Somatic hybridization between potato (*S.tuberosum*) and *S.phureja* to transfer bacterial wilt (*Ralstonia solanacearum*) resistance traits

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ABSTRACT

To sustain potato production in tropical region, new cultivars resistant to a number of diseases must be available. One of most important diseases in the tropical regions is bacterial wilt caused by *Ralstonia solanacearum.* The loss of production due to the disease is up to 90 percent. The possibility to introgress of the resistance genes is come from wild species of *Solanum phureja*. However, crossing between *S.tuberosum* X *S.phureja* is limited by incompatibility between them. Somatic hybridisation is expected to provide a new possibility for increasing genetic variability, and also a means of transferring desirable agronomic traits into potato. The objective of this research is to produce high yield cultivars of potato resistance to bacterial wilt that adapted in the tropical region.

Somatic hybrid plants were produced after protoplast fusion between a dihaploid (2n=2x=24) potato cv. BF15 and a wild tuber-bearing diploid (2n=2x=24) species *S.phureja.* There were 50 plants produced after transferring protoplast-derived microcalli to fresh VKM medium supplemented with 2 mg/l BAP and 0.1 mg/l 2,4-D for 2 weeks followed by transferring into regeneration medium MS supplemented with 0.1 mg/l IAA and 2.0 mg/l zeatin. Emerging shoots were then excised from callus and plantlets were multiplied by subculturing leafly node cutting on hormone-free MS medium. A total 10 somatic hybrid were revealed after confirmed by examining isoenzyme pattern, RAPD analysis, chromosome counting and number of chloroplasts in guard cell. The five of tetraploid (2n=4x=48) of somatic hybrids were then evaluated their performance for resistances by inoculating bacterial wilt races 1 and race 3 *in vitro*, production of microtubers *in vitro*, and their performance in the field.

The cultivated potato cv. BF15 was susceptible to both races tested. All somatic hybrid were resistant or moderate resistant to race 3 strain and three of somatic hybrids were resistant to race 1 strain. All the five of somatic hybrids were able to produce microtubers, however there were variation in the number of tubers, earliness of tuberization, fresh weight as well as dry weight. Tuber production in the field by planting minicutting as propagule revealed that one of somatic hybrid was very promising as compared to Indonesian popular cultivar Granola.

Keywords: *Solanum tuberosum, Solanum phureja,* Somatic hybrids; Bacterial Wilt; *Ralstonia solanacearum*

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Introduction

Potato is a basic staple food crop susceptible to a number of diseases, some of which are widespread and other localized. Bacterial wilt, a severe and devastating plant diseases caused by *Ralstonia solanacearum*, occurs in most tropical, subtropical and warm temperate region (Hayward et al., 1998). In tropical region, the loss of harvest due to the disease is up to 90 percent (Wattimena et al., 2000). The bacterium invade plant vascular tissues from wounded roots or natural opening. Colonization of the stem results in browning of the xylem and frequently leads to partial or complete wilting (Grimault, 1994). There are several race of *R. solanacearum*, but the important race damage potato crop under tropical condition is race 1 and 3 strains (French et al., 1998).

Resistant or tolerant cultivars are mainly utilized for disease management since chemicals are not effective and sanitation measures difficult to apply. Therefore, the worldwide control strategy has consisted of plant breeding for resistance to bacterial wilt (Buddenhagen, 1986). Immunity for such traits was not identified in potato, only tolerant cultivars were selected (Hayward, 1991). To control bacterial wilt, continuous development of resistant or tolerant varieties is needed.

Some wild or related cultivated species are knows to be resistant or highly tolerant to bacterial wilt and thus are potential sources for resistance. *S. phureja* is phylogenically close to *S. tuberosum* and displays resistance traits, dominant and readily transmitted to progeny. Resistance to bacterial wilt derived from *S.phureja* was first described as dominant and controlled by three unlinked genes (Rowe et al., 1979; French et al., 1982). Recently, at least four major genes have been reported to be involved in potato resistance to bacterial wilt (Grimley et al., 1998).

