**FINAL PROJECT REPORT**

**Project Title:** Reduction of generation cycle in apple breeding


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**Cooperators**: Cameron Peace, Fred Bliss, Jim McFerson, Ralph Scorza and ARS team at the Ag Station in Virginia

**Other funding sources**

**Agency Name:** National Science Foundation
**Amt. awarded:** $16,000
**Notes:** The funds support an Undergraduate Research Intern for 10 weeks and cover a stipend, travel and lodging for two years

**Total Project Funding**: $22,942

**Budget History:**

| **Item** | **Year 1:** |
|---|---|
| **Salaries** | |
| **Benefits** | |
| **Wages** | 10,500 |
| **Benefits** | 4211 |
| **Equipment** | |
| **Supplies** | 3000 |
| **Travel** | 1000 |
| **Plot Fees** | 1500 |
| **Miscellaneous** | 2731 |
| **Total** | 22,942 |

**OBJECTIVES**

1. Physiological manipulation of seedling growth and development in the greenhouse for speeding up flowering

For this approach, 2012 seedlings designed specifically for this project which had either Cripps Pink or *M. zumi* as one of the parents, both identified as a source of natural precocity and 2011seedlings selected from crosses that had already been made for the WABP with a reasonable level of diversity including desirable fruit quality traits and seasonality were used.

2. Establish regeneration to facilitate incorporation of quick flowering gene in WA 38

Budget adjustment redirected efforts towards establishment of robust micropropagation protocols, generation of adequate amount of plant material to conduct limited number of regeneration experiments. Acceptable level of regeneration in WA 38 leaf explants was observed which will enable transformation experiments for incorporation of early flowering gene.

**SIGNIFICANT FINDINGS**

Objective 1. Physiological manipulation of seedling growth and development in the greenhouse for speeding up flowering

**The goals of this objective were accomplished successfully.**

1. Achievement of flowering and successful reduction of generation time to 365 days in 8 individuals from 2012 seedling populations obtained using *M. zumi* as donor of genetic precocity crossed with elite WABP selections.

2. While Cripps Pink is a donor of precocity, none of the seedlings derived from crosses with Cripps Pink as a parent have flowered since January 2012.

3. Identification and propagation of floriferous and powdery mildew tolerant seedlings (2011 and 2012 populations).

4. In addition, evaluation of protocols established at WSU Pullman to physiologically reduce generation time is ongoing in the WABP to enable movement of highly desirable seedlings directly to breeding Phase 2 which is expected to enable earlier and more rigorous evaluation of fruit quality and storability saving precious resources.

5. Successful debunking of theory of requirement of 120 nodes to induce flowering in apple seedlings obtained from crosses made in 2010 indicating a strong genetic influence.

Objective 2. Establish regeneration to facilitate incorporation of quick flowering gene in WA 38

**The adjusted goals of this objective were accomplished successfully.** Budgetary adjustments resulted in primary focus on micropropagation of WA 38 and limited plant regeneration experiments.

1. Successful establishment of efficient micropropagation protocols, reasonable level of regeneration and availability of large amount of aseptic material for WA 38 for genetic transformation experiments in order to introduce the early flowering gene was achieved.

**RESULTS & DISCUSSION**

Detailed results and discussion follow Table 1 that summarizes the progress and milestones achieved as an indicator of success in the funded project.

Table 1: Summary of progress and milestones for each of the objectives.

| Time Frame | Objectives | Progress | Milestones |
|---|---|---|---|
| Jan 2013 – Ongoing | 1. Physiological manipulation of seedling growth and development in the greenhouse for speeding up flowering. | Flowering was successfully obtained in 8 individuals derived from 2 crosses with *M. zumi* as one of the parent.<br><br>Crosses were made across the early flowering individuals. | A greenhouse growth model for reducing generation time has been established.<br><br>74 seeds collected and stratified. |
| April 2013 – December 2013 | 2. Establish regeneration to facilitate incorporation of quick flowering gene in WA 38. | Adjusted focus on developing large amount of plant material for regeneration experiments. | Micropropagation and preliminary regeneration procedures established. |

