Radiosensitivity and Cytological Activity at Different Growth Phases of Rice Cells in Suspension Culture

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Introduction

Fundamental aspects of radiation effects on living cells have extensively been accumulated in microorganisms and animal cells cultured *in vitro*[5,13]. By using cultured cells of higher plants as experimental materials, basic information could similarly be provided on the radiosensitivity and mutagenesis of plants in a single cell level. Since pioneer work by MELCHERS and BERGMANN[18] who demonstrated the growth inhibition caused by X-ray irradiation in haploid cell suspensions of *Antirrhinum*, considerable studies have been conducted on the effect of ionizing radiations in cultured cells of *Haplopappus*[9], *Phaseolus*[2], *Nicotiana*[20], *Daucus*[7] and *Arachis*[21]. From these and additional similar experiments it has been made clear that radiosensitivities in cultured cells of plants vary with plant species used and even with the cells originated from different tissues in a plant[1,11,12,22]. Cultured cells of higher plants, however, are nonuniform both in the cytological consittution of cell population and in the physiological state during a culture period. For instance, initial callus tissue derived from plant organ consists of heterogeneous cell population with a wide range of polyploidy and aneuploidy[4,17,23,28,29]. Furthermore, cultured cells exhibit the continuous changes in physiological and metabolic activities during the progress of batch culture from initiation until they reach stationary phase[14,22,26]. These characteristics may drastically affect the radiosensitivity of cultured cells.

In the present experiment, long-term subcultured callus cells of rice were used as experimental materials, which consist of considerably homogeneous cell population and exhibit stable reproduction throughout subcultures. The changes in cytological activity and radiosensitivity were examined at different growth phases of the cell suspension cultures.

Materials and Methods

Culture condition

Culture medium used for induction and subculture of callus tissues was the modified MURASHIGE and SKOOG's[16] supplemented with 10^{-4}M 2, 4-D. Callus cultures were initiated by transferring root-tips obtained from aseptically grown seedlings of rice variety "Sensho" to 0.8% agar-solidified medium. On 30 days after the transferring, induced callus tissues were inoculated into specially designed 200 ml-Erlenmeyer flasks (cf. Fig. 1) containing 30 ml liquid culture medium. A drop of this cell suspension (0.5 ml) was subcultured in a freshly prepared medium every 35 days throughout 2 years. The culture flasks containing free and clamp cells were placed on a reciprocal shaker (85 strokes/min) in dark room at 28 ± 1°C throughout experiments. These culture conditions yielded rapid growth and various sized fragments of callus tissue which were completely pipettable for subsequent subcultures.

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Growth measurement

The growth of callus cells at a given time of culture was evaluated by measuring the fresh weight, dry weight and sedimented cell volume (SCV). As shown in Fig. 1, the SCV was obtained from the reading of the scale on a side-armed glass tube attached to the culture flask, which allowed to estimate continuously and conveniently the growth rate of suspension cultures without disturbance of culture condition. The measurements of the growth parameters are based on an average of 10 cultures.

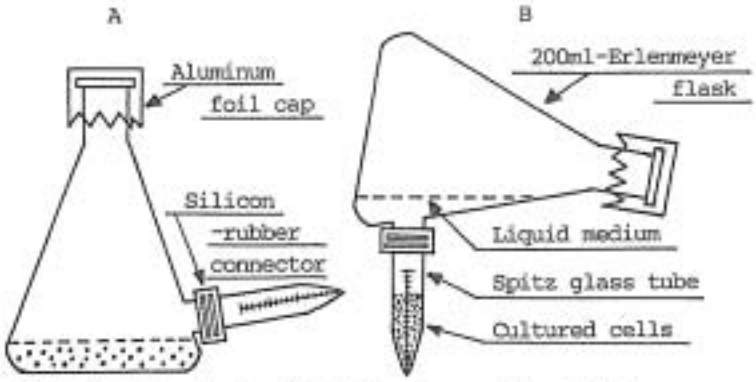

Fig. 1. Specially designed culture-flask with a side-armed glass tube for measurement of the sedimented cell volume of callus culture (A). At a time of the measurement, the glass tube is placed in a vertical position for 15 minutes (B).

Oxygen uptake

For measurement of respiration activity, aliquots of cell suspension in the 23rd subculture were sampled at different growth phases during the culture period of 35 days, and then transferred to Warburg reaction vessels in which the oxygen uptake were determined at 30°C by standard Warburg technique. Manometer readings were taken every 10 min for 1 hr after equilibration. Respiration rates were expressed as oxygen consumption per unit dry weight of the cultured cells.