Introgression of multiple resistance genes from wild *Solanum* species into *S.tuberosum* by classical breeding methods is time consuming, laborious and may encounter difficulties because of sexual incompatibilities, particularly due to different in ploidy level or in endosperm balance number. Somatic fusion is expected to provide a new possibility for increasing genetic variability, and also a means of transferring desirable agronomic traits into potato. The potential use of somatic hybridization has been demonstrated by successful introduction of traits such as resistance to viruses (Roca et al., 1994), salinity (Sherraf, et al., 1994), insect resistance from *S. berthaultii* (Sherraf et al., 1991) and resistance to bacterial wilt from *S. commersonii* (Laferriere et al., 1999). Protoplast fusion between potato and *S.phureja* was reported in the resulting partial elimination of chromosome, particularly chromosome from *S.phureja* (Pijnacker et al., 1987). So far, no information has been available about evaluation of somatic hybrid clones for the introgression of resistance against bacterial wilt from *S.phureja* into potato.

In this study, somatic hybridization between the dihaploid *S.tuberosum* cv BF15 and the diploid *S.phureja* was performed. The somatic hybrids were identified and characterized as well as evaluated from resistance to race 1 and race 3 strains of *R. solanacearum*.

Materials and Methods

Plant material

A clone of dihaploid S.tuberosum L (2n=2x=24 chromosomes), cv BF15, obtained from Morphogenese Vegetatle Experimentale, University of South Paris, France, and a diploid clone of wild tuber-bearing *S.phureja* SVP5 (PH77=1445-2242) provided by the Center for Genetic Resources, Wageningen, the Netherlands, were used. Plants were propagated by subculturing leafly node cuttings, at 4-weeks intervals, on MS basal medium (Murashige and Skoog, 1962). Containing vitamins (Morel and Wetmore, 1951), 20 g/l

sucrose and 7 g/l agar. Environmental conditions were 12 h/day illumination at 62 µE/m^2 /s, 20 ^{o}C and 60% humidity.

Protoplast isolation

Leaves taken from 3-4 week-old cuttings of both species were slightly scarified, then they were transferred into the filter-sterilized enzyme solution containing cell and protoplast washing salts (CPW)(Frearson, Power and Cocking, 1973), 0.5 M mannitol, 1% cellulase R-10 (Yakult, Tokyo, Japan), 0.05% (w/v) pectolyase Y-23 (Sheishin, Tokyo, Japan) and 0.05% (w/v) 2-(N-morpholino)ethanesulfonic acid (MES) buffer at 5.5. Scarified leaves were placed face down on the enzyme solution and incubated overnight in the dark 27 ^{o}C. At the end of digestion period, protoplasts were separated from undigested material through metallic sieves (100 µm mesh), and the resulting suspension was centrifuged at 55 x g for 5 min. The supernatant was removed and protoplasts resuspended in a CPW solution 21% (w/v) sucrose, then centrifuged at 120 x g for 10 min. The floating protoplast were recovered, washed twice in a CPW solution plus 0.25 M mannitol and 0.125 M NaCl by centrifugation at 55 x g for 5 min. Prior to fusion, protoplasts were washed once in a 0.5 M mannitol solution plus 0.2 mM CaCl2, then they were suspended in this solution at a density of 3.5 x 10^5 protoplasts/ml.

Electrical apparatus and fusion procedure

The electrical apparatus and fusion procedure described in Sihachakr et al., (1988) were used. Briefly, the movable multi-electrode were placed in a 15 x 50 mm petri dish a 500-700 µl aliquot of a mixture (1:1) of protoplast from both species. In order to align the protoplast an AC-field at 125 V/cm and 1 MHZ was applied for 15 s; subsequently, one square pulse developing 1.2 kV/cm for 40 µs was applied to achieve protoplast fusion.

Protoplast culture and plant regeneration

After electrical treatments, the movable electrodes were removed, and 6 ml of culture medium were added progressively to the petridish containing the fused protoplast mixture. The culture medium was VKM medium (Binding et al., 1978) supplemented with 250 mg/l PEG, 0.2 mg/l 2,4-D, 0.5 mg/l zeatin, 1 mg/l NAA, 3.3% (w/v) mannitol and 3.2 (w/v) MES. The pH of the medium was adjusted to 5.8 prior to sterilizing by filtration (0.22 µm filter-Millipore). The cultures were kept in the dark for 7 days, afterwards they were exposed to light. On day 15, the culture were diluted 8 times with fresh VKM medium supplemented with 2 mg/l BAP and 0.1 mg/l 2,4-D, pH 5.8. Calli (3-4 mm diameter) were then transferred to the regeneration medium: MS basal medium, vitamins (Morel and Wetmore, 1951), 2 %(w/v) sucrose, 2 mg/l zeatin and 0.1 mg/l IAA and solidified with 7 g/l agar (Difco). Shoots were excised from callus, multiplied by subculturing leafly node cuttings on hormone-free MS medium.

