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**EFFECT OF KINETIN AND
NAPHTHALENEACETIC ACID APPLICATION ON THE
RESPIRATORY METABOLISM DURING CALLUS
DEVELOPMENT IN POTATO TUBER
TISSUE CULTURED *IN VITRO***

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Introduction

When the relatively low concentration of auxin (0.3 mg/l of NAA) was added to the nutrient medium, the explant from potato tuber increased in both fresh and dry weight and the callus culture was established. But no additional application of kinetin was required for callus formation (OKAZAWA *et al.*, 1967). The process of auxin-induced callus formation involved two separate phases of growth, i. e., cell division and cell expansion (OKAZAWA, 1968). As far as the respiratory changes were concerned, the results obtained in the previous papers (AKEMINE *et al.*, 1970; KIKUTA *et al.*, 1971) were as follows: 1) During the early stage of culture when the marked production of DNA occurred, the malonate sensitive respiration was once disappeared and the PP pathway played a dominant role. 2) While the callus was once developed, the massive cell expansion was induced in explants and the rate of the EMP-TCA pathway activity seemed to be proportional to the rate of callus growth.

When the relatively high concentration of auxin (30 mg/l of NAA) was added to the nutrient medium, the explants increased their fresh weight without accompanying by the corresponding increase in dry weight and the friable callus cultures were resulted. However, by the additional

Abbreviations: NAA, α -Naphthaleneacetic acid; 2,4-D, 2,4-Dichlorophenoxyacetic acid; DCPIP, 2,6-Dichlorophenol indophenol; RNA, Ribonucleic acid; DNA, Deoxyribonucleic acid; EMP, Embden-Meyerhof-Parnas; TCA, Tricarboxylic acid; PP, Pentose phosphate; NAD(P), Nicotinamide adenine dinucleotide (phosphate); NAD(P)H, Reduced nicotinamide adenine dinucleotide (phosphate); G6PDH, Glucose-6-phosphate dehydrogenase; 6PGDH; 6-Phosphogluconate dehydrogenase; RQ, Respiratory quotient.

application of kinetin the explants showed marked increases in dry matters and the compact callus cultures were established (KATSURA *et al.*, 1970). In the present investigation, these two systems were used in order to examine whether or not such patterns of growth might reflect the respiratory metabolism in the explants excised from potato tuber cultured *in vitro*.

Materials and Methods

Plant materials

The tubers of potato (*Solanum tuberosum* L., var. Irish Cobbler) stored in a cellar at 2°C in darkness were used as materials. The disk-shaped slices (5 mm in diameter and 1 mm in thickness) for cultural explants were cut and cultured in the same manner as described in the previous paper (OKAZAWA *et al.*, 1967).

Culture conditions

Two slices were explanted onto 30 ml of nutrient medium in a 100 ml-Erlenmeyer's flask after autoclaved at 1.0 kg/cm² pressure for 10 minutes. The nutrient media used were as follows: 1) modified White's medium (OKAZAWA *et al.*, 1967)+0.5% agar (control medium)+0.3 mg/l of NAA (low-auxin medium), 2) control medium+30.0 mg/l of NAA (high-auxin medium, and 3) high-auxin medium+various concentrations (0.3–10.0 mg/l) of kinetin (kinetin medium). The cultures were incubated in a dark room at 25°C. Explants cultured on low-auxin medium, high-auxin medium and kinetin medium are named as low-auxin culture, high-auxin culture and kinetin culture, respectively. Samples were taken at appropriate intervals on the course of 3 week culture for analyses described below.

Measurement of fresh and dry weight

The explants taken from the culture medium were carefully washed with distilled water, and blotted dry with filter paper. The fresh weight was measured immediately after these operations. The dry weight was measured after the explants had been dried in an oven at 80°C for 24 hours.

Determination of protein content

Determination of protein content of explant was carried out according to the previous paper (AKEMINE *et al.*, 1970).

Determination of nucleic acid content

The extraction and estimation of nucleic acid was carried out by the method of OGUR and ROSEN (1950).

Measurement of respiratory rate

The respiratory rate was manometrically determined by measuring O₂ uptake of explant. All measurements were carried out for 1 hour at 25°C after thermal equilibration. Explants (approximately 0.5 g in fresh weight) were placed in a Warburg's flask containing 2.2 ml of *M/15* phosphate buffer, pH 5.0, and the center well contained 0.3 ml of 5 *N* KOH. Carbon dioxide output was determined using the two-flask method with paired samples. Malonate was used as respiratory inhibitor at a final concentration of 5×10^{-2} *M*.

