GENERAL CHARACTERISTICS OF COUMARIN-INDUCED TUBERIZATION OF AXILLARY SHOOTS OF *SOLANUM TUBEROSUM* L. CULTURED *IN VITRO*[1]

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Abstract

Coumarin readily stimulates tuberization of cultured axillary shoots obtained from etiolated potato sprouts. Adequate concentrations of coumarin must be continuously present in the medium to effect tuber initiation. High nitrogen, gibberellic acid, abscisic acid, indole acetic acid, or naphthalene acetic acid reduced or inhibited coumarin-induced tuberization. Gibberellic acid and high concentrations of nitrogen in medium inhibited the uptake of coumarin-3-14C by the axillary shoot. Based on the data of physiological activities by coumarin in other plant species and our data on tuberization, we propose that coumarin may represent in part the unknown inhibitor responsible for tuber initiation with reference to the inhibitor/gibberellic acid ratio theory of tuberization.

Resumen

La aplicación de coumarina estimula la tuberización de brotes axilares obtenidos de brotes etiolados de tubérculos. Coumarina debe estar continuamente presente en el medio de cultivo en concentraciones adecuadas para producir la iniciación de tuberización. Cantidades elevados de nitrógeno, ácido giberélico, ácido absísico, ácido indol-acético y ácido naftalenacético redujeron o inhibieron la tuberización inducida por coumarina. El ácido giberélico y alta concentración de nitrógeno en el medio inhibieron la absorción de coumarina -3-14 C por el brote axilar. De acuerdo a los datos de actividad fisiológica de la coumarina en otras especies vegetales y los datos obtenidos en este trabajo en tuberización, los autores sugieron que la coumarina pueda constituir una parte del inhibidor aún desconocido responsable de la iniciación de la tuberización en referencia a la teoría sobre la proporción inhibidor/ácido giberélico en este proceso.

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Introduction

Coumarins are naturally occurring aromatic compounds which are found in a wide variety of plant species (28, 58). Coumarin and coumarin derivatives are known to have pronounced effects on the physiological processes in both plants and animals. The physiological effects produced by coumarins have been reviewed by Bose, Mayer and Poljakoff-Mayber, Van Sumere *et al.*, and Wolf (8, 36, 77, 80). Coumarin is considered to be primarily a plant growth inhibiting compound. Extensive and detailed research has been conducted on the inhibition of root growth and the germination processes in numerous plant species (3, 5, 60, 68, 70, 81). However, research has also shown that coumarin is a growth stimulant, comparable to Indole acetic acid (IAA) but considered to have a distinctly different mode of action (42, 43). Coumarin has demonstrated a physiological effect on senescence by delaying this process, perhaps by preventing loss of chlorophyll (26). Effects of coumarin on plant tissues and isolated cells of both algae and plants result in chromosomal breakages, increased cell wall plasticity, cell enlargement and changes in cytoplasmic viscosity (10, 18, 55, 59). During the course of evaluating plant growth regulating compounds for effect on tuberization *in vitro,* we reported that coumarin was extremely effective in stimulating tuber initiation (65, 67). The present paper describes in more detail the factors affecting the coumarin-induced tuberization processes.

Materials and Methods

General Culture Methods

Growth of potato sprouts and culture of excised axillary shoots were modified from the methods described by Palmer and Smith, (46). Russet Burbank potato tubers were planted in a sterilized mixture of fertilized sand-vermiculite-peat (2:2:1) and grown at 25 C in the dark. The etiolated sprouts were harvested at 21 days and surface sterilized as previously described (67). Sections 50 mm in length containing a single node were cut from the sprouts, transferred to the agar medium (25) contained in 25 × 150 mm test tubes and incubated in the dark at 25 C for production of axillary shoots. All contaminated potato sprouts were discarded and the axillary shoots from the remaining sterile shoots were excised and transferred directly onto the agar test medium.

Coumarin Incubation Time Study

Excised axillary shoots were initially cultured on the basal medium containing 25 mg/l coumarin. The axillary shoots were then transferred at intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 days to test tubes containing the basal medium less coumarin. The combined time of shoot culture on the coumarin-amended or coumarin-less medium was thirty days.

