PRODUCING VIRUS-TESTED FREE POTATOES BY THERMOTHERAPY AND MERISTEM CULTURE

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INTRODUCTION

Potato breeders are endeavoring to produce new varieties with disease resistances and commercial qualities superior to those currently grown. This is difficult because true seed obtained from flower crosses produces plants with a wide variety of mostly inferior plant and tuber characteristics. Usually, less than one percent of these plants are worthy of further testing so many tends of thousands must be initially evaluated. The breeder must then further test many hundreds of clones derived by saving and planting tubers from promising plants. Since potatoes respond greatly to small environmental differences, the breeder must plant these early generation clones in a wide range of environments to fairly judge their worth.

These early generation screening trials often have viruses present which soon spread to many of the plants and clones, so most lines are seriously diseased and must be discarded by the time they reach advanced performance trial status, where seed increase would be started. If the breeder is fortunate enough to have plants or even whole clones escape this virus exposure during the early generation screening, then he must use every precaution to insure that they remain clean. Because of the hundreds of lines involved this requires an extensive virus indexing program with large, well equipped greenhouses and labs. Also essential is a nuclear seed farm where hundreds of advanced lines can be increased to provide the virus free seed mandatory for meaningful variety performance trials. Acquiring the necessary facilities, equipment, and personnel to operate a seed farm at today's inflated prices is very expensive. With the current low level of funding in agricultural research, such seed increase programs are seldom feasible, so most evaluators are forced to plant virus-infected tubers in their trials. Virus infected clones produce low yields, poor grade, and poor quality and their true value cannot be recognized. Consequently, they are rejected and discarded even though they may be genetically superior.

As part of the Northwest USDA potato breeding program we have a meristem culturing program at Prosser which alleviates some of these disease problems. The objectives of this program are 1) to eliminate virus contamination from promising clones and breeding parents, 2) to maintain the important potato clones free of virus as meristem cultures in tubes and bottles, thus providing a constant, readily renewable source of virus free seed stocks, and 3) to propagate rapidly from meristems to seed-lot sized field plantings to provide the virus-free seed needed for evaluation trials. Objectives No. 2 and 3 are important functions of a nuclear seed farm, but we perform them at greatly reduced cost in test tubes and bottles.

Prosser Meristem Culture Procedures

Potatoes can be cured of virus infection by growing them at high temperatures (98°F) for several weeks and then excising and culturing the meristems, because virus is usually not present in young, heat-treated tissue (1, 4). In 1977 Mellor and Stace-Smith published an article (2) in which several procedures were compared and a recommended protocol for thermotherapy and meristem culturing was suggested. This protocol proved to be successful and suited our circumstances in Prosser. As the methods were implemented some portions were not applicable for processing large numbers of different virus-infected clones in a small space, so experimental adaptations were made and a Prosser meristem culture procedure has evolved. There are several steps as follows:

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Step 1. Breaking dormancy and producing sprouts. We begin with freshly harvested tubers in the fall, so their dormancy has to be broken. Several methods were tried, some of which required the use of the biohazardous compound ethylene chlorohydrin, or overnight incubation in rendite. However, we found that simply dipping tuber eye pieces into a solution of 3 ppm gibberellic acid, planting them shallowly in 3.5" x 3.5" square plastic pots filled with sterile soil, and placing these in a darkened mist chamber, results in 100% sprouting within 10 days. The eye pieces of a few lines that rot in this standard procedure must be suberized first to prevent rotting. This method produces 144 sprouted eye pieces in a 5' x 3' space in 10 days.

Step 2. Rooting of tip cuttings from the sprouts. If plants have any root diseases, they often die in the heat chamber during thermotherapy. We escape the root diseases by making cuttings of the plants to be cured of virus infection. The sprouted plants are removed from the mist chamber when they are 15 cm tall and allowed to leaf out for 2-3 weeks under 1000-3000 lux lighting. A 6-8 cm long tip cutting is then taken from each plant and the lower 2 leaves are removed. It is dipped in a root promoting hormone (Rootone), planted in a 26" x 10-1/2" plastic flat filled with vermiculite and placed back into a mist chamber under supplemental 1000-3000 lux light. When care is taken to keep the cuttings from touching each other, 36-45 cuttings can be rooted per flat. Within 4 weeks the cuttings are well rooted and are transplanted into 10" diameter round peat pots of sterilized soil and placed for 1 week in greenhouse conditions to become established.

Step 3. Thermotherapy of rooted cuttings. The established cuttings are moved to a growth chamber under a light intensity of 3000-4000 lux for a 16-hour day and the temperature maintained at 96 to 98°F. Two weeks into the heat treatment the tops of the plants are pinched off if they look healthy, but not if they appear stressed. The plants are watered twice daily, and maintained in this heat chamber for 6 weeks.