Objective 1. Physiological manipulation of seedling growth and development in the greenhouse for speeding up flowering

Successful induction of flowering in 8 individuals derived from 2 crosses having *M. zumi* as one of the parents was achieved using the accelerated growth and development model established since 2010 (Figure 1A-C; please see at the end of the document). A total of 510 seedlings; 427 WABP seedlings derived from crosses made in 2011 derived from precocious parents and 85 WABP seedlings from crosses made in 2010 derived from non-precocious parents are currently being grown in the WSU Pullman greenhouse facilities.

In order to incorporate precocity in the WABP additional crosses were made between individuals that flowered in 2013. While the juvenile (non-flowering) trees were in dormancy, reciprocal crosses were made across the precocious individuals. Self- crosses were either spontaneous or performed manually. The flowers and pollen were present in the middle of the calendar winter when no pollinizing insects were present therefore the probability of "open" outcrossing is unlikely. Crosses were made by emasculating flowers prior to the "popcorn" stage and the stigmas were coated with fresh pollen taken directly from the anthers of the donor tree's flowers. Additionally pollen was collected from precocious individuals, dehydrated, and stored at -20C for future crosses. These crosses yielded 41 fruit with 74 seeds that have been stratified and are ready for germination (Table 2). The fruit developed normally and there were differences in skin color observed in cross 1127 Figure 2; end of document).

Table 2 Summary of crosses and fruit/seed yield. Key: 1127 = WA5 X *M. zumi* 1123 = 'Honeycrisp' X *M. zumi*

| | Maternal parent | Pollen parent | Number of fruit | Total seeds |
|---|---|---|---|---|
| 1. | 1127-46 | Self | 3 | 3 |
| | 1127-46 | 1123-67 | 4 | 0 |
| 2. | 1127-47 | 1123-67 | 1 | 3 |
| | 1127-47 | Self | 2 | 6 |
| 3. | 1123-67 | Self | 2 | 2 |
| | 1123-67 | 1127-46 | 1 | 3 |
| 4. | 1123-66 | Self | 5 | 9 |
| | 1123-66 | 1127-47 | 1 | 6 |
| 5. | 1123-55 | Self | 5 | 7 |
| | 1123-55 | 1127-46 | 2 | 8 |
| 6. | 1123-15 | Self | 5 | 9 |
| 7. | 1123-59 | Self | 1 | 2 |
| 8. | 1123-48 | Self | 9 | 16 |
| | Total | | 41 | 74 |

Growth and development model developed so far in this project involves fast tracking the trees to enter paradormancy, which is a dormant state obtained after active growth. Further, the plants are fast tracked to enter the endodormancy, a state of dormancy in winter. In addition a state of ecodormancy is maintained where the plants are maintained under quiescent state with temperature and light regulation so that they achieve the requisite number of chilling hours prior to being brought out of dormancy (Figure 3; end of document). Ongoing work will continue to refine this model so that this protocol can be used broadly and efficiently in WSU breeding programs.

Double fruiting: Like all apple trees, the precocious, fruiting trees, continue to grow vegetative branches while the fruit is maturing. In the greenhouse, the restraints of the physical space, including the pot size, can cause stress on the plants. Stressed plants are weak, and prone to disease and infestation by pests. One method to reduce this stress, when up-potting and additional space is not available, is to reduce the canopy by pruning. Additionally pruning allows better air circulation, reducing mildew infestation. The precocious, fruiting trees were pruned in the later part of the growth cycle, when the fruit had reached near full size, and was still unripe. Nearly all the apical buds on the branches had entered a paradormant stage, suppressing lateral growth. When the trees were pruned, mostly with header type cuts, not complete branch removal, the apical bud was removed releasing the lateral buds from paradormancy. Apparently, at least in 'Honeycrisp' X *M.zumi*, sufficient time had passed to allow for full development and maturation of floral meristems within those buds, as evidenced by the fact that a second flowering was achieved from the now growing lateral buds. These flowers underwent self-fertilization in most cases and set a second set of fruit.