Coumarin-3^{14}C Translocation Studies

Excised axillary shoots were incubated on the basic medium amended with 25 mg/1 coumarin and 52 μCi coumarin-3^{14}C per 25 ml tube. After the desired culture period, the shoots were removed from the medium, washed extensively under running tap water, cut into sections, weighed, and placed directly into scintillation vials. The tissue was then crushed in 1.0 ml Soluene-350 (Packard Instr. Co. Inc.) allowed to digest for 3 days, after which 10.0 ml Dimilune-30 (prepared cocktail by Packard Inst. Co. Inc.) was added to the vial and the samples counted in a Packard 3330 scintillation counter.

Results

Axillary shoots cultured on coumarin-amended medium tuberize readily, exhibit little or no elongated growth and produce large numbers of short, thickened roots. The roots produced by the axillary shoots were non-branched, 1.0-1.5 mm in diameter and an average of 12 mm long. In contrast, shoots cultured on basal medium only did not tuberize, demonstrated continuous vegetative elongation, and produced highly branched roots which were approximately 0.1 mm in diameter and in excess of 100 mm in length.

Data on the effects of sucrose concentration, pH and temperature on the coumarin-induced tuberization are given in Table 1. A sucrose concentration range of 6 to 8 percent was optimum, whereas below 4 percent no tuberization was effected and a 10 percent sucrose concentration significantly delayed tuber initiation. Root growth was similarly affected by the sucrose showing no growth below 4 percent and inhibition of growth at a 10 percent sucrose concentration. Tuberization and root growth of the axillary shoots were not affected over a pH range of 4.5 to 7.5. A pH of 8.5 reduced tuberization and a pH of 9.5 completely inhibited tuber formation. Root growth was also reduced in the medium adjusted at a higher pH. Incubation temperatures below 15 and above 30 C were inhibitory to the coumarin-induced tuberization processes. However, at 30 C the coumarin effect on root growth was not inhibited.

Axillary shoots were pre-incubated on coumarin-amended medium and then transferred to basal medium only at daily intervals up to day 12. Shoots which were pre-incubated on coumarin medium up to 9 days and then transferred to basal medium only did not tuberize (Fig. 1). Shoots cultured on coumarin medium after 11 days and then transferred to a basal medium tuberized 100 percent after 30 days in culture.

In a previous report (66) we demonstrated that tuberization of axillary shoots could also be effected by the plant growth regulators IAA and Naphthalene Acetic Acid (NAA). In contrast to coumarin-induced tuberization, the percent tuberization produced by either IAA or NAA rarely ex-

TABLE 1. — *Effect of sucrose concentration, pH, and temperature on coumarin-induced tuberization and root growth of excised axillary shoots (obtained from etiolated sprouts) of* Solanum tuberosum *cultured* in vitro.

	Percent Tuberization		
	Days in culture		Average root
Sucrose Conc. (%)	15	30	length (mm)
0	0	0	0
2	0	0	
4	50	80	12
6	50	100	12
8	50	100	12
10	10	100	5
pH			
4.5	0	100	12
5.5	10	100	12
6.5	30	100	12
7.5	50	100	12
8.5	10	50	5
9.5	0	0	5
temperature			
15	0	10	0
20	50	100	12
25	40	100	12
30	0	0	12

ceeded 50 percent and never reached 100 percent. The coumarin-amended medium was supplemented with either NAA or IAA to observe the interaction on tuber initiation as compared to the effect on tuber initiation resulting from the respective individual plant growth regulators. A combination of coumarin-IAA enhanced tuber initiation over IAA only, at low IAA concentrations and inhibited the process at high IAA concentrations (Table 2). In contrast a combination of coumarin-NAA essentially inhibited tuber initiation as compared to tuber initiation resulting from either coumarin or NAA individually (Table 2).

The tuberization processes induced by coumarin on the axillary shoots were inhibited by abscisic acid (ABA) and Gibberellic acid (GA) (Table 3). The plant growth inhibitor ABA totally inhibited coumarin-induced tuberization 5.0 mg/1, and the plant growth stimulant GA inhibited tuberization at 2.5 mg/1.

