An Isolator Method for Germfree Culture of Higher Plants and the Completion of Life Cycle of Radish

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An Isolator Method for Germfree Culture of Higher Plants and the Completion of Life Cycle of Radish

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Summary

Applying the available methods for germfree rearing of animals, the authors attempted to establish a new method for germfree culture of higher plants by use of plastic isolators.

The improved facilities consist of phytotrons with artificial lights and flexible isolators made of vinyl sheet for agricultural uses or rigid ones made of polymerized methyl meta-acrylate. A convinient device of membrane filtration is attached to the isolator for direct introduction of sterile water. Radish has been used in culture experiments. To secure the sterility of seedlings, sterilized seeds are individually germinated on the agar medium in a test tube and uncontaminated seedlings can be selected for transplanting.

By use of rigid isolators, radish was grown on a soil sterilized by γ-rays irradiation. The radish on the sterilized soil under germfree conditions attained full maturation similarly to that on the original soil, and the seeds thus obtained could yield the second generation of complete life cycle under germfree conditions.

The objects of germfree culture of higher plants are to investigate (a) mineral nutrition, i.e. the effects of microorganisms on the inorganic nutrition of higher plants and mineral deficiencies in plants free from pathogens which may attack the stunted plants, (b) absorption and metabolism of organic compounds as various growth regulators and of soil organic matters, (c) the role that root exudation plays in the physiology and life cycle of the plant itself and (d) synergetic or antagonistic associations between plants and microorganisms.

Various methods under sterile conditions are based on the use of small glass vessels such as flasks, cylinders and test tubes for growing the seedlings and some of the organs or the tissues. These methods involve limitations in connextion with space and ventilation in plant growth conditions. Therefore, it was difficult to grow plants during a long period of time by use of the above tools.

Since the flexible plastic isolator for the rearing and use of germfree animals has been devised by Trexler (1), gnotobiotic culture of animals has been a

productive field of research for several decades. However, examples of the application to germfree plants of plastic isolators have been very few (2, 3, 4, 5).

The present report describes mainly of the technical method for plant culture by use of plastic isolator and its application to germfree culture until full maturation.

Experimental and Results

I. *Facilities for Germfree Culture of Radish*

A. Initial facilities

The initial facilities for germfree culture of higher plants were designed in 1964 (6). They consisted of two parts (1) a large vinyl isolator as the culture chamber of aseptic plants (2) a phytotron equipped with a lamp house and automatic systems for regulating illumination and temperature.

In advance of germfree investigations, preliminary culture experiments were carried out to examine the efficiency of the equipment for plant growth.

Young seedlings of such Cruciferae as radish, rape and mustard exhibited severe chlorosis and malformation of their expanding leaves during the growth in the isolator. These symptoms developed less severely on soybean and hardly on rice seedlings (7). Through a set of cultural experiments, the cause of anomaly of radish leaves was found to be attributable to the harmful effects of the particular vinyl sheet utilized. Therefore, plasticizers presumed to be contained in the vinyl sheet were separately investigated for their effects on radish growth. Among the compounds examined di-isobutyl and di-n-butyl phthalates provoked anomaly for the growth of radish seedlings. Whereas di-(2-ethyl hexyl) phthalate, adipate and sebactate were shown to have no appreciable effect upon radish growth. It was later found that other types of vinyl sheet specified for gricultural use was satisfactorily usable as the material of isolator. Moreover, some modifications should be made on the illumination system and on the device for germfree air supply. So, the initial facilities have been improved as follows.

B. Improved facilities

Figure 1 diagrammatically shows a unit of vinyl isolator. The culture chamber, $60 \times 120 \times 60$ cm, is made of transparent vinyl film of 0.3 mm thickness for agricultural use. Two isolators can be accommodated in the renewed phytotron which has a lamp house with artificial lamps consisting of sixteen 400W mercury lamps, twenty-four 100 W incandescent bulbs and twenty-two 100W fluorescent lamps. Light intensity at plant height is approximately 1.5×10^4 Lux. Air temperature in the culture chamber is controlled at 25 ± 1°C with lights on 14 hours and 18 ± 1°C with lights off 10 hours. Relative humidity in the chamber with plants growing ranges from 75 to 85% without any control.

At present, two phytotrons with the same illumination system are used for the cultivation of germfree plants by use of isolators described above. Radish, Raphanus sativus, has been utilized most successfully in our experiments and the main procedures of its germfree culture are described.