Feeding experiment and assay of radioactivity

Approximately 1.0 g of explants, as intact blocks, were placed in the main compartment of a Warburg's flask. One ml of *M/15* phosphate buffer, pH 5.0, containing 0.1 μ Ci of glucose-U-¹⁴C (2 mCi/mmole), 0.1 μ Ci of glucose-1-¹⁴C, or glucose-6-¹⁴C of equal specific activities (2 mCi/mmole), or 0.1 μ Ci of succinic acid-2, 3-¹⁴C (2 mCi/mmole) was placed in the side arm. For collecting respired CO₂, 0.3 ml of Hyamine hydroxide (10 x) in methanol was placed in the center well. The solution in the side arm was poured into the main compartment at zero time after thermal equilibration at 25°C. After explants were exposed to labeled glucose or succinic acid for 2 hours, they were collected and rinsed with nonradioactive *M/15* phosphate buffer, pH 5.0, then immediately crushed with 80% ethanol in a mortar. Aliquots of the Hyamine-CO₂ fraction, ethanol soluble fraction and ethanol insoluble fraction were transferred to counting vials and assayed the radioactivity with a liquid scintillation counter. The scintillation solvent was prepared according to BRAY (1960).

Enzyme assay

G6PDH and 6PGDH activities were assayed as described in the previous paper (KIKUTA *et al.*, 1971).

Assay for NADP and NADPH

Samples of frozen explants (approximately 1 g in fresh weight) were ground at 3°C in a homogenizer containing 5 ml of 0.1 *N* trichloroacetic acid. The other portion was similarly treated with 0.1 *N* NaOH. Immediately after homogenization, each extract was incubated at 100°C for 90 seconds to denature degradative enzymes. Each homogenate was rapidly cooled to 3°C in an ice bath and adjusted to pH 7.6 before 0.3 ml of 0.2 *N* tris-HCl (pH 7.6) was added. Each extract was centrifuged at 20,000 \times *g* for 20 minutes at 3°C. Immediately after extraction, nicotinamide nucleotide was measured by an assay involving decrease in DCPIP at 620 nm using

a Hitachi spectrophotometer. Each reaction mixture consisted of 160 μ moles of tris-HCl (pH 8.0), 0.24 μ mole of DCPIP, 20 μ moles of $MgCl_2$, isocitrate dehydrogenase, NADPH diaphorase, 2 μ moles of isocitrate and tissue extract to be assayed in a total volume of 3.0 ml (YAMAMOTO, 1963). NADPH diaphorase was prepared from spinach chloroplast by the method of AVRON and JAGENDORF (1956). Isocitrate dehydrogenase was purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Isocitrate solution was prepared from DL-allo-free isocitric acid lactone (Sigma Chemical Co.) as described by CARPENTER and BEEVERS (1959).

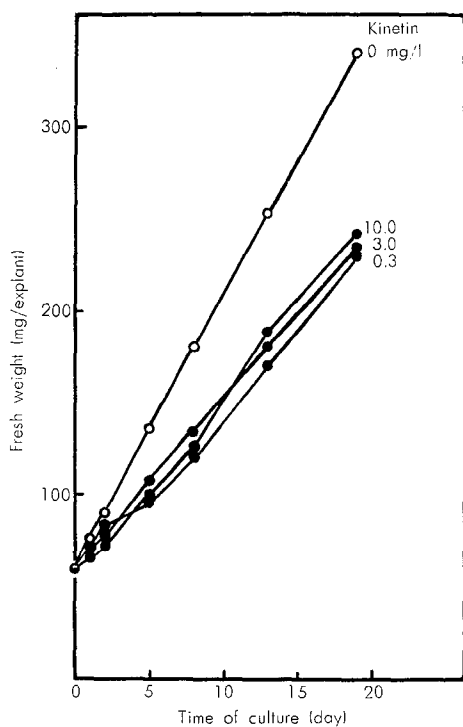


Fig. 1. Effect of kinetin on the fresh weight in explants cultured with 30 mg/l of NAA.

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested at appropriate intervals.