^{3}H-thymidine labeling

To label DNA-synthesizing cells, tritiated thymidine (6-^{3}H-T; specific activity 28 Ci/mM, The Radiochemical Centre, Amersham) was used as a DNA precursor. The ^{3}H-thymidine was dissolved in the culture medium at a concentration of 1 μCi/ml. Callus cells (150 mg of fresh weight) in the 22nd subculture were grown for 25 days under the same condition described above. At different growth phases during the culture period, the cells (about 500 mg of fresh weight) were aseptically sampled and treated with the radioactive medium for 1 hr at 30°C in a water bath with gentle shaking. After this treatment, the cells were washed three times in nonradioactive fresh medium and then reincubated for 2 hr to ensure incorporation of the precursor into nuclear DNA. The treated cells were fixed with a mixture of 3 parts of ethanol and 1 part of acetic acid for 1 hr and then transferred into 70% ethanol for storage in a refrigerator. Following the storage, they were macerated with 10% cellulase and 5% pectinase for 3 hr. Squash preparations were obtained on microscope slides by the method of CONGER and FAIRCHILD[4] and then dehydrated by passing through a ethanol series. The dried slides were dipped into autoradiographic emulsion (Sakura NR-M2, Konishiroku Photo Co. Ltd., Tokyo) at 40°C for 2-3 sec, followed by thorough drying and exposure for 30 days at 4°C in a dark box containing silica gels. After photographic development and fixation, the slides were stained with 0.05% basic fuchsin as described by BERGERON[5] and mounted in Canada balsam. Only the cells with 5 or more silver grains in their nuclei were counted as actually labeled. About 250 cells

were counted from each sample to determine the percentage of DNA-synthesizing cells that were labeled with ^{3}H-thymidine.

X-ray irradiation

The cultured cells in 200 ml-flasks having 30 ml suspension medium were irradiated with 280 kV X-rays at a dose rate of 332 R/min using 1.0 mm aluminum filter. The X-ray dosages and the growth pahses of the cultures at a time of irradiation are described in conjunction with the individual experimental results. The irradiated cells (150 mg of fresh weight) were transferred to freshly prepared medium and then cultured for 35 days under the same condition as described above. Radiosensitivity of the callus cells was estimated by measuring the SCV at 35 days after irradiation. The experiment was repeated once and in each treatment the results are based on the average of them.

Results

Growth pattern during a culture period

Induced callus tissues from root-tips exhibited wide variations both in their colors and in degree of their friability among the test-tubes used for callus induction. From these tissues, whitish and friable cell aggregates which is referred to as SC-cell line hereafter were selected and subsequently subcultured in suspension batch culture for about 2 years. The SC-cell line was characterized by its considerably homogeneous cell population with more than 90% diploid cells. The growth of the cell line was monitored by the SCV measurements throughout subcultures, as shown in Table 1. Figure 2 also shows the growth pattern from initial inoculation into fresh medium to stationary phase in 5th to 20th subcultures. These data clearly indicates that the cell line is kept well in a stable growth even by long term subcultures without any decline of vigor, and that the growht pattern follows the typical sigmoidal curve in each subculture, which is best fitted to the following logistic formula,

$$N_t = K/1+e^{(a-mt)} \quad \cdots(1)$$

where N_t=the SCV at a given time (t; day) of the culture,
K=the SCV at the stationary phase,
a=constant coefficience at the initial culture (t=0), and
m=specific growth rate in the culture.

The parameters in each culture calculated by linear regression analysis are also shown in Table 2. The growth phases of the SC-cell line in batch suspension culture were divided as follows under the conditions adopted in the present experiment; lag phase was a period from the initial to 2 days, log phase from 2 to 30 days and stationary phase from 30 to 35 days. Doubling time (t_d) of the cell population during the log phase was 3.3 days, which was calculated from the following equation using an average value of m shown in Table 2;

$$t_d = \ln 2/m \quad \cdots(2)$$

Three different indices of cell growth, i.e., the SCV, fresh weight and dry weight, were measured during a culture period in the cells of the 23rd subculture, as shown in Fig. 3. As plotted in Fig. 4, the SCV was closely correlated to the fresh weight (r=0.90) and dry weight (r=0.87). Therefore, the SCV measurement seems to be valid method for growth monitoring in cultured cells of plants.

Respiration activity and frequency of ^{3}H-labeled cells

As shwon in Fig. 5, respiration activity measured by oxygen uptake increased rapidly during the initial stages of culture to reach the highest level approximately four times of that observed at culture initiation.

Table 1. Cell growth measured by the sedimented cell volume (ml) in the 4th to 20th subcultures.

