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INHIBITION OF SPINACH CHLOROPLAST PHOTOPHOSPHORYLATION AND ELECTRON TRANSPORT BY SELECTED NATURAL PRODUCTS

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ABSTRACT

The effects of the biflavonoid crassifolin (1), the flavonoid tephrobotin, the Annnonaceous acetogenins squamocin (3), and buliatacin (4) were investigated on different photosynthetic activities in isolated spinach chloroplasts. The results indicated that compounds 1-4 inhibited both ATP synthesis and uncoupled electron transport. In addition, squamocin (3), and bullatacin (4) enhanced basal electron flow and light-activated Mg^{2+} -ATPase. Therefore, acetogenins 3, and 4 behave as uncouplers and Hill reaction inhibitors. Compounds 1, and 2 inhibited basal electron flow and did not affect light-activated Mg^{2+} -ATPase. All the compounds induced a concentration-dependent inhibition of photophosphorylation. Natural products 1-4 did not affect photosystem I (PSI) activity but they inhibited photosystem II (PSII) electron flow. The study of the partial PSII reactions from H_2O to $DCPIP_{ox}$, H_2O to SiMo and diphenylcarbazide DPC to DCPIP established that the site of inhibition was at the oxygen-evolving complex (OEC).

INTRODUCTION

A wide range of chemicals is known to inhibit electron transport process of photosystem II (PSII). Many of these chemicals have become important commercial herbicides. The mechanism of action of many herbicides inhibiting photosynthesis is the blockage of photosystem II (PSII) electron transport by binding to the second stable electron acceptor site (Q_B) at the D1 protein (Pfister and Schreiber 1983; Diner and Petrouleas 1987). This type of inhibition is characteristics of DCMU (diuron) and other herbicides such as *s*-triazines, phenylureas, triazinones, ureas, uracils, biscarbamates, and pyridazinones, often termed as classical diuron-type herbicides (Oettmeier 1992).

The flavonoids are an integral part of the plant kingdom, present in all photosynthesizing cells. Their different biological activities, including antioxidant, antimicrobial, and mutagenic properties, make them interesting object of research (Middleton and Kandaswami 1993). To our knowledge, the effects of biflavonoid crassifolin and flavonoid tephrobotin on photosynthesis have not been investigated.

Annonaceous acetogenins form a wide group of more than 320 natural products that are found only in the plant family Annonaceae. Some of them offer exciting potential for the development of new antitumor and insecticidal agents due to their ability to inhibit complex I (NADH: ubiquinone oxidoreductase) in mammalian and insect mitochondrial electron transport systems (Alali *et al.*1999; Zafra-Polo *et al.*1998). The important insecticidal properties of the Annonaceous acetogeninus have led to the proposal that crude extracts of several Annonaceous species containing a variety of acetogenins could be employed as safe, effective, economical and

environmentally friendly pesticides. The emetic effect induced by these extract in animals is a definite safety factor should someone ingest these pesticidal materials either intentionally or unintentionally (McLaughlin *et al.* 1997). In spite of these considerations, the effect of acetogenins on plant energetic metabolism has not been previously investigated.

Therefore, the objective of this research was to describe the effect of four naturally occurring compounds, crassifolin , tephrobotin, obtained from *Tephrosia crassifolin* and *Tephrosia abbottiae*, squamocin and bullatacin obtained from *Annona purpurea* respectively on several photosynthetic activities in isolated spinach chloroplasts.

MATERIALS AND METHODS

Chemicals

Crassifolin (1), tephrobotin (2), squamocin (3) and bullatacin (4) were provided by Professor J. W. Lewis, Royal Holloway, University of London. Solutions of these compounds were dissolved in absolute ethanol. The final concentration of ethanol was less than 1%, which did not affect the electron transport and ATP synthesis in chloroplast. All biochemicals used in the study were purchased from Sigma Chemical Company, St. Louis, Missouri.

