FINAL PROJECT REPORT

Project Title: Sweet cherry regeneration and transformation system

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Other funding sources: None

Total Project Funding: $30,000

Budget History:

Item	Year 1: 2007	Year 2: 2008	Year 3: 2009
Salaries			
Benefits			
Wages	2250	1729	1729
Benefits		271	271
Equipment			
Supplies	7000	7000	7000
Travel	750	1000	1000
Miscellaneous			
Total	10,000	10,000	10,000

Objectives:

A. We had proposed to employ a three-pronged approach to establish an efficient regeneration system in sweet cherry. Parts of cherry plant to be tested in tissue culture included leaf, internodes and liners. Our specific objectives included:

1. **Identify the best media formulation for each variety being tested**. Test of different explants derived from two sweet cherry varieties in different media combinations. Initially we proposed to test Bing and Rainier cherry explants on media established for Fragaria by one of the PIs. Previously published media will also be tested for their effectiveness.

2. **Identify the best line for regeneration**. We will bring the breeding experiment to a Petri dish. Explants derived from selfed or cross progeny obtained from elite sweet cherry cultivars will be tested in selected media combinations. Our goal is to sample elite material with a wide range of genetic backgrounds as the ability to regenerate easily in culture is likely to require a unique complement of genes. Samples will be made from within the 1,712 existing seedlings available in the breeding program and from the seedlings expected to be germinated from the 17,848 seeds currently in stratification.

3. **Monitor the progression of regeneration using known markers of regeneration**. Expression of regeneration linked genes like *knotted*-1 and *Leaf cotyledon -1* (LCE-1) will be tested to assess and direct progression of regeneration. Sequence information for *knotted*-1 is already available from *Malus* on the GenBank (Accession no. Z71981). This sequence will be used to derive Rosaceae specific primers to be used in our experiments.

B. **Establishment of an efficient transformation system**. Availability of an efficient regeneration system will pave the way for devising Agrobacterium- and particle gun-mediated transformation system for whole tissue explants and PEG-mediated transformation for protoplast cultures.

Significant Findings:

Our final goal is to establish both regeneration and micropropagation capacity in sweet cherry. *Regeneration means developing multiple shoots from individual cells from any part of an existing variety. Micropropagation means propagation from liners or stems with multiple internodes in tissue culture conditions.*

It is an established fact that sweet cherry, peach, plum and other stone fruits are highly recalcitrant in tissue culture. Several protocols have been published on sweet cherry regeneration however; there is rarely a repeat report from the same laboratory. One method that uses seeds as a starting material is well established at Kearneysville Agriculture Research Station. However, the original variety is lost if seeds are used for this process. Thus, this method is not of extensive utility for our goals for the sweet cherry breeding program at WSU and the nursery industry in the PNW.

Recognizing this major resource gap in stone fruit research, three years ago we initiated our experiments in sweet cherry where our aim was to establish a leaf or axillary meristem or liner based regeneration and micropropagation. Some of the significant findings of the project are:

1. Sweet cherry buds and leaf tissues have a heavy pathogen load that is hard to get rid of with bleach treatment alone. We have established a method for decontaminating the explants for successful establishment in tissue culture.

2. The time of collecting the cherry buds determines the survival of the explant and its progression in tissue culture.
3. The effect of GA is unique on sweet cherry tissue culture. It supports development of somatic embryos.

Figure 1: Panel A.
Top – Bing
Second – Lapin shoot regeneration
Third – Rainier
Fourth – Sweetheart forming callus

4. Glucose instead of sucrose is required for sweet cherry explants to respond.

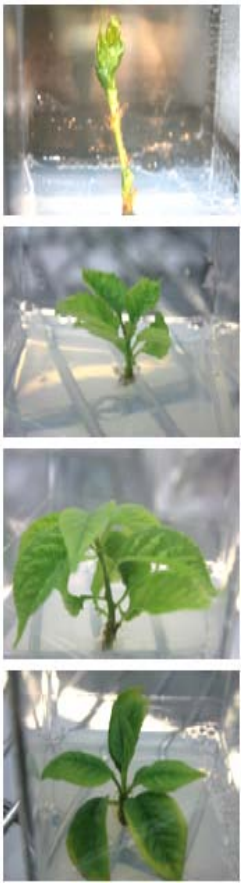

Figure 1: Panel B.
Top – Gisela 5
Second – Gisela 6
Third – Gisela 12
Fourth – Krympsk