Table 3: Additional fruit obtained on precocious individuals (1123 = 'Honeycrisp' X *M. zumi*).

| Tree | Additional number of fruit |
|---|---|
| 1123-48 | 9 |
| 1123-55 | 21 |
| 1123-59 | 4 |
| 1123-66 | 1 |
| Total | 35 |

Mildew infection on RGC or Fast-track apple trees: The trees have endured a variety of pests including powdery mildew. The mildew symptoms are more apparent in late spring when the mildew is prevalent in the environment. Measures have been taken to control and reduce the mildew infection, including fungicidal sprays, oil sprays in sublimation of elemental sulfur. The infection patterns may be partly or entirely attributable to position in the greenhouse as much as genetics. The trees with the most infection are near the evaporative coolers, which may have provided more ideal conditions for the mildew in that proximity. Also the most infested trees are the most distant from the sulfur sublimator, and may not have received the same dose of sulfur as the trees located near the sublimator. Smaller (dwarf) trees tended to have more mildew regardless of genetic cross, possibly due to airflow (or lack thereof) issues, or reduced solar radiation (UV radiation) compared to the taller trees. When the dwarf trees were moved out of the shade of the canopy, and into a better airflow environment, (concurrent with sulfur sublimation) the mildew infection subsided. It is interesting to note that the most precocious seedlings were derived from 1123 cross ('Honeycrisp' X *M. zumi*) and the seedlings from this population are the least affected by powdery mildew under greenhouse conditions (Table 4).

Table 4: Summary of observations made in October 2013. They reflect the level and severity of infection when the mildew was more active.

| **Line** | **Cross** | **Degree of infection** | **Organs infected** | **% coverage on infected leaves** | **Notes** |
|---|---|---|---|---|---|
| 1121 | Cripps pink x Honeycrisp | Minimal | Leaves, older | 10-20 | Minor spots on leaves |
| 1122 | Fuji x M.zumi | Minimal | Leaves, older | 5 (spots only) | Only four trees in this group: all have spots. |
| 1123 | Honeycrisp x M. zumi | Minimal/ slight | Leaves, older | 5-10 | Least infected of all lines |
| 1124 | Sabina x Cripps pink | moderate | Leaves, young and old. Shoots, stems | 100 | Weak trees, not robust. Seem to be least resistant. |
| 1125 | Sabina x M. zumi | Moderate /heavy | Leaves, old and young, shoots, entire branches | 100 | Some branched heavy infected, possibly systemic |
| 1126 | WA5 x Cripps pink | Moderate /light | Leaves, old and young | 40 (spots mostly) | Shoots clean, no bad damage |
| 1127 | WA5 x M. zumi | Moderate | Leaves, old and young | 30-40 | Spots on leaves of most trees. Moderately infested trees have entire infested branches, moderate damage. |

Propagation of precocious individuals: Five fruiting trees were selected for establishment in aseptic tissue culture; 'Honey crisp' X *M. zumi* (trees # 15, 66, 67) and WA5 X *M. zumi* (trees #46 and #47). Material was collected and processed by standard protocol, and cultured in RG media (optimized for 'Royal Gala' apple). Shoots which developed normally were induced to root, and rooted plantlets were transitioned to soil and grown up in the greenhouse. The WA5 X *M. zumi* developed normally and rooted with over 90% efficiency. Currently there are two sapling individual clones of WA5 X *M. zumi* from tree #47, the remainders were discarded. The 'Honey crisp' crosses developed poorly in tissue culture, and many failed to establish. None of the three 'Honeycrisp' crosses have rooted; plants #15 and #66 are still being maintained in tissue culture, as is WA5 X *M. zumi* plant #47.

