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EFFECT OF LIGHT AND SALINITY ON GROWTH OF SOYBEAN CALLUS ESTABLISHED FROM ADAPTED AND UNADAPTED STEM CELLS CULTURE

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ABSTRACT

*Calli of soybean (**Glycine max, L. Maple Arrow**) from cell cultures adapted and unadapted to 85 mM NaCl were established under NaCl stress treatments from 0 to 160 mM. Preadapted calli remain green and attained higher fresh weight. Light induced an increase of 45% in fresh weight of preadapted and nothing in unadapted calli. NaCl decreased growth (represented as fresh weights) with more or less equal rates in preadapted and unadapted calli incubated in dark or light conditions.*

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4 dichlorophenoxyacitic acid; IAA, indol 3-acetic acid; IBA, indolebutyric acid

Key words: *Glycine max* soybean, cell culture, tissue culture, NaCl salinity, light intensity.

INTRODUCTION

Attempts to regenerate intact soybean plants from tissue culture have been unsuccessful until relatively recently

(Lazzeri *et al.*, 1985; Ranch *et al.*, 1985; Barwale *et al.*, 1986). But during the last decade, tissue culture have been recognized as a powerful tool for physiological research including studies on the mechanisms of stress tolerance. Cells and tissues cultured in vitro have been used to study mechanisms of salinity tolerance in halophytes (Smith and McComb 1981) and in nonhalophytes (Orton 1980 and Elhaak and Migahid 1989). Moreover they offers advantages for use in selection of salt tolerant lines (Croughan *et al* ., 1978 & 1981; Zenk 1974; Dix and Street 1975). The present work was done to study the response of soybean calli which were produced from adapted and unadapted cell cultures to salinity and light.

MATERIALS AND METHODS

A cell suspension of soybean (*Glycine mam,* L. Maple Arrow) was established by M. McElwee from callus culture of stem tissues and grown on Gamborg B5 salt medium (Gamborg *et al* ., 1968) with addition of 3.87 g sucrose, 30 g IAA, 0.75 mg kinetin, 1 mg, 2,4-D 5-50 uM per liter and different concentrations of NaCl (0, 85, 170, 340, 510, and 680 mM). Cells were left in continuous NaCl treated subcultures then cells were collected from control (unadapted) and 85 mM NaCl (adapted), a concentration which enhanced fresh and dry weights of soybean cell suspension (El-Sayed and Kirkwood 1992). Callus cultures were established from these cells on MS media (Murashige and Skoog 1962) supplemented with 1 mg BAP, 4 mg IBA, 20 g sucrose, 10 g agar per liter and growth conditions of 25^{0}C and 3000 lux with 14 h photoperiod. After

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15 days equal portions (1 g fresh weight) of the adapted and unadapted calli were inoculated on the same medium supplemented with 0 to 160 mM NaCl. All treatments were incubated at 25 ± 2^{0}C and under continuous illumination with fluorescent light or in darkness. During the stationary phase the tissue cultures were weight and the main of fresh weights were used.

RESULTS AND DISCUSSION

Soybean calli produced from adapted cell suspension cultures attained about 45% higher fresh weight in light- than in dark-incubated calli under all osmotic stress treatments; while those from unadapted cultures attained slightly higher fresh weightin dark than in light incubated ones (Fig. 1 a&b). Apple (Saad 1968) and black cherry (Caponetti *et al.*, 1971) callus cultures were reported to grow better in dark than in light. In contrary results of Thompson *et al.* (1977) indicated about 10% increase in dry weight of tissue culture of *Glycine max* L. with light. However, NaCl enhanced light response in the adapted callus of soybean. Generally calli from adapted cell cultures could survive and acquire higher fresh weight under the used osmotic stress treatments regardless of light or dark incubation. This may indicate higher cell division activity in calli which presurvived slightly osmotic stress (85 mM NaCl). Enhancement effect of low concentration of salt for callus growth which was found by von Hedenstrom and Breckle (1974) and Smith and Mc Comb (1981) could be concluded for preadapted callus. In this concern, Kato and Nagai (1979) and Stavarek and Rains (1984) calculated