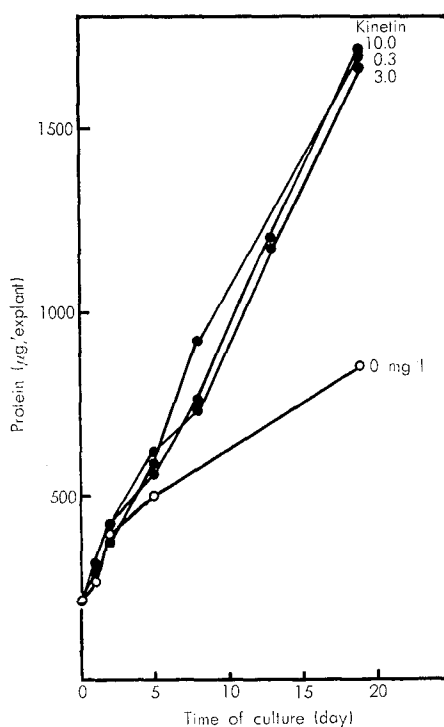


Fig. 2. Effect of kinetin on the protein content in explants cultured with 30 mg/l of NAA.

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested at appropriate intervals. The protein was estimated according to the previous paper (AKEMINE *et al.*, 1970).

Results

(1) *Effects of an additional application of kinetin on the growth of explant in the presence of high level of NAA*

The growth of cultured explants otherwise quiescent tissue excised from potato tubers was estimated by measuring the parameter of fresh weight, protein, RNA and DNA content during 3 week culture period.

Fresh weight The high-auxin culture showed a remarkable increase in fresh weight, attained to 6 fold of an initial weight at the end of 3 week culture (Fig. 1). An additional application of kinetin tended to suppress such enhanced increases in fresh weight.

Protein content As is shown in Fig. 2, the applied kinetin strikingly enhanced the increase in protein content of explant during culture period.

RNA content The changes in the RNA content in high-auxin and kinetin (0.3 mg/l) cultures are shown in Fig. 3. The increasing patterns of RNA content in both cultures were very much similar to those of protein content (Fig. 2).

DNA content The increase in DNA content of the low-auxin, high-auxin and kinetin (0.3 mg/l) cultures is shown in Fig. 4-a and the changes in the fresh weight/DNA ratio in Fig. 4-b. The activity of DNA multiplication was allowed to promote during the initial 5 days after inoculation regardless of whether the explants were cultured with high level of auxin and kinetin added to the medium. Therefore, only the kinetin culture followed a marked increase in the DNA content throughout an entire culture period. This is also clear in Fig. 4-b that the decrease in the fresh weight/DNA ratio was restricted within the initial 2 days in the high-auxin culture indicating auxin-induced water uptake by the explants. The increase in fresh

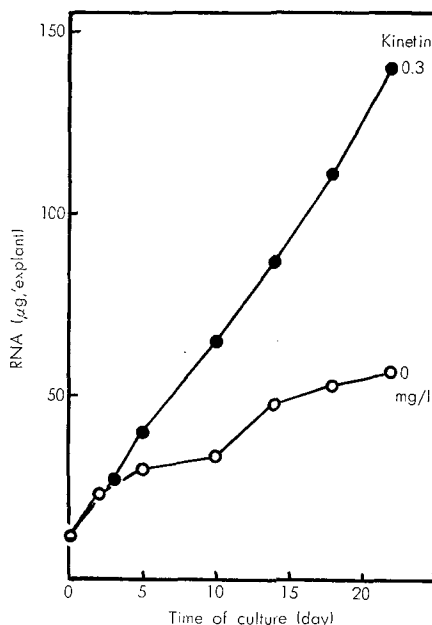


Fig. 3. Effect of kinetin on the RNA content in explants cultured with 30 mg/l of NAA.

Kinetin (0.3 mg/l) was added to the high-auxin medium. The explants were harvested at appropriate intervals. RNA was estimated by the method of OGUR and ROSEN (1950).