2. Establish regeneration to facilitate incorporation of quick flowering gene in WA 38

The budgetary adjustments allowed for fine tuning the micropropagation protocols for WA 38 (Figure 4; end of document). It also allowed for generation of ample amount of plant material that was used for a limited number of plant regeneration experiments. While not the most optimal compared to the regeneration frequencies obtained with Royal Gala the level of regeneration obtained so far is adequate to initiate plant transformation experiments.

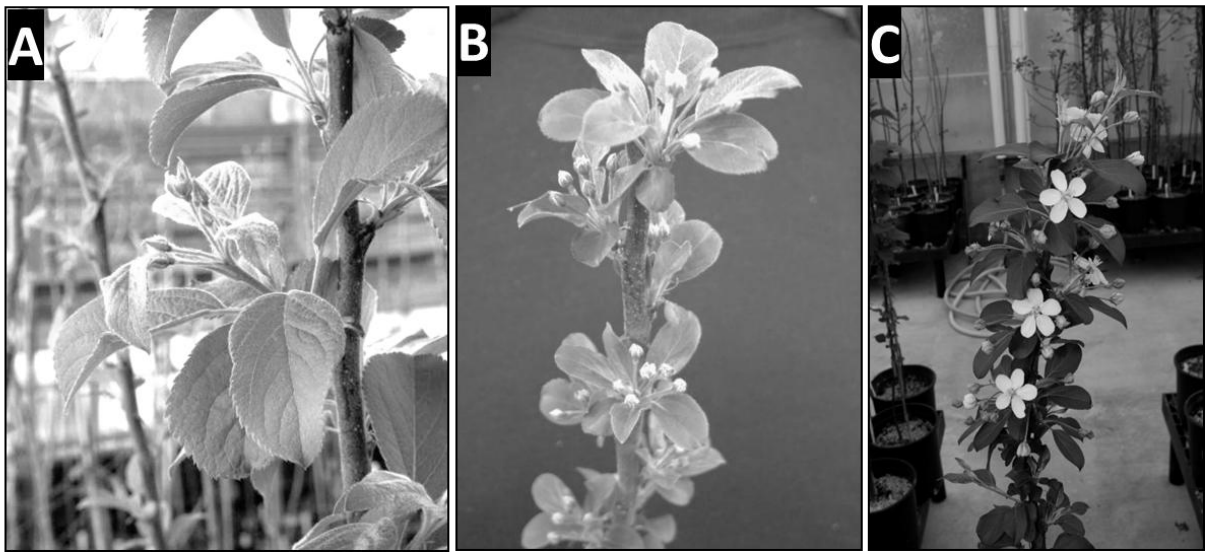


Figure 1: Successful flowering and reduction of generation in apple seedlings. A and B: Floral bud formation in two of the seedlings derived from crosses with M. zumi as one of the parents. C. A floriferous seedling exhibiting flowering within 1 year after seed germination.