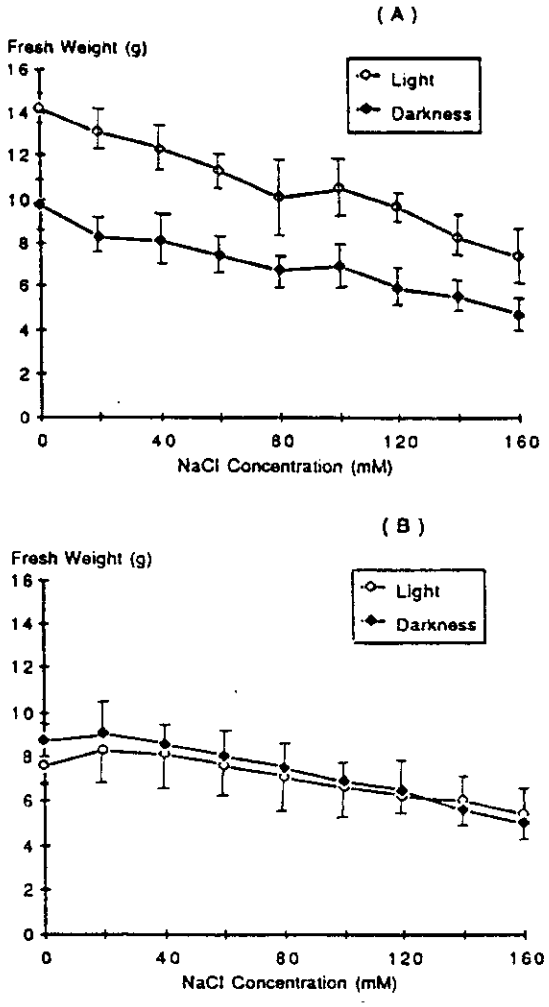

Fig. (1): Influence of light and NaCl concentration on growth in 15 days of callus cultures of soybean (Glycine max L. Maple Arrow) from cells adapted (A) and unadapted (B) to NaCl salinity. Vertical line represent standard deviation of five replicates.

greater dry weight of tissue per weight of sugar consumed (efficiency) for salt-selected than nonselected cells. Further more Croughan *et al.* (1981) referred this salt tolerance to physiological changes connected with ionic transport mechanisms not to genetic changes. However, the growth rates decreased with increasing NaCl concentration in adapted and unadapted calli with more or less equal rates in light and dark- incubated calli. This may indicate that growth inhibition by salt treatments was not through light-induced processes. Therefore, it would possibly by reduced ability for cell wall to expand (Binzel *et al.*, 1985; Iraki *et al.*, 1989) or by restriction of water flow (Boyer *et al.*, 1985). Also the calli from NaCl adapted cells were green in comparison with those from unadapted cells which were yellow. This high level of pigments is perhaps indication of some degree of autotrophy in soybean callus initiated by salt stress. Similar indications of autotrophy in calli were also found by Chong and Taper (1974). The incubation of chlorophyllus calli in light or dark did not affect the rate of decrease in growth by salt stress which confirms that growth inhibition was not through light induced processes. On the other hand, chlorophyll in calli of adapted cells could be the reason for their greater fresh weight especially in light-incubated calli which attained the greatest fresh weights under all osmotic stress treatments. Lee and Strarratt (1972) have shown that green callus of *Euphorbia esula* grow better in light in contrast to poorer growth of non-green callus of *E. cyparissias*. In the present study the reduction in fresh

weights of the calli may be due to expenditure of metabolites for osmoregulation in response to salt stress. In comparison with the work of El-Sayed and Kirkwood (1992), the concentration of NaCl (85 mM) which produce increase in fresh and dry weights of cell suspension cultures, decreased the fresh weight of callus and with greater percentages for adapted callus. This may indicate to greater tolerance for cell over tissue cultures.