Isoenzyme analysis

Leaf extract were prepared with plant materials taken from in vitro plants according to the methods as described in Sihachakr et al (1988). Electroporesis was performed on 7% (w/v) acrylamide gel to separate esterases (EC 3.1.1.2) and peroxidase (EC 1.11.1.7). Staining was done as described in Shields et al (1983).

DNA isolation and PCR analysis

Plant genomic DNA was isolated with mini prep DNA isolation methods. (van Heusden et al., 2000). Optimal conditions, such as temperature, DNA concentration and suitable primers for PCR were checked in primary experiments. The successful PCR was

performed with primer OPA18 (5' GCTATCTGAC3'). The PCR cycle was 94^{o}C for 2 min (1 cycle); 94 ^{o}C for 1 min, 56 ^{o}C for 1 min, 72 ^{o}C for 2 min (35 cycles); 72 ^{o}C annealing extension for 10 min. The reactions were carried out in a Parkin Elmer Cetus Thermocycler.

In vitro tuber production

Six weeks old of in vitro plants were induce for producing in vitro tuber by adding liquid tuberization medium MS containing 90 g/l sucrose, 5 mg/l BAP, 400 mg/l cycocel resulting double layer solid and liquid phases. Cultures were then incubated in the darkness at 20 ^{o}C for 8 weeks.

Determination of in vitro resistance to bacterial wilt

Two strains of *R.solanacearum* (race 1 and race 3) provide by Research Institute for Food Crops Biotechnology, Bogor, Indonesia) were used to inoculate the somatic hybrids and both their parental lines. Inoculations were done using bacterial suspension containing 10^9 colony forming units (cfu) per ml by immersing scissor into the bacterial suspension prior cut the shoot tip. The shoots tip were then transferred into MS medium without hormone and exposed to 14 h/day illumination at 55 µmol/m^2/s, 25 ^{o}C. The test were done by using 20 plants per clone. Plants were observed everyday for the period of incubation, disease symptom and the plant resistance. Disease indexes were calculated according to Winstead and Kelman, as the ration between the sum of the products of each disease number.

Result and Discussion

Protoplast culture and plant regeneration

Cells were developed rapidly into calli after transferring the cultures onto the solidified growth medium. Two weeks later, only 2-3 mm in size were selected to be transferred onto regeneration medium and after 5 weeks on regeneration medium, some calli produced shoots. Only shoot was excised from the regenerating callus and multiplied by subculturing on hormone-free MS medium. Finally, 55 of 322 selected calli produced shoots, representing a percentage of 17.0%. The process of somatic hybridization are showed in Fig. 1.

Isoenzym and PCR analysis

The electrophoretic pattern for esterases and peroxidases confirmed the hybrids nature of the selected putative hybrids. Every isoenzyme system revealed differences between potato BF15 and *S.phureja*. The Isoenzym pattern also distinguished somatic hybrids and their parents (Fig. 1A, 1B). These hybrids nature were also confirmed by PCR analysis. The OPA18 primer (5' GCTATCTGAC3') lead to distinction between the two parents of the fusion experiments (Fig. 1C).

Microtuber production

The five of tetraploid (2n=4x=48) of somatic hybrids were evaluated their production of microtubers *in vitro*. All the five of somatic hybrids were able to produce microtubers, how ever there were variation in the number of tubers, earliness of tuberization, fresh weight as well as dry weight.

