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Crotonic acid as a bioactive factor in carrot seeds (*Daucus carota* L.)

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Abstract

Water extracts from the carrot seed (*Daucus carota* L.) var. Perfekcja exhibit plant growth inhibitory properties against cress, cucumber, onion and carrot in a dose-dependant manner. This property results from the action of low-and high-molecular components of the extract. The low-molecular component was identified as crotonic acid ((*E*)-2-butenoic acid). Its presence was also confirmed in other late varieties of carrot. The determined strong herbicidal properties of crotonic acid and its availability after release to soil combined with its high level in seeds suggest that it might be considered as an allelopathic and autotoxic factor in the seeds.

Keywords: *Daucus carota*; Umbelliferae; Allelopathy; Autotoxic activity; Herbicidal activity; Crotonic acid

1. Introduction

Plants are known to produce secondary metabolites that affect germination and growth of other plants. This is one of a variety of ways in which certain plant can reduce interspecies competition in their natural habitats. Some of these compounds may play an important role in chemical mediation of growth and development of natural plant communities (Dayan et al., 2000; Duke et al., 2000a,b, 2002; Seigler, 1996; Vyvyan, 2002).

Over many years, various types of allelochemicals have been isolated and characterised from a vast number of plants and their organs (Chaves et al., 2001; Hasegawa et al., 1992; Macias et al., 1998, 1999; Ohno et al., 2001; Rice, 1984; Rizvi and Rizvi, 1992). Chemical interference is one of various stresses, which plants must cope with in nature, and therefore it has been studied quite intensively. Allelochemic natural products have been implicated in the patterning of vegetation and weed growth in agricultural systems. It has been also suggested that the release of allelochemicals to the environment may decrease crop yields. Alternatively, these allelochemicals offer a potential as natural herbicides since such compounds constitute standard chemical weapons not only of toxic plants but also common vegetables (Casini and Olivero, 2001; De Feo et al., 1997; Vaughn and Berhow, 1998; Yu and Matsui, 1994).

Carrot (*Daucus carota* L.) is a very popular vegetable cultivated world-wide and popularly used for culinary and cosmetic purposes. Various parts of the plant have also been therapeutically used in folk medicine; e.g. carrot leaves are good diuretics, the seeds are also diuretic and carminative are used for treatment of hangovers, and to stimulate menstruation.

The results of our preliminary study have shown that the spectrum of weeds in carrot cultivars in the rural region of Opole (southwestern part of Poland) is limited to 12 species from among 100 weeds commonly observed in this area. Although carrot is accompanied by a limited number of weeds, the information about its allelochemicals is scarce, with exception of reports concerning carrot–insect interactions (Cole, 1985; Degen et al., 1999a,b; Guerin et al., 1983; Guerin and Städler, 1984). Additionally, it is well established that too dense sowing of carrot seeds results in delay and inhibition of

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their germination. It was therefore of interest to assess the allelopathic potential of the water extracts of carrot seeds under laboratory conditions. Bioassay-directed fractionation and further purification of the aqueous extracts of the seeds afforded a low-molecular weight active compound, identified as crotonic acid.

2. Results and discussion

2.1. Allelopathic activity of crude carrot seed extract

The first effort of the study was to evaluate the herbicidal activity of water extract from carrot seeds. The lyophilised extract was used in tests performed on cress (*Lepidium sativum* L.), cucumber (*Cucumis sativus* L.), carrot (*Daucus carota* L.), and onion (*Allium cepa* L.), in order to evaluate its influence on seed germination and plant growth. The results of the germination test were negative, showing no significant effect of the extract on the germination of all the tested species. On the contrary, carrot seed extract significantly inhibited root and hypocotyl growth of cress, carrot, onion, and cucumber, and this effect was concentration-dependent (Fig. 1) and more pronounced in root growth. Among the four examined species of plants, carrot and cress were most sensitive. In the case of the latter plant, the results show that hypocotyl growth of *D. carota* was much less influenced than that of the root. At concentration of 0.5 mg/ml, the water extract caused 50% inhibition of the root growth of both carrot and cress. At the highest concentration (20 mg/ml) a total inhibition of carrot root growth was observed. It may be due to the fact that the root, which develops first, is affected by the extract of carrot seeds for a longer period of time than the hypocotyl. The observed strong influence of the extract on the growth of carrot root could be attributed to the autotoxic action of allelopathic compound(s) present in the medium. Other tested plants (cucumber and onion) were more resistant to the extract-only concentrations above 5 mg/ml visibly inhibited their growth.