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تأثير الضوء والملوحة على نمو كالوس نبات الفول الذى نمى من خلايا من ساق النبات سبق أو لم يسبق معاملتها بمحلول كلوريد الصوديوم

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أخذت خلايا من سيقان نبات فول الصويا متأقلمة أو غير متأقلمة مع ٨٥ ملى مول من كلوريد الصوديوم وتم زراعتها تحت تركيزات من كلوريد الصوديوم من صفر إلى ١٦٠ ملى مول ٠ وقد أثبتت الدراسة أن الأنسجة التى نمت من خلايا تأقلمت مسبقا مع كلوريد الصوديوم كانت أكثر إخضرار وإحتوت على وزن طازج أكبر ٠ وأدى أيضا تعرض هذه الخلايا للضوء إلى زيادة فى الوزن الطازج للكالوس بالمقارنة بالأنسجة التى لم تتأقلم مع كلوريد الصوديوم ٠ ولكن أدت زيادة تركيز محلول كلوريد الصوديوم إلى معدلات متساوية من النقص فى الوزن الطازج للكالوس سواء كانت الأنسجة من خلايا سبق أو لم يسبق معاملتها بكلوريد الصوديوم أو سواء كانت معرضة للإضاءة أو لم تكن معرضة ٠

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ROLE OF BICARBONATE ON ELECTRON FLOW THROUGH PHOTOSYSTEM II IN PHOTOSYNTHETIC SOYBEAN CELLS

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ABSTRACT

Data presented in this paper show the role of bicarbonate in the acceleration of the electron flow through photosystem II in formate - treated soybean cells. Measurements of variable chlorophyll a fluorescence decay after an actinic flash show that formate treatment slows down the rate of Q_A^- decay (where Q_A is the first plastoquinone electron acceptor of photosystem II). Formate treatment increases the half-times of the fast component of Q_A^- decay from 302 μs to 550 s at (pH 6.5) and the half-times of the intermediate component from 40 ms to 65 ms. Formate treatment has also a big effect on the amplitudes of fast and intermediate components the first parameter decreases by a factor of 3.5 and the second parameter increases by a factor of 2.5. These inhibitions caused by formate treatment are dramatically reversed upon the addition of 2.5 mM HCO_3^-. Oxygen evolution measurements show that addition of 20 mM HCO_3^- to the formate-treated cells enhances the rate of oxygen evolution about 4 times. This stimulation in Hill reaction is not influenced by Co_2 fixation

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as it is observed in presence of 2, 5-dibromo-6-isopropyl -p-benzoquinone which inhibits inter-system electron flow. Thus, HCO_3^- is suggested to play a role in the regulation of electron flow of photosystem II; HCO_3^- stimulates the transfer of electrons from Q_A^- to Q_B (where Q_B is the second plastoquinone electron acceptor of photosystem II) and the plastoquinone pool in photosysthetic soybean cells.

INTRODUCTION

Bicarbonate has been shown to stimulate the electron transport in Hill reaction as suggested earlier by Warburg and Krippahl (1958). The use of inhibitory anions such as formate has been studied by many workers to show the mechanism of the stimulatory effect of bicarbonate on the electron flow of photosystem II. Govindjee and Van Ressen (1978) and Blugaugh and Govindjee (1988a) have indicated that formate inhibits the electron flow due to the further removal of bicarbonate from their binding sites. Bicarbonate reverses, in full, the inhibitory effect of formate. Based on measurements using the absorbance change at 320 nm, siggel at al. (1977) and Farineau and Mathis (1983) proved that the reoxidation of Q_A^- was enhanced to be 10-20 fold faster by bicarbonate readdition to the formate-treated thylakoid membranes. Furthermore, it was demonstrated by Govindjee and Eaton-Rye (1986) that depletion of bicarbonate slows down the electron flow from Q_A^- to Q_B and to the plastoquinone pool in isolated chloroplasts. It was