Chloroplast Isolation and Chlorophyll Determination

Intact chloroplasts were prepared from market spinach leaves *Spinacea oleracea L.* by homogenization and differential centrifugation as described earlier (Macias *et al.* 1999) and suspended in the following medium: 400mM sorbitol, 5mM $MgCl_2$, 10mM KCl, and 0.03 M KOH-tricine at pH 8.0. They were stored as a concentrated suspension in the dark for 1 h at 4°C. Intact chloroplasts were efficiently lysed to yield free thylakoids prior to each experiment by incubating them in the following

electron transport medium: 100mM sorbitol, 10mM KCl, 5mM $MgCl_2$, 0.5mM KCN and 30mM Tricine buffer (pH 8.0 with the addition of

KOH). The chlorophyll (Chl) concentration was measured spectrophotometrically according to Strain *et al.* 1971.

Measurement of Electron Transport and ATP Synthesis

Adenosine 5'-triphosphate (ATP) synthesis was measured as the pH rose from 8.0 to 8.1 with an Orion Mod. 8103 rose microelectrode connected to a corning Model 12 pH meter with expanded scale and registered in a Gilson recorder as reported by Dilley 1972. The reaction medium contained 100mM sorbitol, 10mM KCl, 5mM $MgCl_2$, 0.5mM KCN, 50 µM methylviologen (MV), 1mM KOH-tricine, pH 8.0, 1mM ADP, and 3mM KH_2PO_4 (Calera *et al.* 1995), with a suspension of thylakoids (20 µg of chlorophyll/ml).

Photosynthetic non-cyclic electron transport activity from water to MV was determined with a YSI (Yellow Spring Instrument) Model 5300 oxygen monitor and a Clark-type electrode. The basal electron transport reaction medium was the same as in the ATP synthesis assay, except for the tricine concentration (15mM) and the absence of ADP and KH_2PO_4. For the uncoupled electron transport measurement, NH_4Cl (6mM) was added to the basal electron transport medium. For the phosphorylating electron transport medium 1mM ADP and 3mM KH_2PO_4 were added to the basal electron

transport medium. All reaction mixtures were illuminated for 1 min as described by Macias *et al.* 1999.

Determination of photosystems (PS) I and II electron transport rate

Photosystem I (PSI) electron transport was determined in a similar form to non-cyclic electron transport. The following reagents were added: 10 µM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], 100 µM DCPIP (dichlorophenolindophenol), 50 µM MV, 300 µM ascorbate, and 6mM NH_4 Cl (Macias *et al.* 1999). Throughout uncoupled PSII electron flow 1µM DBMIB (2,4-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone), 50 µM DCPIP/300 µM $K_3[Fe(CN)_6]$ and 6mM NH_4Cl were added to the basal medium but without MV. Partial reactions of uncoupled PSII were measured as follows: (1) from water to silicomolybdate (SiMo) with the same medium (plus 200 µM SiMo and 10 µM DCMU) and the same procedure as for PSII in the absence of DCPIP/300 µM $K_3[Fe(CN)_6]$ as described by Allen and Holmes 1986; (2) from DPC (200 µM diphenyl carbazide) to DCPIP (100 µM) using treated Tris-washed (0.8 M) chloroplasts. The last partial reaction was measure spectrophotometrically as previously reported by Vernon and Shaw 1969.

Mg^{2+}-ATPase activity assays

Mg^{2+}-ATPase activity bound to thylakoid membranes was measured according to Mills *et al.*1980. The amount of released inorganic phosphate was determined as previously described by Taussky and Shorr 1953.

Results are presented as means ±SE. I_{50} values for each activity were determined by linear regression of mean values.

RESULTS AND DISCUSSION

Effects of crassifolin (1), tephrobotin (2), squamocin (3) and bullatacin (4) on photosynthetic activities

Compounds 1-4 inhibited ATP synthesis on freshly lysed chloroplasts isolated from spinach leaves (Table 1). The calculated I_{50} value (concentration required for 50% inhibition) were >60, 32, 42, and 47 µM, respectively.

Table(1): Inhibitory effect of crassifolin, tephrobotin, squamocin, and bullatacin on photophosphorylation from water to methylviologen.