Number of subculture	Time after inoculation (Days) 5	10	20	30	35
4	0.6±0.3	1.8±0.5	2.5±0.3	2.9±0.4	n.m.*
5	0.7±0.2	1.6±0.3	2.1±0.4	2.6±0.3	n.m.
6	0.5±0.2	1.5±0.4	2.4±0.3	2.6±0.3	n.m.
7	0.6±0.1	1.7±0.2	2.5±0.3	2.7±0.3	n.m.
8	0.5±0.1	1.4±0.3	2.2±0.2	2.5±0.2	2.6±0.2
9	0.5±0.1	1.4±0.4	2.3±0.3	2.6±0.3	2.7±0.3
10	0.5±0.2	1.5±0.4	2.2±0.3	2.5±0.3	2.6±0.3
11	0.6±0.2	1.8±0.4	2.5±0.3	2.7±0.1	2.9±0.1
12	0.6±0.1	1.4±0.2	2.2±0.2	2.5±0.3	2.7±0.2
13	0.6±0.2	1.7±0.3	2.2±0.4	2.7±0.4	2.8±0.3
14	0.5±0.1	1.2±0.1	2.1±0.3	2.5±0.2	2.6±0.3
15	0.6±0.2	1.4±0.3	2.4±0.3	2.7±0.2	2.8±0.1
16	0.6±0.2	1.3±0.2	2.2±0.4	2.5±0.2	2.6±0.2
17	0.5±0.1	1.4±0.4	2.4±0.2	2.7±0.1	2.7±0.1
18	0.5±0.1	1.3±0.2	2.1±0.2	2.5±0.2	2.5±0.2
19	0.4±0.1	1.3±0.2	2.1±0.3	2.5±0.3	2.6±0.3
20	0.6±0.2	1.5±0.5	2.4±0.3	2.7±0.4	2.8±0.3

* n.m.; not measured.

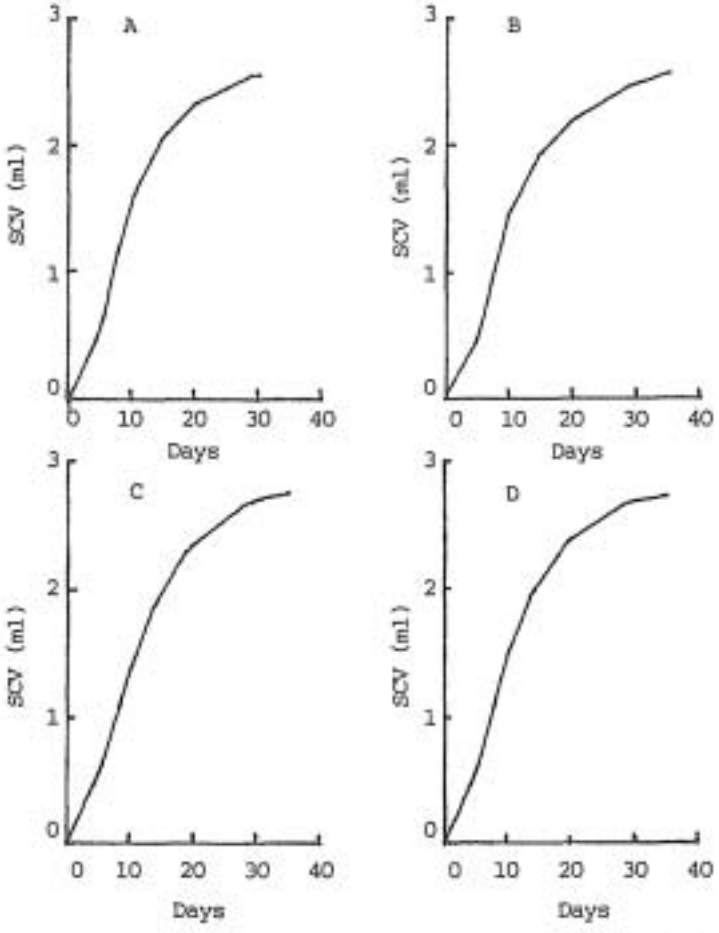

Fig. 2. Growth patterns of callus cells measured by the SCV in 5th(A), 10th(B), 15th(C) and 20th(D) subcultures.

Table 2. Parameters of the logistic formula in the 4th to 23rd subcultures of callus cells.