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suggested by Blugaugh and Govindjee (1988a) that HCO_3^- may have two major sites of action on the acceptor side of photosystem II in chloroplasts, one between Q_A and Q_B near-Fe- and the other in the D_1 - protein of the reaction centre. Eaton-Rye and Govindjee (1988a) have also demonstrated that chlorophyll a fluorescence measurements after a series of single saturated light flashes show a remarkable inhibition of electron flow after the third and subsequent flashes in bicarbonate depleted membranes. El-Shintinawy and Govindjee (1990) suggested the existence of two sites of action of bicarbonate at the acceptor side of photosystem II in spinach leaves: the first site stimulates the electron flow between the hydroxylamine donation site (Z or D) and Q_A while the second accelerates the electron flow beyond Q_A. The role of bicarbonate in controlling the electron flow of photosystem II has also been observed in cyanobacteria by Cao and Govindjee (1988) and in eukaryotic algal cells by Mende and Wiessner (1985). El-Shintinawy et al. (1990) studied the molecular mechanism of anion effect in chlamydomonas cells and demonstrated the presence of a dual bicarbonate - reversible formate effect on the electron flow from A_A^- to Q_B and between D and Q_A. This work was carried out to investigate the mechanism of bicarbonate effect on the electron flow at the Q_A Q_B complex of photosystem II in soybean cells. Formate was used in this study as used earlier in higher plant thylakoid membranes and in algal cells in order to accentuate the bicarbonate effect

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in soybean cells.

MATERIALS AND METHODS

Soybean cells, cell line SB-P (Corsoy) was established by Horn et al. (1983). The cells were cultured in KN^0 medium as described by Rogers et al. (1987) containing organic salts (Murashige et al., 1962), thiamine - Hcl, Kinetin and naphthalene acetic acid as organic components (Rogers and Widholm, 1988). The cells were cultured photoautotrophically for 14 days at 28 ^{0}C in a 5% Co_2 atmosphere under continous light of 300 umoles photons m^{-1} s^{-1} and shaken on a gyratory shaker at 130 rpm. After growth for three weeks soybean cells were collected for fluorescence measurements. Chlorophyll was extracted in 80% acetone (v/v) and its concentration was detected spectrophotometrically using the method of Arnon (1949). The cells were diluted in KN^0 medium to give a concentration of about 10 µg/ml for fluorescence measurements. Formate treatment of soybean cells was carried out according to the method described by El-Shintinawy et al. (1990). Soybean cells were incubated in dark for 3 hours in KN^0 medium containing 25 mM HCO_2^- at 20 ^{0}C under a constant flow of N_2 - gas at pH 5.8. Restored cells were prepared by adding 2.5 mM HCO_3^- to the formate - treated cells. Kinetics of the decay of variable chlorophyll a fluorescence were measured at 685 nm (10 nm bandwidth) by a week measuring flash which could be

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fired at variable times after each of a series of actinic flash (FX - 124, EG and G) were blocked by Corning filters, and were of 2.5 μs duration at half-maximal peak height. An identical instrument has been described by Robinson and Crofts (1983). The half-times ($t_{1/2}$ s) for Q_A^- oxidation were determined according to Joliot and Joliot (1964) after calculating the concentration of Q_A^- (Mathis and Paillotin, 1981):

$$\frac{(F-F_o)}{(F_m-F_o)} = \frac{(1-p)q}{(1-pq)}$$

where, F is the fluorescence yield at time t, F_0 is the fluorescence yield when all Q_A is in the oxidized state, F_m is the maximum fluorescence yield when all Q_A is in the reduced state, p (the connection parameter), is taken as the probability of the intersystem energy transfer and q is the fraction of the closed reaction centres (i.e., q = 1 when Q_A^- is maximum).

Oxygen evolution was measured polarographically using a Yellow Spring Instrument Clark-type electrode in a saturating Yellow light. One mM 2, 5-dimethyl - p - benzoquinone (DMQ) was used as an artificial electron acceptor in presence of 1 mM ferricyanide as another acceptor in order to keep DMQ in the oxidized state. 0.5 μM 2, 5-dibromo-6-isopropyl - p -benzoquinone (DBMIB) was used to block the

electron flow between the two photosystems II and I as reported by Trebst et al. (1970). Soybean cells were diluted in KN^0 medium to a concentration of 20 µg chl/ml for oxygen evolution measurements.

