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FINAL REPORT

SELECTION AND DEVELOPMENT OF VIRUS RESISTANT POTATO BY USING PROTOPLAST CULTURE AND FUSION TECHNIQUE.

A RESEARCH PROJECT

USAID/PSTC PROGRAM

Grant No. 936-5542-G-00-7021-00

Submitted by

Dr. Prasartporn Smitamana
Project Leader

FACULTY OF AGRICULTURE
CHIANG MAI UNIVERSITY
CHIANG MAI 50200
THAILAND

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Project Profile

Country	: Thailand
Grant No.	: 936-5542-G-00-7021-00
Program	: Program on Science and Technology Cooperation
Project Title	: Selection and Developing of Virus Resistant Potato by Using Protoplast Culture and Fusion Technique.
Project Leader	: Dr. Prasartporn Smitamana
Organization	: Faculty of Agriculture Chiang Mai University 50200 Chiang Mai, THAILAND.
Co-investigators	: Dr. Pajchima Smitamana Dr. Danai Boonyakiat Assoc. Prof. Kesinee Ramingwong
Researcher	: Mr. Visnu Somsab Ms. Kaewalin Sinsuwan Ms. Angsana Akarapisan
Project consultant	: Dr. Sherret S. Chase Dr. Stephen L. Sinden
Authorized Officer	: Prof. Kasem Watanachai
Total Project Budget	: 3,816,192 Bath. (149,070 US$)
Project Duration	: 3 Year (August 1987 - July 1990)

Introduction

Potatoes is one of the economic crops and an important class of world food crops with the most varied in its uses and markets. Besides supplying at least 12 essential vitamins, which vitamin C is included, potatoes also provide significant amounts of carbohydrate, protein and iron (Gray and Hughes, 1978). Many old potato cultivars are still widely grown in almost 130 countries where three quarters of the world's population lives (International Potato Centre, 1984) by using asexual propagation method rather than through true seeds. Vegetative reproduction though is the best way to preserve essential varietal characteristics from one generation to the next. At the same time vegetative propagation assures the perpetuation of many infections (including all viruses, mycoplasmas and viroids) from generation to generation too. Meristem culture, either alone or in combination of chemotherapy or thermotherapy has been used to eradicate one or more viruses from the parent stocks in the seed production program. However these virus-free stocks can be re-infected again, therefore breeding for virus resistant is the ideal way to overcome the problem. Since there seem to be no shortage of insect vectors or disease pests in any potential production areas. To solve this problem, breeding for resistant varieties must be seriously concerned. But in potato breeding affords are limited by the nature or frequency with which specific modifications are realized and still problematic. Thus, for vegetatively propagated plants like potato, enhancement through protoplast culture and anther culture methods appears especially promising. Side effects of genetic modifications on sexual fertility are less significant in direct selection programs than in seed crops, and variants can be readily reproduces through tubers. Moreover, there is precedent in another vegetatively propagated crop, sugar-cane, where plant regeneration from single cells led to improved cultivar (Heinz et al., 1977).

Having the tetraploidy character (2n = 4x = 48), potato poses several problems for the plant breeders. This include heterozygosity, the occurrence of male sterility, selection difficulties in the seedling, slow rate of increase in population, and uneasiness of germplasm storage and transportation (Howard, 1978). Thus modern biotechnological plant improvement method for the required gene pooling through the protoplast fusion technique has to be considered as a superior tool for this purpose. Moreover, in order to maintain the tetraploid genotype, only the protoplasts derived from the dihaploid plants (2n = 2x = 24) will be appropriate.

Dihaploid potatoes (2n = 2x = 24) have been successfully obtained from the cultivated tetraploid *S. tuberosum* (2n = 4x = 48) by anther culture (Dunwell and Sunderland, 1973 , Irikura, 1975 , Marunenko and Kuchko, 1985), and can be produced both *in vivo* by pollination of tetraploids with selected diploid *Solanum phureja* genotypes (Hermsen and Verdenius, 1973). However, it has proved difficult to obtain pollen plantlets from the anthers of the cultivated potato (Kohlenbach and Gier, 1972 , Sinha et al., 1976), and this may be due to the genotype of the donor plants (Dunwell and Sunderland, 1973 , Foroughi-Wehr et al., 1977 , Jacobsen and Sopory, 1978 , Sopory et al., 1978 , Wenzel et al., 1979 , Wenzel and Uhrig, 1981). In solving this difficulty many factors have been studied. The components of the culture medium, the stage of development of the microspores have been investigated and suggested (Dunwell and Sunderland, 1973 , Fridborg and Eriksson, 1975 , Sopory et al,1978 , Sopory, 1979).

Protoplasts (plant cells without cell walls) isolated from different genotypes, can be cultured or fused together, then regenerated as hybrid plants. This approach promises to play an increasing role in crop improvement by over

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coming some limitations of conventional breeding methods. Potato is one of the crops to which technology can be applied. After Upadhya (1975) obtained potato protoplasts from leaf mesophyll, grew them in a medium that support callus formation and root differentiation, but not shoot development. Successful plantlet regenerations from potato protoplasts were first achieved by Shepard (1975). The momentum of potato protoplast culture has picked up since then (Haberlach, 1985). Various types of plants were obtained through potato protoplast culture, including autotetraploid commercial cultivars (Schumann *et al.*, 1980, Gunn and Shepard, 1981, Thomas, 1981, Thomas et al., 1982, Shepard, 1982, Bokelmann and Roest, 1983), monoploid lines (Roest and Bokelmann, 1983), dihaploid clones (Binding et al., 1978). Interspecific somatic hybrid plants have been constructed between cultivated potato and *Solanum nigrum* (Binding et al., 1982) or and *S. brevidens* (Austin et al., 1985). This has opened the doors of potato improvement especially for disease resistant (Shepard,1980, Shepard et al., 1980, Austin et al., 1985). Due to the reason that transferring of genes is desirable, the most dramatic and easiest to monitor cell genetic changes are those accomplished by somatic hybridization which are not possible to obtained using conventional breeding methods. Evans et al. (1981) has success in transferring gene for TMV resistant from *Nicotiana nesophila*, a wild tobacco species, to cultivated variety *N. tabacum*. Local lesion type resistance to TMV had been observed in leaves of the somatic hybrid plants. This concept has also applied and success in potato (Austin et al., 1985).

Other way to select virus resistant potato cultivars is through the production of the haploid plants. Potato cultivars differ from other crops in that they are autotetraploid. Since dihaploid can be produced pathenogenetically and anther culture, the idea arose that breeding should be converted from the tetra

ploidy to the dihaploidy or monoploidy and opens the chance to produce the pure homozygous lines by chromosome doubling. Moreover, stepwise reduction of ploidy to simplify inheritance is generally accepted for qualitatively inherited characters. Wenzel *et al.* (1985) has success in showing the clone resistant to potato virus X or potato virus Y increased after the ploidy reduction and stated that qualitatively and quantitatively inherited resistance were kept during haploidization. Having passed the haploid level, a quantitative resistance could become homozygous and, like a qualitative trait, could be transferred , at least to the next generation. Furthermore, the homozygous dihaploid lines can contain valuable monogenic as well as polygenic characters such as disease resistance (Wenzel and Uhrig, 1981) but the combination of these superior genotypes by conventional breeding means is limited by the infertility in these lines (Hermsen and Ramana, 1981). Wenzel *et al.*, (1979) have therefore proposed that, these required characters could be combined by protoplast fusion and the superior heterozygous tetraploid potato cultivar could be derived. Moreover the use of protoplast fusion also allows combinations of dihaploid genomes without meiotic segregation, and could overcome the problems of infertility (Hermsen, 1984, Wenzel *et al.*, 1984). Many tetraploid somatic hybrid of potato have been recovered successfully following by protoplast fusion of two dihaploid clones and also from the protoplast fusion of dihaploid potato with the dihaploid *Solanum brevidens* (Austin *et al.*, *1985* a, b , Ehlenfeldt and Helgeson, 1987). It is therefore clearly proved that the protoplast fusion is a deserving technique in the modern potato breeding program.

Potato leafroll virus (PLRV) is one of the major viruses found in potato world-wide. The PLRV - infected plants generally produce smaller tubers. The depression in yield of secondary infected plants may be considerable : losses exceeding 20 - 87 % of the yield have been reported (Hunnius, 1977).

Primary symptoms of the PLRV infected plants appear mainly in the young leaves at the top of the plants. The infected leaves are usually held upright with pale yellowish color. The leaflets of certain cultivars are often rolled upwards. If the primary infection take place late in the season, the symptoms and yield reduction might not occur. However, the progeny might be partially infected. The severe symptoms can be observed in the secondary infection, which are stunting, rolling of the older leaves and pale. For the basal leaves, stiff and leathery leaves signs are usually found. Tubers from the infected plants show clearly net necrosis, which can be seen when the tuber is cut. Phloem necrosis in stems and petioles can also be detected (Walker, 1952 , Beemster and de Bokx, 1987). Since PLRV can not be transmitted by any mechanical means therefore, mechanical inoculation on the virus indicator or indexing plants is not possible. The best method for PLRV detection in the infected plants nowadays is by using the serological test. Enzyme-linked immunosorbent assay (ELISA) is one of the most sensitive serological tests. After this method had been developed in 1976 (Voller *et al.*, 1976) , it has been reviewed extensively (Clark, 1981 , Torrance and Jones, 1981 , Bar-Joseph and Garnsey, 1981) and a number of modifications and variations have been described (Clark and Adams, 1977). The principle of ELISA technique is that virus in the test sample is selectively trapped and immobilized by specific antibody adsorbed to the surface of the flat-bottomed 96 wells polystyrene microtitre plates. Retained virus is then reacted with further specific antibody to which an alkaline phosphatase enzyme has been linked. The reaction between the trapped virus and the enzyme linked antibody can be detected by adding a suitable enzyme substrate the *p*-nitrophenyl phosphate. Positive reactions are indicated by the substrate turning yellow which can easily be observed by naked eyes. For the quantitative test, the plates can be further measured by 405 nm., the intensity of the yellow color is an indicator of the virus concentration in the test samples.

Since the rich sources of germplasm for disease resistant can be found in many tuber-b aring *Solanum* species and their close relatives, the problem becomes one of transferring or incorporating these resistance genes to commercial acceptable potato cultivars. For PLRV, a new type of resistance has been found in *Solanum acaule*, a wide species of potato. This plants would not become infected with PLRV even though the infective aphids were used to inoculate the plants. If the plants of these accession were grafted with PLRV infected scions, the virus passed from the scion to the plants, but multiplication of virus was prevented or reduced (Anonymous, 1984). Therefore *S. acaule* must be very useful in combining the gene for PLRV resistance into the commercial potato cultivars, through the protoplast fusion technique with both normal tetraploid or dihaploid potatoes. The regenerated plants are expected to maintain the highly immune or resistant to virus and the idea need to be proved through this project.

