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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D C 20523

DATE: 9/23/88

MEMORANDUM

TO: AID/PPC/CDIF/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 6, 401
PR #2

Attachment

PROGRESS REPORT NO. 2

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

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THAILAND

Project Profile

Country	: Thailand
Grant No.	: 936-5542-G-00-7021-00
Program	: Program on Science and Technology Cooperation
Project Title	: Selection and Development of Virus Resistant Potato by Using Protoplast Culture and Fusion Technique.
Project Leader	: Dr. Prasartporn Smitamana
Organization	: Faculty of Agriculture Chiang Mai University 50002 Chiang Mai, THAILAND.
Co-investigators	: Dr. Pajchima Smitamana Dr. Danai Boonyakiat Assoc. Prof. Kesinee Ramingwong
Researcher	: Mr. Visnu Somsab
Project consultant	: Dr. Sherret S. Chase Dr. Stephen S. Sinden
Authorized Officer	: Prof. Avudh Srisukri
Total Project Budget	: 3,816,192 Bath. (149,070 US$)
Project Duration	: 3 Year (August 1987 - July 1990)
Reporting Period	: January - July 1988..
Budget Allocation for this Period	: 452 876.75 Bath (17,690.50 $)

Content

1. Background/Introduction

Of all agricultural crops the potato is 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 Center, 1984) by using asexual propagation method which many kinds of diseases especially viruses can be easily built - up and caused a serious loss every year. 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.

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.

The production of haploid plants using the basic techniques of anther and pollen culture enfolds great promise for plant breeding programs and genetic studies in angiosperms (Bajaj, 1983 ; Maheshwari *et al.*, 1982, 1983). In potato, dihaploid plants can be produced both in vivo by pollination of tetraploids with selected diploid *Solanum phureja* genotypes (Hermsen and Verdenius, 1973) and *in vitro* by anther culture (Mix, 1983 ; Wenzel and Foroughi-Wehr, 1984). Moreover, it has proved difficultly to obtain the pollen plantlets from the anther of the cultivated potatoes, which might due to the genotype of the donor plants (Kohlenbach and Gier, 1972 ; Dunwell and Sunderland, 1973 ; Jacobsen and Sopory, 1978 ; Wenzel and Uhrig, 1981) and this character of responsiveness could be inherited, but still not yet ascertained (Jacobsen and Sopory, 1978 ; Wenzel and Uhrig, 1981) which already discussed in the first report.

Potato protoplast isolation, culture and plantlets regeneration have been accomplished in both various commercially grown tetraploid cultivars and the dihaploid clones (Binding and Nehls, 1977 ; Shepard and Totten, 1977 ; Butenko and Kucho, 1978; Grun and Chu, 1978 ; Wenzel *et al.*, 1979 ; Schumannn *et al.*, 1980). Several different protocols, media, and culturing condition have already established.

As has been discussed, 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.

2. Objectives

2.1. To continue working on anther culture and to induce morphogenesis in the anther deriving calli.

2.2. To find the appropriate conditions for protoplast isolation and culture under the existing laboratory condition.

3. Materials and Methods

3.1. Anther culture

According to the first progress report, anthers from the greenhouse and the field grown potatoes could be induced to form callus on the modified Murashige and Skoog (1962) and modified Nitsch and Nitsch medium. Other media are used in this reporting period 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 are varied (see result and discussion).

3.2. Protoplast isolation and culture.

Potato protoplasts are isolated from both greenhouse and *in vitro* grown (Fig. 1.) 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.). The protoplasts suspension obtained from enzyme digestion was passed through double layer cheesecloth and centrifuged at 600 rpm. for 5 minutes, the supernatant removed, then the pellets resuspended in protoplast wash 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 centrifuge at 1,000 rpm for 5 minutes. The band of protoplasts at the interface of the two solutions was removed carefully using a pasteur pipette and washed twice with wash 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.

4. Results and discussion

Even though the young flower buds have 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 ca. 0.4 ml of culture medium, and kept in a moist chamber. The microbial contamination, mostly bacteria, was easily observed and identified. Only the non-contaminated anthers were transferred to the same media in 35 x 10 mm. Petri dish, and were incubated at 25 + 2° C. with 16 h photoperiod. 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. 1). 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. 2). Out of 576 Russet Burbank's anthers, only 7 could continue growing and produce 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. 3 and 4). 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.