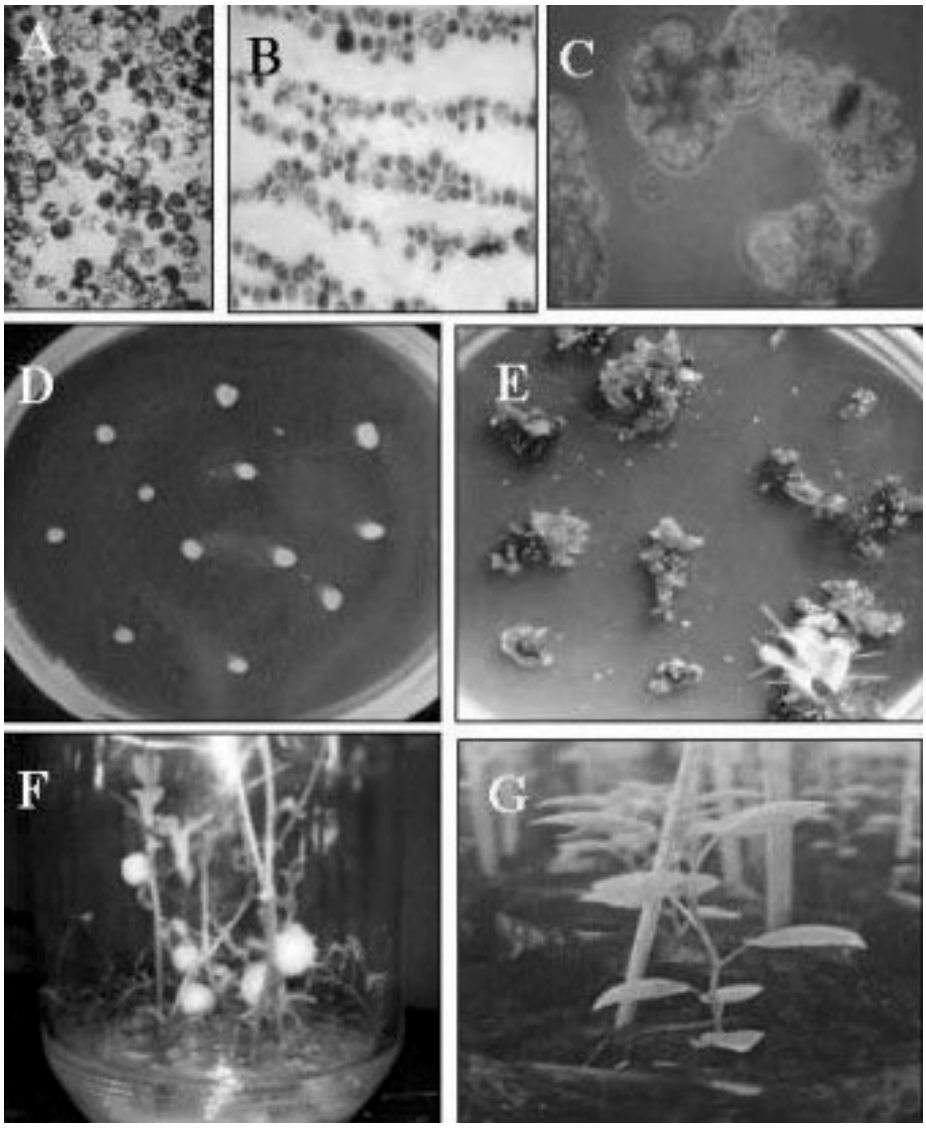

Figure 1. Process for producing somatic hybrids. A. Fresh protoplasts, B. Alignment of protoplasts after application AC field, C. Fusion between protoplasts, D. Microcalli in regeneration medium, E. Producing shoots after 6 weeks cultured in regeneration medium, F. Production of in vitro microtubers, G. Somatic hybrid plants before transferred to the field.

Evaluation of resistance to bacterial wilt in vitro

Solanum tuberosum parental lines showed susceptible to bacterial wilt either race 1 or race 3 strain, while *S.phureja* was resistant to both races. The somatic hybrids tested showed variation to both races in term of incubation period or diseases incidence. All somatic hybrid were resistant or moderate resistant to bacterial wilt race 3 strain, however only 3 of somatic hybrids resistant to race 1 strain, among of them were line BP3, BP4 and BP9. The results indicated that most somatic hybrids showed intermediate to their both parents, as showed in Table 2. Comparing to Indonesian popular cv. Granola, some of the somatic hybrids were better.

Table 1. Production of in vitro micro tuber of tetraploid somatic hybrids and their parental line.

