The Effect of Coumarin Derivatives on Organogenesis and Callus Growth of *Cichorium Intybus* Roots and *Helianthus tuberosus* Tubers in vitro

Coumarins are widely distributed in the plant kingdom, even if they are especially common in the Compositae, Umbelliferae and Rutaceae. These compounds have quite different physiological effects: in some cases they act as inhibitors of plant growth and seed germination, and sometimes as stimulators[1].

Until now the effects of inhibition or stimulation of growth were observed only on the extension of epicotyls, hypocotyls and coleoptiles of different plants[2-4] and on elongation of roots[5,6]. There are very few reports, and only for the parent compound[7,8], on the action of natural coumarins on callogenesis of plants tissues cultivated in vitro.

For this reason, we thought it would be interesting to investigate the effect of coumarins on cellular division and organogenesis of plant tissues grown in vitro, in view of the study of possible relationship between chemical structure and activity of coumarins and its derivatives.

Materials and methods. Explants of *Cichorium intybus* (chicory) roots and dormant tubers of *Helianthus tuberosus* (Jerusalem artichoke) var. OB1, were utilized. Prismatic chicory explants (1.5 cm height) were placed in vitro as previously described[9]. Cylindrical explants (9 mm diam., 1 cm height) of an homogeneous medullary

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Table I. Effect of different coumarins (CM) on growth of *Helianthus tuberosus* dormant tuber and *Cichorium intybus* root explants in vitro

		Helianthus tuberosus				*Cichorium intybus*			
Coumarins	Concentration (*M*)	Dry weight (mg)	Dry wt. (%)	Dry wt. of control (%)	Fresh wt. of control (%)	Dry wt. (mg)	Dry wt. (%)	Dry wt. of control (%)	Fresh wt. of control (%)
CM	0	146 ± 2	11.4	100	100	358 ± 9	13.9	100	100
CM	2×10^{-4}	154 ± 2[a]	9.9	105	121	371 ± 22	11.7	103	123
CM	5×10^{-4}	144 ± 2	12.0	98	92	332 ± 14	10.6	93	120
CM	10^{-3}	110 ± 1[a]	15.0	75	56	262 ± 6[a]	10.2	73	99
3 CH_3-CM	0	147	10.7	100	100	295	11.1	100	100
3 CH_3-CM	2×10^{-4}	134	14.6	91	67				
3 CH_3-CM	3×10^{-4}					276	10.7	93	97
3 CH_3-CM	5×10^{-4}	127	15.7	86	59				
3 CH_3-CM	7×10^{-4}					262	11.6	89	85
3 CH_3-CM	10^{-3}	115	16.2	78	52	233	11.9	79	73
3 OH-CM	0	155 ± 2	11.2	100	100	406	15.3	100	100
3 OH-CM	10^{-4}					338	15.9	83	80
3 OH-CM	2×10^{-4}	147 ± 2[a]	11.8	95	91				
3 OH-CM	5×10^{-4}	135 ± 2[a]	11.9	87	82	353	15.3	87	87
3 OH-CM	10^{-3}	129 ± 2[a]	14.9	83	64	334	14.9	82	84
3 COOH-CM	0	166 ± 5	12.8	100	100	344 ± 11	12.2	100	100
3 COOH-CM	2×10^{-4}	164 ± 3	18.2	99	69	261 ± 43[a]	12.8	76	73
3 COOH-CM	5×10^{-4}	154 ± 3	18.2	93	65	266 ± 12[a]	11.4	77	82
3 COOH-CM	10^{-3}	109 ± 4[a]	16.4	66	51	—	—	—	—
4 OH-CM	0	199 ± 5	15.5	100	100	306	13.0	100	100
4 OH-CM	10^{-4}	170 ± 8[a]	21.2	85	62	301	13.8	98	94
4 OH-CM	5×10^{-4}	119 ± 12[a]	18.1	60	51	252	13.0	82	82
6 Cl-CM	0	199 ± 5	15.5	100	100	250 ± 7	11.1	100	100
6 Cl-CM	10^{-4}	154 ± 8[a]	21.3	77	56	270 ± 8[a]	11.0	108	109
6 Cl-CM	5×10^{-4}	103 ± 10[a]	19.1	52	47	192 ± 6[a]	10.2	77	83
6 Cl-CM	10^{-3}	73 ± 10[a]	13.4	37	42	212 ± 6[a]	10.2	85	92
6 NH_2-CM	0	161	12.5	100	100	355 ± 14	12.4	100	100
6 NH_2-CM	2×10^{-4}	157	12.0	98	101	397 ± 14	11.6	111	119
6 NH_2-CM	5×10^{-4}	154	12.7	96	94	361 ± 19	11.4	101	110
6 NH_2-CM	10^{-3}	146	12.3	91	92	342 ± 11	11.8	96	99
6,7 OH-CM	0	233 ± 23	13.3	100	100	291 ± 19	11.3	100	100
6,7 OH-CM	10^{-4}	231 ± 15	15.8	99	83	290 ± 13	11.4	100	98
6,7 OH-CM	5×10^{-4}	238 ± 9	15.1	102	89	294 ± 20	11.4	101	100
6,7 OH-CM	10^{-3}	250 ± 3	14.6	107	97	321 ± 18	12.0	110	104

