Intra- and Interspecific Allelochemical Effects in Three *Kalanchoe*-Species (Crassulaceae)

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The intra- and interspecific acting allelochemicals of *Kalanchoe daigremontiana, K. tubiflora* and *K. pinnata* (Crassulaceae) were isolated and could be identified as *p*-hydroxybenzoic-, protocatechuic-, gallic-, *p*-coumaric- and coffeic acid. By measuring length of stems and primary roots of *Kalanchoe*-daughter plants the intra- and interspecific inhibitory activities of authentic compounds could be demonstrated.

Introduction

Mutual inhibitory and promoting chemical interference between plants has been described as allelopathy by Molisch (1937). In the meantime allelopathic effects have been investigated under various aspects from many plant systems (Lodhi, 1976; Harborne, 1989; Schildknecht, 1981; Rice, 1984; Rizvi and Rizvi, 1992). In the crassulacean species *Kalanchoe daigremontiana* the inhibitory allelopathic effect is especially impressive (Hibbs and Yokum 1976). In order to establish this model system in practical courses of universities it was necessary to isolate and identify the biologically active natural compounds in conjunction with an allelopathical bioassay. Moreover intra- and interspecific allelopathic effects should be tested qualitatively and quantitatively during long-termed experiments by registrating shoot lenghts of daugther plants depending from their distance to their mother plants. Various amounts of selected authentic allelochemicals should be additionally tested in short-termed experiments by registrating root length of *Kalanchoe*-daughter plants.

Material and Methods

Plant material

The species *Kalanchoe daigremontiana* (Hamet et Perr.), *K. tubiflora* (Hamet) and *K. pinnata* (Pers.) (Crassulaceae), according to Engler (1964) have their original habitats on the Isle of Madagascar and in the southern parts of Africa. Today they are naturalized in the tropics all over the world. The growth of species of the *Kalanchoe* under outdoor conditions was documented on several excursions (W. B., Java, Cook Islands, Seychelles).

While *K. daigremontiana* and *K. tubiflora* were provided by the Botanic Gardens of the University of Bayreuth, leaf material of the species *K. pinnata* was procured from Java (Indonesia). The species can be reproduced vegetatively through breeding buds and cultivated in greenhouses. *K. pinnata* was cloned from meristematic leaf tissue. From leaves that were put on planting pots (15 x 15 cm) filled with vermiculite numerous shoots could be grown at a temperature of 28 °C and a humidity level of about 80%.

Long-term bioassay for the demonstration of the allelopathic effect

A mother plant of about 40 cm size is put with one end into a plastic planting pot (40 x 17 x 14 cm) which is filled with sand. One week later shoots of one clone, which were freshly picked from the leaf edges and have already developed roots, are planted in row at a distance of 3 cm from each other, to examine the intra- respectively the interspecific allelopathy.

After identification of the allelopathically active natural substances, in the long-term bioassay one

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mother plant is replaced by a glass capillary (Pasteur pipette, 150 mm long). During 6 months, every three days 2 ml of an aqueous solution ($8x10^{-6}$ molar) of the allelochemicals are added with the aid of the glass capillary. For quantifying the allelopathic effect, the length of the stems of shoots is registered every 4 weeks.

Short-term bioassay for water soluble allelochemicals

In order to be able to examine the effect of water soluble allelopathic natural substances the following test has been developed: Into each of several glasses with spring lids (45 x 23 mm, volume 10 ml) there is given 1 ml of an aqueous solution of allelopathically active compounds in different concentrations (n = 10; dilution series 10^{-2} to 10^{-6} molar) a freshly picked shoot which has not yet developed roots is put swimming onto the surface of the test solution by a pair of tweezers. The glass is then shut with a plastic lid to avoid evaporation. During a period of 8 days the average primary root lengths of the shoots are registered daily and compared with control plants swimming on water. In case several roots are developed by the breeding buds, the length of the longest root is registered.