Number of subculture	Parameters a	m	k
4	2.014	0.2007	2.9
5	1.481	0.1529	2.6
6	2.522	0.2551	2.6
7	2.249	0.2444	2.7
8	2.261	0.2180	2.5
9	2.376	0.2253	2.6
10	2.227	0.2171	2.5
11	2.169	0.2421	2.7
12	1.885	0.1842	2.5
13	1.956	0.1764	2.7
14	2.255	0.1986	2.5
15	2.255	0.2190	2.7
16	2.108	0.2070	2.5
17	2.484	0.2321	2.7
18	2.175	0.1964	2.5
19	2.446	0.2120	2.5
20	2.181	0.2169	2.7
21	2.294	0.2288	2.5
22	2.194	0.1996	2.6
23	2.168	0.2000	2.8
mean	2.185	0.2113	2.6

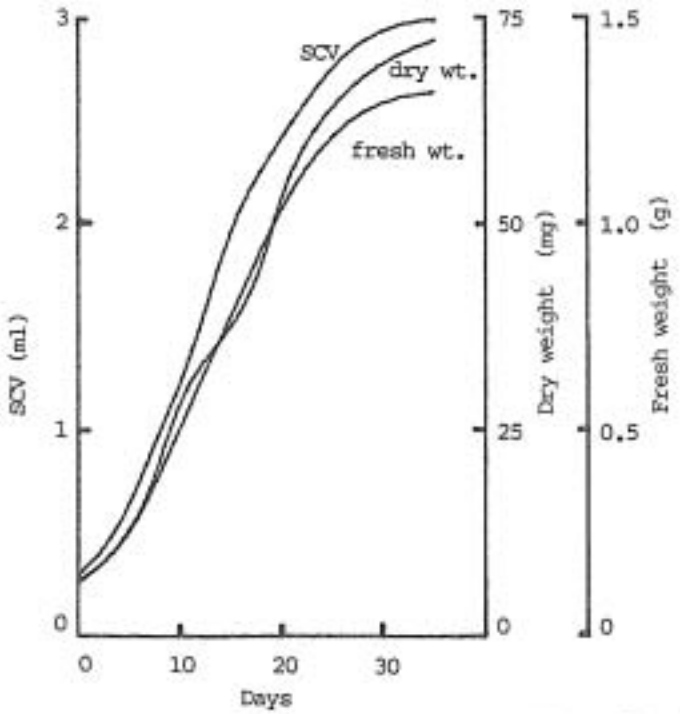

Fig. 3. Growth patterns of callus cells measured by the SCV, fresh and dry weight.

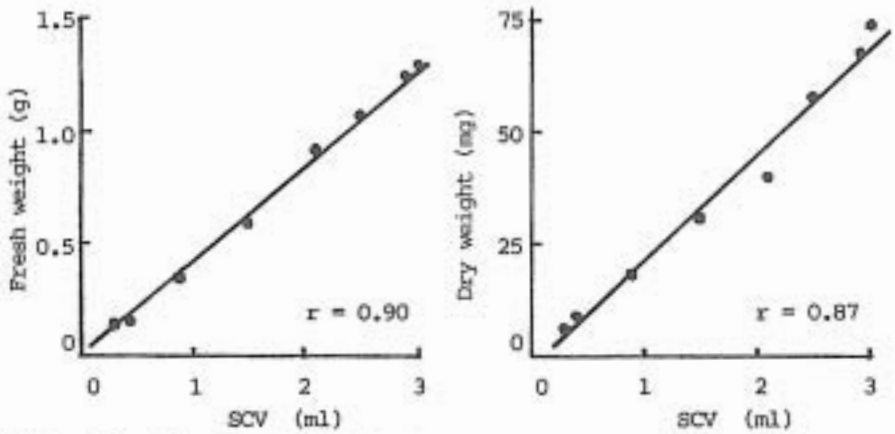

Fig. 4. Correlation of the SCV with fresh weight and dry weight in 23rd subcultured cells.

The peak activity at 7 days after the initiation of culture was followed by a gradual declining to a lower level in stationary phase.

Table 3 shows frequencies of the DNA-synthesizing cells which incorporated ^{3}H-thymidine into their nuclei at different growth phases. The highest frequency (34.3%) was observed at 11 days after the initiation of culture, corresponding to the time of mid log phase. Lag and stationary phases exhibited the lower frequency of the labeled cells (5–7%).

