Determining the Effectiveness of Cryotherapy to Eliminate Plant Virus in Potato

Undergraduate Research Opportunities Program

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Abstract:

Potatoes are vegetatively propagated which allows for the passage of viral diseases from one generation to the next, making it possible for entire clonal populations to become infected with the same pathogen (Kassins et al., 1957). This makes virus eradication an extremely valuable management tool in potato production. It has recently been acknowledged that cryotherapy is an effective method for removing viruses from plant shoot tips (Wang et al., 2003, 2008). Specifically, cryotherapy involves the excision of shoot tips which are frozen in liquid nitrogen, then cultured in a sterile environment until cells regenerate into plants.

This study used ELISA testing to document existing viruses in potato varieties MN02696, MN18747, MN99460-14, COMN04788-04, COMN04788-09, and COMN04692-11 and subjected plant material to two different cryotherapy methods; treatment one (encapsulation method) and treatment two (vitrification method). Treatment one involved three replications; replication two decreased the length of the liquid nitrogen exposure and replication three double coated the beads with a sodium alginate solution. Plant material did not survive in any replications of treatment one, however, plant survival was noted in treatment two. Three weeks after treatment two, plant material was evaluated for survivability. Varieties COMN0477-04, MN 18747, and MN 99460-14 appeared to have the highest levels of survival. Some varieties, MN 02696, COMN 04692-11, MN 18747, and COMN 04788-09 had even developed new shoots. In completing this study, the data collected helped determine which methods allow for the greatest survivability and therefore virus eradication in potato.

Introduction:

Virus control in potato (*Solanum tuberosum*), has been a concern for centuries, due to reduction in yield and quality. Potatoes are vegetatively propagated which allows for the passage of viral

diseases from one generation to the next, possibly infecting entire clonal populations with the same pathogen (Kassins et al., 1957). Potato is affected by more than 20 viruses and is considered to be one of the major food sources of the world. This makes virus eradication for potatoes an extremely valuable management tool in potato production.

It is well documented that the presence of viruses in plants can reduce the yield and quality of the crop. Yield increases of up to 300%, averaging 30%, have been reported following replacement of virus-infected stocks with virus-free plants (Murashige et al., 1980). One of the top virus control principles is eliminating the source of the virus (Diamond et al., 2007). Virus eradication methods such as meristemming (surgical extraction of apical meristem), heat treatments (hot and cold cycles), and viral drug therapy have been the choice methods for virus removal. These methods, though effective, have proven to be time consuming with varying levels of success. Meristemming in particular, requires a high level of skill, patience, and time to complete. Regardless, meristemming has been used extensively to produce virus free clones of crops which are propagated vegetatively (Faccioli and Marani et al., 1998).

Cryotherapy is an emerging science stemming from the use of cryopreservation, a cold treatment used for the long-term storage and preservation of valuable plant materials. It has recently been acknowledged that cryotherapy is an effective method for removing viruses from plant shoot tips (Wang et al., 2003, 2008). Specifically, cryotherapy involves the excision of shoot tips which are frozen in liquid nitrogen then cultured in a five step process in a sterile environment until cells regenerate into plants after thawing and a period of regrowth. With cryotherapy, select conditions are used to ensure the survival of a limited number of undifferentiated cells while eliminating the remaining differentiated and larger cells which have a higher probability of containing virus. Because of this precision, cryotherapy can result in a higher level of virus free

regenerations than traditional methods (Wang et al., 2008). Furthermore, the shoot tip used in cryotherapy can be significantly larger than those used in meristemming, without compromising the success of virus elimination. This speeds up the process of virus eradication significantly (Wang et al., 2003). This study documents virus in plant varieties MN02696, MN18747, MN99460-14, COMN04788-04, COMN04788-09, and COMN04692-11 and subjects plant material to two different cryotherapy methods. Two plant varieties with PVS and four plant varieties with both PVY and PVS were tested. The purpose of this was to see if combinations of viruses play any role in virus eradication through cryotherapy.