5. A particle gun can be used for introducing foreign genes in sweet cherry leaf tissues.

Results and Discussion

Objective A1, A2 and A3

Summary: Objectives A1 and A2 were aimed at identifying the best media formulations for each variety and we had proposed to identify the best line for regeneration from the breeding population. We tested 89 different media formulations defined by previous publications and our own formulations based on observations of sweet cherry biology in tissue culture. We have now identified few media types that support vegetative growth of 5 scions and 4 rootstocks in tissue culture. The procedures with these 9 genotypes have been extremely labor-intensive which precluded us from testing accessions from breeding populations. Looking back the objective A2 seems ambitious and would require an independent project.

Sweet cherry as a tissue culture system is extremely different from apple and pear. Objective A3 was aimed at monitoring regeneration using known genes involved in regeneration. We were unable to reach this stage in this project.

Details
Over the past three years following varieties have been successfully established in tissue culture:

5 Scions: Bing, Rainier, Lapins, Sweetheart, Kiona
4 Rootstocks: Krymsk6, Gisela5, Gisela6, Gisela12
Please see Figure 1 Panels A and B.

Explants:

Vegetative Buds: These were taken at various points during the year with the best results coming from September/October and March/April collections. The spring collections responded much quicker to the media as they were not dormant but the fall collection has not shown significant detrimental effects.

Leaves: mostly younger non-fully expanded leaves

Explant Sterilization

The first bottleneck in establishing a tissue culture/micropropagation system is the preparation of explants to prevent fungal and bacterial contamination. A standard 10 percent bleach solution was used initially and was shown ineffective. The explants were contaminated over various exposure times to the bleach through the point where all of the explants were killed from the bleach while the contamination persisted. Anti-fungal and anti-bacterial additives were added to the media though they had minimal beneficial aspects and as were later determined to also effect explants growth. After further experiments, a combination protocol of ethanol, bleach and mercuric chloride with bud scale removal proved to be effective for decontaminating bud tissues. Although a prolonged procedure, this protocol has provided clean explants that have been maintained in culture for over 1 year and has been used multiple times with tissues from multiple sites. Ethanol followed by 0.1% mercuric chloride treatment for 10-15 minutes are sufficient for cleaning leaf explants of sweet cherry rootstocks and scions while the combination is toxic to pear leaves.

Organogenesis/Embryogenesis

Leaf tissues have been shown in other systems to give rise to adventitious shoots and callus. Our experiments have shown that sweet cherry leaves respond to tissue culture more effectively when the leaves are placed adaxial (top) side down onto the media.

Callus formation

Cutting the leaves into sections approximately 1cm across when measuring along the midrib has provided sections that produce callus at the cut sites along the vasculature. Two weeks of initiation of callus in low light produced multiple callus per leaf while high light and no light produced very few. The media we are using for callus formation was initially discovered after a Bing leaf produced callus and has been repeated with success in Sweetheart.

Callus Maintenance/Proliferation

We have identified a combination of Kinetin, IBA, and GA3 that is promoting growth of callus tissues from the Bing callus. We are experimenting to optimize the growth rate of the callus. The callus from Sweetheart will be moved into these media in after a couple more weeks of initiation.

Shoot Initiation

After callusing, the undifferentiated cells must be given the proper signal to grow into a shoot. Experiments identifying a media for this step are underway since sufficient callus has been generated for experimentation. From the information we gained from sweet cherry to this point we have identified a few combinations of hormones that we expect to lead us toward completion of this aspect of a callus regeneration system.