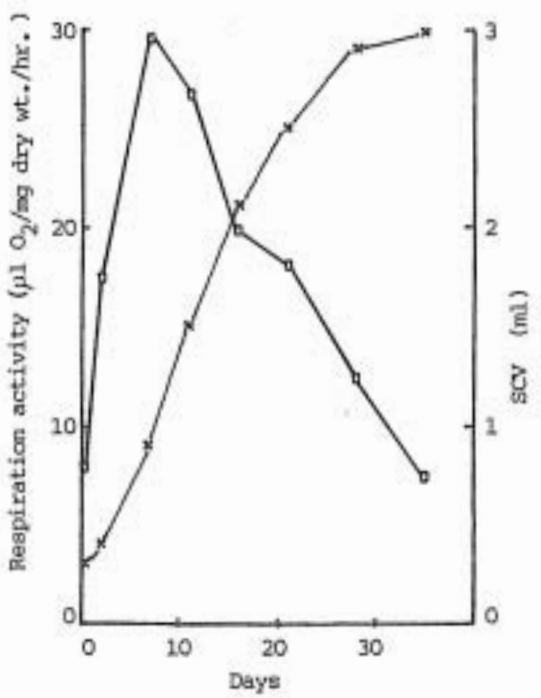

Fig. 5. Respiration activity (○——○) and cell growth (×——×) during a culture period in callus cells.

Table 3. Change in frequency of ^{3}H-thymidine labeled cells during a culture period.

Days after inoculation	2	7	11	16	25
Frequency of labeled cells (%)	7.4	17.6	34.3	24.4	5.2

Changes in radiosensitivities at different growth phases

The SC-cell line subcultured by 22 times was used as experimental material and exposed to X-ray dosages of 2.5 to 10 kR at six different growth phases, i.e., lag pase at 2 days, early log phase at 7 days, mid log phases at 11 and 16 days, late log phase at 25 days and stationary phase at 35 days after inoculation. The irradiated cells which were inoculated into fresh medium were monitored by the SCV measurements until