Materials and Methods:

Plant Material

ELISA Test:

An enzyme-linked immunosorbent assay was used to document the viruses PVY, PVX, PVM, PVS, PVA, and PLRV in the following potato varieties:

MN02696 is infected with PVS

MN99460-14 is infected with PVS

MN18747 is infected with PVY and PVS

COMN04788-04 is infected with PVY and PVS

COMNO4788-09 is infected with PVY and PVS

COMN04692-11 is infected with PVY and PVS.

ELISA Procedure:

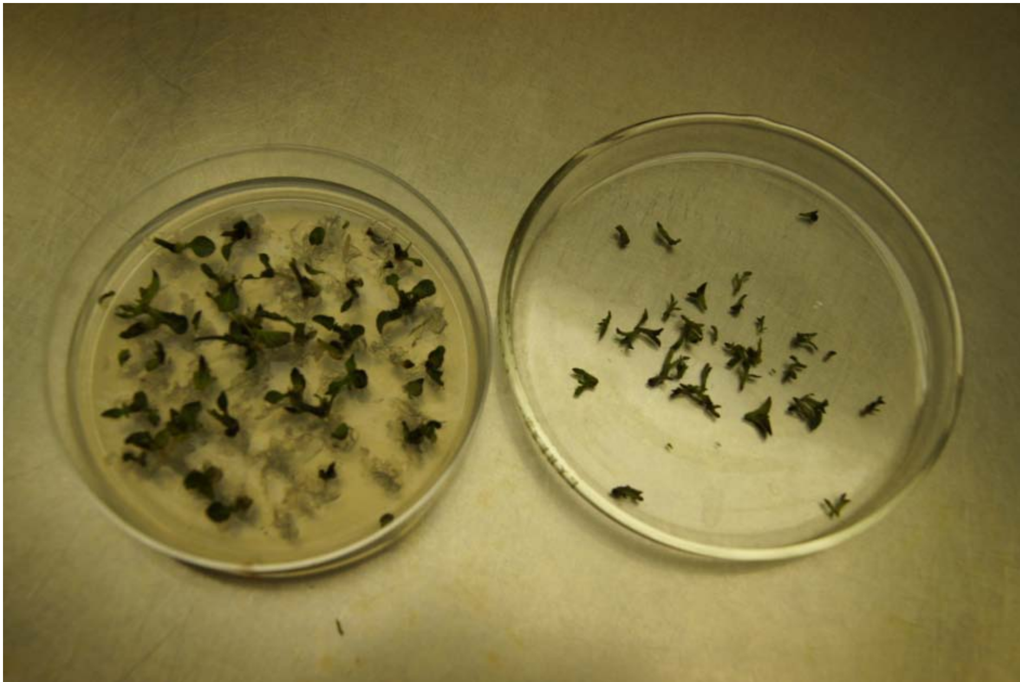
First, a 96-well plate was coated with 100ml of coating buffer per well. The coated plates were placed in a humid box for four hours. After four hours, PBST wash buffer was used to wash plates three times. One gram of plant material was weighed, placed in a mesh bag, and ground with a drill press. Next, 10 mL of general extraction buffer was added to each mesh bag. 100µl of the sap mixture was placed in the corresponding well. Negative controls (plant material with no documented virus and general extraction buffer) and positive controls (plant material with the documented virus) were randomly placed in remaining wells. The plate was refrigerated overnight. Then, PBST wash buffer was used to wash the plates six times. The ECM and ECI buffers were prepared. 2 mL of ECI was placed in a dish labeled PVY. 2mL of ECI was placed in the five remaining dishes, each labeled with a virus type. 20 µl of each enzyme conjugant was added (if both A and B added 10 µl of each) to each dish. 100 µl of each mixture was added in its corresponding wells. The plate sat for two hours. The plate was washed four times with PBST wash buffer. 100 µl of PNP was added to each well. The plate sat for one hour. After one hour the plate was visually analyzed and then analyzed in a plate reader for positive virus results.

Cryotherapy Treatment One Procedure:

1. Culture plants on SM for 12 days.
2. Isolate shoot tips (10mm in length) in a new plate.
3. Pour encapsulation liquid 1 over the shoot tips to coat them and transfer to a new plate.
4. Place additional encapsulation liquid 1 over each individual shoot tip with an eye dropper to ensure all plant material is covered.
5. Pour encapsulation liquid 2 over the shoot tips and let beads set for 10 minutes.
6. Transfer beads to a sterile plate with BM liquid media and place on a rotary shaker at 90 rpm for 16 hours.
7. Transfer beads to a new plate with BM loading solution and place on a rotary shaker at 60 rpm for three hours.

