Clonal propagation and quick detection of virus-free plants of sweet potato, *Ipomoea batatas*

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Sweet potato feathery mottle virus（SPFMV）viral infection of tuber surface of edible sweat potato, *Ipomoea batatas* causes chapped and cracked band symptom called russet crack-like symptom（RC-LS）, and the RC-LS depreciate commercial value and has become a severe problem in agricultural production. This project was undertaken to establish large quantities of virus-free sweet potato from the shoot apex and suckers of the plant using clonal propagation, and to develop a rapid and precise detection method using reverse transcription-polymerase chain reaction（RT-PCR）. The culture of shoot apexes less than 0.3-1.0 mm in size brought plant regenerating rates of 82% and 65% using Komamine and Nomura（1998）medium and Murashige and Skoog（1962）medium, respectively. The results of RT-PCR of RNAs from plants obtained from shoot apex culture and plants of SPFMV sufferer showed that SPFMV virus was clearly removed by shoot apex culture conducted in this study. For clonal propagation, 80-100% of suckers from stems of the virus-free plants detected grew into complete plants after 6 weeks of culture, indicating that the virus-free plants could be routinely propagated 5 times by the short circle. The sweet potato produced in field show no symptom of RC-LS even after two seasons.

Key words: *Ipomoea batatas*（L.）Lam., clonal propagation, reverse transcription-polymerase chain reaction, shoot apex culture, sucker culture.

INTRODUCTION

Sweet potato is one of the important staple foods and cash crop in southern farming region in Japan and cultivated as edible or materials from which the shochu, a clear liquor is distilled. Commercial value of edible sweet potato attached not only to its taste but also to its visual appeal. However, russet crack-like symptom（RC-LS）with chapped and cracked band started to appear on Japanese sweet potato since mid 1970. The RC-LS depreciated commercial value and thus it become a sever problem in agriculture production.

The causative agent was reported to be a *Sweet potato feathery mottle virus*（SPFMV）-S, genus *Potyvirus*（Usugi et al. 1994）of which there are various strains exist in the world（Abad et al. 1992; Usugi et al. 1991）. So far, three strains of SPFMV have been reported in Japan as sever（SPFMV-S）(Usugi et al. 1994), ordinary（SPFMV-O）(Mori et al. 1994）and Tokunoshima（SPFMV-T）(Usugi et al. 1993). Although these strains cause a variety of the various damages on sweet potato, the degree of damage has only been elucidated for SPFMV-S（Nagata et al. 2001）.

The RC-LS occurs recurrent RC-LS due to high rate of virus in stocks. In case of not sever infection, the surface color fades with somewhat degree of horizontal stripes, and the RC-LS can't be detected when tuber surface is smeared with soil during harvest. And the RC-LS usually is observed in tuber surface, but not in the inside of tuber. The RC-LS in stems and leaves can't be recognized and does not inhibit the growth of sweet potato（Nagata 1990）.

To produce virus-free plants, some attempts of shoot apex culture have been carried out using Murashige and Skoog（MS）medium（Nagata 1990, 1991）. However, problems such as low recovery rates of virus-free plants from shoot apex culture, are needed to be addressed. For virus detection, 1）method of naked eye viewing takes a long time to distinguish appearance of RC-LS on the tuber surface, sometime tuber harvested in the first sea-

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The abbreviations used are: BAP, 6-Benzylaminopurine; KN, Komanine & Nomura（1998）; MS, Murashige and Skoog（1962）; NAA, 1-Naphthylacetic acid; RC-LS, russet crack-like symptom; RT-PCR, Reverse transcription-polymerase chain reaction; SPFMV, Sweet potato feathery mottle virus