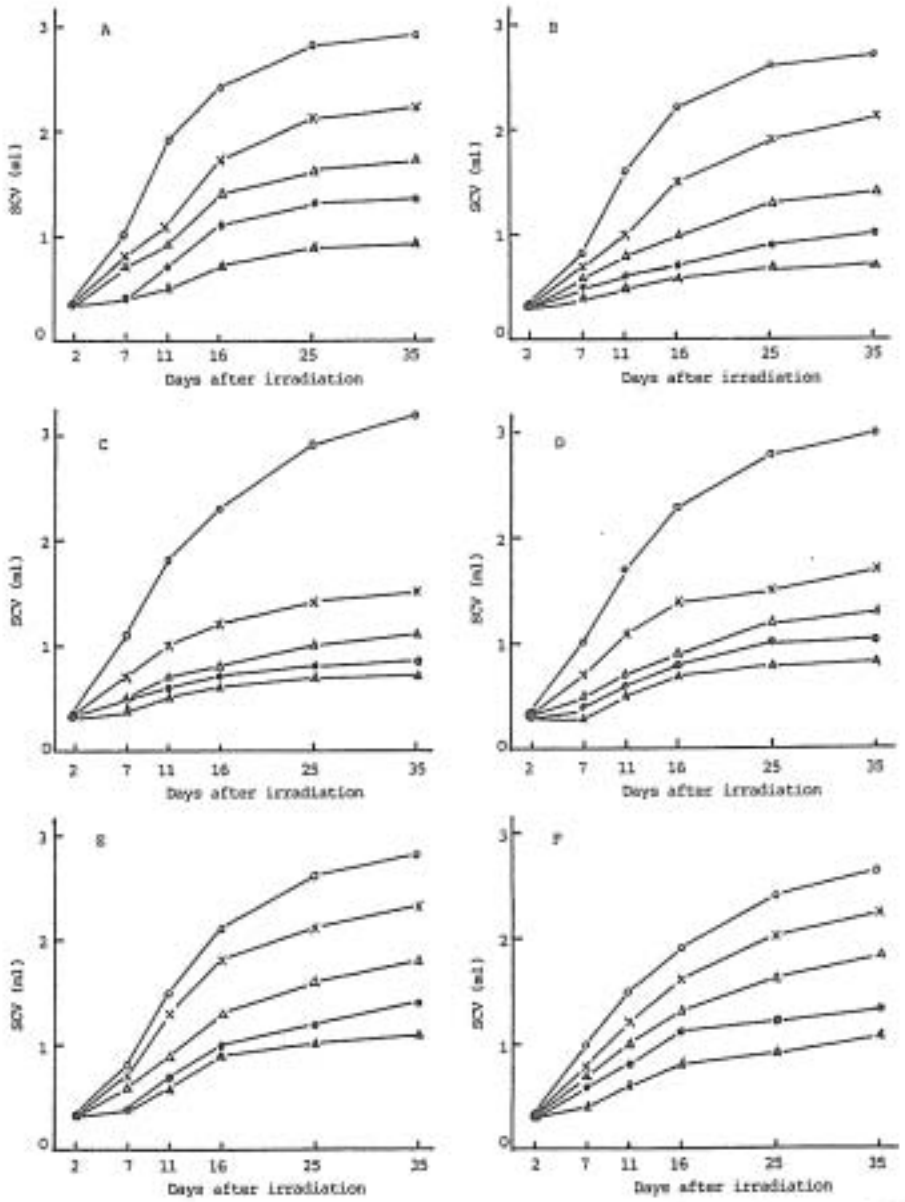

Fig. 6. Cell growth measured by the SCV after X-ray irradiations of 0 kR (Cont., ○), 2.5 kR (×), 5.0 kR (△), 7.5 kR (●) and 10 kR (▲) at 2 days (A), 7 days (B), 11 days (C), 16 days (D), 25 days (E) and 35 days (F) during a culture period.

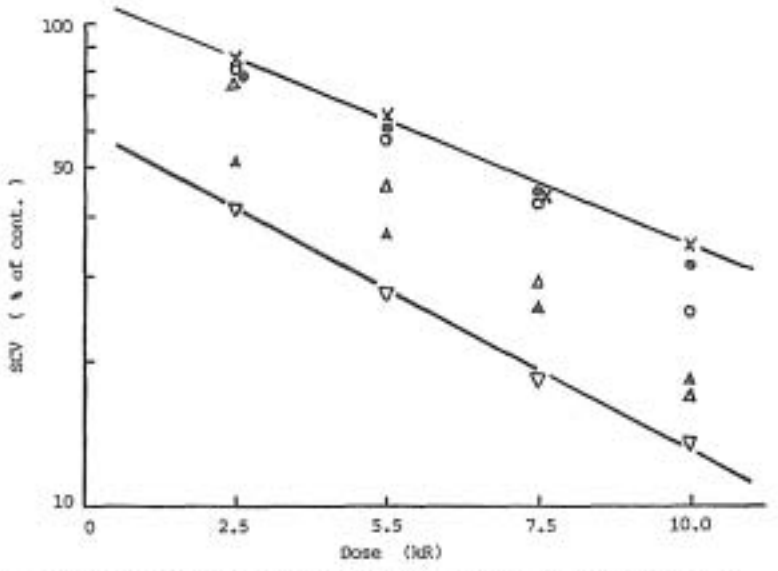

Fig. 7. Dose response of callus cells irradiated at different growth phases, i.e., at 2 days (○), 7 days (△), 11 days (▽), 16 days (▲), 25 days (●) and 35 days (×) after the initiation of culture.

Table 4. Radiosensitivity coefficient (k), constant (m) and D_{50} value at different growth phases of the 22nd subcultured cells.

Irradiated growth phase (days)	k	m	D_{50} (kR)
2	0.1420	1.207	5.83
7	0.1898	1.242	4.47
11	0.1640	0.586	2.23
16	0.1501	0.739	3.31
25	0.1173	1.095	6.46
35	0.1130	1.132	6.91

stationary phase, as illustrated in Fig. 6. From this figure, it is clearly demonstrated that growth rates of the cultures decrease with increasing dosages of X-ray in all growth phases at the time of irradiation. Exposure at mid log phases, however, brought about more prominent reduction of cell growth than those at the other growth phases. For comparison of radiosensitivities at different growth phases, the SCVs at 35 days after irradiation which are expressed as percent of control were plotted on semilogarithmic scale (Fig. 7) in which dose-response curve is well fitted to the following equation;

$$R=1-(1-e^{-kD})^m \qquad \cdots(3)$$

where R=the SCV (% of control),
k=radiosensitivity coefficient,
D=irradiated dose (kR), and
m=constant.

From the equation (3), D_{50} (dose required to reduce the SCV to 50% of control) is calculated as follows;

$$D_{50}=\ln[1-(1-0.5)^{1/m}]/(-k) \qquad \cdots(4)$$

Calculated results of radiosensitivity coefficient (k), constant (m) and D_{37} for each of growth phases are given in Table 4. Based on comparison of D_{37} values, it is clearly noticed that there exists the conspicuous difference of radiosensitivity in growth phases of cultured cells and that mid log phase irradiated at 11th day is most sensitive, i.e., approximately three times as sensitive as stationary phase at 35th day after inoculation.

Discussion

For measurement of growth in cultured cells of plants, many indices have been utilized, e.g., fresh and dry weigth, cell number per unit volume of suspension culture, turbidity of liquid medium and packed cell volume by centrifugation of cultured cells. Methods of these measurements, however, depend upon a sampling manipulation from whole cultured cells which are accompanied by slight disturbance of culture conditions and by if anything unavoidable risk of bacterial contaminations. In the present experiment, the SCV measurement was adopted as convenient and suitable method for suspension culture of plant cells to allow continuous monitoring of cell growth without any disturbance and contamination throughout a culture period. It seems to be valid that the SCV monitoring is appricable for growth measurement of cultured cells in place of above mentioned methods, because of high correlation of the SCV with fresh and dry weight (Fig. 4).