Testing for the virus resistant property in the parent materials is very important in any breeding program either by conventional pollination technique or somatic hybridization which the production of the haploid plants is significantly important as has been discussed in the previous reports. Besides that the morphology of the materials used in the program must also be considered. The growth and development of potatoes, like the other crops, are affected by the environments that exist in the nature at different locations in the world. Though the genetic endowment of a plant (genotype) is essentially constant in comparison with changes in environment that a plant experiences, the expressions (phenotypes) of the genetic information are strongly influenced by environmental changes. Climates with regular and irregular shifts are the determinative components of a given genotype (Langridge, 1963). Morphology

of one population is uniform when grown in their original environment, but showed great deviation in such characters as vigor, growth pattern, height and leaf size when transplanted to other altitudinal locations (Clausen and Hiessey, 1958). The performance of a clone at one transplanting location was no indication of its behavior at another, suggesting that different sets of genes were operating in dissimilar environments. Therefore a successful population in any climate region is one that has evolved its physiological mechanisms to be effective in the light -energy transformation, together with resistance to cold and drought (Cooper, 1963). In order to gain more informations on the adaptability of different potato cultivars to the Thai conditions which is important to the breeding program of the project, detail characterization and description of potatoes need to be studied.

Protein and enzyme banding patterns have been used as a tool for the identification of potato varieties since 1966 (Loschske and Stegeman, 1966, Allicchio *et al.*, 1987). Analysis of the soluble proteins and isozyme were mostly done by using polyacrylamide gel electrophoresis. Desborough and Peloquin (1966) have demonstrated the use of tuber protein patterns in the acid gel system in different ploidy potatoes and conclude that there was no effect of ploidy level on the bands of protein presented in the tested groups (Desborough and Peloquin, 1969). Comparing to the basic gel system, the number of bands detected in the acid gel system was fewer. Therefore it would be easier to identify or classify the phenotypes. Moreover, variation observed in biochemical traits was greater than that in the morphological traits. However, the physiological role of these proteins was yet not known.

2. Objective

2.1. To select potato varieties that are resistant to potato leafroll virus (PLRV).

2.2. To produce the dihaploid plants which can be in the breeding program and somatic hybridization as well.

2.3. To develop new potato cultivars which are resistant to PLRV and suitable for the tropical conditions.

3. Material and method

3.1 Testing for PLRV resistant in potatoes.

In order to get more information on the PLRV resistant character of potatoes grown under the Thai 's conditions, both dihaploid and tetraploid potatoes were grafted with the PLRV infected potato cv. Sieglinde, by using Sieglinde as either scions or stocks. Since the stems of *Solanum acaule* clones were very small, they were therefore grafted on the infected Sieglinde stocks. After grafting the plants were kept in the high humidity incubator at 25 $\pm$ 2 °C. with 16 hr. light for 1 week, to enhance the survival of the grafted scions. Thereafter, the plants were transferred to the screen-house and grown under the normal conditions. In order to warranty the transmission of PLRV from the infected scions to the stocks, in the case of using virus infected Sieglinde as scions, the grafted scions were remained attached to the stocks for 1 month, and then they were cut off with the clean razor blades. In the opposite, if the healthy tested plants were grafted on the PLRV infected Sieglinde, only the axillary shoots from the stock part were removed to elaborate the scions growth rate. Leaf samples were collected 8 weeks after grafting and were extracted in the sample buffer (Boehringer Mannheim GmbH, FRG). Polystyrene plates (Dynatech Laboratories, Inc., Alexandria, U.S.A. or Fa. Greiner, Nuertingen, FRG.) containing 8 x 12 wells were coated with 200 ul coating solution (Boehringer Mannheim GmbH, FRG), and then covered with the polyethylene film and incubated at 37 ° C. for 2 hr. The coating solution was removed and washed 3 times with PBS-Tween by using washing machine (Miniwash, Dynatech Laboratories, Inc., Alexandria, U.S.A.). After washing, 200 ul of plant extracts were pipetted into the wells and incubated over night at 4 °C., as control

standard healthy plant extracted and standard virus suspension were used. The solution was removed and washed 3 times as described, then the plates were filled with 200 ul of diluted 1:2,000 Antibody-AP conjugate solution (Boehringer Mannheim GmbH, FRG) and incubated for 4 hr. at 37 °C. The plates were subsequently washed 3 times with wash buffer and filled with 200 ul of substrate solution (Boehringer Mannheim GmbH, FRG) into each well, incubated for 1 hr. at room temperature. The resulting dephosphorylation was stopped by addition of 3 M NaOH, and the absorbency at 405 nm (A_{405}) was determined for the content of each well with ELISA-plate reader (Minireader II, Dynatech Laboratories, Inc., Alexandria, U.S.A.).

3.2 Anther culture

In order to overcome the physiological problems of the donor plants in which the flowering can be retarded by the tuberization, the potato scions are grafted onto tomato stocks to guarantee vigourous flowering. All the grafted plants are kept in an aphid proof screen house. Flowers buds of size 4 - 6 mm. with the anther length about 3 - 4 mm. are collected and kept in the dark for 48 h in dry test tubes at 6 - 10° C. Surface sterilization of the buds is accomplished by using 70 % ethanol with 0.1 % Tween 20 added. Subsequently, the anthers are dissected in a clean laminar air flow , plated on the following medium Nitsch and Nitsch (1969), Linsmaier and Skoog (1965) , modified Murashige and Skoog (1962) and potato extract medium. Then the plated anthers are incubated at 25 ± 3° C. with 16 h photoperiod (Weatherhead and Henshaw, 1979).

Besides, the modified Murashige and Skoog (1962) and modified Nitsch and Nitsch medium in which anthers from the greenhouse and the field grown potatoes could be induced to form calli, other media were also used to induce both androgenesis and morphogenesis. The basic component of the modified media are as Murashige and Skoog (1962) medium, only the organic constituents and the hormone types as well as the hormone concentrations were varied.

3.3 Shoot formation from cultured roots

The starting material was obtained from the anther culture experiment. The roots derived from anther culture of various potato cultivars were maintained in modified Murashige and Skoog (1962) medium. The experimental media contained the inorganic salts (macro- and microelements) of Murashige and Skoog (1962) and organic constituents of Nitsch and Nitsch (1969) supplemented with 1 ppm Zeatin, 1 ppm IAA, glucose (24.3 g/l), inositol (0.1 g/l) and 0.7 % agar (w/v). The roots were culture under 25 $\pm$ 2 ° C. with 16 hr. light period.

3.4 Ploidy level of the anther derived plants.

Leaves from the *in vitro* grown plantlets either derived from the anther culture from the previous experiments or from the commercial grown tetraploidy plants were cut and the lower epidermis parts were subsequently peeled out in order to make the wet-mount preparation for the light microscopy

investigation. Average numbers of chloroplasts from 40 guard cells were determined and used as an indicator for the ploidy level of the plants.

3.5 Protoplast isolation and culture.

Potato protoplasts are isolated from both greenhouse and *in vitro* grown plants using the two step method and slightly modified Shepard's (1982) method. Non-sterile plant material was surface sterilized by treating with 10 % Clorox solution, containing ca. 0.2 ml. Tween 20, for 15 minutes. After three succeeding wash with sterile distilled water, the leaves were placed in the Petri dishes to retain the high moisture condition, and then the lower epidermis were peeled out and cut into small pieces. The leaf-fragments were subsequently transferred and treated with the enzyme solutions, 0.25 % Macerozyme R - 10 and 0.5 % Cellulase R - 10 in 0.35 M mannitol solution, for 30 x 2 and 40 x 2 minutes respectively, on an orbital shaker (95 rpm., 28° C.). Protoplast suspension obtained from enzyme digestion was passed through double layer cheese-cloth and centrifuged at 600 rpm. for 5 minutes. Subsequently, the supernatant was removed and the pellets were resuspended in protoplast washing solution (0.35 M mannitol, 10^{-6} M $CaCl_2$, 0.5 % PVP).

For the isolation of protoplasts from the *in vitro* grown plant material, modified Shepard's method as adapted in Sinden's Laboratory (personal communication) was used, excised leaves and shoots were cut and floated in the float solution at ca. 8° C. for 48 hours. The plant material was afterward cut into fine pieces and transferred to the soak solution for ca. 24 hours at 8° C., following by 24 hours treated with the enzyme mixture (0.2 % Macerozyme R -10, 0.5 % Cellulose R -10, 2 % PVP, 10.3 % sucrose and 0.95 ml. of MS major

salt stock). The protoplasts were harvested and washed as mentioned above. To remove cell debris, the protoplast suspension was layered either over 30 % Percoll solution or 22 % sucrose solution, and centrifuged at 1,000 rpm for 5 minutes. The band of protoplasts at the interface of the two solutions was removed carefully using a pasture pipette and washed twice with washing solution. The pellet was resuspended in culture medium and plated in medium solidified with 0.4 % agarose (Sigma type VII) or cultured in liquid medium under the dim light condition at 25° C. during the first 24 hr., after that the plates were transferred to low light intensity condition for another 5 -7 days. After microcolonies could be observed, the plates were kept under normal conditions.

3.6 Protoplast fusion and culture.

After isolation protoplasts was purified by centrifuge over 20% sucrose solution then washed 2x with washing medium after that equal number of protoplasts from two origins (one diploid clone or from anther derived commercial cultivars and either Oka 7576 or Oka 3716) were mixed and centrifuge. The protoplast pellet was resuspended in a pre-fusion medium (2.5 g/l $CaCl_2$, 22 g/l KCl) with final concentration of 1.5 x 10^5 protoplasts/ml. Then ca. 0.5 ml of the mixture was transferred to the centre of 6 cm plastic dish and let them settled to the bottom of the dish. Added the fusion medium (200 g/l PEG, 35 g/l $CaCl_2$, 4 g/l glycine, adjusted to pH 10.5 with KOH) carefully to the edge of the protoplasts drop and incubated for 15 min. In order to remove the fusion medium, 4 ml of protoplasts washing solution was added to the dish and incubated for 5 min.. Two ml of the medium was removed after the subsequent gently mixing the medium then another 3 ml of wash medium was added and incubate for 2 min. Mixed the suspension gently then removed 3 ml of the solution and added another 3 ml of culture medium. Finally 3 ml of the solution

was pipetted out and 1 ml of culture modified MS medium (after Ishige, personal communication) was added. The plates were sealed with a strip of paraffin and incubated as previously described. After 7 days of incubation, the medium was removed and new medium without mannitol was replaced. The cells were kept for another 7 days then were transferred to MS medium supplemented with 1 ppm 2, 4 - D for 2 - 4 weeks until microcalli were easily observed with naked eyes.