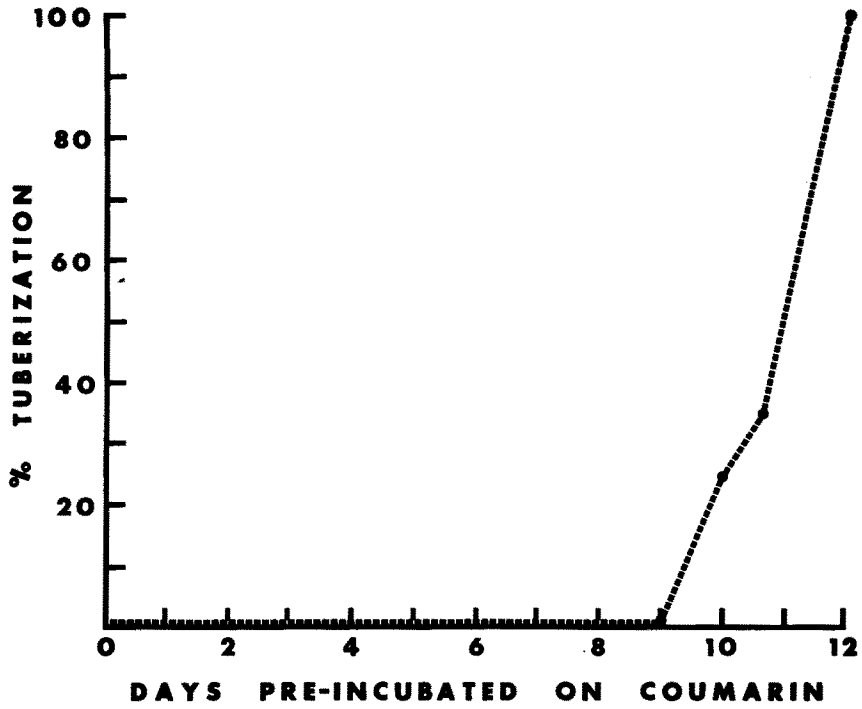

FIG. 1. Effect of time of pre-incubation on coumarin medium on the subsequent tuber formation of excised axillary shoots (obtained from potato sprouts) when cultured on the basal medium only.

Axillary shoots excised from sprouts grown from physiologically younger tubers tuberized more readily than did shoots obtained from tubers which had been stored at 1.7 C for 12 months (Table 4). Tuberization of shoots from young tubers averaged 70 percent in 15 days as compared to 10 percent on shoots obtained from aged tubers.

The effects of GA and high nitrogen concentrations which inhibit coumarin-induced tuberization were studied with respect to their affect on the uptake of coumarin-3^{14}C by axillary potato shoots (Table 5). Results presented in Table 5 are the averages of two experiments in which coumarin-3^{14}C was added to the basal medium which contained 25 mg/l of cold coumarin. The data represent the specific activity of the axillary shoot. Gibberellic acid and high nitrogen concentrations inhibited the uptake of coumarin after 14 days in culture. High nitrogen was more effective in reducing coumarin-3^{14}C than was GA; however, both treatments totally inhibited tuber initiation.

Results not described here showed that coumarin-3^{14}C was taken up at steady rate throughout 30 studies of tuberization. The greatest accumulation of coumarin-3^{14}C occurred in the roots, with the accumulation decreasing

TABLE 2. — *Effect of NAA and IAA on coumarin-induced tuberization of excised axillary shoots (obtained from etiolated sprouts) of* Solanum tuberosum *cultured* in vitro.

Percent Tuberization				Percent Tuberization			
mg/l		Days in culture		mg/l		Days in Culture	
Coumarin	IAA	15	30	Coumarin	NAA	15	30
25	-	40	100	25	-	50	100
25	0.5	40	100	25	0.5	0	20
25	1.0	50	100	25	1.0	0	0
25	5.0	0	80	25	5.0	0	0
25	10.0	0	10	25	10.0	0	0
25	25.0	0	0	25	25.0	0	0
-	0.5	0	50	-	0.5	10	70
-	1.0	0	40	-	1.0	10	60
-	5.0	0	30	-	5.0	0	60
-	10.0	0	30	-	10.0	0	20
-	25.0	0	30	-	25.0	0	20

TABLE 3. — *Effect of abscisic acid (ABA) and gibberellic acid (GA) on coumarin-induced tuberization or excised axillary shoots, (obtained from etiolated sprouts) of* Solanum tuberosum *cultured* in vitro.

mg/l ABA	% Tuberization (30 days)	Average Root Length mm
1.0	10	5
5.0	0	2
mg/l GA		
0.01	100	12
0.1	20	12
1.0	10	12
2.5	0	12

TABLE 4. — *Effect of the age of the mother tuber used as a source of the etiolated sprout on the coumarin-induced tuberization of the excised axillary shoot cultured* in vitro.