	Activity (%)						
	Compound Conc. (µM)						
Tested Compounds	0	10	20	30	40	50	60
Crassifolin	100±2	98±2	84±4	73±3	64±3	60±5	58±2
Tephrobotin	100±3	83±3	66±2	49±4	35±2	21±3	20±2
Squamocin	100±5	96±2	68±4	56±3	50±2	46±3	46±2
Bullatacin	100±2	94±2	88±3	77±5	61±4	48±2	46±3

Note. Details of the experiments are described under Materials and Methods. Control value rates were 294 ± 2, 292 ±3, 290 ± 5, and 291 ± 2 µmol of ATP h^{-1} mg of Chl^{-1} for crassifolin, tephrobotin, squamocin, and bullatacin repectively. Each point represents the mean of five determinations.

To elucidate the mechanism of action of compounds 1-4 on photosynthesis, their effect on electron transport (basal, phosphorylating, and uncoupled) was investigated. Squamocin (3) and bullatacin (4) enhanced basal electron flow from water to MV at 60 µM by 125, and 135% respectively (Table 2). These results suggest that the compounds 3, and 4 act as uncouplers. However, crassifolin (1) and tephrobotin (2) inhibited basal electron flow in a concentration dependent manner (Table 2, 42, and 55%, 60 µM). Compounds 1-4 (Table 3) inhibited uncoupled electron transport from water to MV in spinach thylakoids at the concentration of 60 µM by 96, 67, 52, and 54% respectively. These results indicate that compounds 1, 2, 3, and 4 act as Hill reaction inhibitors. Moreover, compounds 1, 2, 3, and 4 inhibited phosphorylating electron flow (Table 4) at the concentration of 60 µM by 60, 95, 27, and 12% respectively.

Table(2): Effects of crassifolin, tephrobotin, squamocin, and bullatacin on basal electron transport from water to methylviologen.

	Activity (%)						
	Compound Conc. (µM)						
Tested Compounds	0	10	20	30	40	50	60
Crassifolin	100±3	95±2	83±3	78±2	70±3	60±2	58±3
Tephrobotin	100±2	75±2	57±3	45±2	45±4	45±3	45±3
Squamocin	100±2	105±3	110±2	115±3	120±2	124±3	125±2
Bullatacin	100±2	106±2	112±3	118±2	122±3	130±2	135±2

Note. Details of the experiments are described under Materials and Methods. Control value rates were 292.4 ±3, 295 ±2, 294 ±2, and 296.8 ± 3 µmol O_2 mg^{-1} Chl $h^{-1}$for crassifolin, tephrobotin, squamocin, and bullatacin repectively. Each point represents the mean of five determinations.

Table(3): Effects of crassifolin, tephrobotin, squamocin, and bullatacin on uncoupled electron transport from water to methylviologen.

	Activity (%)						
	Compound Conc. (µM)						
Tested Compounds	0	10	20	30	40	50	60
Crassifolin	100±2	96±3	77±3	46±5	20±3	6±2	4±2
Tephrobotin	100±2	75±4	60±4	52±3	43±2	36±4	33±3
Squamocin	100±3	93±3	88±2	82±2	67±4	54±2	48±3
Bullatacin	100±2	92±3	86±2	79±4	65±2	52±3	46±2

Note. Details of the experiments are described under Materials and Methods. Control value rates were 339 ±2, 335 ±2, 340 ±3, and 334 ±2 µmol O_2 mg^{-1} Chl h^{-1} for crassifolin, tephrobotin, squamocin, and bullatacin repectively. Each point represents the mean of five determinations.

To localize the target of inhibition of compounds 1, 2, 3, and 4 on the electron transport chain, their effect on partial reactions (PSI and PSII) was measured using artificial electron donors and electron acceptors as well as appropriate inhibitors (Allen and Holmes 1986). The uncoupled PSI electron transport from $DCPIP_{red}$ to MV was not affected. On the other hand, compounds 1-4 inhibited the uncoupled PSII electron transport from water to $DCPIP_{ox}$ (83, 81, 90, and 28% respectively, at 60 µM) and from water to SiMo (32, 70, 63, and 30%, respectively, at 60 µM) (Table 5). The uncoupled

electron flow was not affected from DPC to $DCPIP_{ox}$ in treated thylakoids. Altogether, the results indicate that the target of the compounds 1-4 was located at the oxygen evolving complex (OEC), in the donor side of PSII. However, uncoupled electron transport from water to DCPIP in the presence of DPC is inhibited by compounds 1-4, because the interacting site of DPC is not available from the intact thylakoids as found by Vernon and Shaw 1969.