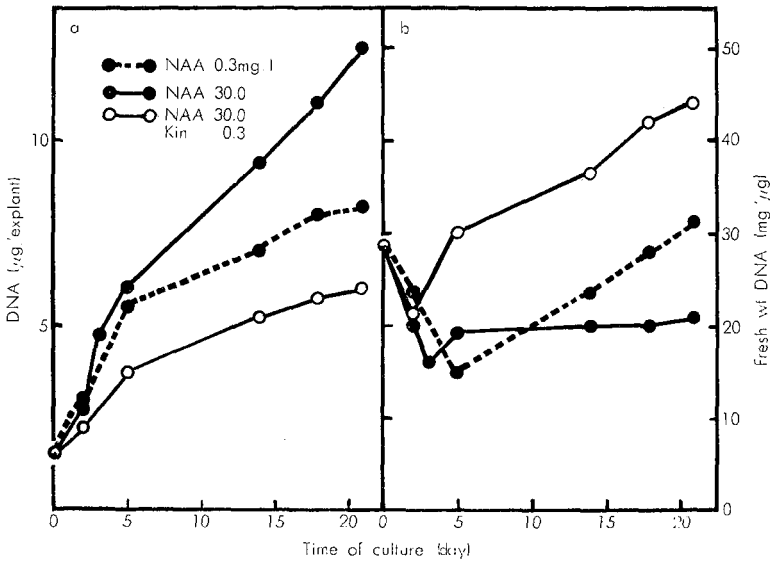


Fig. 4. Changes in the DNA content (a) and the fresh weight/DNA ratio b (in) explants during callus formation.

The explants were cultured with 0.3 mg/l of NAA or 30 mg/l of NAA in the presence and absence of 0.3 mg/l of kinetin and harvested at appropriate intervals. DNA was estimated by the method of OGUR and ROSEN (1950).

TABLE 1. Effect of kinetin on the DNA content and fresh weight in explants cultured with 30 mg/l of NAA

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested at 3 week after inoculation. DNA was estimated by the method of OGUR and ROSEN (1950)

Kinetin conc.	FW mg* (%)	DNA µg* (%)	FW/DNA mg/µg
Initial explant	56.1 (100)	1.75 (100)	32.1
0 mg/l	444.9 (793)	12.4 (709)	35.7
0.3	266.2 (475)	18.6 (1063)	14.3
3.0	298.7 (532)	24.6 (1417)	12.0
10.0	273.0 (487)	23.6 (1349)	11.6

* per explant.

weight/DNA ratio defines the growth phase that the activity of the cell expansion is to predominate over the activity of the cell division. In this sense, the activity of DNA multiplication is allowed to continue the cell division in explants when kinetin was added to the high auxin culture medium.

By increasing the concentration of kinetin added to the medium, the explants were stimulated the activity of DNA multiplication and were suppressed the auxin-induced water uptake (Table 1).

(2) *Effects of an additional application of kinetin on the respiration of explant in the presence of high level of NAA*

Respiratory activity and quotient Figure 5 shows the changes in O_2 uptake per explant (a) and per fresh weight (b) in high-auxin and kinetin cultures. The initial respiratory rise during the first 1 day after inoculation was striking regardless of the presence or absence of additional