Isolation of the allelochemicals

20 g chopped or root material deep frozen at −50 °C are mixed with 300 ml methanol p. a. and suspended with the Ultra-Turrax T 25 (IKA-Labortechnik) at 12 000 rotations/min under cooling by ice. After addition of further 200 ml MeOH the mixture remains in the vibrating funnel (Vibrax IKA-Labortechnik) for three hours. The result is a methanolic suspension of cells separated from its residue by filtration over glass wool and by centrifugation (Labofuge, Heraeus-Christ). After evaporation at a water bath temperature of 40 °C and a pressure of 270 hPa a methanol-free dry extract is obtained. This dry substance is treated with 50 ml of distilled water. The aqueous phase, which besides watersoluble substances also contains disperged lipophilic material, is subsequently subjected to the following four steps of extraction (according to Dey & Harborne, 1989): At first it is extracted for three times with 3 x 100 ml of cyclohexane (Vibrax-VXR). In the separating funnel the cyclohexane phase A and the water phase are separated from each other. The procedure is then repeated with other solvents of different polarities: [Cyclohexane (A) – diethylether (B) – ethylacetate (C) – n-butanol (D)].

The remaining water is removed from the phases A – D by adding twice-distilled benzene and following azeotropic evaporation of the solvent by a rotation evaporator until dryness. The residue is dissolved with 1 ml of the respective solvent and made absolutely free of solvent by nitrogen.

Derivatization

Each water-free sample of the phases A to D is mixed with 1 ml of diethylether and excess of N-methyl-N-trimethylsilyl-trifluoracetamide (MSTFA). After 12 h (room temperature) excess MSTFA and diethylether are subsequently removed by nitrogen.

Identification of the allelochemicals by GC/MS analysis

For separations a capillary gaschromatograph Vega 6000 Series 2 (Carlo Erba) was used with a Unimetrics syringe. 1 µl of each derivatized sample A-D was injected (injector temperature 230 °C). The GC-separation is performed using a fused silica column OV 101 (12.5 m) at the following temperature program: 50 °C → 10 °C / min → 260 °C (3 min) → 10 °C/min → 280 °C (20 min) (carrier: He; 2 ml/min). The gas chromatograph was coupled with a Iontrap (Finnigan ITD 800) in order to record EI-mass spectra (70 eV). For trace analysis of silylated compounds a capillary gaschromatograph Hewlett Packard 5890 Series II with a Silica DB-1 columns (12.5 m) was used. The GC was connected to a mass spectrometer Finnigan MAT 95 (70 eV EI-mass spectra).

Results and Discussion

Allelopathic effects within the test species

At Rarotonga (on the Cook Islands) numerous small shoots of *K. pinnata* were found beneath older Crassulaceae (80 cm high) of the same species (Fig. 1A). In the laboratory beneath mother plants of the three test species shoots will drop off the leaves and take root in the soil. For years they grow up to only limited height. Obviously the numerous shoots are not killed by allelochemicals