8. Transfer beads to a new plate with plant vitrification solution 2 at room temperature for one hour.
9. Remove beads from plate and blot dry with sterile paper towels. Remove excess gel.
10. Divide beads equally between three cryotubes for each plant variety.
11. Immerse cryotubes directly in liquid nitrogen for time treatments of three seconds, 30 seconds, and 60 seconds.
12. Warm cryotubes in a water bath at 40 degrees Celsius for three minutes.
13. Wash beads in ammonium free liquid MS supplemented with 1.2M sucrose for 20 minutes.
14. Remove beads from plate and blot dry on sterile paper towels.
15. Post-culture plant material for survival on ammonium free SM in the dark for three days.
16. Transfer beads on SM and place in light conditions.

Potato plants with documented virus were cultured on survival media (SM) and grown for 12 days. Shoot tips from tissue culture approximately one centimeter in length were cut and placed in a new plate (Picture 1).

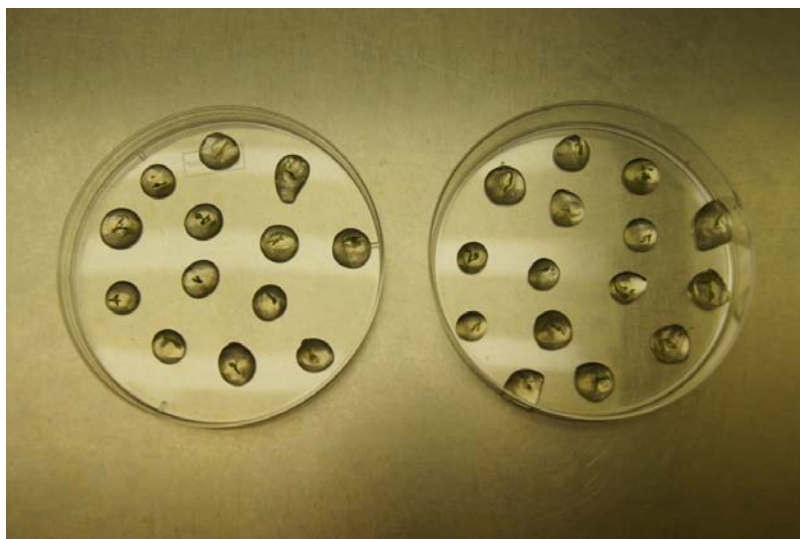


Picture 1

Encapsulation liquid 1 was poured over the shoot tips (Picture 2).

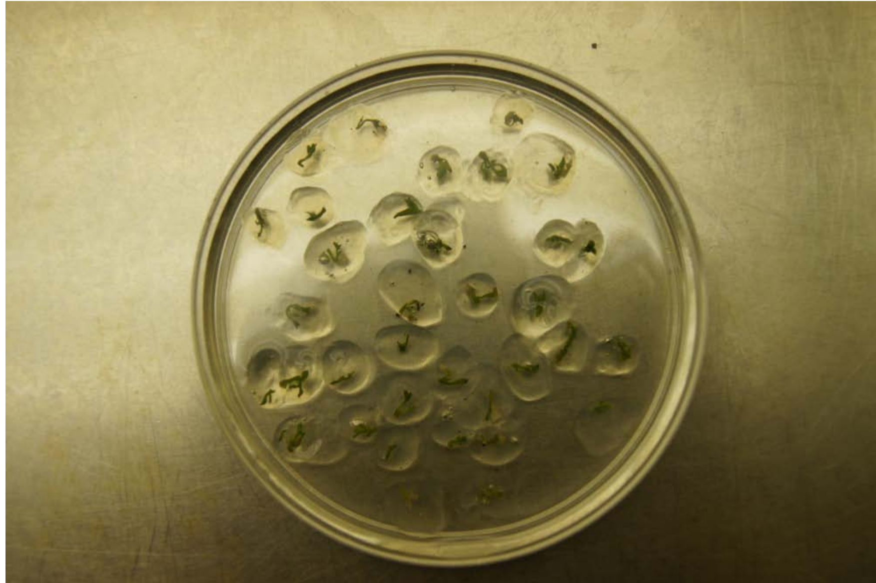
Picture 2

Coated shoot tips were transferred to a new plate. Additional encapsulation liquid 1 was placed over each individual shoot tip with an eye dropper to ensure all plant material was covered (Picture 3).