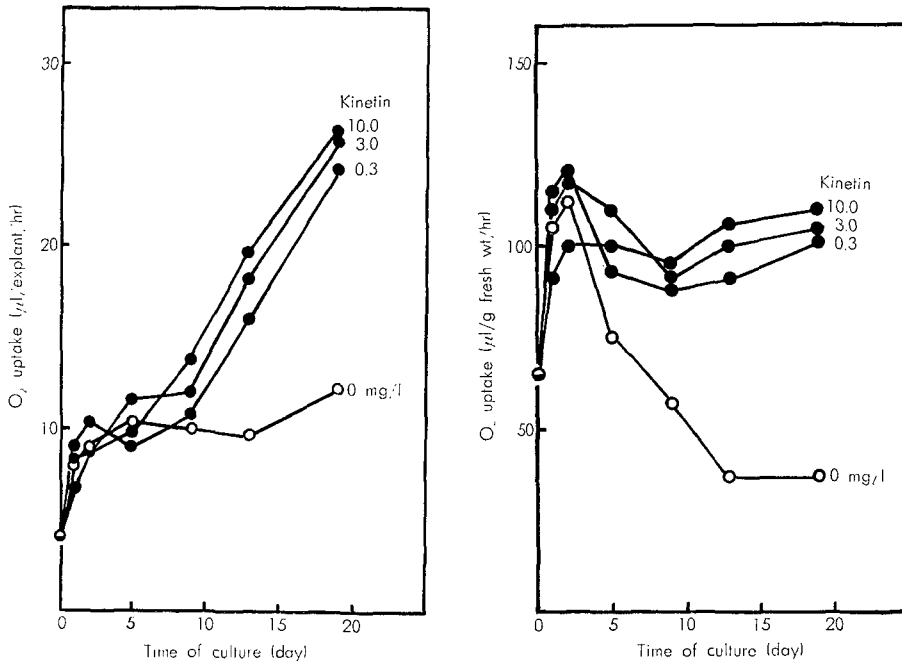


Fig. 5. Effect of kinetin on the oxygen uptake per tissue (a) and per fresh weight (b) in explants cultured with 30 mg/l of NAA.

The various concentrations of kinetin were added to the highauxin medium. The explants were harvested at appropriate intervals. Oxygen uptake was measured manometrically at 25°C for 1 hr.

TABLE 3. Effect of kinetin on the respiratory quotient in explants cultured with 30 mg/l of NAA

Kinetin (10 mg/l) was added to the high-auxin medium. The explants were harvested on the 12th and 18th day after inoculation

Treatment	O ₂ uptake	CO ₂ output	RQ
	μl/g fresh wt/hr		
12-day			
-Kin	60.0	54.6	0.91
+Kin	105.9	96.6	0.91
18-day			
-Kin	53.6	50.2	0.94
+Kin	97.0	92.8	0.96

application of kinetin. While the high-auxin culture rapidly declined the rate of respiratory activity, the kinetin culture still maintained the high level of respiration thereafter.

The initial respiratory quotients of the explant was close to unity (AKEMINE *et al.*, 1970). Table 2 shows the RQ in high-auxin and kinetin (10.0 mg/l) cultures on the 12th and 18th day after inoculation. The RQ of both cultures remained unchanged (ca. 0.9-1.0) throughout culture period.

Malonate sensitivity Malonate ($5 \times 10^{-2} M$) inhibition of O₂ uptake in the high-auxin and kinetin (0.3 mg/l) cultures were examined throughout culture period and the results are shown in Fig. 6. The malonate sensitivity of respiration in high-auxin culture rapidly declined during the first 2 days after inoculation followed by a massive restoration in a subsequent period. The malonate sensitivity in the kinetin culture gradually decreased after inoculation, in which

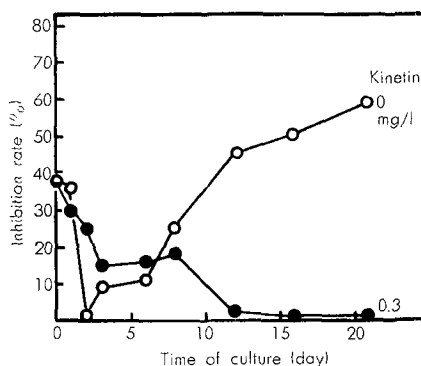


Fig. 6. Effect of kinetin on the malonate sensitivity of respiration in explants cultured with 30 mg/l of NAA.

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested at appropriate intervals. The oxygen uptake was measured manometrically in the presence and absence of $5 \times 10^{-2} M$ malonate at 25°C for 1 hr.

no sign of the restoration was shown in the later culture period. The lack of recovery of respiratory sensitivity to malonate in kinetin culture in the later culture period can be accounted for by prolonged meristematic activity in the explants (Fig. 4).

C6/C1 ratio The changes in C6/C1 ratio in high-auxin and kinetin (1.0 mg/l) cultures are shown in Fig. 7. Both high-auxin and kinetin cultures rapidly decreased in the C6/C1 ratios right after inoculation when the initial respiratory rise was induced. Afterwards, the ratios in high-auxin culture were elevated and kept high level (ca. 0.9) in a subsequent culture period, while such an elevation of the ratio was suppressed to some extent in kinetin culture. Close relationships were observed between the concentration of additionally applied kinetin

and the C6/C1 ratio of the explants during the later culture period. Table 3 shows the effect of kinetin on the C6/C1 ratio of the explants on the 12th

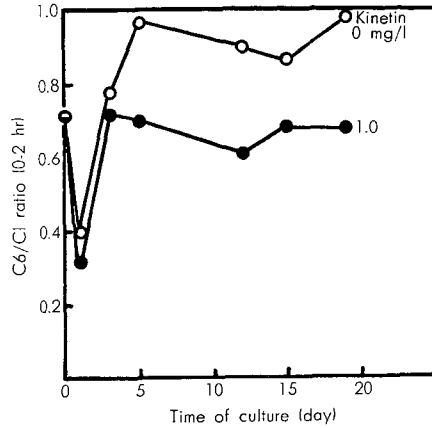


Fig. 7. Effect of kinetin on the C6/C1 ratio in explants cultured with 30 mg/l of NAA.