3.7 Plant Regeneration from the fused calli

Calli size ca. 0.3 - 0.5 cm with the dark green color were transferred to the regeneration medium (see appendix) to promote the shoot formation. The formed shoots which were normally observed within 4 - 5 weeks after cultured were cut and transferred to further culture on MS medium with NAA 1 ppm, BAP 0.5 ppm and GA_3 0.1 ppm.

3.8 Protein pattern analysis

3.8.1 Sample preparation

Leaves and small stems of the *in vitro* grown plants from the cultivars Spunta, Kennebec, Russet Burbank, *S. acaule* (Oka 3716, Oka 7576, PI 472646), and the dihaploid clones (K4, K7, JH 52, JH71) ca. 1 g were homogenized in the extraction buffer (Tris-HCL 0.1 M, 0.014 M 2-mercaptoehanol and PVP 0.1% , pH 7.3) in the hand homogenizers (precooled at - 15° C). The ration sample-buffer was 1:2 (w/v). The homogenates were

centrifuged at 5 ° C at 12,000 rpm for 10 min. Only the clear suspension from the supernatant parts were transferred to the Ependorfs for further step.

3.8.2 Polyacrylamide electrophoresis

Polyacrylamide slap gels were used for the isolation of proteins. The preparation of gel was as described by the Bio-rad standard manual. For running and stacking gel, 10 and 4% gel were used respectively. In order to eliminate all the contaminants in the gel, the gel was pre-run in the Bio-rad Protean™ II Slab Cell at 4 ° C for 30 min before loading and Tris-glycine buffer (Tris 15 g, glycine 72 g, SDS 5 g dissolved in 1 l adjusted pH to 8.3 and made up volume to 5 l) was employed. Each slot was loaded with 60 ul sample mixed with loading buffer (distilled water 4 ml, 0.5 M Tris-HCl, pH 6.8 1 ml, glycerol 0.8 ml, 10% SDS, 2 -mercaptoethanol 0.4 ml, 0.05% bromphenolblue 0.2 ml) in a ratio 1:1 v/v. Separation of protein was performed at 4 ° C at a constant current of 50 mA for 1,000 Volt-Hour.

3.8.3 Visualization of the protein bands

The gel slabs were immediately removed from the cell after stop running and prefixed in the fixative (40% methanol and 10% ethanol, v/v) for 10 min. The gel slabs were further fixed in two changes of fixative (10% ethanol and 15% acetic acid, v/v) for 15 min each. after completing the fixation the gel slabs were treated with Bio-rad oxidizer for 3 min and washed three times with deionized water for 5 min each. Then stained with Bio-rad silver reagent for 15 min, washed again with deionized water for 20 min. The gel slaps were finally

treated with the developer solution for ca. 30 sec, 5 min and 5 min respectively. After the protein bands were clearly observed, the gel slabs were transferred to 5% acetic solution to stop the reaction. To enhance the contrast of some protein bands, 0.1% coomassie blue R - 250 in fixative (40% methanol and 10% acetic acid, v/v), was applied to the gel for ca. 30 min. The gel slabs were destained in a destaining solution (40% methanol, 10% acetic acid, v/v) until the clear bands of proteins could be observed.

3.8.4 Testing for esterase enzymes.

Approximately 500 mg of fresh tissue was ground in 0.1 M Tris-HCl, pH 8.2 with the sample-buffer ratio of 1:2 (w/v) in the -15 ° C homogenizer. After thoroughly grinned 1 ml of 0.1 M Tris-HCL, pH 8.2 with 5% glycine was added. The suspension was centrifuged at 14,000 rpm for 10 min. The clear supernatant fraction was collected and immediately used for the discontinuous gel electrophoresis as described by Laemmli (1970) and Conejero and Semancik (1977) with slightly modification. Ten percent gel without SDS was used for the separation gel and 4% gel was used for the stacking gel in this test. After pre-run for 30 min, 60 ul of sample was loaded in each slot and run in the running buffer (0.025 M Tris-HCl, 0.19 M glycine, pH 8.3) with the constant current at 50 mA for 1,000 Volt-Hour. The gel was stained for 1 h with fast blue B salt solution (0.3 g fast blue B salt in 200 ml 0.1 M phosphate buffer, pH 6.0; filtrated through Whatman 1 filter paper in the dark and added 1% naphthyl acetate in absolute alcohol). The gel slabs were washed 2x with distiled water and kept in 7% acetic acid.

3.8.5 Testing for peroxidase enzymes

Samples preparation and running condition was done as previously described in 3.8.4. After running the gel was stained with 3 amino-9 ethylcarbazole and β naphthyl acetate (20 ml of stock solution : 3 amino-9 ethylcarbazole 840 mg, β naphthyl acetate 580 mg in acetone 400 ml , plus 80 ml 0.1 M Tris HCl, pH4 and 1 ml of 30% H_2O_2). The gel was kept at 4° C for 10 - 12 hr before washed 2 times with distilled water and kept in 7% acetic acid.

3.9 Testing of PLRV resistant in the somatic hybrid plants.

The shoots from the *in vitro* grown somatic hybrid plantlets were used to graft on the PLRV infected Sieglinde potato cultivar by micrografting technique. The grafted shoots were then cultured on the MS medium in the culture vessels (25x150 mm test tube) and kept under the normal laboratory conditions. After 2 weeks only the scion parts were dissected and continue culturing under the same conditions for another 2 weeks. The plants were then transferred to the compost in the environmental chambers for another 2 weeks and further cultured in the screen house for 2 - 3 weeks before samples were taken. Testing for the abundance of PLRV in the plantlets was done by ELISA technique as previously described.

3.10 Field trial

The stem cuttings of the selected somatic hybrids were tested under the field condition during November to the end of February to evaluate the field performance. Plant characters and diseases resistant were observed.

4. Results and Discussion :

4.1 Testing for PLRV resistant in potato varieties.

All of the grafted plants when kept under high humidity condition in the growth chamber for 1 week were survived and new leaves were also developed. The scion could continue growing and became the major part of the plant (Fig. 1). No aerial tubers and other malformation development were detected at the grafted region (Fig. 1).

All of the tested potatoes either dihaploidy or tetraploidy could support the multiplication of PLRV in the plants. This could be notified by the change of the substrate (*p*-nitrophenyl phosphate) from colorless to yellow. However, comparing with the negative and positive controls, all of the tested *Solanum acaule* were highly resistant to PLRV which would be remarked by the slightly yellowing of the substrate, almost non-detectable by the naked eyes, whereas the positive controls and the susceptible cultivars were dark yellow. The detectable virus in *S. acaule* might be the translocated virus from the Sieglinde stocks to the *S. acaule* scions. This presumption was confirmed by the case of the dihaploidy H_3 703, where the H_3 703 were used as stocks, they had only half O.D. value, compared with the same H_3 703 when used as scions grafted on the PLRV infected Sieglinde stocks.

Fig. 1 Growth of potato cv. Kennebec grafted on PLRV infected Sieglinde stock. After 4 weeks of grafting, the scion was removed and the leaf sample was collected for the PLRV test using ELISA technique.

Table 1 Comparison of the multiplication of PLRV in the infected potatoes by using ELISA technique.

Cultivar	O.D. value (A_{405})
Negative control	0.00
Positive control	0.92±0.04
Solanum acaule	
PI 7434	0.03+0.01
Japanese Clone	0.045+0.02
PI 3716	0.057+0.023
Dihaploid potatoes	
H_3 703 (stock)	0.155+0.02
K 4	0.30±0.03
H_3 703 (scion)	0.31±0.023
K 7	0.38±0.02
JH 71	0.42±0.05
JH 52	0.55±0.04
Tetraploid potatoes	
Altlantic	0.12±0.04
Lemhi	0.13±0.02
Russet Burbank (stock)	0.22±0.03
Russet Burbank (scion)	0.24±0.02
Sieglinde	0.256±0.026
Spunta	0.26±0.03
Kennebec	0.325±0.04

Among the commercial cultivars tested in this experiment Atlantic and Lemhi were the most resistant cultivars however they were susceptible to late blight disease caused by *Phytophthora infestan*. Moreover, Lemhi cultivar could not well adapt to the tropical climate like Thailand. It showed growth retardant with the height around 23 - 28 cm. For the most susceptible cultivar to PLRV was Kennebec.

For the tested dihaploid potatoes, the most resistant and susceptible lines were H_3 703 and JH 52 respectively. In the case of the tested *S. acaule* clones all of them were resistant to PLRV infection. The virus found in the plants might translocate from the infected scions to the stocks. The summarization was supported by the results of the H_3 703 where used as stocks and scions grafted with virus infected plants. It was found that the virus could translocate from the infected stock to the H_3 703 scion easily. Anyhow, this phenomenon was not so obvious in the case of Russet Burbank potato where slightly difference in O.D. value of the stock and scion was detected (0.22 ± 0.03 and 0.24 ± 0.02 respectively).

4.2 Anther culture

The potato scions which had been grafted on the tomato stocks (Fig. 2) to avoid the physiological problem, due to the tuberization, which could hinder the flower formation, did form tubers at almost every node especially at the grafted region (Fig. 3). The tubers formed by this mean had the same characters as the normal ones, and the potato plantlets could be developed from such tubers, however some of the aerial tubers were malformed. This phenomenon had strong effect on the flowering, even though could not be clearly

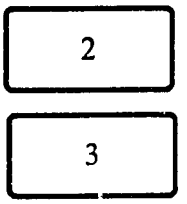
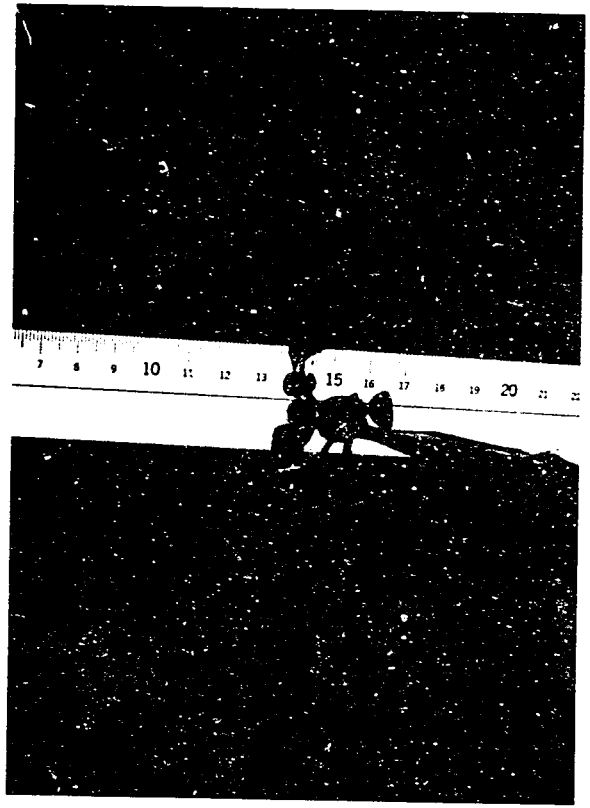


Fig. 2 Grafting of Russet Burbank scion on tomato stock in order to retard the tuberization of the potato.