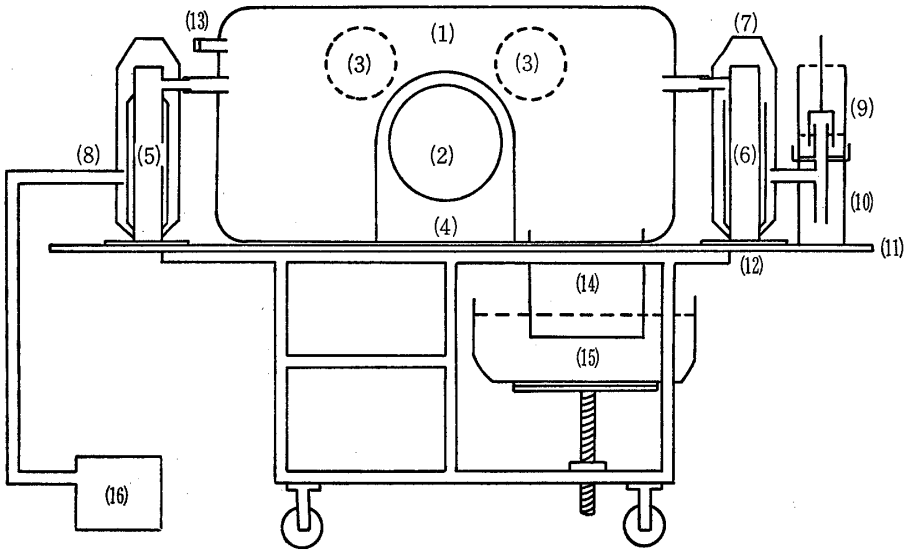

FIG. 1. Vinyl isolator for germfree culture of higher plants.
(1) Culture chamber; (2) Sterile lock; (3) Two rubber gloves; (4) Support frame of sterile lock; (5) Air inlet filter; (6) Air outlet filter; (7) Air retainer; (8) Air pipe; (9) Air exhaust trap; (10) Support rack of air exhaust trap; (11) Isolator set board; (12) Cart; (13) Connection to sterile water supply; (14) Germicidal trap; (15) Germicidal solution; (16) Blower

II. *Procedures of Germfree Cultue of Radish*

1) Sterilization of the isolators — An isolator is sterilized by spraying 2% peracetic acid (150–200 ml) and an hour later is flowed with filtered air for 24 hours. Alternatively and more conveniently the isolators can be sterilized by Epon 12 gas (12% ethylene oxide on 88% trifluromethane). The culture chamber is filled with the gas for 18 hours and then flashed by filtered air for 24 hours. This procedure allows sterilization of such materials as culture vessels and stainless goods in the chamber and also the filter systems of air and water. Autoclavable materials and equipments such as water, nutrient solution and tweezers are placed in a stainless steel cylinder sealed with mylar film and autoclaved for 15 minutes at 121°C (pressure, 1.1–1.2 kg/cm^2). Prior to this treatment, it is good for a vacant cylinder to be autoclaved for 10 minutes at 121°C. After autoclaving, the sterilized cylinder cooled to room temperature is connected to the sterile lock by a rubber sleeve. The connecting sleeve and entry port are sprayed with 2% peracetic acid. After an hour the seal on the cylinder is broken and the materials are introduced into the isolator. All the manipulations inside the isolator are done throughout glove ports on the other side of the isolator.

2) Checking for microorganisms contamination — The sterility of the isolators and growing plants is confirmed by the following method. The floor and wall of the vinyl chamber are mopped up by a piece of cotton and the cotton is rubbed over the testing agar media commonly utilized in microbiology. These media are fluid of thioglycolate (bacteriological use, Nissui), malt extract (agar powder for fungi, Nissui) and plate count agar medium containing brom cresol purple (bacteriological use as lactic acid, Nissui). Culture solution, soil and macerates of plant tissue under investigation are also placed on the agar media. Petri dishes containig the media are taken out through germicidal trap and incubated at 30°C and 37°C for 2 weeks to examine any contamination by bacteria or fungi.

3) Seed sterilization — Radish seeds are successively soaked in 70% ethanol for 5 minutes, in 10% calcium hypochlorite for 20 minutes and in 200-fold diluted alkyldimethyl benzyl ammonium chloride for 5 minutes. Following sterilization the seeds are transported into the isolator through the germicidal trap and rinsed four times with sterile distilled water. Washed seeds are sown on the salan net floating on water in a large petri dish. Seven days after sowing, uniform seedlings are utilized for cultural experiments. The method was quite adequate with the seeds in a can named Comet which was purchased in 1968. We can say this was fortunate. With other lots of seed purchased later, this simple method could not satisfy to secure the perfect sterility of all seedlings treated in the same way. Therefore, additional method for obtaining germfree seedlings was inevitably required.