Shoot Elongation

Post cleaning of bud tissues and shoot initiation of leaf tissues, elongation of the shoot is next important step. Several media have been tested to obtain elongation with modifications to: carbon source, osmolality, photoperiod, incubation temperature, auxins, cytokinins, and gibberellic acid levels. Recently, a modification to media has resulted small amounts of elongation though the effect

needs to be further isolated and optimized and could possibly be variety specific. The stem elongation was not a lasting effect, however, which expected to be the result of slow loss of GA3 to levels ideal for growth and continued loss until the effect was removed again. This has lead to a trial of multiple levels of GA3 at very low concentrations to reproduce this phenotypic change.

Micropropagation

Once elongated shoots are grown in axenic conditions, we expect that they can be propagated using a method similar to that used with pear and apple where the shoots are laid horizontally to stimulate axial bud growth by reduction apical dominance. The axial buds will produce shoots that can be separated, elongated and used to produce more shoots cyclically.

Rooting/Acclimatization

This is a step that we have yet to explore; however, grafting of the materials we produce in tissue culture could provide an intermediate step between tissue culture and common propagation techniques where losses during rooting can be circumvented. We plan to explore this possibility with NNII to help them in establishing a method in obtaining virus free cherry stocks and scions.

Following media combinations were tested during the course of this project. These media formulations were derived from published work as well base on our observations. A total of 89 media types were tested.

1. Basal nutrition media after Murashige and Skoog (27 variations)

We used various intensities and combinations of the following hormones.

Ascorbic Acid	None, 100-200mg/L
BAP	1-3 mg/L
TDZ	None
GA3	None
GA4+7	None, .5-1, 2, 3 mg/L
IBA	None, .1mg/L
PPM	1-2 ml/L

2. Woody Propagation Media (7 variations)

We used various intensities and combinations of the following hormones.

Charcoal	500 mg/L
Ascorbic Acid	None
BAP	None, 1, 7 mg/L
TDZ	.25, 1 mg/L
GA3	None, 1 mg/L
GA4+7	1 mg/L
IBA	.1mg/L
PPM	2 ml/L

3. Variation of basal nutrition media– **Sucrose** (21 variations)

We used various intensities and combinations of the following hormones.

TC Agar	5.6, 6 g
Ascorbic Acid	None

BAP	None, .25-1mg/L
TDZ	None
GA3	None, 1,10 mg/L
GA4+7	None, .5-1 mg/L
IBA	None, .1mg/L
PPM	None
Kinetin	2, 4, 8 mg/L
DDT (1 Sample)	150 mg/L

4. Basic nutrition media – **Combination of Glucose, Sorbitol, Fructose** (20 variations)

We used various intensities and combinations of the following hormones.

TC Agar	6 g
Ascorbic Acid	None
BAP	None
TDZ	None, 1 mg/L
GA3	None, 125, 500 microgram/L
GA4+7	None
IBA	5 microgram/L, .1mg/L, 4 mg/L
PPM	None
Kinetin	4, 8, 12, 16 mg/L
DDT	None

5. Basic nutrition media with different nitrogen source – **Glucose** (14 variations)

We used various intensities and combinations of the following hormones.

TC Agar	6, 7 g
Ascorbic Acid	None
BAP	None, 1mg/L
TDZ	None, 125 micro, .2, .5mg/L
GA3	None, .1, .2, .4 mg/L
GA4+7	None, .1, 1, 2, 4, 8mg/L
IBA	None, .1-.4 mg/L
PPM	None
Kinetin	None, 2, 4, 8 mg/L
DDT	None

Objective B

In order to establish a sweet cherry transformation system we needed to test the method for DNA delivery into leaf or other explants. We preferred to use the particle gun method. The rationale behind using this method is that we don't have to worry about the specificity of the Agrobacterium strain for sweet cherry.

Leaf explants were used for standardizing DNA delivery into sweet cherry leaf material. Different parameters like distance between target and microcarrier launch assembly and pressure were used to test the DNA delivery system. A schematic of the particle gun is shown in Figure 2A. A cell expressing the red fluorescent protein in sweet cherry leaf cell is shown in Figure 2B.