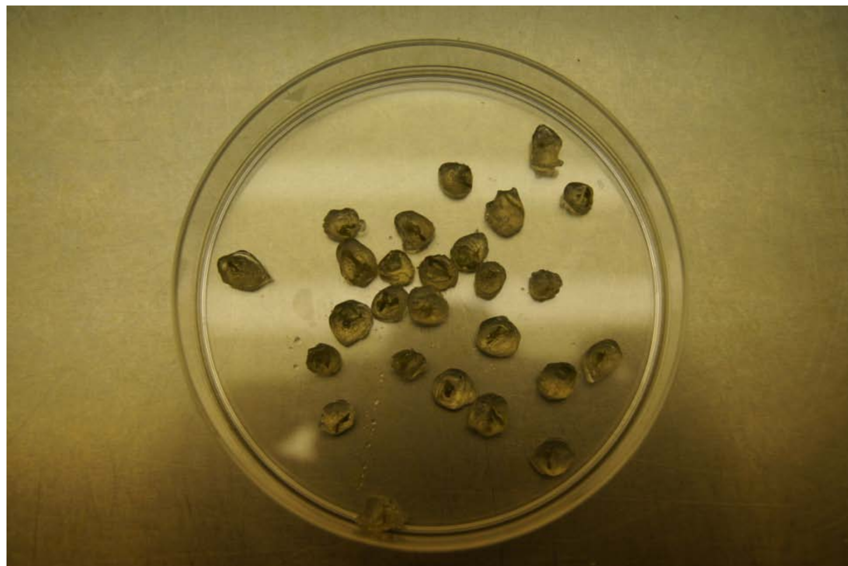
Picture 3

Then, encapsulation liquid 2 was poured over the shoot tips. Encapsulation liquid 2 reacts with encapsulation liquid 1 and stabilizes the coating to form a bead (Picture 4).



Picture 4

The beads set in the solution for 10 minutes. The finished beads were transferred to a sterile plate (Picture 5).



Picture 5

BM liquid media was added and the plates were placed on a rotary shaker at 90 rpm for 16 hours. This further enriched the beads with nutrients and high sugar to help with the vitrification process. After 16 hours, the beads were removed from the BM liquid media and placed into the BM loading solution which precultures the plant material to promote plant survival. The plates were then placed on a rotary shaker at 60 rpm for three hours. The BM loading solution was removed from the plates and plant vitrification solution 2 was added, furthering the vitrification process of the beads. The beads were left in this solution at room temperature for one hour. Then the plant vitrification solution was removed from the plate. Beads were blotted dry with sterile paper towels and excess gel was removed. Beads were then transferred to new sterile plates.

The beads were divided equally between three cryotubes for each variety. Cryotubes were then immersed directly in liquid nitrogen for time treatments of three seconds, 30 seconds, and 60 seconds. After immersion in liquid nitrogen, the cryotubes were warmed rapidly in a water bath at 40 degrees Celsius for three minutes. After warming, beads were washed in ammonium free liquid MS supplemented with 1.2M sucrose for 20 minutes. The beads were surface dried by blotting on sterile paper towels. The beads were post-cultured for survival on ammonium free SM in the dark for three days. After three days beads were transferred and cultured to light conditions on SM.

Due to the lack of plant material survival the procedure was modified and repeated. All plant material was immersed in liquid nitrogen for three seconds (modification to step 11 in procedure). The reduction of time exposed to liquid nitrogen unfortunately did not improve survivability.