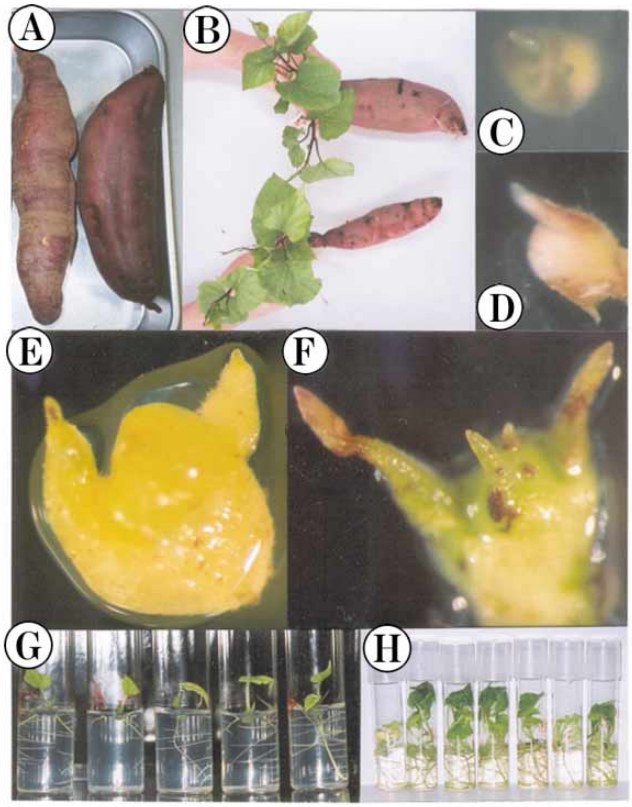

Fig. 1. Recovery of sweet potato feathery mottle virus (SPFMV)-free sweet potato using cultures of shoot apex. A) Sweet potato with russet crack-like symptom (RC-LS) (left) and healthy one (right) used in this study; B) New plants growing from healthy (right) and RC-LS (left); C) Shoot apex 0.1-0.3 mm in size token from RC-LS plant and cultured on Komamine & Nomura (1998) medium supplemented with 0.05 μM NAA and 4.44 μM BAP; D), E), F), G) and H) Growing of shoot apex after 3, 6, 9, 12 and 16 weeks of culture, respectively.

son does not show RC-LS even the virus exits there, it will surface in 2nd season; 2) observation of string-like virus particle using transmission electronic microscopy requires a lot of work to prepare material for observation and costly equipment and expertise. Recently, as a PCR based molecular biological method were used to detect virus, for example, reverse transcription-PCR (RT-PCR) (Nagata et al. 2001).

The objectives of this study were to establish an efficient culture system of virus-free sweet potato using shoot apex culture, to rapidly and precisely detect the presence of virus in plants by RT-PCR, and to ultimately develop a clonal propagation system in large quantities using culture of suckers from the tested virus-free plants.

MATERIALS AND METHODS

Plant materials. Sweet potato, *Ipomoea batatas* (L.) lam. cv. "Miyazakibeni", mainly cultivated in southern Kyushu in Japan was provided kindly from Horticulture branch, The Miyazaki Agricultural Experimental Station (Miyakonojo, Japan) with tubers of SPFMV infected and healthy ones (Fig. 1A). The tubers were planted in vermiculite sterilized under 120℃, 15 min. and cultivated in a growth chamber of 25℃, 3.3 μmol m^{-2} s^{-1} on 16 h photoperiod. Buds of infected and virus free tubers were visually indistinguishable (Fig. 1B).

Table 1. Effects of sodium hypochlorite solution (SHS) on shoot apex culture of sweet potato (15 min of sterilization, observed after 3 weeks of culture)*

SHS %	No. shoot apex plated	No. surviving of shoot apex	Surviving rates %
0.1	60	48±1.5	80
0.2	60	52±1.2	90
0.3	60	60±1.2	100
0.4	60	60±0.5	100
0.5	60	60±0.7	100
0.6	60	60±0.3	100
0.7	60	60±0.3	100
0.8	60	60±0.7	100
0.9	60	56±1.2	93
1.0	60	48±1.8	80
1.1	60	48±1.5	80
1.2	60	36±2.0	60
1.3	60	24±1.5	40
1.4	60	6±1.8	10
1.5	60	6±2.0	10

* Data shown represent means±SD derived from four experiments.