Table (4): Effects of crassifolin, tephrobotin, squamocin, and bullatacin on phosphorylating electron transport from water to methylviologen.

	Activity (%)						
	Compound Conc. (μM)						
Tested Compounds	0	10	20	30	40	50	60
Crassifolin	100±3	98±3	96±2	61±4	48±3	40±3	40±3
Tephrobotin	100±3	31±2	15±4	7±3	6±2	5±2	5±2
Squamocin	100±3	93±3	84±3	78±2	75±4	73±4	73±2
Bullatacin	100±2	95±2	94±2	92±3	90±3	88±2	88±3

Note. Details of the experiments are described under Materials and Methods. Control value rates were 460 ± 6, 456 ± 5, 462 ± 4, and 464 ± 3 μmol O_2 mg^{-1} Chl h^{-1} for crassifolin, tephrobotin, squamocin, and bullatacin respectively. Each point represents the mean of five determinations.

Many flavonoids have been reported to interact with biomembranes, and their effectiveness has been found to be related to their incorporation rate into cells and to their orientation in biomembranes (Thomas *et al.* 1992; Kaneko *et al.* 1994). Flavonoid capacity to modify membrane-dependent processes (such as electron transport in thylakoids) and ability to interact and penetrate lipid bilayers(causing variations in their structure and fluidity) are documented (Saija *et al.*1995; Santos *et al.*1998). In this regard, the ability of compounds crassifolin (1) and tephrobotin (2) to inhibit the electron flow in thylakoids can be understood, but are needed to clarify their molecular mechanism of action. On the other hand, acetogenins, squamocin (3), and bullatacin (4) influence their potency as OEC inhibitors or uncouplers. These results are consistent with those previously reported by McLaughlin *et al.* 1997; Lotin-Hennsen *et al.* 1998.

Mg^{2+}-ATPase activity

Some uncouplers such as tricolorin, NH_4Cl and FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) stimulate the activity of the Mg^{2+} -ATPase (Achnine *et al.* 1999). However, crassifolin (1) tephrobotin (2), and squamocin (3) at all concentrations tested did not enhance significantly the light dependent Mg^{2+} -ATPase bound to membranes. On the other hand, bullatacin (4) slightly stimulate the enzyme activity by 22 and 38% at 150 and 200 μM, repectively (Table 6). Although, the mild stimulatory effect of bullatacin (4) on the Mg^{2+} -ATPase activity could be related with its uncoupling properties, by interacting with the soluble ATPase (CF_1). The overall results suggest that crassifolin (1) tephrobotin (2), squamocin, and bullatacin (4) act as uncouplers by perturbing the thylakoid membranes.

Table(5): Effects of crassifolin, tephrobotin, squamocin, and bullatacin on uncoupled PSII electron transport from water to DCPIP and from water to SiMo.

Concentration	Inhibition%	
	H_2O to DCPIP[a]	H_2O to SiMo[b]
Crassifolin		
0 µM	0	0
10 µM	22 ± 2	7 ± 0.2
20 µM	39 ± 3	12 ± 0.3
30 µM	52 ± 4	18 ± 0.6
40 µM	68 ± 2	26 ± 2
50 µM	80 ± 4	29 ± 3
60 µM	83 ± 5	32 ± 2
Tephrobotin		
0 µM	0	0
10 µM	19 ± 0.5	25 ± 2
20 µM	23 ± 2	37 ± 3
30 µM	34 ± 3	60 ± 4
40 µM	62 ± 6	65 ± 4
50 µM	79 ± 4	70 ± 5
60 µM	81 ± 2	70 ± 4
Squamocin		
0 µM	0	0
10 µM	13 ± 0.2	8 ± 0.2
20 µM	27 ± 2	15 ± 0.3
30 µM	54 ± 4	40 ± 3
40 µM	81 ± 6	55 ± 3
50 µM	87 ± 3	61 ± 2
60 µM	90 ± 4	63 ± 2
Bullatacin		
0 µM	0	0
10 µM	8 ± 0.3	9 ± 0.2
20 µM	14 ± 0.2	11 ± 0.3
30 µM	19 ± 0.4	18 ± 0.8
40 µM	21 ± 0.8	20 ± 0.6
50 uM	25 ± 2	26 ± 2
60 µM	28 ± 2	30 ± 4