2.2. Isolation and identification of the active component from seeds

A preliminary approach to fractionate the crude extract resulted in isolation of a low-molecular weight active component and determination of its structure. The first attempt was to isolate hydrophobic components soluble in *n*-hexane. Active components were not found and extraction with hexane did not influence the activity of the water fraction. Sequential extraction with ethyl acetate resulted in isolation of (*E*)-2-butenoic acid (crotonic acid), which in turn appeared to exert strong allelopathic activity.

Crotonic acid was reported as a main phytotoxic factor of *Croton tiglium* L. but it was later shown that this finding was erroneous one and that the phytotoxic activity of this species results from the action of the structurally related tiglic (*E*-2-methyl-2-butenoic acid) acid (Lloyd, 1898). Also 18-acetoxy-*cis*-cloeroda-

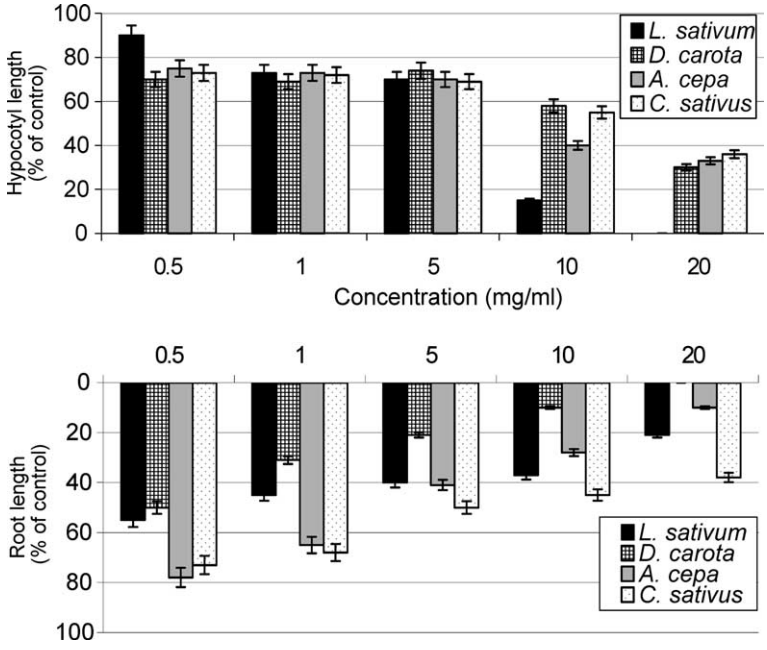


Fig. 1. Effect of varying concentrations of the water extract from carrot seeds on the lengths of hypocotyl and root of tested plants. Means ± SE from four independent experiments with 15 plants for each determination are shown.

3,13*E*-dien-15-oic acid, the steroidal derivative found in some plants, is mistakenly named as crotonic acid (Borquez et al., 1995; Tojo et al., 1999). To our best knowledge, our paper reports for the first time the isolation of this acid as a natural bioactive agent. It is worth noting that phytotoxic activity of very high concentrations of crotonic acid, understood as *E*-2-butenoic acid, against *Lemna minor* was reported earlier (Fromm, 1955).

The remaining extract still exhibited significant allelopathic activity. This activity is attributed to a fraction most likely containing oligosugars of a molecular mass lower than 3 kDa, as indicated by NMR and MS studies (data not shown).

2.3. Herbicidal activity of crotonic and tiglic acid

The herbicidal effects of crotonic acid (both isolated from seeds and purchased) and tiglic acids were determined using the four species of plants mentioned above. Also, in this experiment the influence on seed germination and plant growth was evaluated.

Contrary to an earlier report (Yatagai and Unrinin, 1989) crotonic acid did not influence the germination of any of the studied plants, whereas it exhibited a strong growth inhibition activity (Table 1). The activity of crotonic acid isolated from carrot seeds was compared with that exhibited by a commercial compound. In both cases the inhibitory action on the studied test plants was identical within experimental error. Hypocotyl growth was less affected than root growth inhibited only at the highest concentrations applied up to 1 mM. Our results clearly indicate (Table 1) that carrot plants appear to be most sensitive to the action of crotonic acid and therefore crotonic acid fits the general rules of autotoxic properties (Chon et al., 2002; Robles et al., 1996; Yu, 1999). Thus, crotonic acid exhibits relatively low herbicidal activity, but it is produced by seeds in sufficient quantity to have an ecological effect.