Shoot apex culture. The young runner including growing point (meristem) and some leaves were cut, treated with 0.1-2.0% concentration of sodium hypochlorite solution in 5, 10, 15 and 20 min, respectively, and washed with sterilized water. Then, shoot apex including growing point and one small leaf with 0.1-0.3 mm in size was taken out, with the aid of a dissecting microscope, and cultured on MS (Murashige and Skoog 1962) medium supplemented with 0.05 μM 1-naphthaleneacetic acid (NAA) and 4.44 μM 6-benzylaminopurine (BAP) and KN (Komamine and Nomura 1998) medium supplemented with 0.05 μM NAA and 4.44 μM BAP (Table 2) in a growth chamber at 25℃, 3.3 μmol m^{-2} s^{-1} on 16 h photoperiod. After 3 months of culture, the recovery rates of plants were investigated.

Acclimatization of recovery plants. The recovery plants from shoot apex were taken out from experimental tubes, and acclimated with one of the following 3 methods. 1) Directly transplanted into pot of sterilized soil; 2) Directly transplanted into pot of sterilized vermiculite; 3) Directly transplanted into sterilized vermiculite of pot sealed up with vinyl bag. After transplanting, the pots were cultured in a growth chamber at 25℃, 3.3 μmol m^{-2} s^{-1} on 16 h photoperiod.

Detections of recovered virus-free plants by RT-PCR. The RT-PCR is performed using the following protocol. Mature leaves (500 mg), were used to isolate total RNA of infected plants or recovery plants of "Miyazakibeni", respectively, following the manufacture's protocol of ISOGEN RNA extraction kit (Nippon Gene, Toyama, Japan). The concentrations of RNAs were checked by electrophoresis on 2% agarose gels.

Primer C1 (upstream): GGACAAGCCCCATCAAATG

Table 2. Effects of sterilizing time (sodium hypochlorite solution = 0.3%) on shoot apex culture of sweet potato (observed after 3 weeks of culture)*

Sterilizing time (min.)	No. shoot apex plated	No. surviving of shoot apex	Surviving rates %
5	60	36±1.8	60
10	60	60±0.3	100
15	72	72±1.2	100
20	78	72±2.0	92.3

*Data shown present means±SD derived from four experiments.

(bases 1024 to 1043) and primer C2 (downstream): CTGGAATGGTTGCGGGTTGC (bases 1265 to 1284) (Mori et al. 1994, 1995) were used for RT-PCR of SPFMV-S and SPFMV-O RNA as the nucleotide sequence in this region is well conserved between the two strains.

RT-PCR was performed with DNA Engine PTC 200 (MJ Japan). Takara RNA LA PCR™ Kit (AWV) Ver. 1.1 (Takara, Japan) was used for RT-PCR reaction. RNAs of 150 ng were used in infected and recovery plants, respectively. RT-PCR reactions were performed according to the manufacture's protocol. RT-PCR products were subjected to electrophoresis on 2% agarose gels for visualization.

Propagation of the recovery plants by sucker culture in large quantities. On the assumption of practical production, it becomes important that how the pathogen free plants can be propagated in large quantities in a fast and efficient manual. To propagate in large quantity, the recovered plants which were confirmed by RT-PCR to be virus-free, the suckers of the plants were chosen as plantlets for in vitro culture. The recovering plants with 4-5 leaves (Fig. 1H) were cut in suckers of upper, middle and lower, and cultured on hormone-free MS medium. And they were cultured onto bio-pots in a growth chamber under the conditions of 25℃, 3.3 μmol m^{-2} s^{-1} on 16 h photoperiod. The propagation rates of the plantlets from different parts of the recovery plants were investigated after 3 weeks of culture.