RESULTS AND DISCUSSION

It was suggested by Duysens and Sweers (1963) that the maximum intensity of chlorophyll a fluorescence is reached when Q_A s are fully reduced since Q_A is a quencher of chl a fluorescence. Therefore, the decay of chlorophyll a fluorescence yield is related to the concentration of Q_A population in the sample. In higher plant thylakoids, Q_A^- decay is slowed down greatly after three actinic flashes in bicarbonate-depleted membranes compared to control or restored samples as reported by Eaton-Rye and Govindjee (1988 a, b). Chlorophyll a fluorescence decays, monitoring the oxidation of Q_A^- following the third actinic flash, at pH 6.5 in formate-treated restored and control soybean cells are presented in Fig. 1. Adramatic decrease in the Q_A^- reoxidation rate as a result of formate treatment is noticed. However, adding 2.5 mM HCO_3^- to the formate - treated, cells enhanced the Q_A^- oxidation rate and produced restored curve that is similar to control.

The kinetics of chlorophyll a fluorescence after an

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actinic flash indicate the presence of two different components. A fast component of chlorophyll a decay (half-time in the μs range) and an intermediate component (half-time in the ms range). Both components are suggested to be related to the equilibration of $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ as suggested earlier by Robinson and crofts (1983, 1987). Two components extracted from the Q_A^- decay curves of soybean cells were analyzed to study the mechanism of both formate and bicarbonate effect. Table 1 shows the half-times and amplitudes of fast (f) and intermediate (i) components of chlorophyll a fluorescence decays after three actinic flashes in control, formate-treated and restored soybean cells at pH 6.5. The half-time of the fast component for control was 302 ms and the half-time of the intermediate component was 40 ms. These half-time were increased by about 1.8 and 1.6 times in formate-treated cells (550 μs and 65 ms). However, adding 2.5 mM HCO_3^- restored both half-times to almost control (260 μs and 36 ms) showing a remarkable reversibility. Formate treatment has a remarkable effect on the amplitudes of both fast and intermediate components; it decreased A(f) about 4 times and increased A(i) about 2.5 times. However, these changes were relieved by adding 2.5 mM HCO_3^-.

The effect of anion treatment (formate and bicarbonate) on the steady state electron transport from H_2O to DMQ (DMQ is an artificial electron acceptor) in the presence of

ferricyanide is presented in table 2. Incubation of soybean cells for 3 hours in formate - containing media (25 mM HCO_3^-, pH 6.5), produced an oxygen evolution rate of 40.2 μmoles O_2 (mg chl a)$^{-1}$ h^{-1}. Addition of 20 mM HCO_3^- (pH adjusted to 6.5) to the treated cells enhanced the rate to 257.5 μmoles O_2 (mg chla)$^{-1}$ h^{-1}, reversing the inhibition of the electron transport by formate treatment. However, in the absence of the two acceptors DMQ and $K_3Fe(CN)_6$ the oxygen evolution rate was 85.4 μmoles O_2 (mg chla)$^{-1}$ h^{-1} indicating that addition of bicarbonate stimulates the electron flow of photosystem II and increases the rate of CO_2 fixation. Thus, the net stimulation in the electron flow rate by bicarbonate addition was 4.3 fold. DBMIB, an inhibitor which blocks the electron transport between photosystem II and phtosystem I, was added to the reaction mixture to be sure that the above results are not affected by CO_2 fixation. Addition of 20 mM HCO_3^- to the formate - treated cells in the presence of 0.5 μM DBMIB and absence of the acceptors produced no oxygen evolution while in the presence of both the acceptors and the inhibitor a rate of 159 μmoles O_2 (mg chla)$^{-1}$ h^{-1} was recorded. Thus, addition of bicarbonate enhances the electron transport rate by 4.2 fold showing that HCO_3^- stimulates the electron transport of photosystem II and this role is independent of its role in CO_2 fixation.