4) Selection of germfree seedlings – Seed sterilization and the selection of germfree seedlings are the most important and difficult steps in the procedure. It is no exaggeration to say that the success of germfree culture of plants depends on clean seeds free from microorganisms. In the case of purchased seeds in general, individual seeds must be examined for their sterility during germination. The method is as follows. After radish seeds are treated with sterilants as above described, they are introduced into the isolator and washed four times with sterile distilled water. Usually, 140 seeds are separately sown in each test tube (3×10 cm). Each tube contains 20 ml of one-third concentration of BCP agar medium whose composition is in g per liter, yeast extract 2.5, peptone 5.0, glucose 1.0, Tween-80 1.0, L-cysteine 0.1, brom cresol purple 0.06, and agar 15.0, pH 6.8–7.0. The concentration of agar, 0.5% has been found to be most suitable both for root growth and for checking microorganisms (8). After the test tubes are capped with aluminium foil, the seeds are allowed to germinate at 25°C for three days in the dark. Contaminated seedlings are distinguished by detectable colonies of microorganisms on the agar medium. Aluminium caps of the test tubes free from microorganisms are removed and the seedlings are further grown for four days under light conditions as above described. In this manner, seedlings

can be selected for uniformity and used for transplanting in further experiments (8). This method is also adaptable to obtain germfree rice seedlings.

5) Direct introduction of sterile water by filtration — A large quantity of sterile water is indispensable for germfree culture of higher plants in the isolator. Sterile water can be obtained by use of autoclaving, but it takes long time and much labor to put autoclaved water into the isolator. In order to solve this problem, a convenient method which allows direct introduction of membrane-filtered water into the culture chamber of the isolator has been investigated (9). As Plate 1 shows, the isolator is connected with a device consisting of a filter holder, a pressure vessel (20 liter) and an air compressor. The filter holder is loaded with a piece of Millipore membrane GSWP, pore size 220 nm and 47 mm in diameter. The filter system is sterilized at the same time when the isolator is treated with Epon 12 gas as above mentioned. Distilled water previously filtered through another unsterilized membrane (pore size 220 nm) is pooled into the pressure vessel and is passed the filter system at a pressure of about 4 atm into containers in the isolator. It requires approximately 40 minutes to obtain 15 *l* of sterile water.

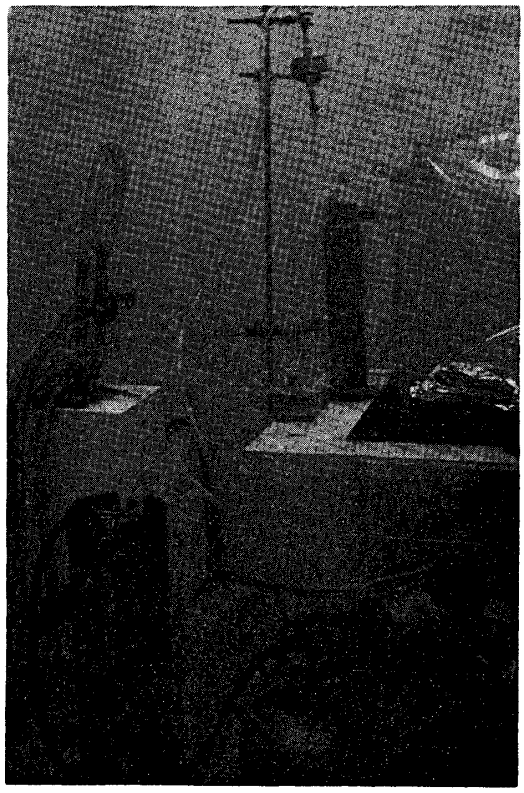

PLATE 1. Apparatus for obtaining sterile water by Millipore membrane filtration.

III *Completion of two life cycles of radish under gnotobiotic conditons*

As an application of the method described above, germfree culture of radish was attempted to verify if the completion of its life cycle could normally be

attained for two generations under gnotobiotic conditions. For this purpose, cultivation on a sterilized soil in rigid isolators was employed to facilitate the long-term experiments.