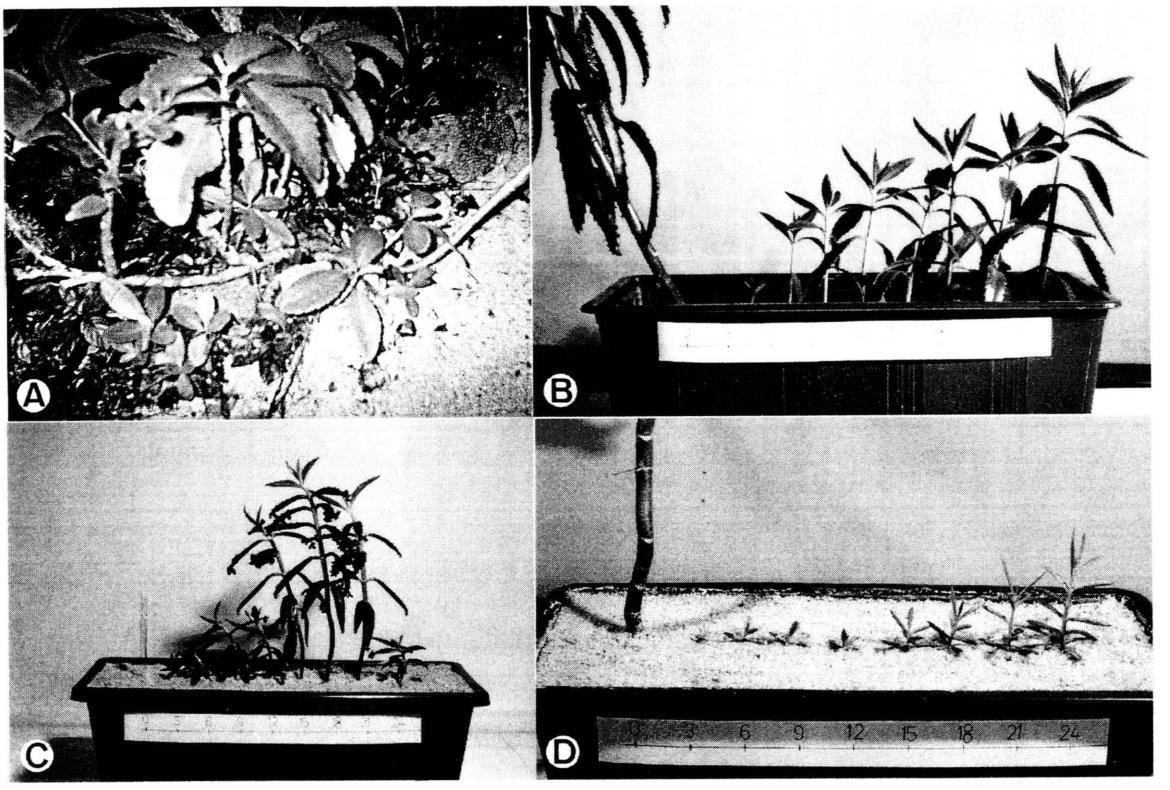

Fig. 1. A. Intraspecific allelopathic effect of a mother plant of *Kalanchoe pinnata* on surrounding plants in the field (Cook Islands). B. Gradual intraspecific allelopathic effect of a mother plant of *K. daigremontania* (left) on daughter plants planted at different distance (3–24 cm) from mother plant 150 days after starting the experiment in the laboratory. C. Allelopathic activity of a mixture containing the five identified aromatic acids ($8x10^{-6}$molar) after 12 months. The compounds were continuously introduced into soil by a glass capillary. D. Interspecific allelopathic effect of a mother plant of *Kalanchoe daigremontiana* on surrounding plants of *K. tubiflora* in the laboratory after 120 days. Fig. 2.

emitted by their mother plants, but only are strongly inhibited in their growth. Therefore all genetically identic shoots that have dropped on the soil can survive and are subjected to the same abiotic and biotic conditions in the ecosystem.

Evaluation of the long-term bioassays for the demonstration of the allelopathic effect

As shown in Fig. 2 first allelopathic effect is noticeable already within one month after planting the shoots of the *K. daigremontiana* species beneath their mother plant. The greater their distance from their mother plant the higher grow their stems. Over a period of six months of observation it can be seen that those shoots which are growing in direct neighbourhood (about 3 cm) to their mother plants are increasing in height from month to month.

Fig. 1B illustrates the intraspecific allelopathic effect after 150 days. The allelochemicals from the mother plant diffuse in the sandy soil of the test biotope over to the shoots and cause an intraspecific inhibition effect. As the plants in the experiment were watered once a week regulary from all sides, the allelochemicals released by the mother plant must be of a polar nature, respectively water soluble.

Isolation of the allelochemicals by successive extraction with the solvents A-D

2 mg of solvent-free dry extract obtained from 38.6 g freshly chopped root material are extracted

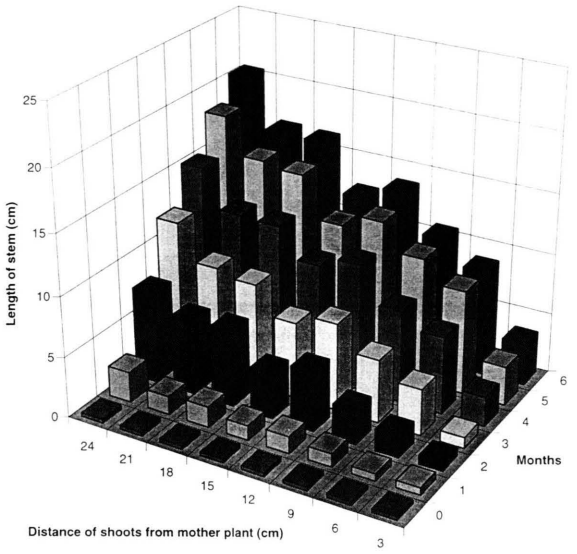

Fig. 2. Long-term bioassay to demonstrate intraspecific allelopathy in *K. daigremontiana* by registrating shoot length (cm) of daughter plants in the laboratory during 1–6 months after starting the experiment.

with the solvents A-D. The fractions obtained A to D are each mixed with 10 ml of distilled water dissolved in an ultrasonic bath and centrifugated. The aqueous fractions are tested in the bioassay for an allelopathic effect.