Control of *Helianthus tuberosus* = nutritive medium + IAA $2\times10^{-6}M$. Control of *Cichorium intybus* = nutritive medium without IAA. Average values ± SE were made on about 20 explants of 20 days old. [a] The difference of each average with its control is significant at least at 5% (Student's *t*-test). 4 OH-CM $10^{-3}M$ does not allow the gelification of medium. 3 COOH-CM $10^{-3}M$ makes the gelification of medium difficult.

Table II. Effect of different coumarins (CM) on bud neoformation in *Cichorium intybus* root explants in vitro

Coumarins	Concentration (M)	No. of neoformed buds	No. of buds (%)	Max. height of leaves (mm)	Max height of leaves (%)
CM	0	4.8 ± 0.5	100	20.0 ± 6.2	100
CM	2×10^{-4}	3.0 ± 0.7[a]	63	10.7 ± 2.4	54
CM	5×10^{-4}	2.9 ± 0.7[a]	60	6.1 ± 1.5[a]	31
CM	10^{-3}	1.3 ± 0.8[a]	27	3.3 ± 0.6[a]	17
3 CH_3-CM	0	12.5 ± 0.8	100	15.5	100
3 CH_3-CM	3×10^{-4}	13.5 ± 0.6	108	13.3	86
3 CH_3-CM	7×10^{-4}	11.6 ± 0.6	94	11.8[a]	76
3 CH_3-CM	10^{-3}	9.2 ± 0.6[a]	74	9.3[a]	60
3 OH-CM	0	13.9 ± 0.8	100	12.2	100
3 OH-CM	10^{-4}	12.0 ± 0.7	86	10.5	86
3 OH-CM	5×10^{-4}	12.6 ± 0.8	90	10.2[a]	84
3 OH-CM	10^{-3}	12.2 ± 0.6	88	9.9[a]	81
3 COOH-CM	0	6.1 ± 0.9	100	38.4 ± 6.2	100
3 COOH-CM	2×10^{-4}	6.5 ± 0.9	106	37.2 ± 3.4	96
3 COOH-CM	5×10^{-4}	6.5 ± 0.8	106	33.9 ± 3.7	88
4 OH-CM	0	8.2 ± 0.6	100	—	—
4 OH-CM	10^{-4}	8.4 ± 0.8	102	—	—
4 OH-CM	5×10^{-4}	6.7 ± 0.5[a]	82	—	—
6 Cl-CM	0	10.5 ± 0.5	100	—	—
6 Cl-CM	10^{-4}	10.5 ± 0.6	100	—	—
6 Cl-CM	5×10^{-4}	8.6 ± 0.5[a]	82	—	—
6 Cl-CM	10^{-3}	8.2 ± 0.6[a]	78	—	—
6 NH_2-CM	0	4.1 ± 0.6	100	39.4 ± 3.8	100
6 NH_2-CM	2×10^{-4}	3.7 ± 0.8[a]	90	40.5 ± 5.3	103
6 NH_2-CM	5×10^{-4}	5.0 ± 0.7	121	40.2 ± 4.2	102
6 NH_2-CM	10^{-3}	4.8 ± 0.8	117	36.1 ± 5.1	92
6,7 OH-CM	0	8.4 ± 1.0	100	48.1 ± 3.9	100
6,7 OH-CM	10^{-4}	10.2 ± 1.1	121	53.5 ± 3.3	112
6,7 OH-CM	5×10^{-4}	6.0 ± 0.8	71	41.9 ± 4.3	87
6,7 OH-CM	10^{-3}	7.4 ± 0.8	88	35.8 ± 3.4[a]	74