RESULTS AND DISCUSSION

Effect of sterilization method for the plantlet surviving. Even though there have some methods established concerning the shoot apex culture (Nagata 1990, 1991) a critical and detailed procedure is still needed for practical application in agricultural production of sweet potato with virus-free seedlings. Here, we re-corrected every step for the culture procedure. At first, effect of sodium hypochlorite solution concentration on shoot apex culture was investigated (Table 1). 100% of taking rates were achieved in the treatments of 0.3 to 0.8% among the range of 0.1 to 1.5% with 15 min. Then, the effect of sterilization time with 0.3% concentration on shoot apex culture was investigated (Table 2). 100% of taking rates were gained in 10 and 15 min. among the range of 5 to 20 min. Therefore, it is clear on shoot apex culture that the treatment with 0.3% sodium hypochlorite solution and 10 min. can stop the infection of unwanted bacteria perfectly, when young seedlings cultured in artificial conditioner were used.

Table 3. Comparison of chemical components between KN and MS media*

KN medium Components	mgl^{-1}	MS medium Components	mgl^{-1}
KNO_3	5,560	KNO_3	1,900
NH_4Cl	268	NH_4Cl	1,650
$CaCl_2 \cdot 2H_2O$	220	$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	185	$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	68	KH_2PO_4	170
H_3BO_3	2.4	H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	7.14	$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	4.05	$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.375	KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.127	$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.01	$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_4 \cdot 6H_2O$	0.01	$CoCl_2 \cdot 6H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	27.8	$FeSO_4 \cdot 7H_2O$	27.8
$Na_2 \cdot EDTA$	37.3	$Na_2 \cdot EDTA$	37.3
Nicotinic acid	0.5	Nicotinic acid	0.5
Pyridoxine · HCl	0.05	Pyridoxine · HCl	0.5
Thiamine · HCl	0.3	Thiamine · HCl	0.1
Sucrose	20,000	Glycine	2
		myo-inositol	100
		Sucrose	30,000

*KN: Komamine & Nomura medium (1998); MS: Murashige & Skoog medium (1962).

Comparison of different media. The effects of three kinds of media on shoot apex culture were compared among hormone-free MS, MS medium complemented with 0.05 μM NAA and 4.44 μM BAP and KN medium supplemented with 0.05 μM NAA and 4.44 μM BAP. Here, we used KN medium with the same hormone combinations to that of MS medium used by Nagata (1990). The taking rates of 10% (8/80), 65% (51/78) and 82% (81/99) were obtained from the three media, respectively. 82% of taking rate in KN medium was highest among tested.

When compared the components of KN with that of MS (Table 3), we found that there don't contain Glycine and *myo*-inositol, in KN but MS medium, and nitric ammonia in MS is changed to chlorine ammonia in KN medium. When compared the dosages of KN with that of MS, we found that 1) there are no changes in $FeSO_4 \cdot 7H_2O$, $Na_2 \cdot EDTA$ and Nicotinic acid between the two media, but KNO_3 and Thiamine in KN are 3 times of that in MS. It is well known that the components and dosages of various compounds varied for different kinds of plants. KN medium compared with that of MS medium, gave the best result of taking rate (82%), indicating that KN medium is more suitable to shoot apex culture of sweet potato.

Shoot apex culture. Shoot apex including growth point and one small leaf was cultured on KN medium (Fig. 1C), and shoot apex began to grow after 3 weeks of culture (Fig. 1D). Two leaves with growth point