Kinetin (1.0 mg/l) was added to the high-auxin medium. The explants harvested at appropriate intervals were exposed to 0.1 μ Ci of glucose-1- 14 C or glucose-6- 14 C of equal specific activities (2 mCi/mmmole) at 25°C for 2 hr.

TABLE 3. Effect of kinetin on the $^{14}\text{CO}_2$ liberation from glucose-1- ^{14}C and glucose-6- ^{14}C in explants cultured with 30 mg/l of NAA

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested on the 12th day after inoculation and exposed to 0.1 μ Ci of glucose-1- ^{14}C or glucose-6- ^{14}C of equal specific activities (2 mCi/mmmole) at 25°C for 2 hr

Kinetin conc.	$^{14}\text{CO}_2^*$		C6/C1
	C-1	C-6	
0 mg/l	15.5	13.8	0.90
0.3	24.7	18.3	0.74
3.0	30.8	20.9	0.68
10.0	36.0	23.0	0.59

* % of absorbed ^{14}C (0-2 hr)

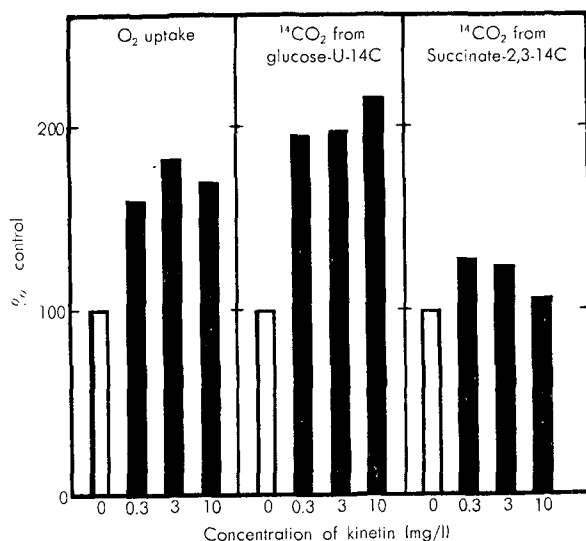


Fig. 8. Effect of kinetin on the oxygen uptake and ¹⁴CO₂ liberation from glucose-U-¹⁴C and succinic acid-2,3-¹⁴C in explants cultured with 30 mg/l of NAA

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested on the 16th day after inoculation and exposed to 0.1 uCi of glucose-U-¹⁴C (2 mCi/mmole) or succinic acid-2,3-¹⁴C (2 mCi/mmole) at 25°C for 2 hr.

TABLE 4. Effect of kinetin on the activities of G6PDH and 6PGDH in explants cultured with 30 mg/l of NAA

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested on the 13th day after inoculation. The assays were made according to the previous paper (KIKUTA *et al.*, 1971)

Kinetin conc.	Protein*	Absolute**		Specific***	
		G6PDH	6PGDH	G6PDH	6PGDH
0 mg/l	206 (100)	0.110 (100)	0.070 (100)	272 (100)	175 (100)
0.3	265 (129)	0.170 (155)	0.106 (151)	325 (119)	204 (117)
3.0	354 (172)	0.200 (182)	0.140 (200)	283 (104)	198 (113)
10.0	326 (158)	0.196 (178)	0.136 (194)	300 (110)	208 (119)

* $\mu\text{g/ml}$ enzyme ** OD change/min/ml enzyme *** (unit the amount of enzyme which produces one μmole NADPH/min/mg protein)

day after inoculation. The higher the concentration of kinetin was added to the medium, the lower the C6/C1 ratio of explants was found.