Average values ± SE were made on about 15 explants of 20 days old. [a] The difference of each average with its control is significant at least at 5% (Student's t-test). 4 OH-CM $10^{-3}M$ does not allow the gelification of medium. 3 COOH-CM $10^{-3}M$ makes the gelification of medium difficult.

parenchyma of *H. tuberosus* tubers were placed in vitro in a nutritive medium[10] with glucose 4%, indol-3-acetic acid (IAA) $2\times10^{-6}M$ and purified agar 1% (Fluka). The cultures were randomized in a culture room at 24 °C in alternating light (1,800 lux) or in the dark. The coumarins, used at concentrations between 0.1 mM and 1 mM, were: parent compound (CM), 3-methylcoumarin (CH_3-CM), 3-hydroxycoumarin (3OH-CM), coumarin-3-carboxylic acid (3 COOH-CM), 4-hydroxycoumarin (4 OH-CM), 6-chlorocoumarin (6 Cl-CM), 6-aminocoumarin (6 NH_2-CM) and 6,7-dihydroxycoumarin (esculetin) (6,7 OH-CM). The purity of these compounds was verified by means of paper chromatography[11,12]. Chlorophyll extraction and determination in chicory was made according to SMITH and BENITEZ[13]. The experiments were repeated at least twice at different times with similar results.

Results and discussion. The results show (Tables I and II), on the whole, that the coumarins studied, among 0.1 and 1 mM concentrations, exert generally an inhibition either on natural cellular proliferation and organogenesis of *Cichorium intybus*, or on cellular proliferation induced by IAA in explants of *Helianthus tuberosus* dormant tubers. Only coumarin on *H. tuberosus*, and 6-chlorocoumarin on *C. intybus* respectively at 0.2 or 0.1 mM conc., showed a very weak growth effect. Only this datum on coumarin agrees with the results of DUPLESSY-GRAILLOT[7], which are hardly comparable with ours.

The range level of such inhibitions changes according to plant tested, to the coumarin used, to its concentrations and sometimes to the conditions of light or dark. An example of complete inhibition was shown by 6 Cl-CM at mM concentration which completely blocked the stimulation of *H. tuberosus* by IAA: the explants are equal to a control without IAA. There are never necrosis and the vitality of inhibited explants was also shown by an evident synthesis of chlorophyll, even though reduced, in respect to the control, at the most of 50%.

Besides it was possible to demonstrate that a coumarin derivative exercises a reversible inhibition of growth and that it can also inhibit a tissue, previously stimulated by IAA, very strongly. For this purpose 2 sorts of contemporaneous experiments were carried out (Table III).

Bud formation is generally inhibited in *C. intybus* explants by coumarins, either as average of bud number for explant, or as height of leaves (Table II). The bud inhibition was maintained also 36 days after the beginning of the experiment.

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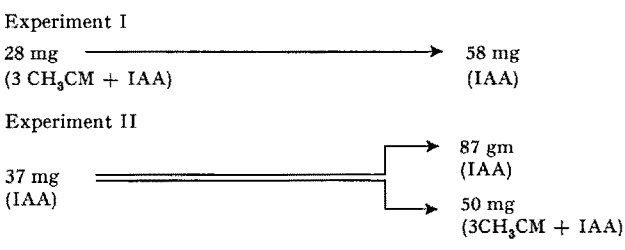
Table III. Control of 3-methylcoumarin inhibition on *Helianthus tuberosus* dormant tuber explants in vitro



Each datum is dry weight average (mg) of 20 explants grown first for 21 days and after another 26 days. Concentrations are: 3CH_3CM $10^{-3}M$ and IAA $2\times10^{-6}M$.