The SC-cell line used in the present experiment consisted of homogeneous cell population with almost all diploid cells and proliferated in accordance with the standard sigmoidal curve during a culture period. The cell line, however, required the considerably longer doubling-time in the log phase, 78.7 hr (3.3 days), when compared with other suspension cultures of plants, for example, 60–70 hr in *Acer pseudoplatanus*[14], 48 hr in *Nicotiana tabacum*[18], 36 hr in *Rosa* sp.[21], 24 hr in *Phaseolus vulgaris*[16] and *Lycopersicon esculentum*,[22] and 22 hr in *Haplopappus gracilis*[6].

The frequency of mitotic cells, in general, is very low in cultured cells of a plant as compared with meristematic tissues of an intact plant. Whereas, ^{3}H-thymidine incorporation into nuclei of cultured cells is easily detected by autoradiography with a high accuracy and closely correlates with mitotic activity. In the present experiment, O_2 uptake and percentage of ^{3}H-labeled cells were therefore examined during a culture period as indications of physiological and cytological activities, respectively. SHIMIZU et al.[25] reported in suspension culture of *Acer pseudoplatanus* that lag phase is characterized by intensive metabolic activity with high level of respiration activity and energy charge, followed by high frequency of cell division and decline of energy charge in log phase. Changes in respiration and cell proliferation activities observed in the present experiment agree with those reported in *Acer* [25]and *Lycopersicon*[22].

Figure 8 shows the changes in radiosensitivity of the SC-cell line during a culture period together with those in the SCV and in respiration activity and ^{3}H-thymidine labeled cells. This figure indicates that the higher frequency of ^{3}H-labeled cells is concomitant with the higher radiosensitivity, while the respiration activity does not directly affect the sensitivity. Furthermore, cells at mid log phase were the most sensitive to X-rays, stationary phase cells were the most resistant and lag phase cells were intermediate in radiosensitivity. Similar result was reported by TIPPINS and PARRY[27] in the mutant strain of yeast, i.e., that stationary phase cells were more resistant than exponential phase cells. They also suggested that stationary phase cell is in G_0 phase which is outside the normal mitotic cell cycle, rather than in early G_1. PINON[24] has shown that cells at stationary phase have a folded genome which is a distinct and characteristic structure as opposed to G_1 cells. The present experimental results, therefore, suggest that nuclear and/or cytological factors such as mitotic and DNA-synthetic activities greately take part in determination of the differential radiosensitivity at various growth phases of cultured cells in plants. MIKAELSEN[19] examined the physiological and metabolic activities during a germination period of barley seeds irradiated with fast neutrons, and showed that the reduction in seedling-growth, respiration and protein synthesis were caused by primary inhibitory effect of the radiation on DNA synthesis. The present suggestion also supports the MIKAELSEN's findings.