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In summary, the data show a remarkable bicarbonate effect in the photoautotropic soybean cells; bicarbonate depletion or formate treatment slows down the Q_A^- decay of chlorophyll a fluorescence (Fig. 1). This change can be used as an evidence for the existence of bicarbonate effect in soybean cells as it was used earlier to show the same phenomenon in higher plant thylakoid membranes. Oxygen evolution measurements show clearly that bicarbonate accelerates the rate of electron flow of photosystem II which is a different role of that in CO_2 fixation as it is seen in presence of the inhibitor (DBMIB) that blocks the electron flow between the two photosystems II and I. These results, taken together with the fluorescence measurements act to establish the stimulatory role of bicarbonate on the electron flow from Q_A^- and Q_B and to the intersystem quinone pool in the photosynthetic soybean cells.

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TABLE 1. Effect of anion treatment on the half-times ($t_{1/2}$) and amplitudes (A) of the fast (f) and the intermediate (i) components of chlorophyll a fluorescence decay (after three actinic flashes) of soybean cells. Formate treatment was 3 hours and the reaction medium was KN^0 at pH 6.5. $t_{1/2}$ and A were calculated from Q_A^- decay curves of control, formate - treated and restored cells. The standard error was calculated from 4 measurements for each treatment.

Parameters	Treatment		
	Control	Formate-treated	Restored
$t_{1/2}$(f) (μs)	302 ± 5	550 ± 1	260 ± 4
$t_{1/2}$(i) (ms)	40 ± 1	65 ± 1	36 ± 1
A (f) (%)	68 ± 1	15 ± 2	65 ± 1
A (i) (%)	32 ± 1	85 ± 2	35 ± 2

TABLE 2. Effect of anion treatment on the oxygen evolution rate in soybean cells. KN^0 medium was used as the reaction medium at pH 6.5. Formate (25 mM) treatment was 3 hours. One mM DMQ and 1mM $K_3Fe(CN)_6$ were used as electron acceptors and 0.5 μM DBMIB was used as an inhibitor. Cell suspension

containing 20 µg chl a/ml were used. The standard error was calculated from 4 measurements for each treatment.

Treatment	Rate of oxygen evolution µmolesO_2 (mg chl)$^{-1}$ h^{-1}	Net stimulation by HCO_3^- (B-C)	Ratio $+HCO_3^-/HCO_3^-$ (B-C)/A
A_1. $-HCO_3^-$ +DMQ+$k_3Fe(CN)_6$	40.2 ± 4		
B_1. + HCO_3^-+QMD+$K_3Fe(CN)_6$	257.5 ± 17	172.1	4.3
C_1. + HCO_3^-, no acceptors	85.4 ± 14		
A_2. A_1 + DBMIB	38 ± 2		
B_2. B_1 + DBMIB	159 ± 15		
C_2. C_1 + DBMIB	00.0 ± 1	159	4.2

FIGURE/ LEGENDS

Figure 1. Decay of chlorophyll a fluorescence yield after three actinic flashes in control (Δ), formate-treated (□) and cbicarbonate restored (0) soybean cells at pH 6.5. F_0 is the chlorophyll a fluorescence yield from the measuring falsh with all Q_A^- oxidized and F is the yield at the indicated time after the actinic flash.