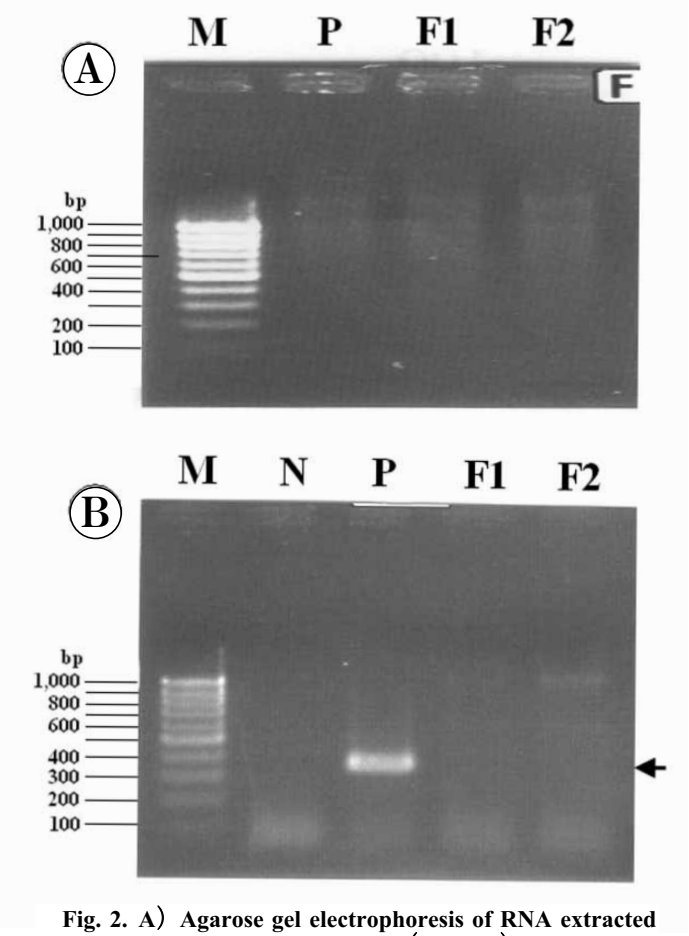

Fig. 2. A) Agarose gel electrophoresis of RNA extracted from leaves of SPFMV sufferer (Lane P) and healthy plants (Lanes F1 and F2) of "Miyazakibeni". Lane M: 100 base ladder marker; B) Agarose gel electrophoresis of DNA fragments amplified from RNAs using RT-PCR with primer C1: GGACAAGCCCCATCAAATG and primer C2: CTGGAATGGTTGC-GGGTTGC. The arrowhead indicates SPFMV-specific fragment. Lane M: 100 base ladder marker; Lane N: negative control (neither DNA nor RNA presents in the reaction mixture); Lane P: positive control (DNA amplified from RNAs of sufferer of SPFMV leaves of "Miyazakibeni"; Lanes F1, F2: DNAs amplified from RNAs of leaves of healthy plants of "Miyazakibeni".

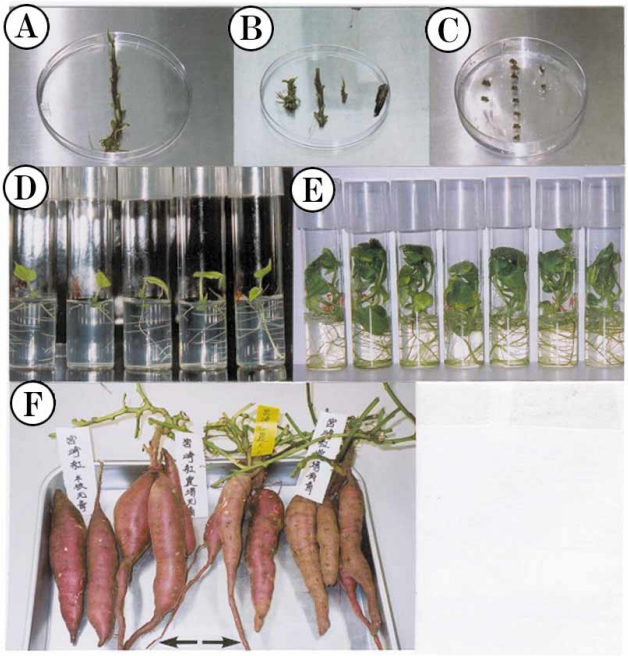

Fig. 3. Clonal propagation in large quantity using sucker culture of stems of detected virus-free plants. A) Stem; B) Stem cut into upper, middle and lower parts; C) Suckers cut from the upper (right), middle (center) and lower (left) parts; D) and E) Plant growth from the suckers after 4 and 6 weeks of culture, respectively; F) Tubers obtained from recovered SPFMV-free plants after cultivation of two seasons, left for SPFMV-free ones and right for SPFMV sufferer ones from shoot apex culture directed with allow heads, respectively.

were observable after 6 weeks (Fig. 1E). And four leaves, shoots with rooting and complete plants were obtained after 9, 12 and 16 weeks, respectively (Fig. 1F, G, H).