Fig. 3 Aerial tuber formation on the grafted region of potato and tomato, all of the tubers were green with developed shoots and could be used as planting material.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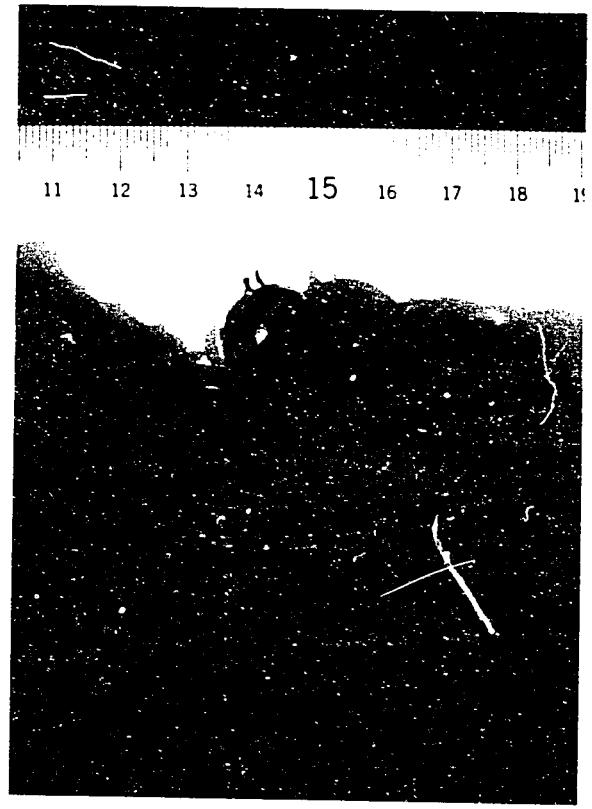
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explained the causes, but it could be probably the short photoperiod in the cool season under the northern Thailand conditions.

Callus induction was generally evidence 4 to 10 weeks after anther inoculation (Fig. 4 and 5). Though the pro - embryoid stage can be observed from the anther cultured in many kinds of media after 2 to 4 weeks culture (Fig. 6 and Table 2), but non of the true embryoid which normally can be observed rupturing the anther wall within 3 to 5 weeks after plating (Sopory et al., 1978 , Wenzel and Foroughi-Wehr, 1984), could be detected. Some anthers had developed the green callus at the basal end of the anther (Fig. 6). Even though the microspores at the uninucleate or late uninucleate stage had been selected in this experiment to enhance more chances of embryoid production (Dunwell and Sunderland, 197 . Sopory et all, 1978 , Weatherhead and Henshaw 1979), still the true embryoid had not been observed. This might be caused by the influence of the genotype of the donor plant. Since it has long been noticed that some potato cultivars and dihaploid breeding lines responded more readily than the others (Dunwell and Sunderland, 1973 , Foroughi-Wehr et al., 1977 , Jacobsen and Sopory, 1978 , Wenzel et al., 1979 , Wenzel and Uhrig, 1981 , Powell and Uhrig, 1987).

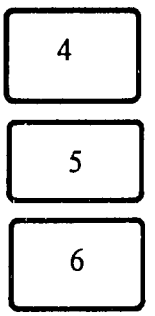
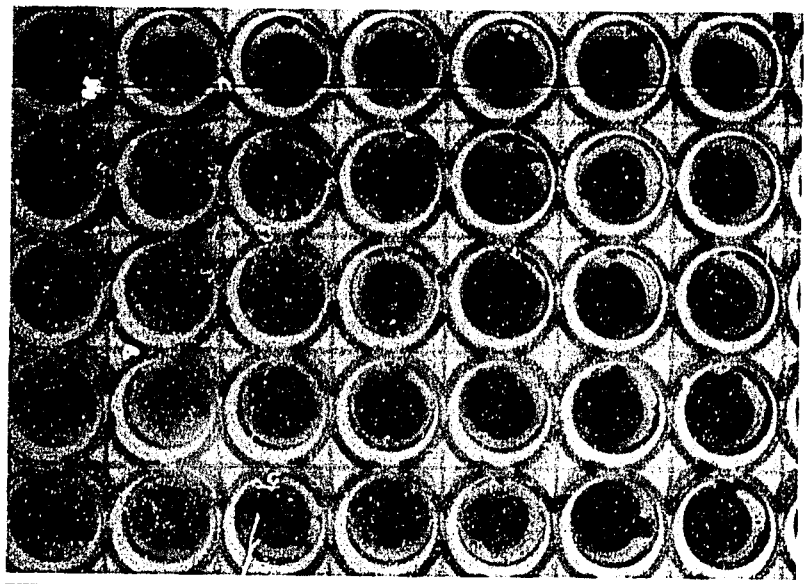


Fig. 4 Anthers cultured in the 96 wells plate to avoid the contamination of the microbes during the first period of culture. The non-contaminated anthers were transferred to the 35x10 mm. plates for further development.

Fig. 5 Callus formation in some anthers from Russet Burbank cultivar after 3 weeks of cultured in modified Nitsch & Nitsch (1969) medium.

Fig. 6 Green callus development from Spunta cultivar anthers after 4 weeks of cultured.



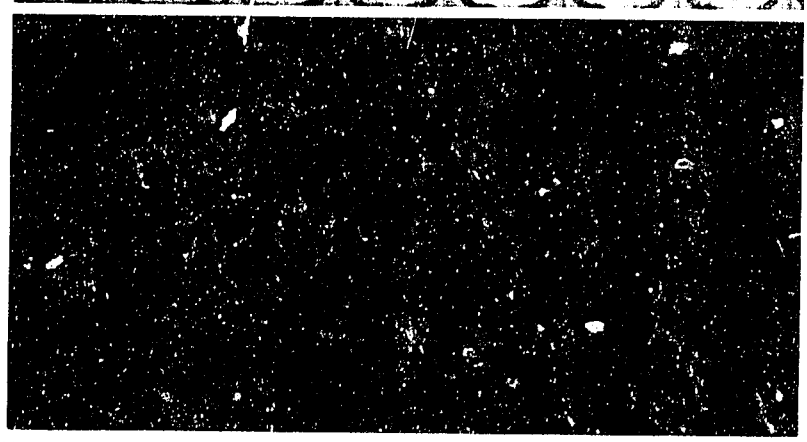


Table 2 Effect of media on callus development and pro-embryoid formation of the cultured anthers.

Media	Response		
	Browning[a]	Callus	Pro - embryoid
MS - PE*	14 - 21	+	+
N & N	3 - 5	-	-
N & N + 10% CW	14 - 28	+	+
L & S	7 - 12	-	+
P & U	7 - 10	-	-

* MS PE = Murashige & Skoog (1962) + 10 % potato extract

N & N = Nitsch & Nitsch (1969)

L & S = Linsmaier & Skoog (1965)

P & U = Powell & Uhrig (1987)

CW = Coconut water

[a] = days after inoculation

+ = produce callus/pro-embryoid after 3 weeks inoculation

- = not produce callus/pro-embryoid after 3 weeks inoculation.

Even though the young flower buds had been carefully surface sterilized with the Clorox solution, some contamination were still observed. To minimized the contamination, the 96 wells microtiter plates were used at the first week of culture. Therefore each anther was transferred to the separated well which contain 400 ul of culture medium, and kept in a moist chamber. The microbial contamination, mostly bacteria, was easily observed and identified (Fig. 4 and 5). Only the non-contaminated anthers were transferred to the same media in 35 x 10 mm. Petri dish, and were incubated at 25 $\pm$ 2° C. with 16 h photoperiod (Fig 6). Some friable calli were derived from the Russet Burbank anthers after transferred from the modified Nitsch and Nitsch medium to the MS medium supplemented with 100 mg inositol, 0.5 mg pyridoxine , 1 mg nicotinic acid, 1 mg glycine, 0.4 mg thiamine, 1 mg NAA, 30 g sucrose and 0.7 % agar, pH 5.7 (Fig. 7). The same type of calli could be observed from the anthers cultured on the MS medium enriched with 0.5 mg NAA, 0.3 mg BAP, 100 mg inositol, 50 g sucrose and 0.7 % agar. However, some anthers produced the compact calli after culturing on the same medium for 8 weeks (Fig. 8). Out of 576 Russet Burbank's anthers, only 7 could continue growing and produing calli, compared to the Spunta and the V - 2 (CIP 375335.1) varieties which ca. 1.05 and 1.42 % of calli could be obtained respectively.

The first morphogenesis could be observed when the calli were transferred to the MS medium (0.5 mg BAP, 1 mg NAA, 1 mg GA, 0.7 % agar , pH 5.7) for 5 weeks, some meristem-like regions and the roots could be observed (Fig. 9). In this experiment there was no real embryoid directly developed from the anthers like in the case of Sopory *et al* (1978) or the others (Dunwell and Sunderland, 1973 , Weatherhead and Henshaw, 1979 , Wenzel *et al*, 1979 , Wenzel and Uhrig, 1981 and Powell and Uhrig, 1987), which could properly caused by the different potato cultivars or lines.

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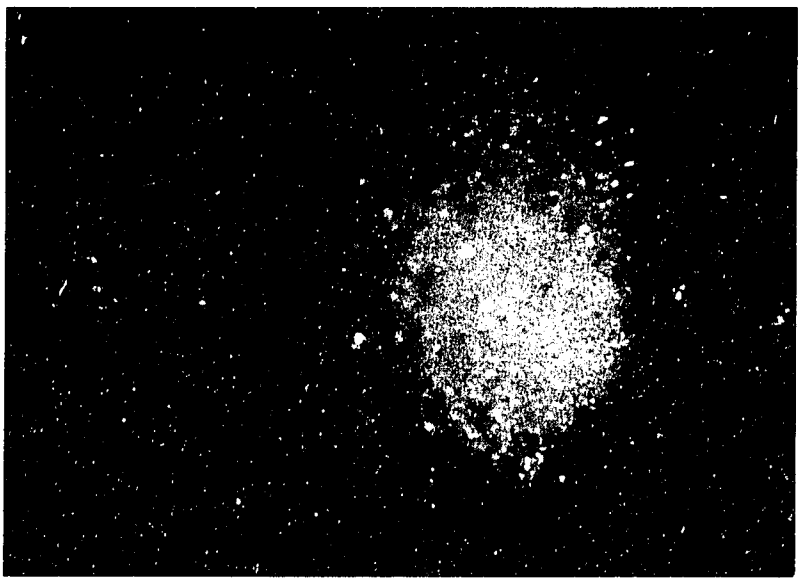
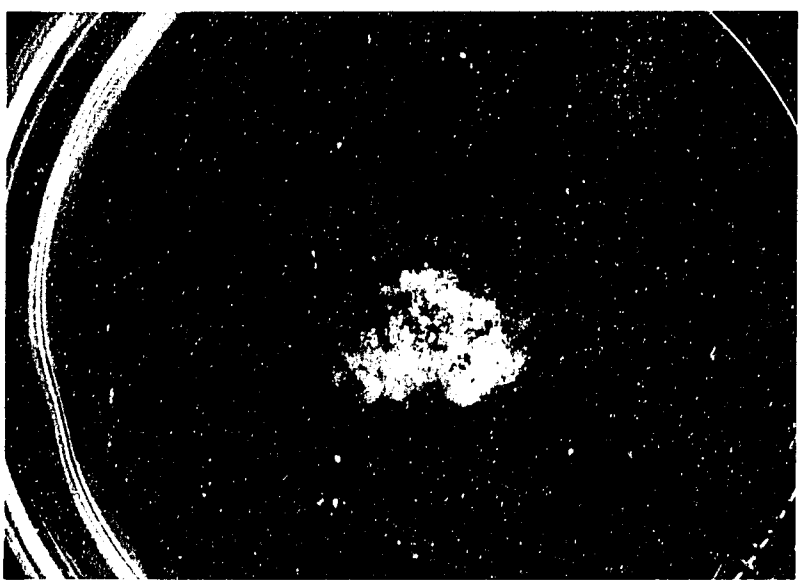
Fig. 7 Friable callus derived from Russet Burbank anther cultured on modified MS medium.