Tuber Age (months in storage)	% Tuberization days in culture	
	15	30
12	10	100
3	70	100

TABLE 5. — *Effect of Gibberellic (GA) and nitrogen on the uptake of coumarin-3^{14}C by excised axillary shoots of potato sprouts when cultured* in vitro.

Days in culture	Specific Activity of Coumarin-3^{14}C		
	Coumarin	Coumarin + GA[1]	Coumarin + high nitrogen[2]
3	21,000	23,000	23,500
7	45,000	56,000	45,000
10	34,000	33,000	31,000
14 tuber 5,700	79,000	45,000	40,000
21 tuber 7,200	225,000	112,000	80,000
30 tuber 7,800	267,000	178,000	83,600

[1]GA used at 2.5 mg/1.

[2]Nitrogen concentration was increased to 62.5 mM N/1 by the addition of NH_2NO_3. Basal medium contained 2.5 mM N/1.

toward the shoot tip. Significant coumarin-3^{14}C was always noted in all developing tubers.

Coumarin was not metabolized by the shoots during the 30 day tuberization studies. Shoot and tuber tissues were extracted in 95 percent ethyl alcohol and chromatogrammed on silica gel G in cyclohexane/ethylacetate (1:1 v/v). The tissue extracts were co-chromatographed with cold coumarin and the plates scraped in 10 mm sections, placed in scintillation vials and counted on a Packard 3330 counter. A single radioactivity zone identical to the Rf of coumarin was read indicating that coumarin is not degraded or substituted during the tuberization processes.

Discussion

Coumarin, cis-o-coumarinic acid lactone readily stimulates tuberization of excised axillary shoots of potato sprouts cultured *in vitro*.

Histological data by Reeve *et al.* (54) indicate that tuber initiation and growth are the result of cell enlargement and shifts in the planes of cell division. Possible support for the role and explanation of coumarin-induced tuberization can be suggested by pulling together diverse data on the effects of coumarin in other plant systems.

Cell Division

The direction of cell division (longitudinal or lateral) is considered to be in part due to the endogenous sulphydral, disulfide (SH/SS) ratio within the cell. It is thought that the SH/SS ratio affects the microtubules which make up the spindle fibers, causing a reorientation of the chromosomes, and also affects the orientation of the cellulose microfibrils, the components of the cell wall structure. Data by Svensson (68) suggest that the

coumarin effect on wheat and corn roots with respect to cell volume and polarity of cell division may be mediated through the influence of coumarin on the SH/SS ratio within the cell. In a subsequent paper, Svensson (69) suggests that this effect is a direct influence of coumarin, and not due to coumarin influences on other endogenous plant growth regulators. Earlier work by Thimann and Bonner (71) suggests that the inhibitory action of coumarin on oat coleoptiles is by means of the coumarin reacting with a sulfhydryl enzyme.

Cell Enlargement

Supporting data for coumarin possibly affecting cell enlargement during tuberization may be considered from the data by Harada *et al.*, Ron and Mayer, and Sharma and Chaudhuri (18, 56, 59), which suggest that coumarin may directly affect cell volume and the viscosity of the cellular sap of individual plant cells. The effects of coumarin on cell volume may be explained in part by possible inhibition of cellulose synthesis as described by Hogetsu *et al.* (22).

Ethylene

Experiments by Garcia-Torres (15) and in our laboratory showed that the ethylene releasing compound Ethrel, (2-chlorethyl) phosphonic acid demonstrated a low degree of tuberization stimulation. An interaction between coumarin and ethylene is presented in data by Morgan and Powell (40) which indicated a coumarin-induced increase in ethylene synthesis in etiolated bean segments. Data supporting a possible role of ethylene in tuber initiation can be taken from the papers by Apelbaum and Burg, Nee *et al.*, and Ridge (1, 41, 55) who demonstrate that in ethylene-treated pea stems, there is an effect to reorient the microfibrils, thus shifting the plane of cellular division, and also possibly influencing cell wall chemistry. Svensson, however, (69) does not feel that coumarin action is mediated through increase in ethylene synthesis, and in contrast data by Mingo-Castel *et al.*, and Staden and Dimalla (38, 39, 64) showed that ethylene inhibited *in vitro* tuberization of potato stolons and on sprouts of aged tubers under the conditions of their experiments.