1) Sterilization of soil by γ-rays irradiation

An alluvial soil of paddy field near Sendai was utilized in pot culture of radish. One kg of air-dried soil was packed in polyethylene bag and wrapped with three layers of aluminium foil. After incubation at 30°C for two days the soil was irradiated with γ-rays by use of Electron Linac at the laboratory of Nuclear Science, Tohoku University. Total dose of absorbed γ-rays with the soil was approximately 5 Mrad. The soil was transported into a small vinyl isolator ($60\times60\times60$ cm) through its germicidal trap. After the confirmation of its sterility, the sterilized soil was again transported into a rigid plastic isolator ($52\times120\times75$ cm) made of polymer of methyl meta-acrylate. As a comparison, sterilized soil was also prepared by autoclaving. Table 1 indicates the effects on soil properties of sterilization by γ-rays irradiation or by autoclaving. It is evident that some changes occurred but the soil irradiated by γ-rays was affected far less than the autoclaved soil, as for soil acidity in particular (10). Previous experiments clearly demonstrated better growth of radish seedlings in the former than in the latter (11). Therefore, sterilized soil treated with γ-rays was utilized in the following cultivations of germfree radish.

TABLE 1. *Physical and Chemical Properties of Original Soil and Sterilized Soils.*

Soil properties	Original soil	Sterilized Soil	
		Irradiation by γ-rays	Autoclaving
Water (%)	5.4	6.2	3.4
T-C (%)	1.55	1.46	1.30
T-N (%)	0.19	0.20	0.19
C/N	8.2	7.5	6.8
pH(H_2O)	5.2	5.6	4.9
Max. Water Capacity (%)	63	56	53
Exchange acidity (Y_1)	0.22	0.21	1.46

2) Germfree cultivation of radish for two generations — The rigid plastic isolator was sterilized by 2% peracetic acid instead of Epon 12 gas. Materials and equipments were sterilized by an autoclave and introduced into a rigid isolator after the same method as with the vinyl isolator. Two of germfree radish seedlings were transplanted on each pot containing 200 g of soil sterilized by γ-rays, and 12 pots were prepared. Nutrients were supplied as solutions (Table 2), and 10 to 20 ml of sterile water were supplied every day to each pot. The cultural conditions of the

phytotron were same as described above. At the same time, a conventional radish culture as the control was undertaken in another rigid isolator. In this case, the isolator was not sterilized and the original soil was used. Other procedures were the same as in the germfree culture.

The schedule for cultivating the first generation is shown in Table 2. Some of the radish seedlings transplanted on the sterilized soil exhibited temporary retardation in their growth and a few died at the early stage of cultivation most probably due to poor water penetration and a high level of available manganese in the soil resulted from γ-rays irradiation (12). However, most of the seedlings soon recovered their growth thereafter. The absence of microbial contamination through the germfree culture was confimed every two weeks. After four months of cultivation, the plants attained full maturation both under the germfree and the conventional conditions. Though there existed some variations in their individual plant sizes, no appreciable difference in their growth patterns including

TABLE 2. *Cultivation of Radish on a Soil Sterilized by γ-Rays under Germfree Conditions.*

		1973
March,	22	Seeds individually sown on agar medium in test tubes.
	29	Two germfree seedlings were transplanted into a pot.
April,	11	NH_4NO_3 (N 20 mg) in solution was supplied to each pot.
	12	Na_2HPO_4 (P_2O_5 20 mg) was supplied likewise.
	13	KCl(K_2O 20 mg) was supplied likewise.
May,	11	One seedling was thinned out per pot.
	20	Bolting began.
	25	Artifiical pollination was operated by hand at flowering.
June, at the middle;		Fruting began.
July, at the end;		Plants were fully matured.

flowering and fruiting was observed between the germfree plants and the control ones. The seeds obtained from the former were inspected for their sterility by placing them on BCP agar medium above mentioned. All of 30 seeds examined were confirmed to be free from bacteria and fungi and 28 of them normally germinated. Then the seeds were used for consecutive germfree culture for the second generation which attained full maturation at the middle of December. Radish seeds thus yielded were again free from microorganisms and 32 seeds out of 35 examined could germinate without any anomaly. Plate 2 shows the flowering and fruiting of radish under the first gnotobiotic conditions by use of a rigid isolator (10).

General Discussion

The report of Lindsey (2) was the first to indicate that plastic film isolators have great utility in growing higher plants under gnotobiotic conditions. Previous

methods for growing sterile plants consisted chiefly in techniques employing confining glass vessels or apparatus which sealed only root system (13, 14, 15, 16). These methods are still of much use for plant growth in a short term experiment under aseptic conditions.