Fig. 8 Compact callus derived from Russet Burbank anther after 8 weeks of culture on modified MS medium.





4.3 Induce shoot formation from root culture.

Green calli formation on the cultured roots was first observed after 7 days of sub-culturing. The developed calli were normally compact. After this phase ca. 20 - 25 days, adventitious shoots were induced from the Russet Burbank and Lemhi cultured roots (Fig. 10). The other clones (Spunta and V2) could not develop to form any shoot like structure, only green tissue could be observed. Anyhow, the regenerated plantlets had to be tested for the chromosome number and other characteristic. Since there was the callus phase involved in the induction process, therefore some variations of the plants could be expected. This was not the same as the result from Espinoza and Dodds (1985) where the root cultures of the diploidy and tetraploidy potatoes were used and no callus stage had been associated. Moreover, these variations were eventually useful for the breeding program in this experiment.

4. 4 Ploidy level of the anther derived plants

Direct chromosome counting from the stained root tip could be one way of estimating the ploidy level of potatoes, but it needed practice and experiences. Under our conditions, the root tips were very small and difficult to handle, though the root from young sprouts could be obtained and stained with Feulgen reagent and counter stain with acetocarmine, the exact chromosome numbers were so variable and in some cases not so clear chromosome could be obtained. Since the chloroplast numbers in the guard cells of the plants could easily be observed and counted without using any staining procedure and the number of chloroplast in the guard cells could represent the ploidy level. Therefore, counting the number of chloroplasts in the guard cell was selected for

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Fig. 9 Morphogenesis of meristem and root like structures from the anther derived callus after 5 weeks of cultured on modified MS medium supplemented with 0.5 ppm BAP, 1 ppm NAA and 1 ppm GA_3.

Fig. 10 Adventitious shoot formation on the cultured potato roots which derived from anther culture. Small compact green calli could be first observed before the real morphogenesis evolved.

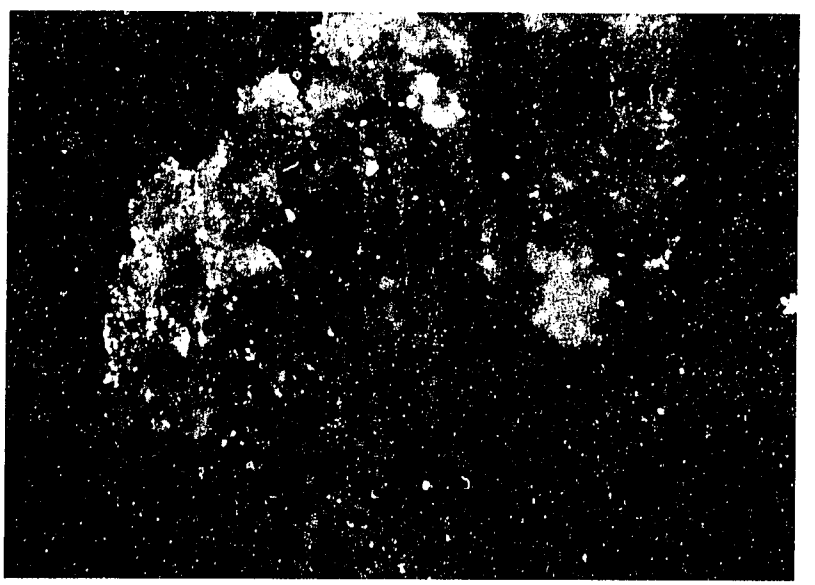

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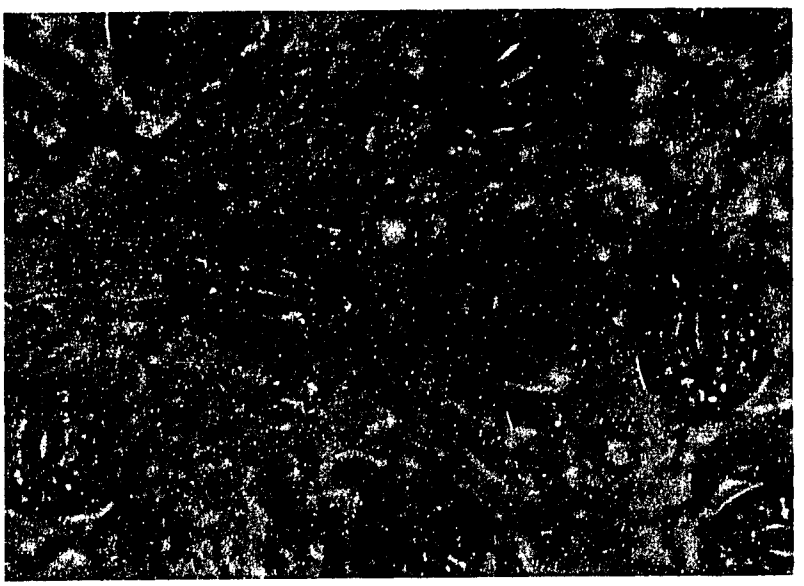
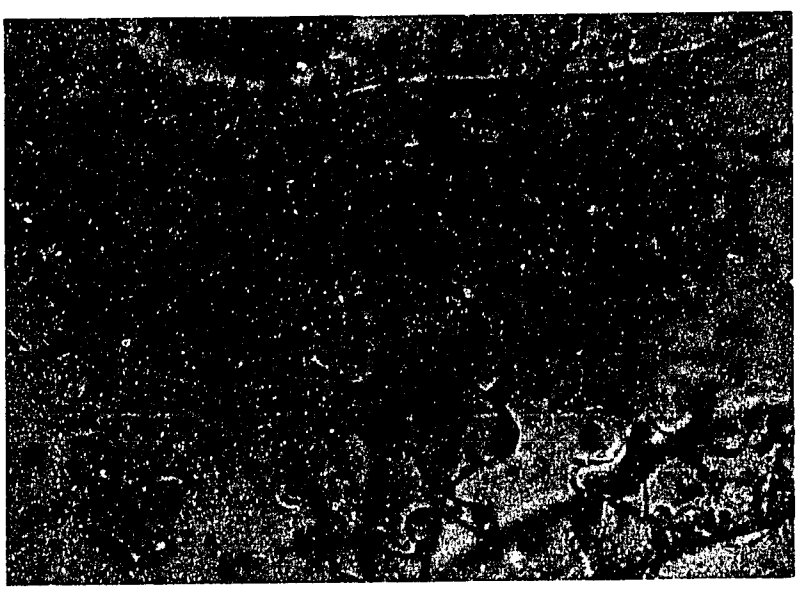
Fig. 11 Chloroplasts in the guard cells from the 4x Russet Burbank cultivar.

Fig. 12 Chloroplasts from the anther derived Russet Burbank plant, showed the different in number of chloroplast abundance in the guard cells.





the ploidy level identification. The anther derived Lemhi and Russet Burbank plantlets contain less chloroplasts than the normal commercial grown one (Fig. 11) and could be identified as the diploidy clones (Fig. 12 and Table 3).

Table 3 Average and standard deviation of chloroplasts in the guard cell of the selected potatoes clones.

Cultivar	Avg. No. of Chloroplasts	Std.
Atlantic	18.256	2.361
Kennebec	16.575	2.084
Lemhi	11.268	1.415
Norchip	16.325	2.285
Russet Burbank	11.051	1.616
Spunta	16.375	1.812
Lemhi	8.325	0.627
Russet Burbank clones (from anther)	9.700	2.100
H - 3703	10.325	1.902
JH52	9.125	1.187
JH71	6.925	1.058
K4	8.200	1.417
K7	7.675	1.233
S. acaule	11.135	1.651

4.5 Protoplast Culture

The protoplasts derived from the greenhouse grown plants could easily observe the chloroplasts which were located on the surface of the cells (Fig. 13) and started to divide after 5 - 8 days of culture in the VKM (Binding and Nehls, 1977) or in the CL medium, connected by a horizontal slot to the reservoir medium in the tri-compartmented 9 cm Petri dish (Fig. 14). The 8 p medium (Kao and Michayluk, 1975), though widely used to cultivate the protoplasts and cell from many plant species did not support any cell division and most of the protoplasts died after 7 - 10 days of culturing in this medium. Moreover, the protoplasts derived from the *in vitro* shoots reformed visible cellwall within 2 days after isolation. The plating efficiency was 1.8 % in both VKM and CL medium, where as RM 5 (Creissen and Karp, 1985) was only 0.1%. This plating efficiency was better when protoplasts were cultured in Ishige medium which could promote the cell division up to 40% especially when the filtrates from the cell culture medium was added during the first week.