Acclimation of recovering plants. All of the three methods (see "M & M") conducted here for acclimation of recovering plants gave the same taking rates (100%) in final. However, methods of 1) directly transplanted into pot of sterilized soil and 2) directly transplanted into pot of sterilized vermiculite needed a long time (2-3 weeks) for recovery as the plant growth environments (humidity and airtight)were changed too much from in vitro to in vivo. On the other hand, the method of 3) directly transplanted into sterilized vermiculite of pot sealed up with vinyl bag showed quick growth of bud, and needed only one week for recovery. This method was conducted successfully in other plants, bahiagrass (*Paspalum notatum*)(Chen et al. 2001), guineagrass (*Panicum maximum*)(Chen et al. 2002). Therefore, the 3rd method was thought to be the best one for acclimation of recovery plants with short time, and easily operating in large quantity.

Virus detection. The recovered candidate virus-free plants were provided for virus detection using RT-PCR method. As a control the infected of SPFMV plant was also used for RNA extraction. Total RNAs were extracted efficiently using ISOGEN RNA extraction kit (Fig. 2A) from both of the leaves. The result of RT-PCR indicated that 200-300 bp of the specific fragment of SPFMV was amplified (Fig. 2B) in sample of leaves of P (a sufferer of SPFMV), and there were not specific band of the fragment in F1 and F2 (the leaves of recovery plants by shoot apex culture). The above results of RT-PCR have made it clear by the method described in this study that SPFMV could be precisely detected, and together, that whether the recovery plants have already been removed or not could be confirmed exactly.

Nagata et al. (2001) reported that RT-PCR method could be used to detect the nucleotide sequence of SPFMV-S and -O, which were bought from Takara chemicals (Takara, Japan). In this study, we practice the method of RT-PCR on the recovery plants from shoot apex culture of the sufferer of SPFMV, "Miyazakibeni" , a popular variety in southern Japan. This result has provided a rapid and precise method to detect virus-free recovery plants, meaning that you do not need to cultivate the candidate virus-free plants two seasons to detect if the virus is removed or not. And it was somewhat exciting for farmers because it is available for agricultural production.

Our results agreed with that of Nagata（2001）that SPFMV-S and -O could be checked efficiently using the primers based on the nucleotide sequence of SPFMV-S and -O. However, as indicated by Usugi et al.（1993）, that there must be different strains of SPFMV in the field, the universal primers for detecting SPFMV strains are being develop as much haste as possible.

Propagation method in large quantities. For the practical purpose, a large scale production of virus-free seedlings is necessary. As shown as in Fig. 3A, B, C, suckers were cut from upper, middle and lower parts of recovery plant stems, which were confirmed to be SPFMV-free, are cultured on hormone-free MS medium in a growth chamber at 25℃, 3.3 μmol m^{-2} s^{-1} on 16 h photoperiod. The suckers of virus free were shown after cultures of 4 and 6 weeks, respectively（Fig. 3D, E）. After 6 weeks of culture, 60%（36/60）, 100%（99/99）and 80%（64/80）of plants were obtained from the upper, middle and lower parts of SPFMV-free plants, respectively.

The above results indicated the possibility that about 5 seedlings per plant（5 suckers/ SPFMV-free plant）could be obtained by sucker culture within 6 weeks of cycle. Using this method, virus-free plants obtained from shoot apex culture can be propagated by sucker culture in short cycle and in large quantities, which is available to apply in practical production.

After two seasons of cultivation of SPFMV-free plants in farm, the tubers were obtained and observed for symptom analysis. There were no RC-LS detected by naked eye, and the tubers looked smooth and shiny could be potentially increased for its high commercial value（Fig. 3F）. In general, the SPFMV-free plants could be kept and used continually for over five seasons of cultivation. Using the rapid and precise clonal propagation system of virus-free recovery plants established in this study, the production of high quality and commercial value of sweet potato will be more possible not only in southern Japan but also in eastern Asia, moreover in the world.

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