¹⁴CO₂ liberation from glucose-U-¹⁴C and succinic acid-2, 3-¹⁴C

Figure 8 shows the rates of O₂ uptake and of ¹⁴CO₂ liberation from glucose-U-¹⁴C or succinic acid-2, 3-¹⁴C in high-auxin and kinetin cultures on the 16th day after inoculation. The application of kinetin enhanced O₂ uptake of explant (Fig. 5). This promotion of O₂ uptake by added kinetin was accompanied by the corresponding promotion of the activity of ¹⁴CO₂ liberation from glucose-U-¹⁴C, but merely from succinic acid-2, 3-¹⁴C.

G6PDH and 6PGDH activities Table 4 shows the activities of G6PDH and 6PGDH in high-auxin and kinetin cultures on the 13th day after inoculation. Although the application of kinetin stimulated the absolute enzyme activities (*ca.* 100% above control), the specific activities of both dehydrogenases were not so promoted (5-20% above control) by applied kinetin.

NADP level The availability of NADP can regulate the activity of the PP pathway in plant cells (AP REES and BEEVERS, 1960; BUTT and BEEVERS, 1961; YAMAMOTO, 1966). Figure 9 shows the changes in oxidized NADP level in high-auxin and kinetin (10.0 mg/l) cultures throughout culture period. Kinetin culture showed consistently higher level than high-auxin culture during an entire culture period. The estimation of NADP+NADPH level was made in both cultures on the 12th day after inoculation (Table 5). The NADP levels were found to be increased as the concentration of added kinetin was raised. The NADPH levels were also increased by rise in kinetin application, but the magnitude of NADPH was not so marked than that of NADP, and therefore, the NADPH/NADP ratio was consequently lowered in kinetin culture.

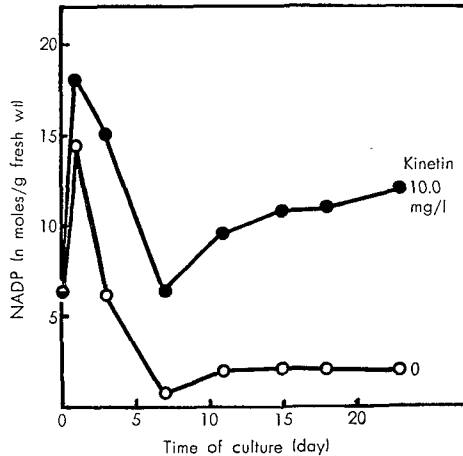


Fig. 9. Effect of kinetin on the level of oxidized NADP in explants cultured with 30 mg/l of NAA.

Kinetin (10 mg/l) was added to the high-auxin medium. The explants were harvested at appropriate intervals. NADP was estimated by the method of YAMAMOTO (1963).

TABLE 5. Effect of kinetin on the NADP level in explants cultured with 30 mg/l of NAA

The various concentrations of kinetin were added to the high-auxin medium. The explants were harvested on the 12th day after inoculation. NADP estimation was made by the method of YAMAMOTO (1963)

Kinetin conc.	NADP	NADPH	NADP+NADPH	NADPH/NADP
	<i>n</i> moles/g fresh wt			
Initial explant	9.3	5.7	15.0	0.6
0 mg/l	5.5 (100)	6.6 (100)	12.1 (100)	1.2
0.3	12.8 (233)	12.9 (195)	25.7 (212)	1.0
3.0	16.3 (296)	13.3 (202)	29.6 (245)	0.8
10.0	18.3 (333)	12.9 (195)	31.2 (258)	0.7

Discussion

The explants cultured with high level of NAA alone showed a rapid increase in fresh weight accompanied by only small increases in protein, RNA and DNA. Consequent on the additional application of kinetin to the culture medium the fresh weight increase was suppressed to some extent, but the other growth parameters were increased rapidly. On the basis of these findings, it is reasonable to say that the high level of NAA stimulated the active water uptake in large portion of cells in the explants and that the additionally applied kinetin promoted the activity of meristematic growth of explant. The callus induced by high level of NAA in the presence of kinetin seems to have a phase of the prolonged duration of active cell division on the course of callus development compared with the one induced by low concentration of NAA alone.