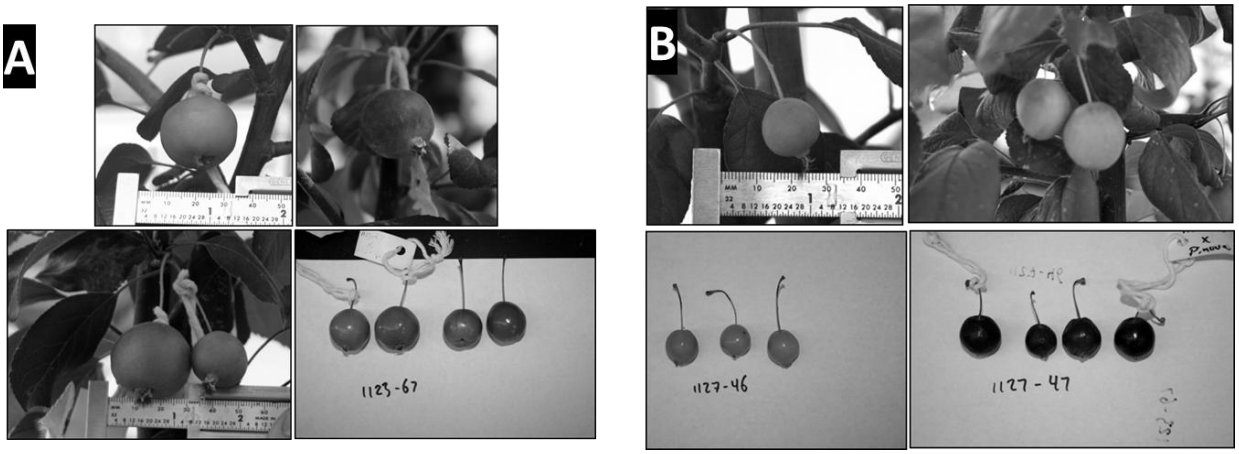


Figure 2: Fruit production on individuals of 1123 and 1127 crosses.
A. 1123: Unripe and ripe fruit. B. 1127: Unripe and ripe fruit. Note the difference in skin color.

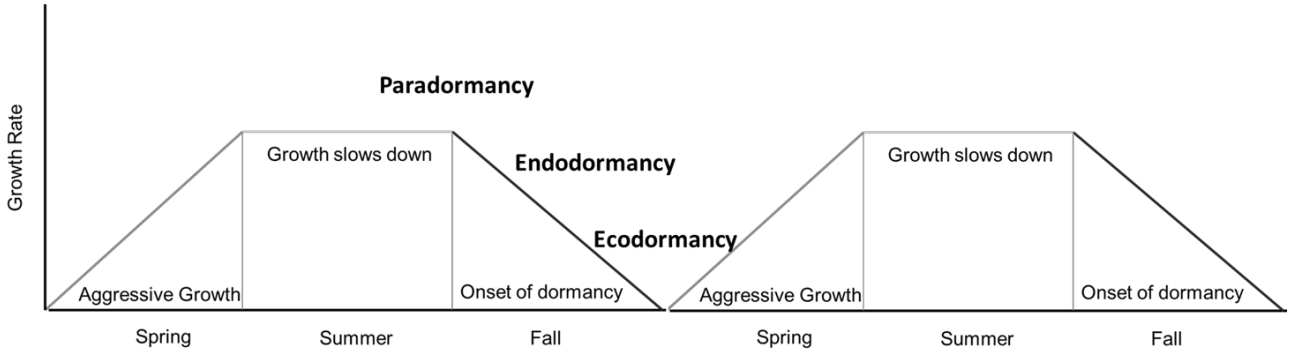


Figure 3: Growth development model representation incorporating paradormancy, endodormancy and ecodormancy.

Figure 4: Robust plants of WA 38 available in the laboratory to be used for plant transformation experiments.

## EXECUTIVE SUMMARY

Significant progress: Successful flowering and reduction of generation was achieved in 8 individuals derived from two crosses that used *M. zumi* as one of the parents. An evolving model of greenhouse based growth and development of WABP seedlings has the potential of moving most promising seedlings directly to Phase II saving previous resources.

Summary of findings: Genetic component is critical for the induction of flowering besides physiological manipulation of growth and development. *M. zumi* is a known donor of precocity and the trait was able to express itself when crossed with 'HoneyCrisp' and 'WA5'. Cripps Pink another donor of precocity failed to produce flowers over the last 2 years. The flowers produced in the greenhouse are viable and produce viable fruit with seeds. Several of the flowering trees produced two crops and in particular the trees derived from 'HoneyCrisp x *M. zumi*' cross were found to be tolerant to powdery mildew under greenhouse conditions.

Future directions: The non-fruiting trees are being maintained in the greenhouse and cycled through accelerated growth and development to obtain flowering. Seeds obtained in 2013 will be germinated to assess the inheritance of M. zumi derived precocity and floriferousness and moving these trait into the next generation so that they can be utilized in the WABP.