From shoots of the same mother plant the growth of primary roots in water serves as a control. As shown by the bioassay (Fig. 3), the allelochemicals can be found especially in solvent fraction B (diethylether phase), but as well in C (ethylacetate phase).

Compared with the controls, fraction B shows a very significant (**: $\alpha \leq 0{,}01$; t test), fraction C a significant (*: $\alpha \leq 0{,}05$; t test) inhibition effect on the growth of the roots of the shoots.

Fractions A (cyclohexane phase) and D (*n*-butanol phase) do not show a significant inhibition effect (n.s. = non-significant) compared to the water-control and therefore are less taken into consideration in the following GC/MS analyses for the identification of the allelochemicals.

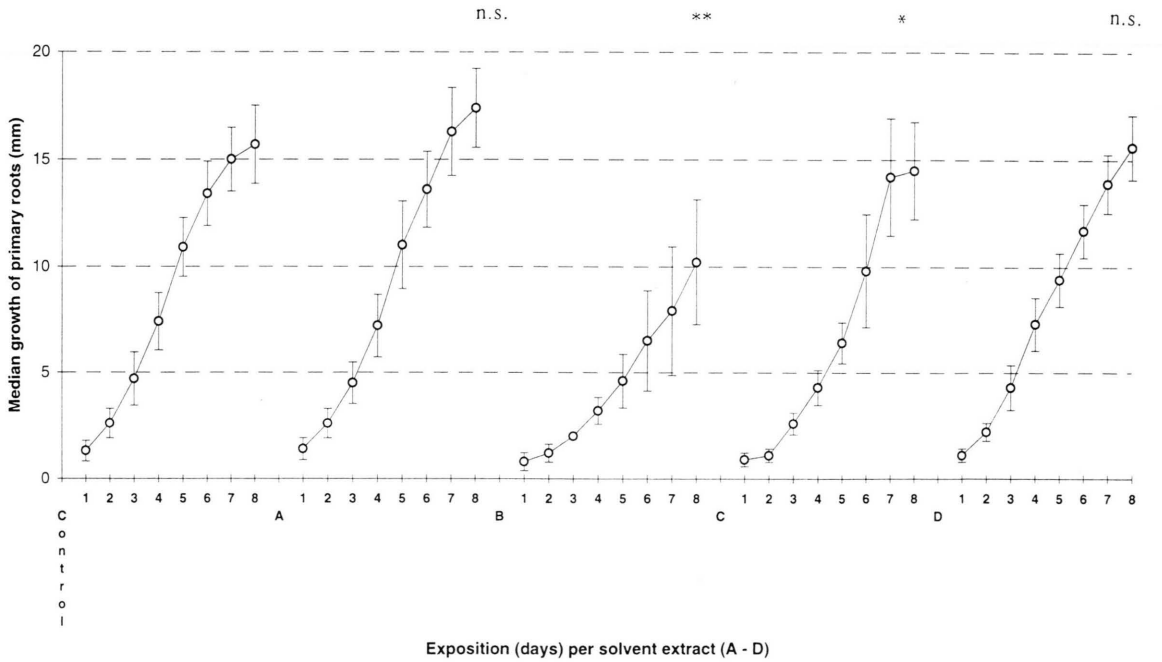

Fig. 3. Time dependent allelopathic acitivity (1–8 days) of different aqueous solvent fractions (A – D; control) from root extracts of *K. daigremontiana* against daughter plants of *K. daigremontania* in the laboratory (water control: left). The length of primary roots of daughter plants were registrated. n.s.: not significant; *: significant ($\alpha \leq 0.05$); ** highly significant ($\alpha \leq 0.01$).