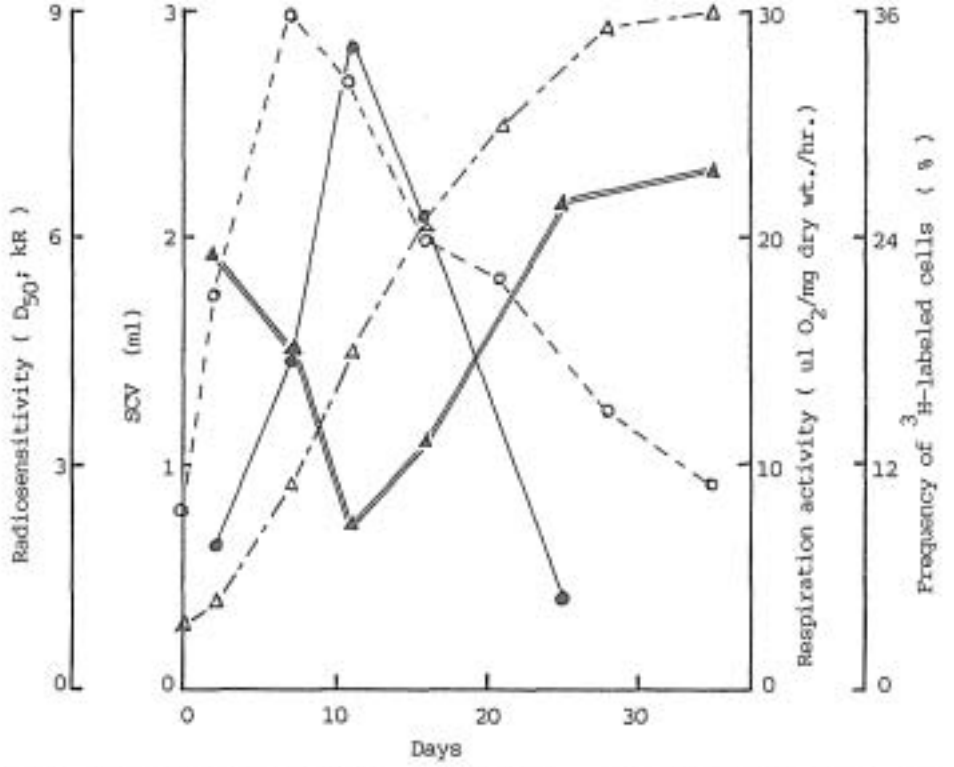

Fig. 8. Changes in radiosensitivity (▲═══▲), SCV (△----△), respiration activity (○----○) and ^{3}H-thymidine labeled cell frequency (●——●) during the culture period in subcultured cells.

Bajaj *et al.*[2] observed in *Phaseolus vulgaris* that callus cells cultured *in vitro* were more radioresistant than seeds and seedlings, i.e., D_{50} being 10 kR for callus cells, 8 kR for seeds and 3 kR for seedlings. Callus cells used for their experiment, however, exhibited various levels of polyploidy and were not defined as to growth phase at a time of gamma-irradiation. The present study reveals that D_{50}s in the SC-cell line are ranging from 2.2 kR to 6.9 kR depending upon the growth phase (Table 3), and not so greately differ from that in the seedlings (4.8 kR) but do from that in seeds (31.1 kR) which were examined by Kowyama and Shibata[15]. The radiosensitivity of the seedlings is mainly decided by radiation damage in the apical meristematic cells which consist of interphase cells in large part. Therefore, it is concluded that the cultured cells at stationary phase exhibit the same degree of radiosensitivity as cells in the apical meristem of seedling.

Summary

To examine the changes in physiological activity and radiosensitivity of cultured cells during a culture period, the long-term subcultured callus cells of the rice viriety "Sensho" which refer to as SC-cell line were used as experimental materials. The SC-cell line was characterized by considerably homogeneous diploid cell population and by sigmoidal growth pattern with a long doubling time (78.7 hr) in log phase.

In the SC-cell line, the mid log phase was the most sensitive to X-rays, the stationary phase the most resistant and the lag phase intermediate. Differential radiosensitivities in various growth phases coincided

with change in the frequency of ^{3}H-thymidine labeled cells, indicating that the higher frequency of DNA-synthesizing cells correlates with the higher radiosensitivity. Respiration activity measured by O_2 uptake did not directly affect the radiosensitivity. The cultured cells at stationary phase exhibited the same degree of radiosensitivity as the seedlings. The present results suggest that cytological factors such as the frequency of DNA-synthesizing cells and mitotic activity take part in determination of the radiosensitivities at different growth phases of cultured cells in higher plants.

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摘　　要

イネ培養細胞の各生育相における放射線感受性と細胞学的活性との関係

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イネ品種“戦捷”の根端から誘導し2年以上にわたって長期間液体培養をしたカルス細胞系統（SC-細胞系統）を用いて，培養期間中の種々の生育相における放射線感受性と細胞学的・生理学的活性の変化を調査した。供試したSC-細胞系統は比較的均一な2倍体細胞集団から成り，25回以上の継代培養においても旺盛な増殖を示す細胞系統であり，対数増殖期における増殖量倍化時間は78.7時間であった。

このSC-細胞系統において，2.5～10 kR のX線を照射した後，細胞増殖量によって放射線感受性を調べた結果，対数増殖中期の感受性が最も高く（D_{10}=2.2 kR），定常期は最も抵抗性であって（D_{10}=6.9 kR），約3倍の感受性の差異が認められた。さらに種々の生育相において，^{3}H-チミジンの細胞核内取り込み頻度をミクロオートラジオグラフィー法によって調査し，DNA 合成期細胞の頻度を推定した。またワールブルグ法により各生育相の呼吸活性を調べた。その結果，DNA 合成期細胞の頻度が高い生育相ほど放射線感受性が高いこと，および呼吸活性と感受性との間には直接的な関係は認められないことが明らかとなった。以上のことから，高等植物の培養細胞において細胞核のDNA 合成や細胞分裂などの細胞学的活性が放射線感受性の主な支配要因として作用していると考えられる。