Table IV. 3-hydroxycoumarin effect on root neoformation in *Cichorium intybus* explants in vitro

3 OH-CM (M)	No. neoformed roots	No. roots (%)
0	7.1 ± 0.7	100
10^{-4}	5.1 ± 0.4[a]	72
5×10^{-4}	5.4 ± 0.6[a]	76
10^{-3}	0.5 ± 0.1[a]	7

Average values ± SE were made on 28 explants, 25 days old, growing in continuous light. [a] The difference of each average with its control is significant at least at 5% Student's t-test.

Root neoformation in chicory is inhibited (more than 90%) particularly by mM 3 OH-CM (Table IV). Also 3 CH_3-CM causes a similar effect. The rhyzogenetic activity also of *H. tuberosus* explants was verified according to TRIPATHI[10] and TRIPATHI and GAUTHERET[14]. Unfortunately our explants were not able to form roots on TRIPATHI medium probably because this phenomenon is related to variety.

Two parallel experiments were made in alternating light or in the dark, with 3 CH_3-CM and 4 OH-CM, at concentrations between 0.1 and 1 mM, on *C. intybus* and *H. tuberosus*. The data show, referred to the controls, no significant differences of growth except for *C. intybus* cultivated on 3 CH_3-CM in the dark, which is more inhibited. 3 CH_3-CM shows a greater effect on bud formation in the dark than in the light, while 4 OH-CM exerts the same effect in both cases. However, the number of buds formed by explants grown on the same coumarin concentrations, was constantly lower in those grown in the dark (about 33%).

The results were also examined to discover a possible relationship between the type and position of substituting radicals in the molecule and its action. The coumarin (CM) generally inhibits the callogenesis to the same degree as 3 CH_3-CM and the organogenesis more than the other coumarins essayed. The methyl substitution in position 3 has a greater inhibitory effect, at mM concentrations, than hydroxylation and carboxylation in the same position on bud formation in *C. intybus*. The hydroxy-groups in position 3 and 4 cause a similar effect on chicory, while the 4 OH-CM exerts a greater inhibition on *H. tuberosus*. The substitution in position 6 with amino or 6, 7 with hydroxy-groups has very little effect on callogenesis of *H. tuberosus* and *C. intybus*, while 6-chloro-group substituted coumarin is more inhibiting.

Probably the plants do not metabolize coumarins simply by detaching the radicals: in fact the effects of various derivatives are often very different from those of parent compound. Generally *H. tuberosus* appears to be more sensitive than *C. intybus* to coumarin action, especially expressed as fresh weight. Besides chicory does not show significant variations of dry weight percentage. It is known that the IAA activation of dormant tissues as *H. tuberosus* raises its hydration. In such explants, treated with increasing coumarin concentrations, the dry weight percentage generally grows parallel. This fact could indicate that, in addition to the inhibitory effect on callogenesis, these compounds, at suitable concentrations, probably exercise some inhibition also on extention growth as confirmed by numerous authors[2,4-6], perhaps modifying the cellular permeability[15].

In conclusion, the inhibitory effect on growth and organogenesis of coumarins essayed are obviously different according to the tissues utilized, but overall they are dependent on position and type of substituting radicals[16].

Riassunto. La cumarina ed alcuni suoi derivati (3 metil-, 3 idrossi-, 3 carbossi-, 4 idrossi-, 6 amino-, 6 cloro- e 6,7 diidrossi-cumarina), specialmente alla concentrazione mM, hanno effetto inibitorio sia sulla callogenesi di tuberi di *Helianthis tuberosus* che sulla organogenesi (neoformazione di gemme e radici) e callogenesi di radici di *Cichorium intybus* coltivati in vitro. Il grado di inibizione dipende dal tipo di radicale sostituente della cumarina e dalla pianta utilizzata come test.

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Antimicrobial Activity of Cyclacillin against *Escherichia coli* in vivo and in vitro

Cyclacillin [6-(1-aminocyclohexanecarboxamido)penicillanic acid] is a semisynthetic penicillin with a wide antibacterial spectrum; it resembles ampicillin in being effective against a wide range of gram-positive and gram-negative pathogens, but, unlike ampicillin, is also effective against the penicillinase-producing staphylococci[1-3]. However, in laboratory experiments in which cyclacillin was tested against a number of gram-positive and gram-negative pathogens, an inconsistency was encountered between the in vivo and in vitro susceptibility[4]. In vitro, cyclacillin was less active than ampicillin against both gram-negative and gram-positive bacteria, while in vivo,