β-inhibitor and Abscisic Acid (ABA)

Tissues of plants and fruits, extracted in acidic ether, when separated by chromatography exhibit a zone, Rf 0.6-0.85 which is characterized by having a strong inhibiting effect on the standard coleoptile growth test. This zone was originally termed the β-inhibitor zone by Bennet Clark and Kefford (4). Subsequent research by Milborrow (37) resulted in the identification of ABA from the β-inhibitor complex in numerous plants including potato tubers. Data by Okazawa (45) showed that potato plants under induced conditions have higher inhibitor and lower GA levels, while in plants

grown under non-induced conditions, just the opposite is true. Most authors agree that tuberization is the result of a critical GA/inhibitor ratio, possibly effected to some degree by an auxin. This feeling stimulated numerous papers dealing with the effects of the growth inhibitor ABA on tuberization in potatoes, since it was a component identified as a natural inhibitor found in β-inhibitor complex. Applications of ABA to intact potato plants and stolon tips have been reported to stimulate tuberization El-Antably *et al.*, Kraus and Marschner (12, 30). Conversely, data by Claver, Palmer and Smith, Smith and Rappaport, (9, 47, 61) and in the present paper indicate that ABA did not stimulate tuber initiation of stolons, or sprouts and inhibited both kinetin and coumarin-induced tuberization. Okazawa (45) indicates support for a component of the β-inhibitors complex as being the inhibitor involved in tuberization, and Krauss (29) feels that a ABA/GA may play a role in tuberization. From the data to date we would tend to agree with Claver (9) in his assessment that ABA is not the inhibitor responsible for tuberization. We do feel, however, that there is room for the consideration of a β-inhibitor component to be responsible for tuber induction, namely coumarin.

It is pertinent to mention that while coumarin has not been identified as a component of the β-inhibitor complex isolated from potatoes Hemberg (20), Housley and Taylor (23) present data indicating the presence of the coumarin scopoletin in the potato β-inhibitor complex. Coumarin and the coumarin precursors cinnamic acid and o-coumaric acid have been identified as components of the β-inhibitor isolated from fruits by Varga (78). Blumenthal-Goldschmidt and Rappaport (6) do not believe coumarin to be the active component of the β-inhibitor complex responsible for the bud rest in potatoes. However, there remains the possibility for the existence of coumarin in the β-inhibitor complex in potato tubers and a role in the Gibberellín/inhibitor tuberization phenomenon since numerous active components have not yet been identified, Holst (21).

Auxin-like Role of Coumarin

It is often suggested in papers on tuberization that while the process of tuber initiation is not a direct response of an unknown auxin, the initiation is somehow mediated via auxin activity.

Coumarin effects on plant growth prior to 1961 as reviewed by Mayer and Poljakoff (36) indicate that coumarin can both stimulate or inhibit plant growth processes. Neumann (42) suggests that coumarin be considered an auxin. Studies by Neumann (42, 43) and Knypl (27) show that coumarin can stimulate sunflower, bean, pea segments, and oat coleoptiles similar to IAA; however, it appears that the mode of action is distinctly different. Jansson and Svensson (24) studying *in vitro* growth of soybean hypocotyls showed that coumarin induced root growth and stimulated an increase in fresh weight of the explant.

Coumarin in Potato Tubers

To date we have only tentatively identified coumarin from extracted tuber tissues. Ethanol extractions of tissues from developing tubers (5-10 mm dia) were separated by thin layer chromatography. Chromatographs were spotted with authentic coumarin and sprayed with base to observe coumarin fluorescence. A yellow fluorescing spot identical to the R_f of coumarin was observed. Unpublished results of Austin and Clarke (2) also suggest the presence of coumarin in potato tubers.