Identification of the allelochemicals by GC/MS analyses

Fig. 4 shows the total ion current chromatogram of the diethylether phase B of the silylated root extract of *Kalanchoe daigremontiana*. According to literature data possible allelochemicals are marked with Roman numerals (**I** to **V**). The identification of the aromatic allelochemicals (substances **I** to **V**) was achieved by comparing the EI-mass spectra obtained with spectra from the NBS spectra library (National Bureau of Standards) and with spectra of authentic substances.

The GC/MS analysis of **I** (scan 153) resulted in a mass spectrum with the molecular ion at *m/z* 282 (26%) and a base peak at *m/z* 267. Due to further characteristic fragments at *m/z* 223 (41%), 193 (32%), 147 (1%) and 73 (7%) compound **I** could be identified as benzoic acid, 4-[(trimethylsilyl)-oxy]- trimethylsilylester ($C_{13}H_{22}O_3Si_2$).

The EI-mass spectrum of **II** (scan 321) was characterized by a molecular ion at *m/z* 370 (100%). Further characteristic ions were recorded *m/z* 355 (52%), 311 (25%), 281 (14%), 193 (78%), 147 (9%) and 73 (19%), indicating that **II** is identical with benzoic acid, 3,4-bis [(trimethylsilyl)-oxy]-trimethylsilylester ($C_{16}H_{30}O_4Si_3$).

The spectrum of **III** (scan 453) was characterized by a molecular ion at *m/z* 308 (88%) and a base peak *m/z* 293. Further characteristic ions were recorded at *m/z* 249 (49%), 219 (91%), 147 (6%) and 73 (55%), indicating that **III** is identical with cinnamic acid, *p*-[(trimethylsilylsil)-oxy]-trimethylsilylester ($C_{15}H_{24}O_3Si_2$).

The spectrum of **IV** (scan 474) was characterized by a molecular ion at *m/z* 308 (88%) and a base peak *m/z* 458. Further characteristic ions were recorded at *m/z* 443 (37%), 399 (7%), 281 (83%), 147 (4%) and 73 (20%), indicating that **IV** is identical with benzoic acid, 3,4,5-tris [(trimethylsilyl)-oxy]-trimethylsilylester ($C_{19}H_{38}O_5Si_4$).

The EI-mass spectrum of **V** (scan 696) was characterized by a molecular ion at *m/z* 396 (base peak). Further characteristic ions were recorded