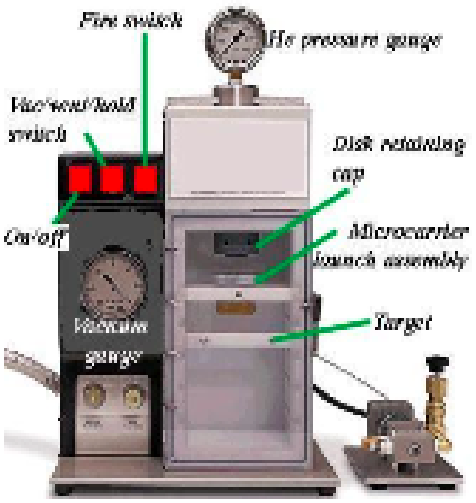

Figure: 2A. Particle Gun used for standardizing DNA delivery in to sweet cherry explants.

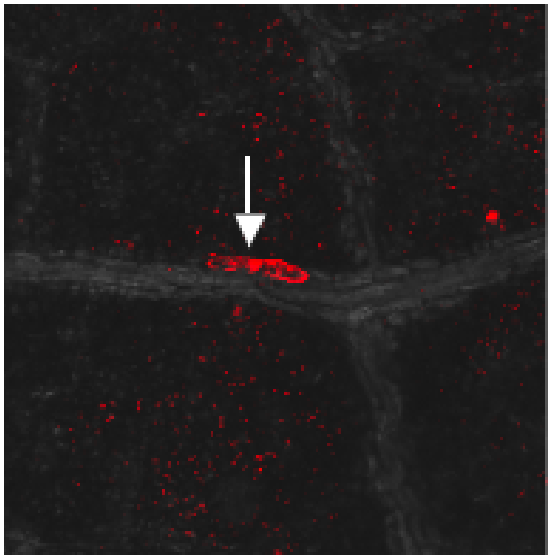

Figure: 2B. Arrow indicates a fluorescent sweet cherry cell expressing red protein

Thus we have a good system for DNA delivery that we can now utilize for creating targeted mutations or controlled sports induction for improving certain aspects of existing varieties. Our efforts continue to fine-tune the regeneration system. A combination of good regeneration system and DNA delivery approaches can greatly benefit the sweet cherry research.

Executive Summary

The time involved in propagation of tree species is a major factor controlling the amount of time between variety release and widespread adoption of that variety. In other tree fruits, apple and pear, we have used micropropagation to successfully increase the propagation rate, thereby decreasing the amount of time invested to provide growers with adequate amounts of material. Sweet cherry is a member of the Prunus family where attempts at tissue culture and micropropagation have resulted in minimal success.

Sweet cherry has a narrow genetic diversity therefore extensive crosses will not yield large diversity in traits. The methods developed in this project are expected to enable us in creating random and directed sports generation thereby increasing the diversity. Mutations are a safe way of improving existing variety or creating novel varieties. These are not considered GMOs.

After extensive experimentation we have developed media for 9 genotypes – 5 scions and 4 rootstocks. We have also established preliminary methods for introducing DNA into sweet cherry using particle gun.

One overarching observations is that sweet cherry is unique compared to apple and pear. In tissue culture there are not many successful reports available for either regeneration or micropropagation. Here we have created a large repository of media formulations for enabling these techniques, which undoubtedly require further work.

Broader impact of our methods developed here will be its integration with Clean Plant Network activities coordinated by Bill Howell. Our methods can be used to clean scions and stocks of viruses. This project was carried out as part of Ph.D. work of Tyson Koepke who has been supported by an NIH protein Biotech training program, ARCS fellowship and now USDA-SCRI support. The project provided an opportunity for the training of three undergraduate students – Cory Druffel, Ashley Koepke and Matt Allan. These students have been successful in obtaining undergraduate research fellowships from CAHNRS and Matt has recently been awarded the Auvil Fellowship. The work done with support of this project has been presented at several national and international forums in the form of poster presentations.