Note. Details of the experiments are described under Materials and Methods. [a]Control values for the electron flow were 388.5 ± 4, 374 ± 5, 382 ± 2, and 377.8 ± 3 µmol O_2 mg^{-1} Chl h^{-1}, respectively. [b]Control values for the electron flow were 327.5 ± 2, 326 ± 3, 326.5 ± 2, and 326 ± 3 µmol O_2 mg^{-1} Chl h^{-1}, respectively. Each point represents the mean of five determinations.

Table (6): Effects of crassifolin, tephrobotin, squamocin, and bullatacin on Mg^{2+} -ATPase Activity.

	Activity (%)						
	Compound Conc. (µM)						
Tested Compounds	0	10	20	30	40	50	60
Crassifolin	100±2	99±2	98±3	99±2	99±3	97±2	99±3
Tephrobotin	100±3	98±2	99±2	99±3	99±2	96±2	99±2
Squamocin	100±3	100±3	107±3	109±2	111±4	116±4	117±2
Bullatacin	100±2	102±2	108±2	110±3	118±3	122±2	138±3

Note. Details of the experiments are described under Materials and Methods. Control values for the rate of ATP hydrolysis by Mg^{2+} -ATPase were 370 ± 2, 376 ± 4, 359 ± 5, and 366 ± 3 µmol P_i mg^{-1} Chl h^{-1} , respectively. Each point represents the mean of five determinations.

The results presented here indicate that possible interference of flavonoids and Annonaceous acetogenins with the photosynthetic processes may occur in the plant cell. Because the OEC is unique to plant chloroplasts and cyanobacteria, flavonoids and Annonaceous acetogenins represent good candidates for the development of new specific, biodegradable, and environmentally safe herbicides.

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تثبيط عملية الفسفرة الضوئية والأنتقال الألكترونى فـــى كلوروبلاســتيدات أوراق نبات السبانخ بواسطة بعض المركبات المستخلصة من مصادر طبيعية.

شفيقة أحمد الكسبانى

المعمل المركزى للمبيدات – مركز البحوث الزراعية – الصبحية – الاسكندرية

تم دراسة تأثير أربعة من المركبات المستخلصة من مـصادر طبيعيـة وهـى كراسـيفولين مـن البيوفلافيونويدات وتفروبوتين من الفلافونويدات وكل من سكواموسين وبيولاتاسين من الأسيتوجينينات علـى بعض التفاعلات الضوئية المختلفة فى سلسلة الانتقال الالكترونى فى الكلوروبلاستيدات المعزولة مـن اوراق نبات السبانخ.

وقد أظهرت النتائج أن المركبات الأربعة تثبط عملية تخليق الأدينـوزين ثالـث الفوسـفات وتمنـع البروتونات الناتجة من أكسدة مواد التفاعل فى سلسلة الانتقال الالكترونى من الوصول الى أنزيم الأدينـوزين ثالث الفوسفاتيز. وقد وجد ان كل من سكواموسين وبيولاتاسين تؤدى الى زيادة تدفق الألكترونات الأساسـية ونشاط انزيم الأدينوزين ثالث الفوسفاتيز (الماغنسيوم)، مما يثبت أن هذة المركبات تعمل كمثبطـات لعمليـة تخليق الأدينوزين ثالث الفوسفات ولتفاعل هيل. ولم يحدث تأثير لهذة التفـاعلات بواسـطة كراسـيفولين و تفروبوتين.

وقد وجد ان المركبات الأربعة تثبط عملية الفسفرة الضوئية ولاتؤثر على النظـام الـضوئى الأول ولكنها تثبط تفاعلات النظام الضوئى الثانى.

ومن دراسة التفاعلات الضوئية يمكن اعتبار تفاعلات النظام الضوئى الثـانى هـدف هـام لهـذة المركبات ، وبالتالى من الممكن استخدامها كمبيدات حشائش آمنة.