Due to the lack of survival of plant material, the procedure was again modified and repeated. The beads were double-coated with the encapsulation liquid 1 and 2 (modification to steps 3, 4 and 5

in procedure). Encapsulation liquid 1 was poured over the shoot tips. Coated shoot tips were transferred to a new plate. Additional encapsulation liquid 1 was placed over each individual shoot tip with an eye dropper to ensure all plant material was covered. Then, encapsulation liquid 2 was poured over the shoot tips. The beads set in the solution for 10 minutes. The beads were transferred to a new plate and encapsulation liquid 1 was poured over the shoot tips. The coated shoot tips were transferred to a new plate where additional encapsulation liquid 1 was placed over the individual beads using an eye dropper and encapsulation liquid 2 was poured over the shoot tips. The beads set again for 10 minutes. To increase plant survival, all cryotubes were placed in liquid nitrogen for a time treatment of three seconds.

Cryotherapy Treatment Two Procedure:

Due to the lack of survival of plant material in treatment one, a different method of cryotherapy was also tested.

1. Prepare a petri dish with liquid 0.5M sucrose MS medium and one filter paper.
2. Isolate the shoot tips (3-4mm in length) and place in the petri dish.
3. Incubate for 24 hours at 24 degrees Celsius.
4. Place 10µL droplets of PVS2 on sterile aluminum foil strips.
5. With a forceps, add a shoot tip to each drop.
6. Place an additional 5 µL drop of PVS2 over the shoot tip to ensure coverage of plant material.
7. Transfer aluminum foil strips with shoot tips to precooled cryotubes.
8. Immerse cryotubes directly in liquid nitrogen for one hour.
9. Open the tubes, and transfer aluminum foil strips to liquid MS medium at room temperature.
10. Place shoot tips on semisolid MS medium for regrowth.

A petri dish was prepared with a 0.5M sucrose liquid MS medium and one filter paper. Shoot tips 3 to 4mm in length were isolated and placed in the petri dish and incubated for 24 hours at 24 degrees Celsius. Next, 10 µl droplets of PVS2 were placed on sterile aluminum foil strips. A shoot tip was added to each drop with a forceps. An additional 5 µl drop of PVS2 was placed over each shoot tip to ensure coverage of plant material. The strips were held at room temperature for 30 minutes. The aluminum foil strips with the shoot tips were transferred to precooled cryotubes. The cryotubes were immersed in liquid nitrogen for one hour. The aluminum foil strips were then rapidly rewarmed by transferring the strips to liquid MS medium at room temperature and gently shaking them. The shoot tips were then placed on MS medium for 20 days at 24 degrees Celsius.

Results:

Treatment one:

Plant material was subjected to a cryotherapy technique involving an encapsulation-based procedure. One, three, and six weeks after each replication, plant material was evaluated for survival.

Replication one: The plant material did not survive.

Replication two: The procedure was modified and the length of exposure to liquid nitrogen was decreased to three seconds for all treatments. It was thought that exposing plant material to liquid nitrogen for longer than three seconds was unnecessary and possibly harming the survivability of the plant material. However, even with a decreased exposure time to the liquid nitrogen, the plant material did not survive.

<u>Replication three</u>: The procedure was modified. The treatment time in liquid nitrogen was decreased to three seconds for all treatments and also the beads were double coated with encapsulation liquids 1 and 2. It was thought that perhaps the plant material was not surviving because it wasn't protected enough from the liquid nitrogen. Double coating the beads was done to ensure that plant material was sufficiently protected from the liquid nitrogen. Unfortunately, even with a decreased exposure time to liquid nitrogen, and a double coating of the sodium alginate, plant material failed to survive.

<u>Treatment two:</u>

Since treatment one was proving to be unsuccessful, it was decided to try another method of cryotherapy known as a vitrification method. One and three weeks after treatment, the plants were evaluated for survival. After one week, the majority of the plant material across all varieties appeared to have not survived, however some shoots still appeared green and looked as though they might survive (Figure 1). Varieties MN 99460-14, MN 02696, and COMN 04692-11 appeared to have the highest amount of plant survival.