The activity of the structurally related tiglic acid, an allelochemical isolated from *Croton tiglium* L., was also tested. This compound was less inhibitory towards cress.

2.4. Screening for the presence of crotonic acid in other Umbelliferous species

The level of crotonic acid found in carrot seeds of the Perfekcja variety is exceptionally high and reaches up to 1% of dry mass of the seeds. To determine if this compound is a standard allelochemical present in seeds of other carrot varieties and in seeds of related vegetables, we screened for its presence additional nine varieties of carrot, two varieties of parsley and two varieties of dill (Table 2). The occurrence of crotonic acid in the seeds of these plants was studied by GC-MS, while its level was determined by isolation. Crotonic acid was detected only in the seeds of four varieties of carrot and in the seeds of parsley var. Berlinska. Quite interestingly, all these species belong to late-maturing varieties. As seen in Table 2, crotonic acid constitutes a high portion of dry mass of seeds of late carrot species with var. Perfekcja being the best producer of this compound. Its level in parsley var. Berlinska is 10 times lower, and this acid may not play a significant allelopathic activity during the development of this plant.

2.5. Crotonic acid in exudates of germinating carrot seeds

In order to determine if crotonic acid is really released from seeds upon germination and thus plays an allelochemical role, we examined exudates from germinating seeds of those of species Umbelliferae, which were found to be producers of this acid. Crotonic acid turned out to be the main component of *D. carota* germinating seed exudates detectable by GC-MS. Its quantity in exudates, determined by isolation, ranged from 70% to 90% of the quantity found in the dry mass of the seeds (Table 2). This means that under the experimental conditions applied in this work the concentration of crotonic acid released from seeds of the Perfekcja variety of carrot varied from 500 to 800 μg/ml, which was at least 10 times higher than the determined IC_{50}. Crotonic acid released from seeds of the Dolanka variety (the weakest among producers of this acid) still reached a concentration higher (72 μg/ml) than its IC_{50} determined in this work.

Table 1
Herbicidal activity of crotonic acid from carrot seed, commercial crotonic acid and tiglic acid in comparison with the crude extract against *L. sativum*, *C. sativus*, *D. carota* and *A. cepa*

Tested compounds	*L. sativum* IC_{50} (μg/ml)		*C. sativus* IC_{50} (μg/ml)		*D. carota* IC_{50} (μg/ml)		*A. cepa* IC_{50} (μg/ml)	
	Root	Hypocot.	Root	Hypocot.	Root	Hypocot.	Root	Hypocot.
Crotonic acid from carrot seeds	50	>175	72	>175	45	50	68	45
Crotonic acid	40	>175	68	>175	45	58	66	45
Tiglic acid	>175	>175	Non-toxic	Non-toxic	>175	>175	Non-toxic	Non-toxic
Crude extract	800	>5000	>5000	>5000	500	>5000	400	>5000

Table 2
The occurrence of crotonic acid in the seeds and exudates from germinating seeds of tested varieties of plants from the family Umbelliferae

	% of dry mass of seeds	% of dry mass released to the cotton wool	% of dry mass released to the silty clay	% of dry mass released to the sandy soil
Varieties of carrot (*Daucus carota* L.)				
Perfekcja	0.8–1	0.5–0.8	0.7–0.8	0.8–0.9
Nectarina	nd[a]	nd	–	–
Flacoro	0.21	0.18	–	–
Amsterdamska	nd	nd	–	–
Dolanka	0.12	0.09	–	–
Lenka	nd	nd	–	–
Koral F_1	0.43	0.26	–	–
Trophy	0.23	0.18	–	–
Nantejska	nd	nd	–	–
Kalina	nd	nd	–	–
Varieties of parsley (*Petroselinum sativum* L.)				
Berlinska	0.03	nd	–	–
Vistula	nd	nd	–	–
Lenka	nd	nd	–	–
Varieties of parsley (*Petroselinum sativum* var. *foliosum*)				
Paramont	nd	nd	–	–
Karnaval	nd	nd	–	–
Varieties of dill (*Anethum graveolens* L.)				
Szmaragd	nd	nd	–	–

[a] nd = not detected.

Carrot allelopathy can be evoked if an allelochemical released into the soil is not deactivated either by fast bacterial degradation or by sorption on soil particles. Thus, we have also studied availability (recovery) of this compound from two significantly different and well defined samples of soil, namely sandy soil and silty clay, used routinely for carrot cultivation and collected in the Opole region. The quantity of crotonic acid recovered from both types of soil was exactly the same (within the experimental error) as the recovery from cotton wool, thus showing that this allelochemical might easily act as a herbicide under natural conditions (Table 2).

