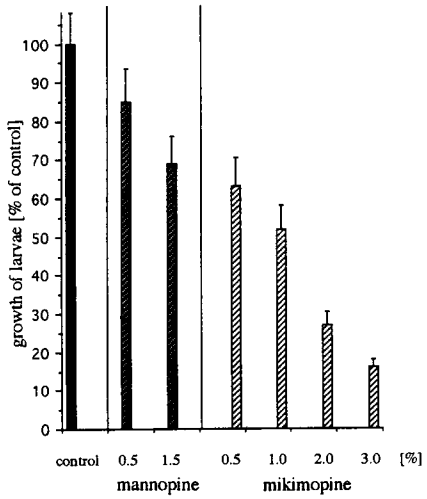

Fig. 6 b: Growth retardation in comparison to control animals after 3 days. Opines were added to artificial diet in the concentrations given and offered in no-choice experiments. Bars indicate the standard error, (n = 24).

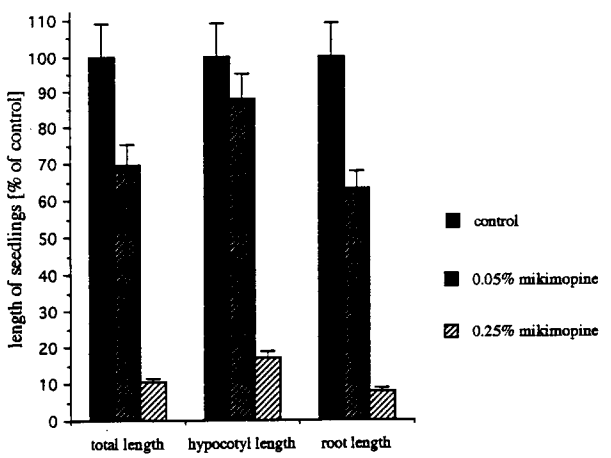

Fig. 6 c: Allelopathic activity in *Lepidium sativum* seeds. Seedlings were exposed to mikimopine in aqueous solution for 5 days. Bars indicate the standard error, (n = 200).

The finding that opines serve as specific growth substances for the pathogenic bacteria [Lippincott et al., 1973; Tempé et al., 1979; Petit et al., 1983] led to the opine concept, which proposes that the pathogen induces the formation of opines to create an environment favourable for its own growth. Accepting this concept, the reduction of alkaloid biosynthesis in the normal roots would facilitate the penetration of the bacteria in uninfected roots, since tropane alkaloids are antimicrobial to some degree. On the other hand, the stimulation of alkaloid production in already infected roots might prevent a new infection by other bacteria. In addition, our data provide first evidence that the opines exhibit a certain toxicity and deterrency against insects and probably competing plants. The bacteria thus creates an environment that favours its own growth, and in addition it might protect its host against herbivorous insects or other pathogens. We suggest, therefore, that *Agrobacteria*-plant interactions are probably of symbiotic rather than pathogenic nature. Whether the allelochemical properties of opines are effective under natural conditions in the field needs to be tested.

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