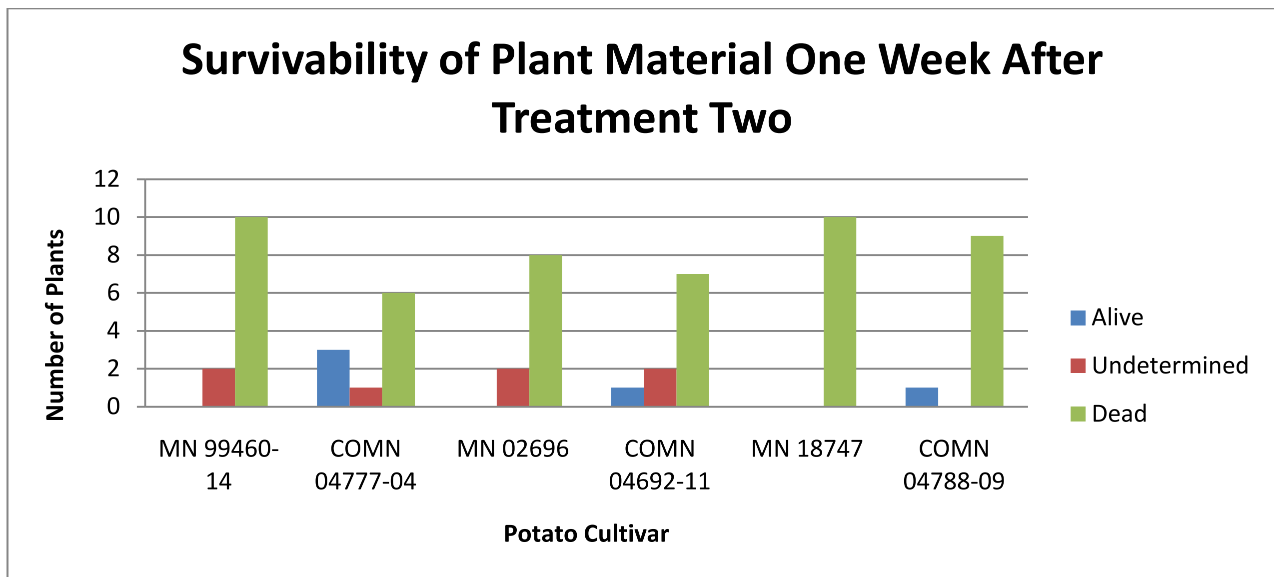


Figure 1: Figure one shows the various cultivars tested, and the number of surviving shoots documented for each one week after treatment. Varieties MN 99460-14, MN 02696, and COMN 04692-11 appeared to have the highest amount of plant survival.

Three weeks after treatment, plant material was evaluated again for survivability. The amount of plant survival appeared to be much greater. Varieties COMN0477-04, MN 18747, and MN 99460-14 appeared to have the highest levels of plant survival. Varieties MN 02696, COMN 04692-11, MN 18747, and COMN 04788-09 had developed new shoots (Figure 2). After one week, it may have been too early to determine if plant material had survived the treatment. A liquid nitrogen treatment puts the plant material under a lot of stress and the plant material needed more than one week to recover.