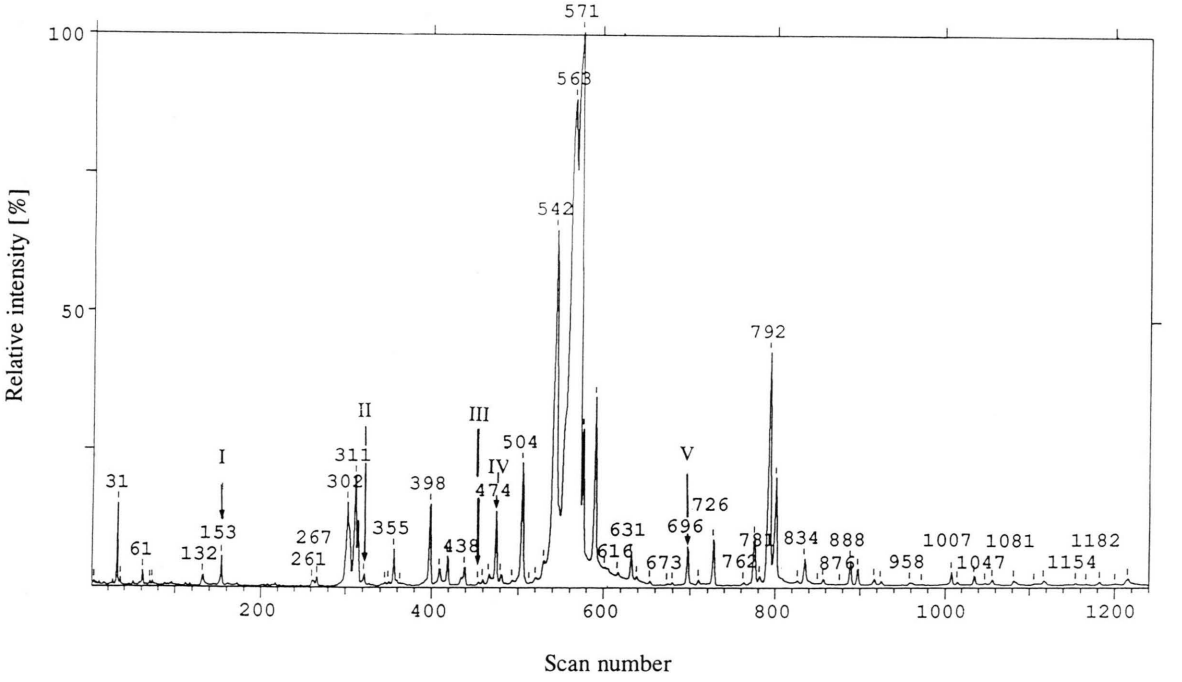

Fig. 4. Total ion chromatogram of silylated root extract of *K. daigremontiana*. Aromatic allelopathical chemicals are marked as **I** – **V**). Other compounds (from solvent, stabilisators, softening agents) are indicated by their scan number.

at *m/z* 381 (23%), 307 (11%), 219 (70%), 147 (4%) and 73 (36%), indicating that **V** is cinnamic acid, 3,4-bis [(trimethylsilyl)-oxy]-trimethylsilylester ($C_{18}H_{32}O_4Si_3$).

Furthermore these derivatives of benzoic acid [*p*-hydroxybenzoic-(**I**), protocatechuic- (**II**) and gallic acid (**IV**)] and cinnamic acid [*p*-coumaric- (**III**) and coffeic acid (**V**)] could be detected in the root extracts of *Kalanchoe daigremontiana*, *K. tubiflora* and *K. pinnata*.

Short-term bioassay with the authentic allelochemicals

Are the benzoic- and cinnamic acid derivatives from the root extracts of *Kalanchoe daigremontiana* allelopathically effective agents? In order to answer this question intraspecific bioassays are performed with cloned shoots of the species *K. daigremontiana* using various amounts of purchaseable authentic compounds dissolved in water. Freshly picked shoots of a clone, with no roots yet, in the bioassay are exposed to aqueous solutions with different concentrations of the aromatic acids (Fig. 5a-e). In the bioassay derivatives of both benzoic (*p*-hydroxybenzoic-, protocatechuic- and gallic acid) and cinnamic acid (*p*-coumaric- and coffeic acid) proved to be effective allelochemicals. When primary roots of average length (in mm) of the shoots had been exposed for 8 days to authentic allelochemicals of different concentrations and are compared with water-controls a very significant (** $\alpha \leq 0.01$) allelopathic inhibition effect on the growth of the roots is seen with the 5 allelochemicals at 10^{-2} and 10^{-3} molar concentrations of acids. If the shoots are exposed to the polar gallic acid, a very significant inhibition effect can be noticed over the whole range from 10^{-2} to 10^{-6} molar concentrations. Exposure of shoots without roots in 10^{-5} molar *p*-hydroxybenzoic acid also results in a very significant inhibition effect on the growth of the roots. A statistically significant (* $\alpha \leq 0{,}05$) inhibition effect on root lenghts was demonstated after exposure in 10^{-4} and 10^{-5} molar *p*-coumaric acid solution. As compared with the water control the inhibition effect of 10^{-6} molar *p*-hydroxybenzoic and p-coumaric acid is statistically nonsignificant (n.s.). However, the solutions of protocatechuic- and coffeic acid in 10^{-4}, 10^{-5} and 10^{-6} molar concentrations, respectively, have a statistically non-significant (n.s.) inhibition effect on the growth of the roots compared to the control tests in water.

Long-term bioassay with the authentic allelochemicals

In the long-term bioassay a mother plant producing allelochemicals is replaced by a glass capillary by way of which allelochemicals are supplied continuously. Every three days 2 ml of a aqueous $8 \cdot 10^{-6}$ molar solution of the identified allelochemicals are added with the aid of the glass capillary. Fig. 1C shows the influence of the allelochemicals on the growth of the shoots of the species *Kalanchoe daigremontiana*.