4.6 Protoplast fusion and culture.

The use of PEG as a chemical induction in the fusion process though it could induce the protoplast fusion, but this method did need a lot of caution. If the PEG was left in the fusion medium too long, the cell would be clumped together and form a large cell. Moreover, many dead cells could also be observed after the fusion process. However, the average percentage of the fused protoplasts was 22.76 $\pm$ 2.35 by this mentioned protocol. The protoplasts which were adhered to the bottom of the plates could further growth and form the small colony. Microcalli could be observed within 5 - 10 days after transferring

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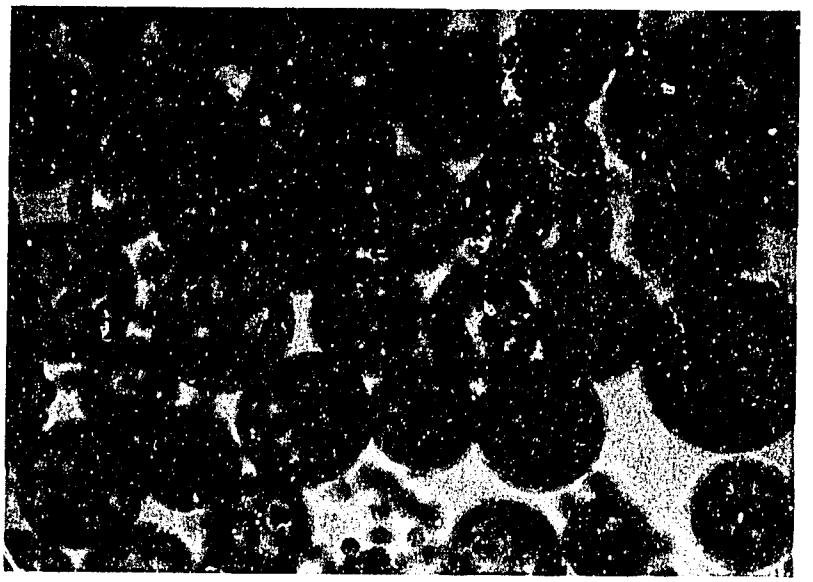
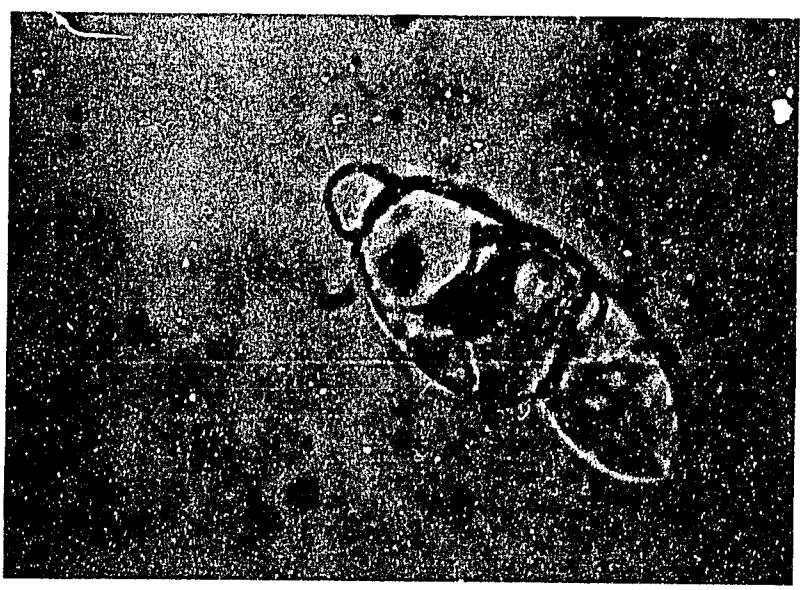
Fig. 13 Protoplasts isolated from the green house grown potato after cleaning and washing, 20x.

Fig. 14 First cell division of potato mesophyll protoplasts cultured in CL medium, 20x.





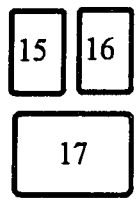
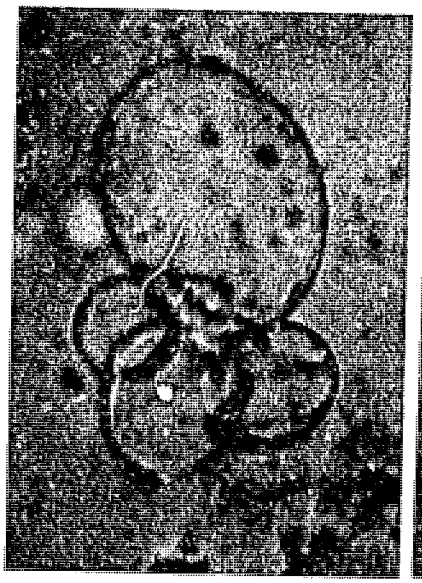
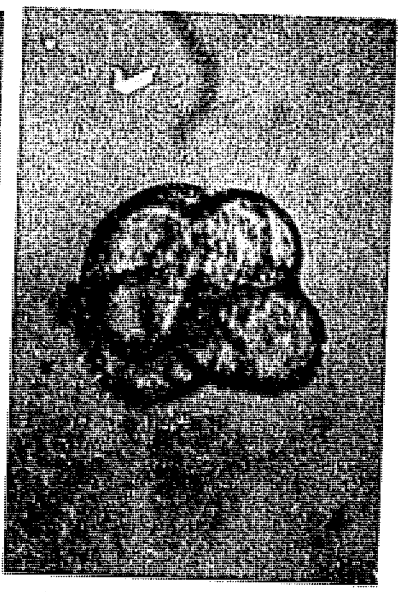


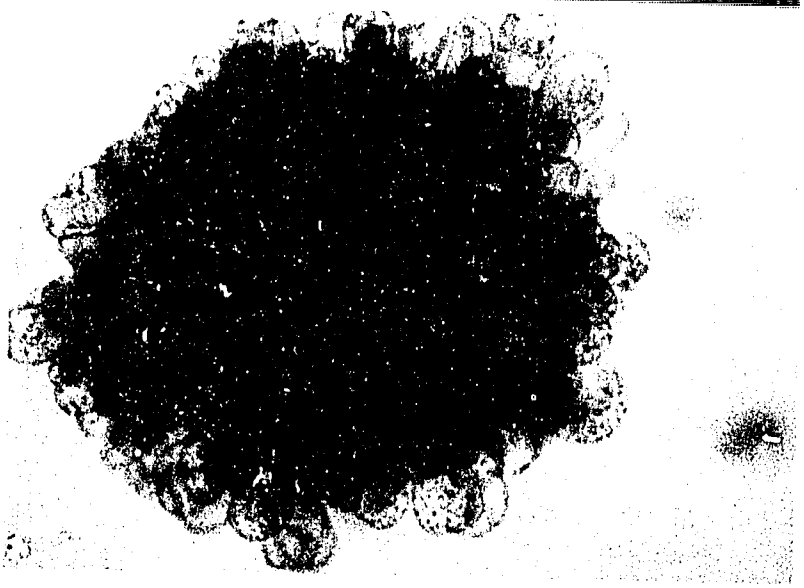
Fig. 15 Early stage of microcallus formation of the fused potato protoplasts after 14 days of culture in Ishige medium, 20x.

Fig. 16 Microcallus from the fused potato protoplasts after transferred to MS medium supplemented with 1 ppm 2,4 - D, 20x.

Fig. 17 Callus from the fused potato protoplasts after 4 weeks cultured in MS medium supplemented with 1 ppm 2,4 - D, 4x.







to the Ishige medium (Fig 15 and 16). To stimulate the growth of the calli 1 ml of fresh medium without mannitol was added after 7 days of culture. The calli could develop and see by naked eyes within 25 - 30 days (Fig. 17 and 18) after culturing in the MS + 1 ppm. 2,4 - D medium.

4.7 Plant regeneration from the fused calli.

After 30 days of culture, the white to green cluster of cell was formed (Fig. 18). Among the *S. acaule* clones used in the fusion experiment, the Oka 3716 was superior than the other clones, in terms of better source of good quality protoplasts and higher plating efficiency. The colonies grown in liquid Ishige medium were normally white but the cells were very compact. The colonies would turn green after transferred to the MS medium supplemented with 0.04 ppm BAP and 0.3 ppm NAA (Fig. 19) and some shoot could also be observed (Fig. 19). The fusants between the Oka 3716 and JH 52, Oka 3716 and Russet Burbank, Oka 7576 and USW, and PI 472646 and JH 52 were obtained. However, 161 clones of the Oka 3716 and JH52 and 37 clones of Oka 3716 and Russet Burbank could continue to form the whole plantlets. The others could get only to meristem-like structure and could not form the real plantlets. Some plantlets from Oka 7576 and USW were obtained but they were albino plants and could keep only on the synthetic medium and were lost after 6 months of cultures due to very week, tiny stem and leaves.

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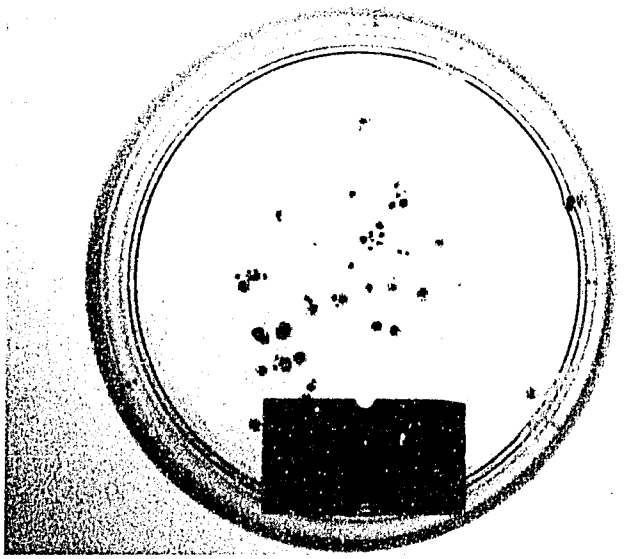
Fig. 18 Small calli developed from the fused Oka 3716 and JH 52 protoplasts after cultured in modified MS + 1 ppm 2,4 -D.

Fig. 19 Compact and green callus with a shoot development after 25 days of cultured in regeneration medium.



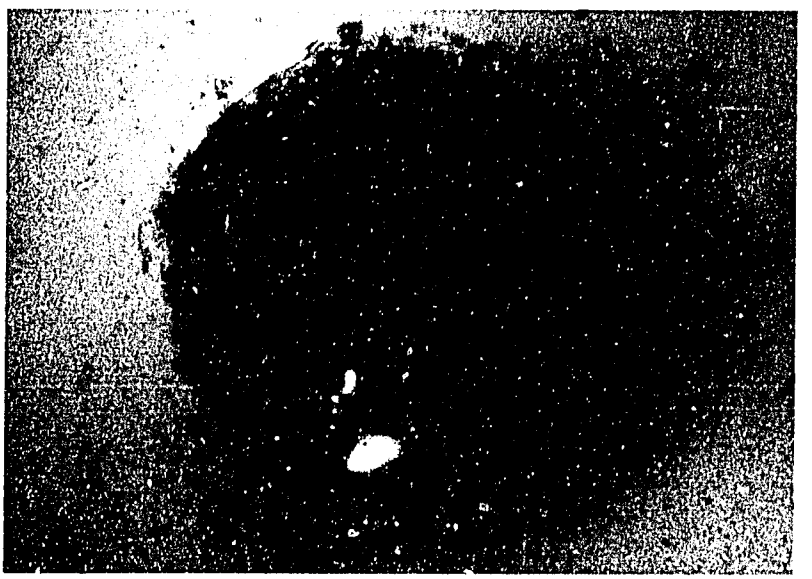


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Fig. 20 Shoot formation of the somatic hybrid between Oka 3716 and Russet Burbank derived from anther culture, after 35 days of culture on the regeneration medium.