Step 4. Cutting meristems. At the end of the thermotherapy period a cutting 10 to 20 cm long is taken from the top of each plant and placed in damp paper toweling. The plants are usually so stressed they die after several weeks of heat treatment so the remaining portions of the plants are discarded after cuttings are taken.

The wrapped cuttings are taken to a laboratory and kept damp in a plastic bag. For meristem excision (Fig. 1A), the leaves are removed from the stem leaving small petiole stubs and the cuttings are divided into several stem sections, each with a bud. We encountered bacterial and fungal contaminations in early work so began surface sterilizing these stem sections for 1 minute in 10% bleach, followed by 4 rinses in sterile water. A stem section is placed on sterile filter paper on the stage of a binocular dissecting scope in a laminar flow hood. The bud is viewed through the microscope and outer leaflets are removed with an ethanol dipped and flamed scalpel blade having a very sharp point (Fig. 1B). When only the last 2-4 leaf primorida and meristematic dome remain, a 0.5 to 0.7 mm piece of the tip is excised (Fig. 1C) with the freshly-sterilized scalpel blade and placed in a 12 x 100 mm tube partially filled with 3 ml of sterile modified Mellor-Stace-Smith (2) liquid media (Table 1). The tubers are then covered with a sterile plastic cap that allows free air exchange.

Step 5. Meristem culturing. We tried several containers, media and covers for culturing the meristems, including the above described tubes with plastic caps. Others tried included 95 x 23 mm shell vials containing 5 ml liquid media with and without a filter paper bridge, or containing 5 ml of solidified agar media. We also tried 250 ml erlenmeyer flasks with 50 ml of solidified agar media. All these were covered with sterile Saran wrap.

The most rapid and healthy growth of meristems is induced by placing the freshly-excised meristem in a 12 x 100 mm tube as described above. After 4 weeks the resulting plantlet is transferred to a shell vial on to 5 ml of solidified agar media and the vial covered by Saran. This transfer is made earlier if the plantlet sinks to the bottom of the liquid media. Each month the developing plantlet is transferred to a fresh vial and when 7 cm tall, with good root development, it is transferred into a flask for maintenance.

Step 6. Propagation of plantlets. We tried a number of methods of propagating the single plantlet produced by growth of the meristem into a large number of plantlets in culture bottles. We tried making cuttings in culture by removing plantlets from the tubes or vials, cutting into nodal sections, and placing each section in a new vial, but bacterial or fungal contamination often occurred. We finally adapted a technique (Brent McCowen personal communication) where a decapitated plantlet is placed full length on the surface of agar media in a 250 ml flask. Each node produces a new shoot and in most cases one plantlet develops into 8-12 new plantlets with very little contamination. Each of these new plants is easily transferred to soil, vials or other flasks. Thus, once we produce the single plantlet from the meristem, we are able to produce hundreds of virus-free plantlets of any promising line in a relatively short period of time and maintain them in sterile conditions and have them available at any time.

Step 7. Movement of plantlets to soil. The movement of the plantlet to soil is a critical step, as reported by Mellor and Stace-Smith (2). We lost several plantlets before we adapted a procedure used in an asparagus meristem program being conducted at Prosser. The well-rooted, 5-7 cm plantlet is transferred to a 2" x 2" square peat pot filled with a peat/vermiculite mixture and placed in a plastic tray in a mist chamber. This method allows the processing of many plantlets in a small amount of space and 100% of the plantlets survive. When the plantlets are 10-15 cm tall they may be transplanted into soil to grow a crop of virus-free tubers in the greenhouse, a screenhouse or seed farm. Starting with 30-50 culture-produced plantlets, sufficient tubers are produced in one generation on a nuclear seed farm to supply the seed needed for meaningful evaluation trials. The culture-produced plants can also be transplanted into soil pots and used as mother plants in a stem cutting program; however, plants can be produced more economically in test tube cultures with no danger of recontamination with viruses.

Step 8. Long-term storage of plantlets in vials and flasks. Once the plantlet is moved from the vial to the soil it can become reinfected by many diseases so it is much better to maintain the cured lines as plantlets in vials and flasks. As more and more lines are being cured of virus in our program, the periodic transferring of them to new media has become time consuming so we investigated several methods of storing the plantlets for longer periods with minimal care. We tried using media deficient in phosphate, using media with 3 times the normal amount of sugar to induce tuberization and dormancy, and placing plantlets at cooler temperatures. These methods have all shown promise and we are now able to wait at least 4 months between transfers. Recent literature (5, 6) indicates that long-term storage of disease free lines in vials and flasks will be possible. Success in inducing tuber formation in vials or flasks has differed greatly with genotype and we don't know yet how difficult it will be to break their dormancy, but we plan continued research in this area.

It seems clear at this point that we will be able to maintain hundreds of clones in cultures at a small fraction of the cost of renewing the tubers each year on a seed farm, and there will be no danger of recontamination with virus.