Interspecific allelopathy with shoots of the species Kalanchoe tubiflora

In order to demonstrate an interspecific allelopathic effect of *Kalanchoe daigremontiana* on shoots of *K. tubiflora*, a one-year-old mother plant of the species *K. daigremontiana*, about 40 cm high, is potted in a planting pot filled with sand. One week later shoots of a clone of the species *K. tubiflora* are planted in row at a distance of 3 cm from each other. Fig. 1D shows that the allelopathic agents obviously also act interspecifically after 120 days.

Allelopathy in Kalanchoe-species

The biosynthesis of the detected allelochemicals in plants is achieved by the shikimic acid pathway. According to Hess (1981) shikimic acid – apart from the production of phenolic substances – is important to provide the aminoacids phenylalanine, tyrosine and tryptophan. According to Rice (1984) allelopathy also includes transport and incorporation of the produced allelochemicals by neighbouring organisms in the biotope. Schlee (1992) characterizes following different phases of allelopathy: the production of secondary natural compounds in the plant, their release by evaporation, washing out, decomposition of plant parts by destruents and active root exsudation, the spreading in the biotope and the incorporation of substances by neighbouring organisms.

After an exposure period of four days at 10^{-6} molar respectively 10^{-5} molar concentrations the

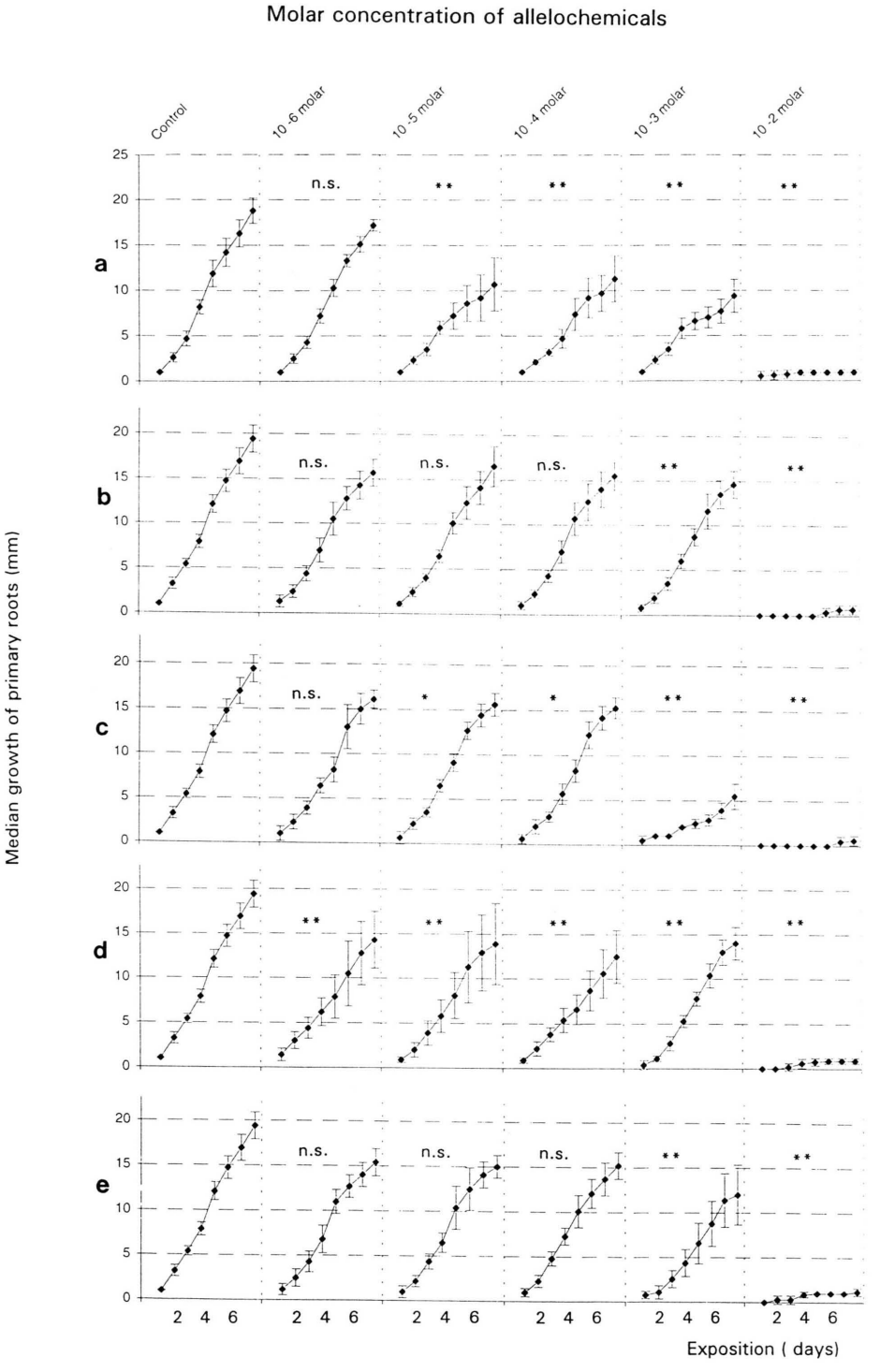

Fig. 5. Time dependent allelopathic acitivity (1–8 days) of different aqueous solutions of aromatic acids (10^{-6}–10^{-2} molar) **I** – **V**(a: *p*-hydroxybenzoic acid; b: protocatechuic acid; c: *p* – coumaric acid; d: gallic acid; e: coffeic acid) against daugther plants of *Kalanchoe daigremontiana* as compared with the water control (left). n.s.: not significant; *: significant ($\alpha \leq 0.05$); ** highly significant ($\alpha \leq 0.01$).

5 identified allelochemicals exhibited a weak allelopathic inhibition effect on the growth of the primary roots of the shoots (Fig. 5). The question has not been answered so far whether the amounts of eventually present allelochemicals in the leaf interior of the leaf (detection by GC/MS analysis of leaf extracts) already may have an influence on the size of the shoots on the leaf edges, respectively on the root lengths of the shoots. When ripe the shoots fall off their mother plants and root in the soil. Here, however, the substances **I** to **V** emitted by the mother plant by way of root exsudation are already present.

