Sensitivity of vibrios to sanguinarine

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Summary. The sensitivity to sanguinarine of various strains belonging to 4 vibrio biotypes was investigated. *Vibrio cholerae* (classical) was most sensitive, and *Vibrio parahaemolyticus* least sensitive to this alkaloid. Statistical analysis revealed significant differences between the 4 biotypes in their sensitivity to sanguinarine.

Sanguinarine, a benzophenanthridine alkaloid, has been reported to possess antitumor and antimicrobial activity[2]. Recently, it has been found to form a molecular complex with DNA by intercalation[3]. The sensitivity of various gram-positive bacteria to sanguinarine has been investigated[2], but similar data on the vibrios, gram-negative bacteria and the causative agent of cholera, are lacking in the literature. In the present communication we present a brief account of our investigation of the sensitivity to sanguinarine of a number of strains belonging to *Vibrio cholerae* (classical), *Vibrio cholerae* (El Tor), nonagglutinable (NAG) vibrio and *Vibrio parahaemolyticus.*

Materials and methods. The media used for the cultivation of vibrios included the following (w/v): a) peptone agar (pH 8.0) containing 3 g bactopeptone (Difco); 0.5 g NaCl and 2 g bacto agar (Difco) in 100 ml distilled water, b) peptone water (pH 8.0) containing 3 g bactopeptone; 0.5 g NaCl in 100 ml distilled water. Vibrios were grown in 100-ml Erlenmeyer flasks containing 20 ml of culture media so as to provide enough surface for satisfactory aeration[4]. For all purposes 0.5 ml of an overnight culture in peptone water media was inoculated into 20 ml fresh media. Phosphate buffered saline (pH 6.8) containing 0.34 g KH_2PO_4, 0.445 g Na_2HPO_4 and 0.91 g NaCl in 100 ml distilled water was used as dilution fluid. Treatment with sanguinarine chloride of different concentrations was done on log phase bacterial culture ($\sim 10^8$ cells/ml) and absorbance and viable counts of treated and untreated bacteria were determined as previously described[5,6]. The minimal inhibitory concentration (MIC) was estimated by the tube dilution method as described[7]. Briefly, 0.2 ml of a 1-fold dilution of log phase bacteria ($\sim 10^8$ cells/ml) in peptone water medium was inoculated into 3 ml of a solution of sanguinarine prepared with the same medium and incubated at 37 °C. The concentrations of sanguinarine tested were 100, 80, 60, 50, 40, 25, 20, 15, 12.5, 10, 8, 6, 5, 4, 3, 2, 1 and 0.5 µg/ml. MIC was expressed by the lowest concentration at which no turbidity occurred after 18 h at 37 °C.

Results and discussion. The survival of different vibrio biotypes after treatment with increasing concentrations of sanguinarine gave a straight line in a semilogarithmic plot (data not shown). It has been observed that *V. cholerae* (classical) strains are most sensitive and *V. parahaemolyticus* strains are least sensitive to sanguinarine treatment on a comparative scale. The sensitivity of different strains of vibrio biotypes according to the MIC values of sanguinarine are presented in table 1. The results indicate that the MIC values of sanguinarine against *V. cholerae* (classical) and *V. cholerae* (El Tor) strains are 5 times lower as compared to the alkaloid berberine[8]. Data on the survival of different strains belonging to each vibrio biotype are summarized and also presented in table 1. The average sanguinarine dose required for 50% survival the organisms varied from 1.2±0.2 (*V. cholerae* classical) to 17.06±0.5 (*V. parahaemolyticus*) µg/ml. These data are comparable to the sensitivity of vibrios to furazolidone[9], which has found useful application in human therapy for cholera.

Statistical tests[10] were performed to establish the significance of the differences between 4 vibrio biotypes with respect to their sensitivity to sanguinarine treatment (survival after 1 h treatment). The F value in the variance ratio test was calculated to be 16.0. The greater variance estimate has 6 degrees of freedom and the lesser estimate 12 degrees. The table of variance ratio reported by Armitage[10] shows that the corresponding value of F at the 1% level is 5.76 only, demonstrating convincingly that there are significant differences between the 4 biotypes with respect to their

Table 1. MIC values of sanguinarine and sanguinarine doses required for 50% survivals of the different vibrio strains after 1 h treatment at 37 °C

Strains of different biotypes		MIC (µg/ml)	Dose for 50% survivals Sanguinarine concentration (µg/ml)	Average (µg/ml)
V. cholerae (classical)				
OGAWA	154	5	1.3	
	T_5	4	1.2	
	T_3	4	1.2	
	116/71	5	1.3	1.2±0.2
	447/70	5	1.2	
INABA	569B	3	1.1	
	C16/71	3	1.1	
V. cholerae (El Tor)				
	OE 65	8	3.3	
	OE 64	10	3.8	
	E 373/69	10	3.6	3.5±0.25
	E 435/70	10	3.6	
	MAK 757	8	3.2	
NAG vibrio				
	W-50	20	6.5	
	W-12	20	6.7	
	PS-55	15	5.4	
	PS-135	20	5.7	6.0±0.5
	PS-80	20	5.8	
	PS-57	20	5.9	
	PS-175	20	6.6	
V. parahaemolyticus				
	3/80	50	17.7	
	4/80	50	16.4	17.06±0.5
	154/80	50	17.1	