Step 9. Testing to determine if lines are cured of virus infection. We usually know what viruses are present in a plant before it goes into therapy, based upon results of an extensive virus testing program. But to be certain of virus cure, three times during the meristeming process virus indexing is done; when the plant is placed in thermotherapy, when the plantlet is transferred into a vial, and when it is moved into soil. A leaf sap sample is taken, inoculated onto Gomphrena globosa, at the 8 to 10 leaf stage; Nicotiana debneyi, at the 3 to 5 leaf stage; Nicotiana tabacum var. xanthi, at the 3 to 5 leaf stage; and Datura tatula, at the 2 leaf stage. Clean sap and known virus standards are included in each test. The host plants are examined every 10 days for 40 days; if any symptoms are seen in the last two indexings the meristemed plantlet is discarded.

ACHIEVEMENTS OF PROSSER MERISTEMS CULTURE PROGRAM

Over 60 virus-infected lines are currently being meristemed, 36 have completed the program, been virus tested, cured, and are being maintained using the methods described. These lines are now available for trials with the assurance that:

1. A source of clean seed of these lines will be available year after year with no chance of their being reinfected.

2. Many clean plants of any given line can be produced in a relatively short period of time (6-10 weeks) and in any season of the year.

3. A safe place is available to store important breeding parents and promising new clones for long period with minimal effort.

4. The expensive and time-consuming job of producing seed for trials each year will involve a minimal threat of virus infection.

5. Performance tests of promising lines will produce more meaningful results so superior genotypes can be recognized.

6. Tests for virus and other disease and pest resistances can be safely conducted in early generations without jeopardizing subsequent seed increase of promising clones. Essentially, we have a nuclear seed farm in bottles which can be easily and inexpensively maintained and expanded.

These same storage and maintenance methods provide a way for virologists to place and maintain pure virus stocks into culture and greatly ease their efforts in maintaining these stocks for research purposes.

In his final report on one aspect of his work, variety testing, to the Washington Potato Commission in 1979, Dr. R. Kunkel related the failure in 22 years of variety performance trials to identify a potato variety superior to Russet Burbank. He said this failure resulted because virus contamination in seed stocks of new lines "made yield data useless". He believed superior lines never received a fair test and were discarded because of virus contamination. He also believed it was "useless to go on" with variety performance trials until the virus problem could be solved, and he strongly suggested the establishment of a nuclear or foundation seed farm in Washington to help turn around the years of small plot testing failures. We think we have the solution to the virus problem in operation now.

By using the more technical, but less expensive methods described herein breeder clones lost to virus contamination are being retrieved; meaningful performance trials can be conducted and good, clean, seed can be made available to commercial seedsmen for larger increases. This will make possible commerical scale trials so the strengths and weaknesses of potential cultivars can be more accurately identified and superior new lines recognized. This will greatly improve the chances of finding superior disease, pest and stress resistant cultivars. There is a great need to replace the currently used cultivars with their many problems, which cause multi-million dollar losses each year.

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Table 1. Preparation of stocks solutions for meristem culture media[b] as used in Prosser.

Stock Solution	Constituents	Concentration gm/1
A	$NH_4 NO_3$	82.5
B	KNO_3	95.0
C	$H_3 BO_3$	1.24
	$KH_2 PO_4$	34.00
	KI	0.166
	$Na_2 MO O_4 \cdot 2H_2O$	0.05
	$Co Cl_2 \cdot 6H_2O$	0.005
D	$Ca Cl_2 \cdot 2H_2O$	88.0
E	$Mg SO_4 \cdot 7H_2O$	74.0
	$Mn SO_4 \cdot H_2O$	3.45
	$Zn SO_4 \cdot 7H_2O$	1.72
	$Cu SO_4 \cdot 5H_2O$	0.005
F[a]	Na_2 EDTA	7.45
	$Fe SO_4 \cdot 7H_2O$	5.57
G	Thiamine HCl	0.08
	Nicotinic acid	0.1
	Pyridoxine HCl	0.1
	Glycine	0.4
H	Kinetin	0.4 mg/10 ml 2% NaOH

a) Dissolve Fe SO_4 in 200 ml distilled water, dissolve Na_2 EDTA in 200 ml distilled water, add together with heating and continuous stirring. Cool, adjust volume to 1000 ml.

b) To 800 ml add 20 ml each of A and B, 5 ml each of C, D, E, F, G, 0.1 ml of Stock H, 30 gm sucrose, 100 mg inositol. Adjust pH to 5.7 with 2% Na OH. 7 gm bactoagar was added to 1000 ml of this media for solid media. Autoclave 15 minutes at 15 lb pressure.

Figure 1. Potato plant sections showing bud, young leaves, and meristematic dome. A. Stem piece showing leaf petiole stub and axillary bud. B. Bud showing young leaves, meristematic dome. C. Meristematic dome with 2 youngest leaves -- indicates where tissue is cut with scalpel blade.