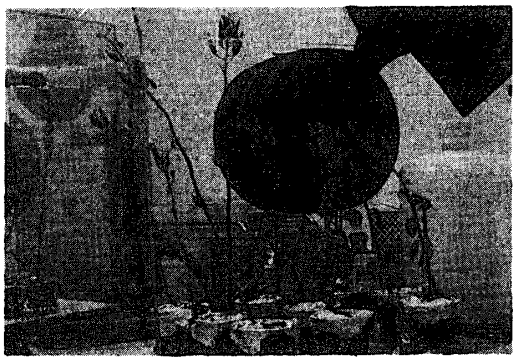

PLATE 2. Fruiting of germfree radish in a rigid isolator.

In order to investigate the physiology and nutrition of germfee higher plants, it is desirable to develop a technique which enables a prolonged aseptic culture of entire plant in a large scale. In our laboratory, the initial facilities for growing germfree plants were designed after the vinyl isolator system for germfree rearing of animals, especially of rats by Tanami and his associates (17). The vinyl sheet utilized, however, brought about harmful effects on radish growth because of some sorts of plasticizer contained in it. Modifications on the initial facilities were undertaken as for the kind of vinyl sheet and the light sources of phytotron (18). At present vinyl isolators ($60\times120\times60$ cm) made of vinyl sheet for agricultural uses and of rigid plastic are rountinely utilized for cultivation of germfree plants. Smaller isolators ($60\times60\times60$ cm) are also employed, when necessary, for selecting germfree seedlings or confirming the sterility of soils. In view of the available space and ventilation efficiency, the isolator method seems to have more advatntageous than the techniques employing glass vessels.

Seed sterilization and selection of seedlings free from microorganisms are most important for the cultivation of germfree higher plants. It sometimes appeared that seed sterilization alone was not sufficient to secure the complete sterility of all seedlings after germination. This seemed to be the case particularly with the seeds obtained and stored under humid climate as in Japan, and we realized the necessity to inspect individual seeds against microbial contamination, especially with fungi. It might be said that the surest way to succeed in germfree culture of higher plants would depend on how to obtain clean seeds free from microorganisms. To overcome these limitations, we introduced an additional step of seedlings selection as described above. Uncontaminated seedlings could be selected by germinating each sterilized seed on BCP agar medium in a test tube and by visual inspection of the absence of microbial colonies on the agar.

The procedure of obtaining a large quantity of water into the isolator is a hard task as well as an essential step in germfree culture of plants, especially in hydroculture. This was solved by attaching a membrane filter system to the isolator. The system is of much use not only to facilitate direct introduction of sterile water but to save time and labor. Germfree plants have been used to investigate the relation between microorganisms and the inorganic nutrition of higher plants. Barber (15) reported that the uptake of phosphate in clover and tomato are attributable in part to microbial activity. Asanuma (16) revealed the effects of soil microorganisms on the absorption of nutrient elements especially of nitrogen and phosphate by rice seedlings. Both experiments were carried out under sterile conditions in glass vessels.

To study the effects of soil microorganisms on plant growth over a long period of time, isolator method is far better than the method by glass vessels. Lindsey (2) showed that bean grew well for two consecutive generations both in the absence and the presence of microorganisms. Hale (3) demonstrated the completion of life cycle of peanut under germfree conditions. The culture techniques used by them were sand culture with Knop solution or hydroculture with Hoagland solution. There are only two reports about the perfect life cycle of germfree plants.

Applying the improved facilities, the authors attempted to cultivate germfree radish till its full maturation. Rigid isolators and sterilized soil by γ-rays irraditon were used in the experiment. Evidences have been presented that a soil irradiated by γ-rays is better than autoclaved soil when radish growth on two treated soils was compared (11, 12). Rovira and Bowen (19) emphasized that phytotoxic conditions may exist in the soil following steam sterilization. It was shown that the soil treated with γ-rays was less influenced than the autoclaved soil as for its physical and chemical properties (Table 1). The growth of radish on the sterilized soil under germfree conditions was almost comparable with that on the untreated soil under conventional conditions. The only difference observed with the former was a temporary growth inhibition of young radish probably due to an increased level of exchangeable manganese in the sterilized soil (12). The germfree radish attained to full maturation and the seeds thus obtained could yield the second generation for its perfect cycle. The fact that such plants as beans, tomato, peanut and radish are able to complete their life cycle under germfree environments does not mean that microorganisms are simply unnecessary to plant growth. The problem whether soil microorganisms have a detrimental, beneficial or neutral effect on plant growth remains to be solved by use of germfree plant culture. The isolator method described here seems to be an advantageous tool to elucidate the relationship between plant and microorganisms (20).

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