3. Concluding remarks

Aqueous extracts of carrot seeds exhibit remarkable herbicidal activity. This effect can be attributed to a major low-molecular component – crotonic acid, and unidentified oligosaccharide. The high level of crotonic acid in carrot seeds, var. Perfekcja (around 1% of dry mass), as well as in other carrot varieties, supports its allelopathic function. To our best knowledge this is the first report on the is activity of crotonic acid, if the misidentification of this acid as the major phytotoxin of croton (*Croton* sp.) is excluded. This finding is of some interest also because the presence of crotonic acid was earlier documented in a crude wood distillate (Goos and Reiter, 1946), in sponges (Venkateswarlu et al., 1998), and in defensive secretions of several beetles (Kanehisa and Murase, 1977).

It is not yet known whether allelochemicals are actively released by plants or if it occurs passively. Despite the release is active or passive, the influence of external factors may be important. It is well documented that in same cases plants growing under stress conditions produce higher concentrations of allelochemicals (Einhellig, 1996). This could possibly be a result of competition during the process of evolution. Quite interestingly, we have detected crotonic acid only in late-maturing varieties of carrot and parsley. It is worth mentioning that sowing and seed harvesting seasons of carrot are the same for both late and early varieties. Because of this, the presence of crotonic acid only in late-maturing carrot varieties could not be ascribed to the action of environmental conditions but seems to be of genetic origin.

4. Experimental

4.1. Seed material

Seeds of the most popular late variety of carrot *Daucus carota* L. Perfekcja, collected in 2002, were purchased from Torseed Co., Poland. Seeds of all other varieties of carrot seeds (Dolanka, Nectarina, Amsterdamska, Lenka, Koral F_1, Nantejska, Kalina and Trophy) and of parsley (Berlinska, Lenka, Vistula, Karnaval, Paramont) and dill (var. Szmaragd) were purchased from Toraf s.c., Poland.

Seeds of plant species used in plant growth regulation tests, namely cress (*Lepidium sativum* L.), cucumber

(*Cucumis sativus* L.) cv. Polan F1, carrot (*Daucus carota* L.) and dicotyledonous and monocotyledonous onion (*Allium cepa* L.) were obtained from Torseed Co., Poland.

4.2. Carrot seed water extract

Carrot seeds (30 g) were finely ground and then shaken for 3 h at room temperature (25 °C) with 100 ml of water purified with a Milli-Q system (Millipore, Bedford, MA, USA). The suspension was filtered (Whatman No 2 filter paper) and then centrifuged (15,000 rpm, 25 °C, 10 min) to remove any solid particles. The pH of the obtained water extracts was between 6.3 and 6.5. Crude extract was lyophilised and stored at −15 °C.

4.3. Extraction and isolation of the active compound from seeds

The crude water extract of carrot seeds (25 ml) was extracted with *n*-hexane (3 × 15 ml) followed by extraction with ethyl acetate (5 15 ml). The combined ethyl acetate fractions were dried over anhydrous sodium sulphate and concentrated at 40 °C in vacuum to 2 ml volume, which allowed crystallisation of the active component (4 °C for 24 h). The isolated creamy crystalline substance was weighed and analysed by GC-MS and ^{1}H and ^{13}C NMR spectroscopy. Isolations from other varieties of carrot and various plants from the Umbelliferae family were performed in the same manner.

4.4. Isolation of a low-molecular weight bioactive component from exudates of germinating seeds

In order to determine if crotonic acid is released into the environment during inhibition and germination processes the following procedure was performed. Seeds of *D. carota* varieties (5 g), in which the presence of crotonic acid was detected by GC-MS, were placed on cotton wool in glass Petri dishes (20 cm ∅) and wetted with water purified with the Milli-Q system (50 ml). Seeds were incubated for five days at 26 °C in the dark. The aqueous medium from the Petri dish was collected and combined with cotton wool washings (three times with 25 ml). Water fractions (about 100 ml) were filtered through filter paper (Whatman No. 2) in order to remove remaining particles and then crotonic acid was isolated as described above. The isolated exudate was weighed and analysed by GC-MS and ^{1}H and ^{13}C NMR spectroscopy.