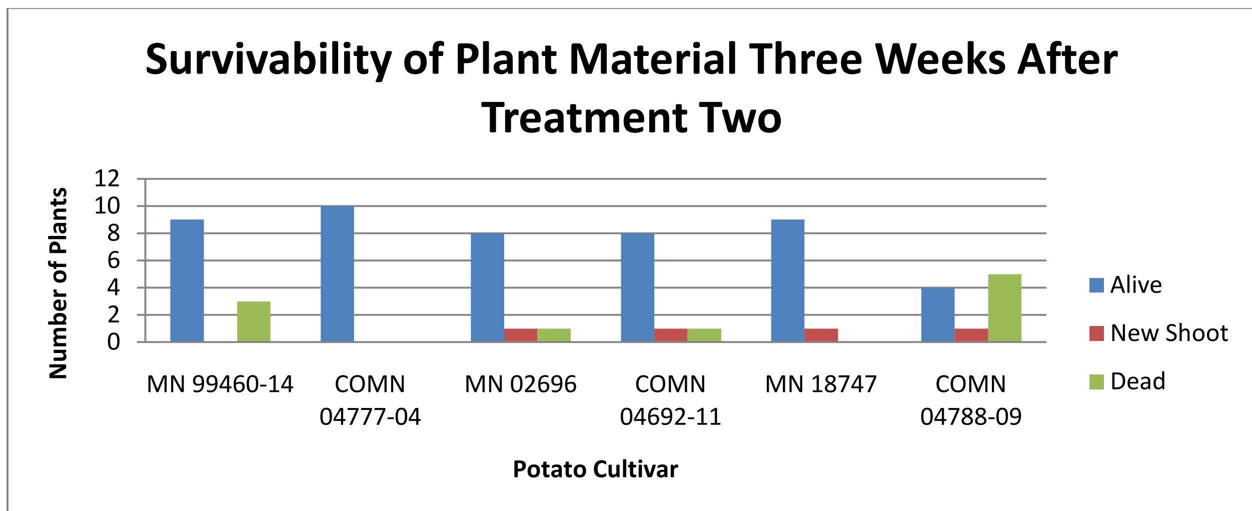


Figure 2: Figure one shows the various cultivars tested, and the number of surviving shoots documented for each three weeks after treatment. Varieties MN 02696, COMN 04692-11, MN 18747, and COMN 04788-09 had developed new shoots.

Discussion:

Treatment two resulted in better plant survival. This is thought to be due to the lack of preculturing on a high sucrose medium, the rapid vitrification on aluminum foil strips, the longer exposure to liquid nitrogen, and the less complex procedure.

Cryotherapy involves the basic steps of preconditioning, preculturing, encapsulation, and vitrification to prepare plant material for exposure to liquid nitrogen. The following documents how the basic steps of preconditioning, preculturing, encapsulation, and vitrification were done for both treatment one and two. This is done as a comparison to show how treatment one and two differ and could explain why treatment two produced better results.

Preconditioning of plants is the culture of the mother plant material under conditions that prepare the plants to survive cryotherapy. This may include culturing the mother plants at low temperature conditions or in a medium with high-sucrose content.

> Treatment one: Plants were preconditioned for 12 days on a high-sucrose medium. This preconditioning method appeared to cause plant stress.
>
> Treatment two: Plants were not preconditioned. It was noted in the procedure that the highest survivability of plant material was found in treatments where preconditioning was not used.

Preculturing is a set of conditions that the plant material is exposed to after excision and before encapsulation.

> Treatment one: The plant material was precultured in liquid BM and BM loading solution after the encapsulation process. It is believed that preculturing is more effective when performed before the encapsulation process.

Treatment two: The shoot tips were precultured in 0.5M sucrose MS medium for 24 hours at 24 degrees Celsius. This technique was very effective in promoting plant survival.

Encapsulation involves the formation of alginate beads. The plant material is suspended in a calcium-free liquid basal medium with 3% sodium alginate. The coated plant material is then coated with a liquid culture medium high in calcium chloride. This induces polymerization of alginate which produces beads around the explants.

Treatment one: Beads were encapsulated using encapsulation liquid one and two. It was believed that the plant material was not fully covered and protected. The procedure was modified. In replication three, the plant material was double coated with encapsulation liquid one and two.

Treatment two: The procedure did not involve encapsulation.

Vitrification is a liquid-solid phase transition that transitions directly from a liquid to amorphous glass, avoiding crystallization. Pure water is very difficult to vitrify because the growth rate of crystals is very high. Highly concentrated cryoprotective solutions, like glycerol, are viscous and are easily supercooled. The cryoprotective solution coating the beads is supercooled to low temperatures and solidifies into a metastable glass without undergoing crystallization.

Treatment one: The beads were placed in PVS2 solution for one hour. This length of exposure to PVS2 may have been too long and contributed to the low levels of plant survival.

Treatment two: The procedure noted that the optimal time for PVS2 incubation was 15 minutes. Exposing the beads to liquid nitrogen in aluminum foil allows for faster vitrification when compared to beads exposed directly to cryotubes.

It should also be noted that treatment one is a sweet potato cryotherapy method and treatment two is a potato method. These methods could be species specific, which could explain plant survivability. Also, after treatment one had been completed, further research was done that stated for optimal plant survival and virus eradication the liquid nitrogen treatment should be one hour. In all replications of treatment one, the length of liquid nitrogen exposure was three minutes or less. This also could explain the low plant survivability levels.