Table 2. Statistical differences observed in the survivals of different vibrios (t-tests) after treatment with sanguinarine for 1 h. The probability values correspond to the test of differences between the organisms in the row and column to which the particular value belongs

Organisms	*V. cholerae* (classical)	*V. cholerae* (El Tor)	NAG vibrios	*V. parahaemolyticus*
V. cholerae (classical)	$p=1$	$p<0.001$	$p<0.001$	$p<0.001$
V. cholerae (El Tor)	$p<0.001$	$p=1$	$p<0.001$	$p<0.001$
NAG vibrios	$p<0.001$	$p<0.001$	$p=1$	$p<0.001$
V. parahaemolyticus	$p<0.001$	$p<0.001$	$p<0.001$	$p=1$

sensitivity to sanguinarine, i.e. they are not drawn from the same parental population. Student's t-tests were also performed taking any 2 biotypes at a time and the results demonstrating the degrees of significance are presented in table 2. Table 2 thus reveals that the difference between any 2 vibrio biotype is also statistically significant.

The present study has shown that *V. cholerae* (classical), *V. cholerae* (El Tor), NAG vibrios and *V. parahaemolyticus* may be differentiated from each other on the basis of their sensitivity to sanguinarine and may thus be of importance for the taxonomy of vibrios.

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Phytotoxic effects of cadmium in leaf segments of *Avena sativa* L., and the protective role of calcium

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Summary. It is shown that ethylene production can be stimulated in peeled oat leaf segments in the dark by cadmium at concentrations below 0.01 mM, and that cadmium-stimulated stress ethylene accelerates senescence processes. Higher cadmium concentrations cause membrane deterioration and inhibit ethylene production and senescence. These effects of cadmium are antagonized by calcium. This indicates a possible protection of plant cells against cadmium toxicity by calcium.

Cadmium is an environmental contaminant of increasing importance because of industrial processes and refuse incineration. It is readily taken up by plants from soils[2] and nutrient solutions[3], and accumulates in various tissues during the growing season. This accumulation is toxic to plants. Reduced rates of photosynthesis[4], transpiration[5] and respiration[6] have been observed, as well as stimulated or inhibited enzyme activities[6,7]. Among the early effects of excess cadmium uptake in beans, short-term stimulation of ethylene biosynthesis was reported in a recent paper[3]. Such stimulation is limited to a relatively short incubation period. Changes in peroxidase activity and phenolic materials accompany the subsequent decline. Cadmium is known to bind to macromolecular cell constituents[8]. In bean leaf discs, membrane damage seems to be the cause of the inhibition of a membrane-involving step in ethylene biosynthesis[9]. The interaction with membranes and soluble proteins may be responsible for the impact of cadmium on cellular metabolism. Membranes function as important regulators of specific pathways, such as ethylene biosynthesis, and developmental processes, such as senescence. Stress-induced senescence in oat leaves includes a rapid increase in protease activity leading to an enhanced content of α-amino nitrogen, ethylene biosynthesis and chlorophyll loss[10]. In the present study, the oat leaf-system has been used to show that membrane damage induced by cadmium is associated with inhibited ethylene production and an altered senescence rate, and that calcium protects the cells against cadmium damage.

Materials and methods. The 1st leaf of 14-day-old seedlings of *Avena sativa* L. (var. Victory) was used for all experiments. The seeds (Swedish Seed Company, Ltd, Svalöv, Sweden) were grown in vermiculite in controlled growth chambers at 25 °C with a 16-h photoperiod (about 12,000 lux). Leaf sections were excised and sterilized as described before[11]. The abaxial epidermis was peeled off with fine forceps. Five segments of 50 mm length each were rinsed in sterile distilled water and floated in glass bottles, stripped side down, on 5 ml of Na-phosphate buffer (1 mM, pH 5.7). Cadmium was added as $CdCl_2$ at concentrations in the range 0–1 mM. Where indicated, $CaCl_2$ (1 mM) was included. The bottles were sealed with serum rubber caps and incubated for 48 h in the dark at 25 °C. At the end of the incubation period, air samples were withdrawn with a 5 ml syringe and injected into a gas chromatograph (Perkin Elmer Model F-11) equipped with an activated alumina column and a flame ionization detector. Ethylene was identified and quantified by comparison with the retention time and peak height of ethylene standards. After taking the air samples, the bottles were opened, samples of the incubation solution were removed and their UV-absorbance at 280 nm was determined spectrophotometrically (Aminco DW-2a). The presence of UV absorbing materials in the incubation solution was used as measure for leakage due to membrane damage[9]. For the determination of chlorophyll and α-amino nitrogen, leaves were extracted with hot 80% ethanol and extracts were analyzed as described before[12].

Results and discussion. Peeling off the epidermal cells of oat

Effect of cadmium (0.01 mM) and aminoethoxyvinylglycine (AVG, 0.1 mM) on dark-induced chlorophyll loss during 48 h of incubation

	Chlorophyll loss (in % of initial value)
H_2O	60 ± 4
Cadmium	71 ± 5
Cadmium + AVG	52 ± 4
AVG	55 ± 5

Values are means (N = 4) + 1 SD.