The protoplasts derived from the greenhouse grown plants could easily observe the chloroplasts which were located on the surface of the cells (Fig. 6) 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. 9). 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 cell wall within 2 days after isolation (Fig. 8). The plating efficiency at this reporting period was 1.8 % in both VKM and CL medium, where as RM 5 (Creissen and Karp, 1985) had only 0.1 %.

5. Conclusion/Remarks

Within this reporting period, the organogenesis of the calli derived from the anthers of various potato cultivars was achieved. Even though the true shoots or plantlets could not ready be obtained at this current time, the meristem dome and some roots were already observed, thereby the dihaploid plantlets could be expected to evolve from such calli. By the mean time, the *Solanum phureja* clones, which were kindly supplied by Dr. Hermsen and Dr. Schilde-Rentschler (personal contact), have been grown in the local made control environment chamber, and expected to bloom in the near future. This could be pollinated with other commercial potato cultivars and the dihaploid seeds could be obtained by this crossing too (Hermundstad and Peloquin, 1987 ; Frusciante and Peloquin, 1987 ; Estrada, 1987).

The protoplast isolation and culture work could be now standardized, both *in vitro* shoots and greenhouse grown plants could be used for this purpose. The protoplasts could be successfully isolated, and the cultivated protoplasts could further divide, but the plating efficiency was fairly low. Even though Fish and Karp (1986) had reported the same rate, it ought to be improved to get more chance of attaining the fusion product. This might be achieved by using the bead culture method as stated by Schilde-Rentschler *et al.* (1987). Nevertheless, this method need to be verified and standardized to fit our laboratory conditions. According to the result, the protoplasts could be

easily isolated from the greenhouse grown plants, but the *in vitro* shoots have the superior advantages in term of no contamination problem and giving the better quality protoplasts. This might cause by the difference between the method used, whereby one need more time and thus the plant cells could probably better adapt themselves to the culture conditions during the succeeding changes of various solutions in the isolation steps. This could be noticed by the structure and the appearance of the protoplasts which some were ready to divide almost instantly after the isolation.

6. <u>Workplan for the Next Period</u> :

1. Continuing the anther culture and the protoplast experiments to get more dihaploid plants and more information to improve the culture conditions and the plating efficiency.

2. Starting protoplast fusion experiment to standardize the fusion methodology and conditions. The derived dihaploid plants will be used at this step.

3. Starting the isozyme pattern analysis of various potato cultivars used in the proposal. This information will be very important for the selection of the fusion products.

4. Starting the ELISA work on the detection of the PLRV multiplication in the potato cultivars and various *S. acaule* clones.

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8. Annex/Pictures :

Fig. 1. A friable callus derived from the Russet Burbank anther cultured on modified Murashige and Skoog (1962) medium. (x 8)

Fig. 2. A compact callus derived from Russet Burbank anther after 8 weeks on modified Murashige and Skoog (1962) medium. (x 8)

Fig. 3. Morphogenesis of potato callus after 5 weeks transferred on the modified Murashige and Skoog (1962) medium supplemented with 0.5 ppm BAP, 1 ppm NAA and 1 ppm GA. (x 8)

Fig. 4. Some meristem-like dome and root formation could be observed from the callus cultured on the same medium as Fig. 3. (x 4)