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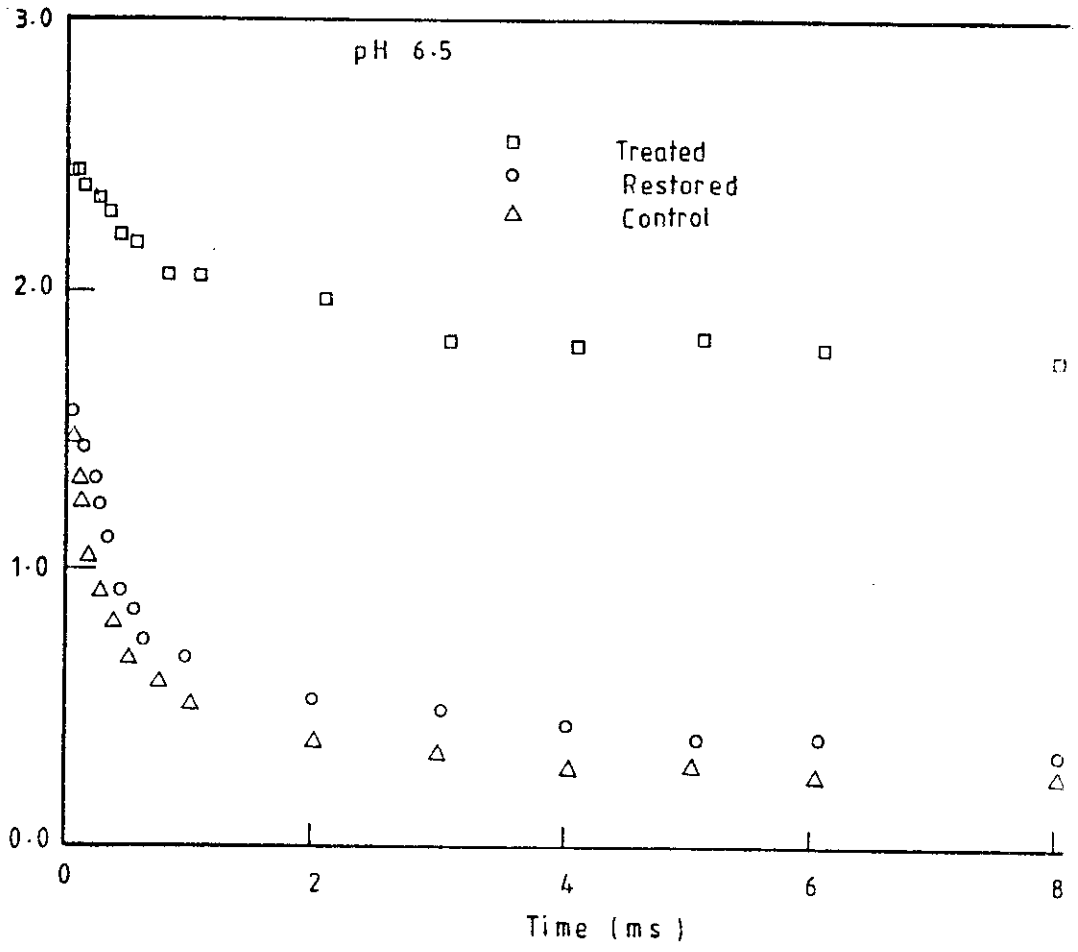

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تأثير أنيون البيكربونات على تفاعلات الإنتقال الإلكتروني خلال النظام الضوئي الثاني لعملية البناء الضوئي باستخدام خلايا نبات فول الصويا

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يهدف هذا البحث الى بيان تأثير أنيون البيكربونات على تفاعلات الإنتقال الإلكتروني في النظام الضوئي الثاني لعملية البناء الضوئي وذلك باستخدا مخلايا نبات فول الصويا المستنبتة .

أظهرت نتائج تجارب قياسات التفلور بإستخدام طريقة الوميض الثنائي لخلايا فول الصويا المعاملة بأنيون الفورمات إنخفاضا ملموسا في معدل إنحسار التفلور للكلورفيل أ وذلك نتيجة تقليل معدل أكسدة المستقبل الإلكتروني الأولي بواسطة المستقبل الإلكتروني الثانوي .

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إضافة أنيون البيكربونات للعينات السابقة أدى إلى زيادة معدل إنحسار التفلور للكلوروفيل أ وذلك نتيجة زيادة معدل أكسدة المستقبل الإلكترونى الأولى ؛ كما أثبتت قياسات الأكسجين المتصاعد أن آيون البيكربونات يزيد من تفاعل هيل أربعة أضعاف فى وجود مثبط لعملية التثبيت الكربونى مما يدل على أن دور أنيون البيكربونات على تفاعلات الإنتقال الإلكترونى للنظام الضوئى الثانى يختلف عن دوره فى عملية التثبيت الكربونى .