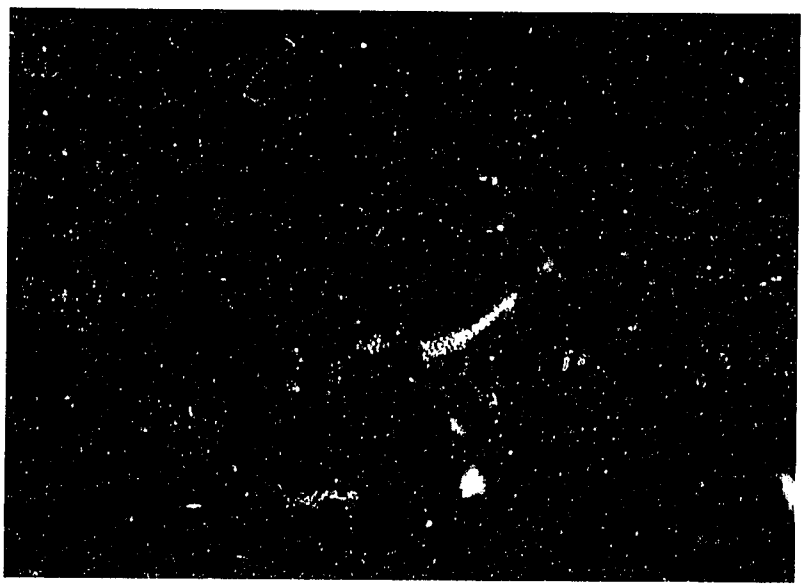

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Fig. 21 Somatic hybrid plantlets (2 tubes in the center) comparing with the Russet Burbank (left) and Oka 3716 (right) plantlets.

RAW + 3716

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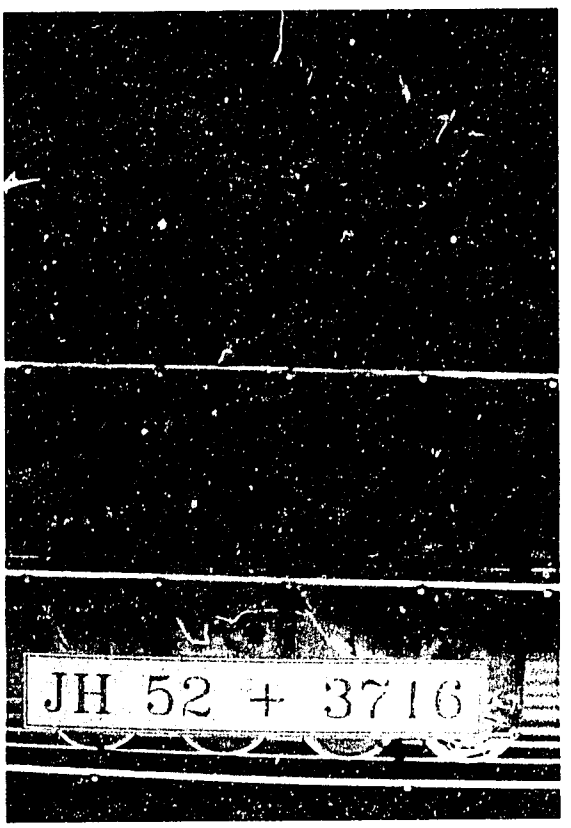
Fig. 22 Somatic hybrid plantlets (2 tubes in the centre) comparing with the JH52 (left) and Oka 3716 (right) plantlets.



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4.8 Total Protein electrophoresis.

In the protein bands pattern analysis, all of the *S. acaule* clones could be identified by the clear bands of protein at the top of the gel slab. None of the commercial cultivars (Russet Burbank, Kennebec and Spunta) as well as the dihaploid clones had the same pattern of bands at this regions, except the Russet Burbank and the Kennebec had the only one light band at the bottom of the protein group (Fig. 23). Silver stain would not clearly stain the protein bands at this region, therefore the light or translucent bands could be detected. All of the clear bands were dark stained with the Coomassie blue solution and gave better pattern of the proteins. Moreover, the pattern of the total protein bands was stable when the *in vitro* grown plantlets were used. Thus this technique was applied for identification of the somatic hybrid as shown in fig. 23.

4.9 Testing for esterase and peroxidase enzymes.

The study on the esterase enzymes analysis could be used to identify the Oka 3716 from the other clones because 3 clear bands could be detected after 30 min of staining, where as the other cultivars had 2 bands (Fig. 24). Moreover, the position of each band of different cultivars was also dissimilar. It was also used for rapid confirming of the fusants.

Peroxidase though had been reported as the useful tool for identification of potato cultivars (Mollers and Wenzel, 1992), but in our condition these enzymes were not so effective and depended on the gel quality in each experiment. Some greenish black bands were obtained after staining but there was no clear significant different among the cultivars used in this experiment.

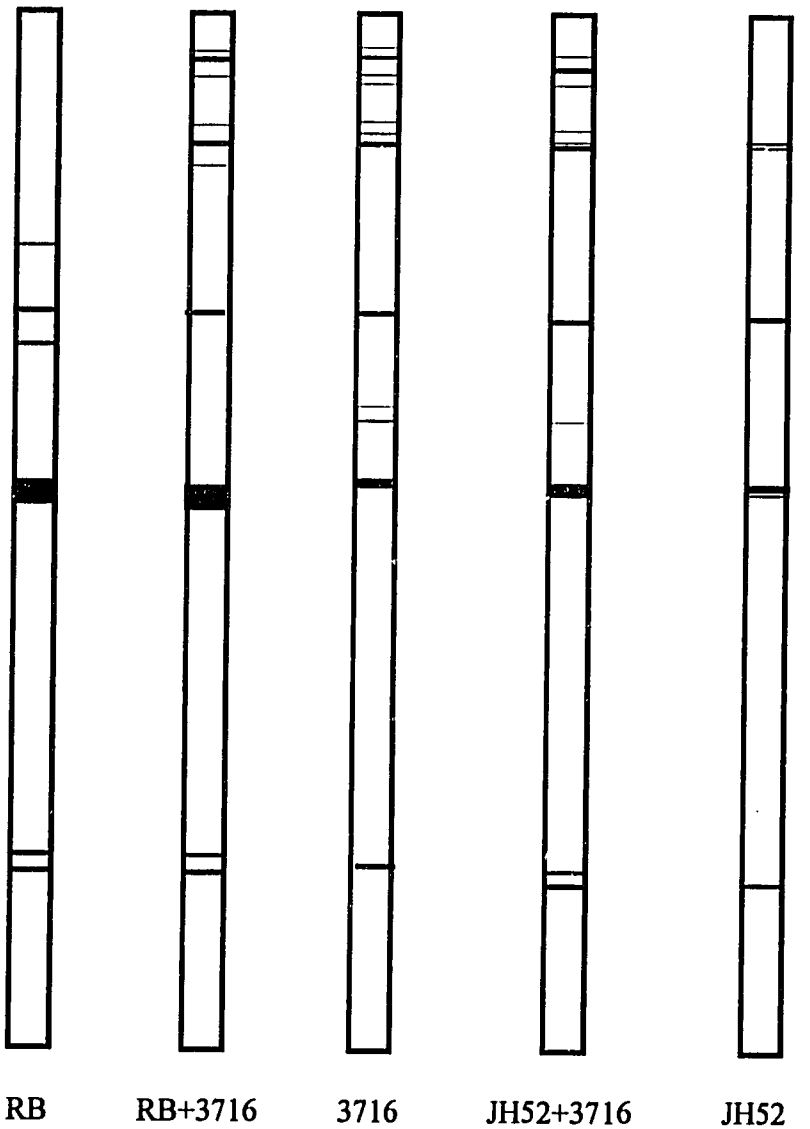

Fig. 23 Major protein bands used for identification of fusants.

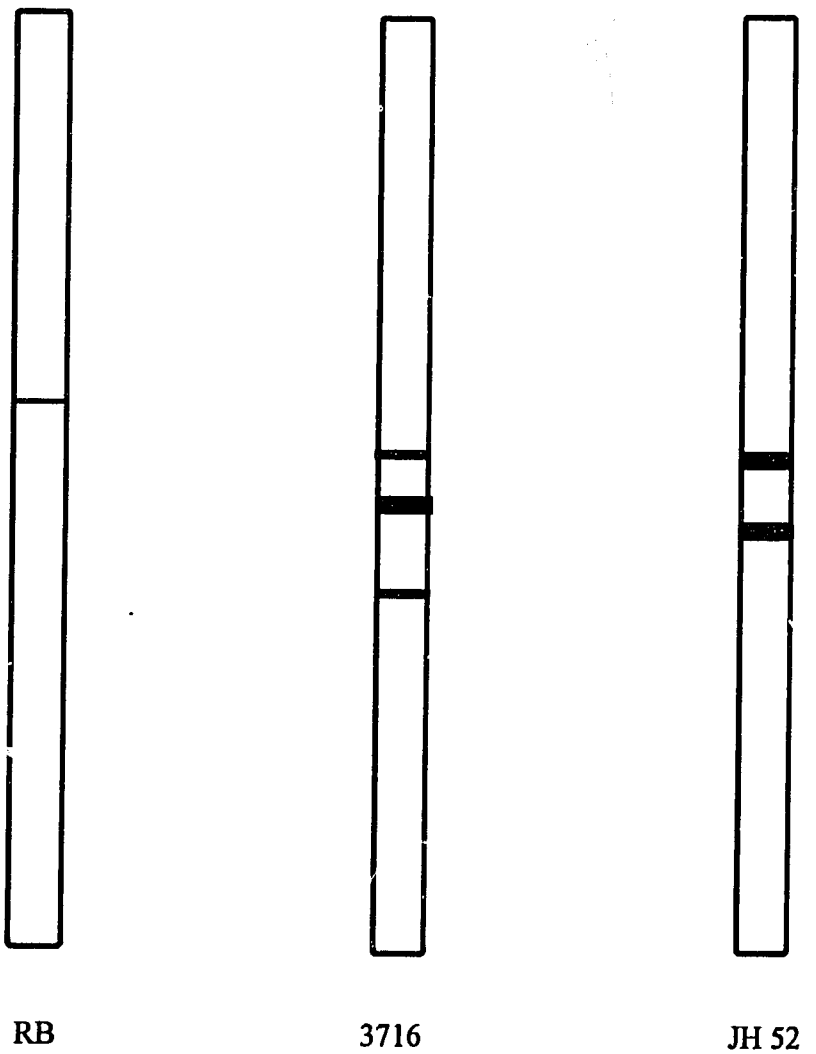

Fig. 24 Esterase pattern of some potato cultivars used in the experiments.