Genotype	Number of tuber per plant	Average fresh weight per tuber (mg)	% dry weight
BP3	2.1b	125b	12.1±1.7
BP4	3.2c	149c	15.2±0.9
BP6	1.4a	112a	13.4±2.1
BP9	1.1a	97a	10.2±1.2
BP15	2.2b	131b	12.7±0.7
BP16	1.6a	119b	14.2±2.2
S.phureja	3.1c	87a	15.7±3.7
BF15	2.4bc	92a	14.2±1.6
Granola	2.2b	137b	11.7±1.3

Values followed by the same latter are not significantly different at P=0.05

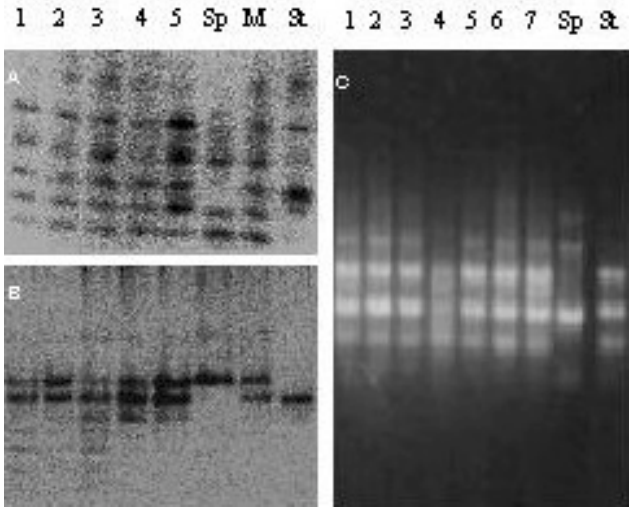

Figure 2. A, B= Electrophoresis binding pattern of esterases (A) and peroxidases (B) for *S.tuberosum* cv BF15 (St), *S.phureja* (Sp), a mixture of parental extract (M) and their somatic hybrids BP3, BP4, BP6, BP15 and BP9 (lanes 1-5 repectively). C=Electrophopresis profiles of PCR amplification product using OPA18 primer (5' GCTATCTGAC3'). DNA from *S.tuberosum* (St), *Sphureja* (Sp) and their somatic hybrids BP3, BP4, BP6, BP1, BP9, BP15, and BP16 (lanes 1-7 respectively).

Performance somatic hybrid in the field

All of the tetraploid somatic hybrid tested showed normal growth and produce tubers. The propagules used were mini cutting. Mini cuttings were produced from in vitro cuttings after 4 week acclimatization. Mini cuttings were shoot tip cuttings with 3-4 leaves. The cuttings were then rooted to produce seedling. The seedling were then transfer to the field. Usually, the tuber produce from the seedling are smaller tuber comparing with normal seed tuber. Numbers of stolon are usually also less. So, to demonstrate the potential yield of somatic hybrid, there was also commercial cultivar planted as control. The result indicated that two of somatic hybrids were better than control in term of height of plant, number of tuber, fresh weight as well as dry matter, although the results should be further elucidated.

Table 2. The rate of resistance of somatic hybrids after shoot inoculation by race 1 strain of *R.solanacearum*

Clones	Incubation periods (days after inoculation)	Diseases incidence (%)	Degree of resistance
R. solanacearum race 1 strain			
BP3	18	19.2	Resistant
BP4	16	16.6	Resistant
BP6	6	42.1	Susceptible
BP9	14	17.6	Resistant
BP15	8	57.3	Susceptible
BP16	8	66.4	Susceptible
S.phureja	17	15.8	Resistant
BF15	5	100.0	Susceptible
Granola	5	87.5	Susceptible
R. solanacearum race 3 strain			
BP3	14	37.6	Moderate Resistant
BP4	13	17.7	Resistant
BP6	17	16.3	Resistant
BP9	10	23.7	Moderate Resistant
BP15	10	27.3	Moderate Resistant
BP16	6	10.3	Resistant
S.phureja	15	17.5	Resistant
BF15	5	100	Susceptible
Granola	7	75.5	Susceptible

Table 3. Performance of tetraploid somatic hybrids and their parental line in the field.

Genotype	Height of plant (cm)	Number of tuber per plant	Fresh weight of tuber per plant (g)	% Dry matter
BP3	56.7a	2.2a	33.7a	15.4±1.9
BP4	72.8c	8.2cd	211.7	17.8±1.5
BP6	77.4cd	3.9a	67.2b	13.7±2.2
BP9	43.7a	5.1b	94.7bc	12.2±1.3
BP15	60.7b	4.2ab	75.9bc	14.2±0.9
BP16	82.3d	6.4bc	117.3c	15.1±2.7
S.phureja	73.4c	9.6d	96.4bc	17.1±1.9
BF15	61.7b	5.7b	121.7c	16.2±1.8
Granola	68.9b	7.4c	177.4d	14.3±1.4

Values followed by the same latter are not significantly different at P=0.05

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