4.5. Recovery of crotonic acid from soil samples

Soil samples collected in the Opole region were a generous gift from Dr. Izabela Pisarek (Department of Land Protection of the Opole University). These soils are well characterised with respect of their physicochemical properties (pH, organic N and micro and macro elements) and content of organic matter (Pisarek, 2000, 2003).

Glass Petri dishes (20 cm ∅) were filled with 200 g per dish of sandy soil (pH 3.6), and silty clay (pH 6.4). Seeds of *D. carota* variety Perfekcja (5 g) were sown at a depth of 1 mm in each dish. Immediately after sowing, the soil surface was sprayed with 50 ml of water purified with Milli-Q system. Seeds were incubated for five days at 26 °C in the dark. After this time, the soils were individually extracted with H_2O (three times with 50 ml). Water fractions (about 150 ml) were centrifuged at 15,000 rpm, 25 °C, 10 min in order to remove remaining particles and then crotonic acid was isolated as described above.

4.6. Germination test

The lyophilised extract from carrot seeds was dissolved in water to give the final concentrations of 0.5, 1, 5, 10, and 20 mg/ml. Crotonic acid (a sample isolated from seeds and the commercial compound) and tiglic acid ((*E*)-2-methyl crotonic acid) were dissolved in water to give the final concentrations of 0.5, 0.75, 1 and 10 mM. Pure water was used as a control. Five millilitres of the appropriate solution was transferred to a 9-cm diameter Petri dish lined with filter papers (Whatman No. 2) containing 50 seeds for cress and carrot or 15 seeds for cucumber. Seeds were incubated in the dark at 26 °C. The germination test took 2 days for all the species with the exception of carrot (5 days). Seeds were considered germinated when the root length exceeded 2 mm.

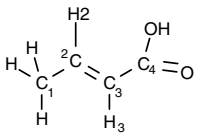
4.7. Plant growth regulatory activity of the seed extract, crotonic acid and tiglic acid

Fifteen uniformly pre-germinated seeds of the test plants with 1–2 mm roots were transferred to Petri dishes containing solutions of the seed extract or crotonic/tyglic acids. Dishes were placed in a growth chamber at 26 °C for 6 days (for carrot 8 days) with a 12-h photoperiod. Then, the separated hypocotyl and root lengths (or weights in the case of cucumber) were measured. All the experiments were performed four times, with three replications.

4.8. Structural studies

The NMR spectra were recorded at 300.13 MHz for ^{1}H and 75.46 for ^{13}C using a BRUKER AVANCE DRX300 spectrometer with TMS (tetramethylosilane) as an internal standard. FT-IR spectra were recordered in KBr pellets using a PU9800FT-IR Philips Analytical spectrometer. GC analyses were performed with a

Table 3
^{1}H and ^{13}C NMR data for crotonic acid (in D_2O)

	^{1}H δ (ppm)	^{13}C δ (ppm)	Integration
1	1.9	18	3H
2	7.1	147	1H
3	5.9	122	1H
4		172	

J_{1-2} = 6.72 Hz, J_{2-3} = 15.50 Hz.

Hewlett Packard 6890 gas chromatograph equipped with an FID detector. Diethyl ether solution (1 μl) was injected onto an HP-1 capillary column (30 m × 0.32 mm bonded-phase fused silica). The initial oven temperature was maintained at 60 °C for 2 min and then raised at 10 °C/min to 280 °C. Helium was used as a carrier gas. MS analyses were performed on a quadrupole Hewlett Packard 6897 instrument with ionisation at 70 eV. The structure of the active compound was determined by peak matching library search to published standard mass spectra and by comparison with an authentic reference compound (Sigma–Aldrich Chemical Co.).

The structure of this bioactive factor was determined as by means of GC-MS, FT-IR and NMR and the obtained data were compared with those received for an authentic sample of crotonic acid. Three signals (Table 3) were found in ^{1}H NMR spectrum taken in D_2O, whereas ^{13}C spectrum contained four signals (Table 3) corresponding to crotonic acid. The IR spectrum showed signals for –OH (broad peak centred at 2920 cm^{-1}) and >C═O (1704 cm^{-1}) and MS analysis yielded the molecular ion at *m/z* 86, which confirmed this assignment. The purity of the acid was determined by GC chromatography, where only one peak (R_t = 3.86 min) was observed.

4.9. Statistical analysis

Dixon's *Q*-test was used to reject the unreasonable results of length or weight measurements of root and hypocotyl. The mean values for samples and controls were compared by the null hypothesis test at the 5% significance level (Miller and Miller, 1984). For all the data mean values ± SE were calculated.

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