The explants cultured with high level of NAA in the absence of kinetin showed the high level of malonate sensitivity of respiration and of C6/C1 ratio throughout culture period except in the early stage of culture. While added kinetin tended to suppress such high level of malonate sensitivity of respiration and of C6/C1 ratio. These results indicated that the high level of NAA caused to rise the participation of the EMP-TCA pathway and that the additional application of kinetin suppressed the EMP-TCA pathway participation and stimulated the PP pathway activity. The lowered values in malonate sensitivity of respiration and in C6/C1 ratio in the early stage of culture (Fig. 6, 7) were also observed in low-auxin

culture, but not in control culture (explants cultured on control medium) (AKEMINE *et al.*, 1970; KIKUTA *et al.*, 1971). During the first few days after inoculation the first wave of DNA multiplication followed by cell division occurred when the explants were cultured in the presence of NAA.

Judging from the above evidence, it seems to be considered that the PP pathway and the EMP-TCA pathway play a significant role in metabolism during the phase of cell division and the phase of cell expansion on the course of callus development in potato tissue culture, respectively.

The reduced malonate sensitivity of respiration, the low value of C6/C1 ratio, and the relatively lower activity of $^{14}\text{CO}_2$ liberation from labeled citric acid were observed in the callus tissue derived from epicotyl of *Stizolobium hassjoo* when compared with the epicotyl tissue (MOROHASHI *et al.*, 1965). In the case of callus induction from carrot root tissue by the application of 2, 4-D and kinetin, the EMP-TCA pathway was remarkably increased during the first 4-6 days after culture, and as the callus developed, the relative participation of the PP pathway gradually increased and simultaneously alcohol fermentation occurred (KOMAMINE *et al.*, 1969). This observation did not necessarily support the results of the present study. The potato explants cultured with NAA and kinetin (kinetin culture) did not show any significant rise of the EMP-TCA pathway activity during the early stage of culture and any alcohol fermentation in the later culture period. However, the PP pathway activation seems to be a common metabolic feature during the active callus development (mainly due to the phase of cell division) in both potato and carrot tissue cultures.

The magnitude of increases in the PP pathway dehydrogenase activities to account for the pathway participation of glucose metabolism was not sufficiently deduced by kinetin application. AP REES and BEEVERS (1960), BUTT and BEEVERS (1961) and YAMAMOTO (1966) found that the participation of the PP pathway could be increased by addition of NADP or of electron acceptors for reduced NADP. Therefore any reaction whereby NADP is formed, either by synthesis or by oxidation of NADPH, can regulate the participation of the PP pathway. As expected, the kinetin culture had higher level of oxidized NADP than high-auxin culture (Fig. 9, Table 5). Simultaneously the added kinetin lowered the NADPH/NADH ratio in explant (Table 5), which suggested that NADPH reoxidation system operated efficiently in the kinetin culture. These increased availability of NADP may be an important factor in accounting for an increase in the PP pathway activity in kinetin culture.

YAMAMOTO and OHYAMA (1962) found increased levels of the oxidized nicotinamide nucleotide in the leaves of spinach and tobacco after treatment with kinetin. Then, cytokinin, such as kinetin, which stimulates the meristematic growth of plant cells, may play an important role in the regulation of plant metabolism through regulating the nicotinamide nucleotide levels (KURAIISHI *et al.*, 1968).

The kinetin culture maintained high level of respiration throughout culture period (Fig. 5). This stimulated respiration in kinetin culture could be resulted from the oxidation of NADPH generated from the dehydrogenase reactions in the PP pathway.

Summary

The effects of an additional application of kinetin (0.3–10.0 mg/l) on the growth and respiration of potato tuber explant cultured with relatively high concentration of NAA (30.0 mg/l) were investigated on the course of 3 week culture. 1) Consequent on the application of kinetin to the nutrient medium the fresh weight increase of explant was suppressed to some extent, but the increases of protein, RNA and DNA were markedly stimulated. 2) The explants cultured in the presence of applied kinetin showed the high rate of O₂ uptake, which was accompanied by an increase in ¹⁴CO₂ liberation from glucose-U-¹⁴C, but merely from succinic acid-2, 3-¹⁴C. Furthermore, 3) the applied kinetin caused the explants to decrease their malonate sensitivity of respiration and the C₆/C₁ ratio. 4) Though the specific activities of G6PDH and 6PGDH were not so promoted by kinetin application, the NADP+NADPH level, especially that of NADP, was much increased by added kinetin. These findings suggest a conclusion that an additional application of kinetin promoted the meristematic growth of the explant and simultaneously stimulated the PP pathway activity by increasing the availability of NADP in the explant.

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