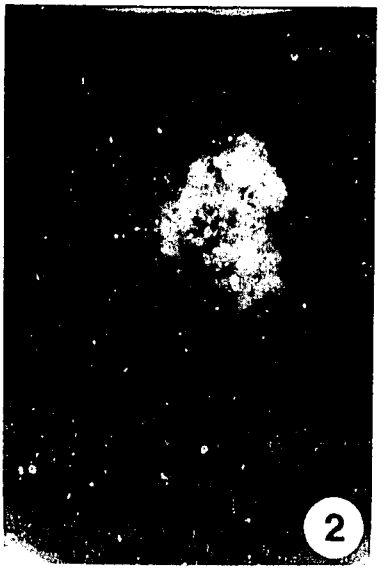

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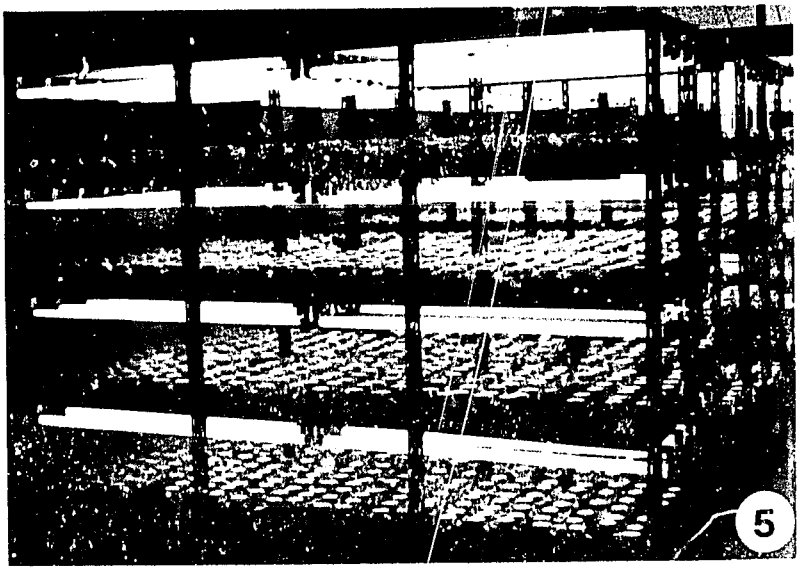
Fig. 5. Rapid multiplication of *in vitro* shoots through the single node cutting method for the protoplast isolation.

Fig. 6. Fresh isolated mesophyll protoplasts from the greenhouse grown plants. (x 20)

Fig. 7. Cell wall formation of potato protoplast after 2 days of culture in VKM. (x 20)

Fig. 8. First cell division of the mesophyll potato protoplasts cultured in CL medium. (x 20)



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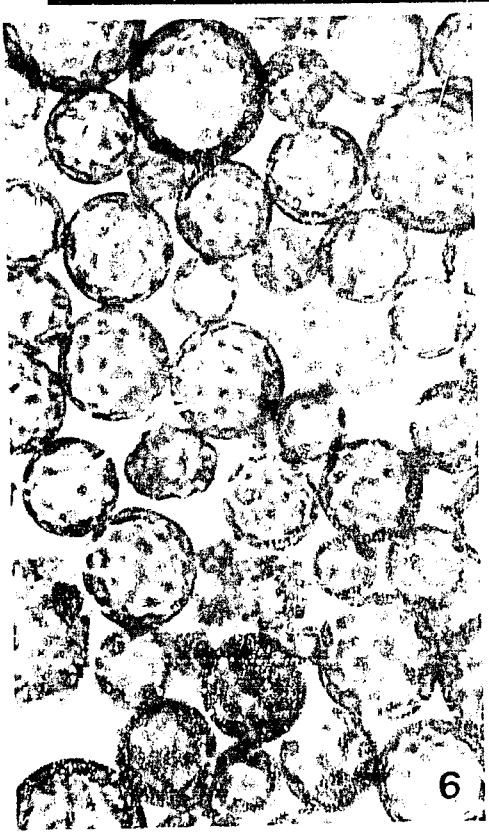

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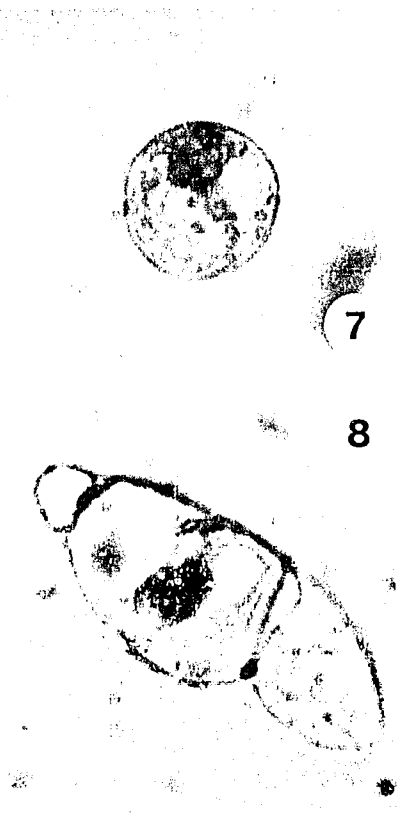

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