Whether the root growth of the shoots at a 10^{-4} molar allelochemical solution is more inhibited by *p*-coumaric-, gallic- and *p*-hydroxybenzoic acid than by exposure in coffeic- and protocatechuic-acid can not be assessed due to the statistical variations. At 10^{-3} molar allelochemicals solutions, in contrast to the control in water, significant inhibitory effects on the root growth of the test species were registrated. Especially 10^{-2} molar solutions inhibit the root growth of the shoots nearly completely, although that a high concentration of acids is normally not found in soil.

If conspecific inhibitions on shoots of the species *K. daigremontiana* and *K. tubiflora* in the biotest after a 4 days period of exposure are compared to each other, it can be stated that the species *K. tubiflora* at all concentrations of the allelochemicals is more inhibited compared to *K. daigremontiana* (Bär, 1995). Presumably this is due to the fact that the shoots of the species *K. tubiflora* have a smaller volume and maximal length than those of the species *K. daigremontiana*. Obviously a minor concentration of allelochemicals is sufficient to produce an inhibition effect in *K. tubiflora*. An influence of the polarity of the allelochemicals on the inhibition on the growth can not be detected with statistic reliability. Comparing the growth of the stem lengths of the shoots used in the long-term experiments it can be noticed that in experiments for intraspecific allelopathy the inhibition effect is weaker than in experiments for interspecific allelopathy. The decisive factor for this difference presumably is the different genetic material of the test species.

If the mother plants of the 3 test species are replaced by glass capillaries through which continually allelochemical solutions are pouring into the planting pot the allelochemicals can spread by diffusion in the sandy ground (Fig 1C). If you assume a 8 x 10^{-6} molar concentration in the pot at the beginning of the experiment, the amount of allelochemicals after 3 months – with 30 applications – would have reached about 10^{-7} molar. Given a sand volume of 9,5 litres, this concentration of every acid would correspond to about 10^{-8} molar pot content. After 6 months consequently in the long-term experiments a maximum theoretical concentration of about 2 x 10^{-8} molar per acid would have been achieved. Hereby a possible adsorption of the allelochemicals to soil colloids or a microbial degradation of the allelochemicals over the 6 months test period were not taken into consideration. In order to evaluate the probably low actual concentrations of *Kalanchoe* allelochemicals in the field the various types of soils present in different geographic areas must be additionally considered.

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