Comparison to Kinetin-induced Tuberization

One of several objectives in the present study was to compare coumarin-induced tuberization with that of the kinetin-induced tuberization reported by Palmer and Smith, and Smith and Palmer (46, 62). Results show that a minimum concentration of at least 4 percent sucrose is required for either coumarin or kinetin to effect tuber initiation, while temperatures of 15 C or above 30 C are inhibitory (Table 1). Data by Palmer and Smith (48) indicate that isolated stolons preincubated on kinetin-amended medium for a 3-4 day period will initiate tubers if then transferred to a medium without kinetin. The data presented in this study show that the stolons must be continuously incubated in a coumarin medium for at least 11-12 days to effect the tuberization response (Fig. 1). Since coumarin has an internal lactone ring structure which opens under alkaline conditions, we incubated the shoots in coumarin-amended medium over a wide pH range. Results showed that coumarin-induced tuberization was inhibited if the shoots were cultured in a medium buffered at pH 8.5 or higher (Table 1). Applications of kinetin-8-14C as described by Smith and Palmer, resulted in the accumulation of labeled kinetin at the stolon tip prior to and during tuber initiation, results also indicated that the kinetin was metabolized or modified during uptake by the stolon. Incorporation of coumarin-3-14C into the culture medium resulted in a continuous and increasing uptake during the 30 day study (Table 5). Labeled coumarin also readily accumulated in the developing tubers. Extraction and subsequent thin lay chromatograph showed that the coumarin molecule remains intact and unchanged throughout the 30 day study.

Perhaps the most striking difference between kinetin and coumarin-induced tuberization *in vitro* is the fact that while high nitrogen concentrations do not affect tuberization induced by kinetin it totally inhibits coumarin-induced tuberization, Stallknecht (67).

Addition of either high nitrogen concentrations or GA to the medium significantly reduced the uptake of labeled coumarin after 10 days in culture (Table 5). These results support the data for a need of continuous uptake of coumarin for at least 11-14 days as shown (Fig. 1).

General Discussion

Results on the effects of various compounds on coumarin-induced tuberization showed that kinetin slightly stimulated, whereas 2,4-Dichlorophenoxy acetic acid, (2,4-D), 2-choroethyl-trimethylammonium chloride (CCC), Succinic Acid-2, 2 dimethyl hydrazine (ALAR), Triiodobenzoic acid (TIBA) had no effect, with the exception to TIBA which resulted in loss of root polarity of the shoot.

During the course of our tuberization studies we investigated over thirty substituted coumarins, phenolic acids, and growth regulators for evaluation of tuber initiation stimulus. Tuberization was stimulated by application of Herniarin (7-methoxy-coumarin), o-coumaric acid, Ethrel, IAA, NAA, 2,4-D, and ancymodol [α-cyclopropyl-α-(p-methoxyphenyl)] 5-pyrimidine-methanol, (AREST).* No root stimulation or growth was promoted on the shoot by any of the compounds listed above; however, IAA, NAA, and 2,4-D did stimulate callus development at the base of the shoot.

Applications of compounds such as kinetin, NAA, IAA, 2,4-D, CCC, p-coumarin acid, ferulic acid, caffeic acid, maleic acid hydrazide, TIBA, and Ethrel have been reported to stimulate *in vitro* tuberization of potato stems, sprouts, or stolons (45, 47, 49, 50, 74). At present it is not known whether the chemicals affect tuberization processes directly acting as a stimulus, or by influencing an endogenous gibberellin concentration within the plant. A review of data and hypotheses by numerous authors can be summarized by stating that tuber induction is stimulated by short days, cool nights, low nitrogen and adequate carbohydrate nutrition, and a delicate balance between a graft transmissable stimulus and endogenous gibberellin levels (7, 11, 13, 14, 16, 17, 19, 31, 32, 33, 34, 35, 39, 44, 51, 52, 53, 57, 72, 73, 75, 78, 79).

Summary

If indeed coumarin acts as an anti-gibberellin agent as suggested by Berrie *et al.* (4) then considering that most authors agree that tuberization is the result of a Gibberellin/inhibitor ratio it is tempting to speculate that coumarin be considered a component of the inhibitor fraction. Supportive of the possible role of the involvement of coumarin in the tuberization processes are the following:

1. Tentative identification of coumarin in tuber tissues
2. The effects of coumarin on cell wall plasticity and enlargement
3. The effect of coumarin on the reorientation of the plane of cell division
4. The effect of high nitrogen and GA on the translocation of coumarin within the axillary shoot cultured *in vitro*.
5. The presence of coumarin in the β-inhibitor complex

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6. The auxin-like activity of coumarin

While one cannot emphatically state that coumarin is involved in the tuberization processes, results of data presented in the present paper and by authors studying effects of coumarin on other plant processes allow us to suggest a possible role for coumarin in the tuberization processes of the potato *Solanum tuberosum.*

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