Further Studies:

Further studies will include testing surviving plant material after the cryotherapy treatments for the presence of virus. This would allow for the determination of if the cryotherapy treatment was successful in virus eradication. Further studies also might include treating plant material with combinations of virus eradication procedures including heat treatments, meristemming, and chemical treatments to determine greater effectiveness in virus eradication. It would also be beneficial to study whether or not certain methods more effectively remove one type of virus over another. In completing this study, the data collected helped determine which methods allow for the greatest extent of survivability and virus eradication in potato. This knowledge can be used to more efficiently remove virus from seed potatoes stocks in a laboratory setting. This study is extremely beneficial in determining the most efficient and cost effective method of virus removal in potato.

Identifying the most effective viral removal method will be a key component in the continued success of the potato industry and allow potato producers to more efficiently produce a healthy crop.

Materials and Methods Continued:

ELISA:

Coating Buffer

200 µl 10x carbonate coating buffer

1800 µl DI water

10 µl capturing antibody

PBST Wash buffer

500 mL DI water

5g wash buffer powder

General Extraction Buffer

16.5g Buffer powder

500mL DI water

10 g Tween-20

ECM Buffer

.4g dry nonfat milk

100mL PBST wash buffer

ECI Buffer

.5g ECI buffer

15mL DI water

PNP

3mL 5x PNP substrate

12mL DI water

3 PNP tablets

Cryotherapy Treatment One:

1L SM

4.3 g MS Salts

30.10 g sucrose

1.76 ascorbic acid

1.46g Ca(NO3)2

0.9061g L-arginine

0.193286g C4H12N2-2HCL

495.7 microliters of BAP stock solution at 0.01g/ml

10g Agar

Encapsulation Liquid 1

2.5g Sodium Alginate

14.629 ml of glycerol

13.69 g sucrose

Complete volume to 100ml

Encapsulation Liquid 2

1.1098 CaCl2

14.629ml of glycerol

13.69g sucrose

Complete volume to 100ml

1L BM Liquid Media

4.3g MS Salts

10.267g sucrose

1.76g ascorbic acid

1.46g Ca(NO3)2

0.190616g Calcium Panthotenate

0.99294 L-arginine

0.193286g C4H12N2-2HCL

Adjust pH to 6.0

1L BM Loading Solution

4.3g MS Salts

10.267g sucrose

1.76g ascorbic acid

1.46g Ca(NO3)2

0.190616g Calcium Panthotenate

0.99294 L-arginine

0.193286g C4H12N2-2HCL

+14.629ml glycerol

+5.476g sucrose

Adjust pH to 5.7

Plant Vitrification Solution 2

20g glycerol

15g ethylene glycol

15g dimethylsulphoxide

13.69g sucrose

4.3g MS Salts

1L Ammonium Free MS Liquid

4.4g Ammonium Free MS Powder

1ml MS vitamins

25g Sucrose

10g agar

+41.07g sucrose

1L Ammonium Free SM Media:

4.3g MS Salts (use ammonium free MS powder)

10.267g sucrose

1.76g ascorbic acid

1.46g Ca(NO3)2

0.190616g Calcium Panthotenate

0.99294 L-arginine

0.193286g C4H12N2-2HCL

495.7 microliters of BAP stock solution at 0.01g/ml

10g Agar

Adjust pH to 5.7

1L shoot regrowth media

4.3g MS Salts (not ammonium free SM powder)

10.267g sucrose

1.76g ascorbic acid

1.46g Ca(NO3)2

0.190616g Calcium Panthotenate

0.99294 L-arginine

0.193286g C4H12N2-2HCL

10g Agar

29 microliters of Gibberellic Acid or GA3

Cryotherapy Treatment Two:

0.5L 0.5M Sucrose Liquid MS

85.5 g Sucrose

2.1g MS +vitamins

1L MS

4.3g MS+ vitamins

10g Agar

25g Sucrose

1L MS Liquid Medium

4.3g MS+vitamins

25g Sucrose

PVS2

20g glycerol

15g ethylene glycol

15g DMSO

13.69g sucrose

4.3g MS Salts

Adjust pH to 5.8

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