4.10 Multiplication of PLRV in the regenerated plants.

The micro-grafting technique (Fig. 24) was used for transferring of PLRV from the Sieglinde infected plantlets to the regenerated fusants and the scions were cut and cultured under the screen house conditions. The plants showed no detectable symptom of leafroll or other deforming of leaves unlike in the normal PLRV infected potato. Five plants were used to test for PLRV abundance in the somatic hybrid plants by ELISA. Comparing with the parent lines, all of the selected clones were highly resistant to PLRV, especially the somatic hybrids derived from JH52 and Oka 3716 (Table 4).

Table 3 Multiplication of PLRV in the somatic hybrids

Cultivar	O.D (A_{405}) range	No. of Clones
Russet Burbank (RB)	0.24	2
JH 52	0.57	2
Oka 3716	0.06	2
RB + Oka 3716	0.07-0.09	14
RB + Oka 3716	0.10-0.12	18
RB + Oka 3716	>0.12	5
JH52+Oka3716	0.07-0.09	49
JH52+Oka3716	0.1-0.12	96
JH52+Oka3716	>0.12	22

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Fig. 25 *In vitro* micrografting of the somatic hybrid on the PLRV infected Sieglinde before cultured on MS medium.

4.11 Field trial

The somatic hybrids when planted under the field condition could grow better than the parent lines. The plants carried Oka 3716 characters in term of semi-dwarf height (25 $\pm$ 4.53 cm), stem ; round with pale green color, thin around 2.47 $\pm$ 0.13 mm in diameter, white flower color. The different between somatic hybrid derived from JH52 and Oka 3716 and Russet Burbank and Oka 3716 were shown in Fig. 26 and 27 respectively.

For the JH 52 and Oka 3716 leaves were dark green and better spread than the Russet Burbank and Oka 3716 which normally erected like Russet Burbank type cultivar. Moreover, they had more simple hairs and shorter internode (30.4 mm long) than the other hybrid. The somatic hybrids between Russet Burbank and Oka 3716 had the tendency to form more runner than the JH52 and Oka 3716 (Fig. 27) and could form a colony of plants surround the original plant.

Tubers were small size 1.2 - 3.7 cm (Fig. 28), white fresh with thin white skin and very low yield comparing with any commercial cultivars.

No PLRV symptom was detected during the planting period. The other character that was superior than the commercial cultivar planted in the same area was resistant to *Phytophthora infestans* by showing only small black spot on the infected leaves where as the others showed severe spread of pathogen with necrotic lesion on leaves and stems and death of infected plants.

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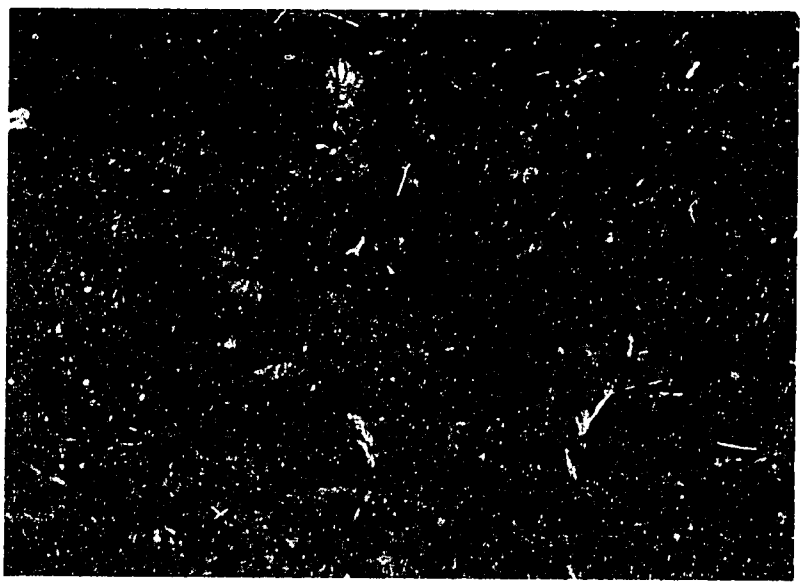
Fig. 26 Somatic hybrid plant from JH 52 and Oka 3716 grew under field conditions with the following description.

Plant decumbent, moderate compact, branched with 3 primary stems; flowering in 45 -50 days after transplanting. Stem green, round, wing. Leaves horizontal; lamina dark green in color, scarcely dissected; terminal leaflet shape ovate, apex acute, margin entire. Flowers whit. Stolons scarcely present.

Fig. 27 Somatic hybrid plant from Russet Burbank and Oka 3716 grew under field conditions with the following description.

Plant decumbent, almost compact, branched with 5 primaty stems; flowering 45 - 50 days after transplanting. Stem pale green, round. Leaves semi-erect; laminar pale green in color, scarcely dissected; terminal leaflet shape ovate, apex obtuse, margin undulate. Flower white. Stolons profusely present.



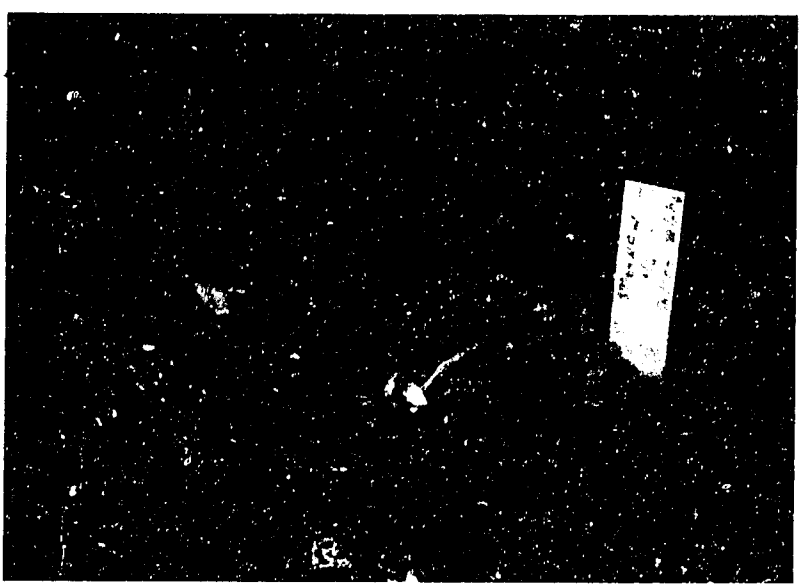


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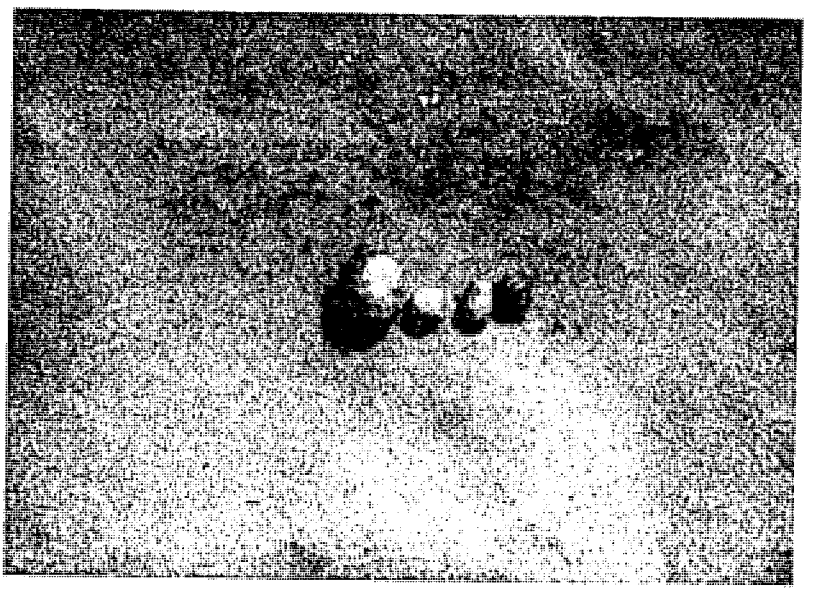
Fig. 28 Tubers obtained from the somatic hybrids with white fresh and skin.



5. Conclusion

5.1 The suitable commercial cultivars for the Thai conditions in term of PLRV resistant could be divided in to the following groups a) tolerance : Atlantic and Lemhi cultivars b) moderate resistant : Russet Burbank, Sieglinde and Spunta cultivars and c) susceptible : Kennebec cultivar. Even though the Atlantic cultivar was more resistant to PLRV but it was very susceptible to late blight disease compare to the others especially Spunta which was more resistant to late blight than every cultivars grown in Thailand. Lemhi and Russet Burbank cultivar could not well adapt to the hot and humid climate and the yield was lower than other cultivars. Thus they were not so popular in this area.

5.2 Grafting transmission was very effective in transferring PLRV from infected plants to the non-infected ones. Moreover, micrografting could be directly used in the *in vitro* grown plants.

5.3 Chloroplast number in the guard cells could be effectively used for the ploidy level identification in potato.

5.4 *In vitro* grown plantlets could be very good source of protoplasts in term of no contamination and more healthy protoplasts were obtained.

5.5 PEG could be used for the protoplast fusion though it took longer time and more complicate than electro - fusion. The factors involved in the successfulness of this technique was the time used in each fusion step and the lowering of the PEG concentration after the fusion process. Moreover, the media used in protoplast culture were also very important. Furthermore, lowering of the osmotic stabilizer in the cultured medium was another important factor in protoplast culture. It should be gradually decrease related to the culturing period.

5.6 Protoplast fusion technique was a good way for the gene pooling because all of the genes located in both nucleus and cytoplasm could be simultaneously combined together. However, this could be the disadvantage in certain plant improvement method. Due to the total genes pooling from two origins by this technique, the unwanted inferior characters could also be presented in the somatic hybrids. In this experiment, the plant characters and yield were strongly influenced by the genes from *S. acaule*. Anyhow, the PLRV resistant could be virtuously transferred to the somatic hybrids. At present, all of the derived somatic could not yet used for the commercial production or did not have the commercial value but they could be a good source for the PLRV resistant clones. Moreover, the unexpected desire character that could obtain from the protoplast fusion among *S. acaule* and the commercial cultivars was resistant to *Phytophthora infestans* which cause serious loss and was one of the important disease in the tropical region.

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Acknowledgements

This work was financially supported by the USAID/PSTC Program. Grant No. 936-5542-G-00-7021-00. Due to many unexpected and uncontrolled environmental factors, this report has to delayed for years, we are really sorry about this and would like to apologize to USAID/PSTC. We would like to thank Dr. Stephen S. Sinden and Dr. Sherret S. Chase for their help and useful comments and finally to Faculty of Agriculture, Chiang Mai University for supplying the working space.

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