# Adventitious shoot regeneration and genetic transformation of *Prunus serotina* (black cherry) for reproductive sterility

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## Abstract

Black cherry (*Prunus serotina* Ehrh.) is a valuable hardwood in the eastern United States and Canada. There has been an increase in demand for high quality black cherry wood and a need to establish plantations with improved black cherry. Genetically improved trees containing foreign genes will be subject to government regulatory guidelines because of the potential for dispersal of transgenic pollen, thus requiring the need for sterility. The objective of this research was to develop a reliable system for genetic modification of black cherry for reproductive sterility. An improved method for adventitious shoot regeneration from leaves was established for three genotypes (F, # 3, and # 4; # 3 and # 4 are mature trees). The highest regeneration efficiency for F, #3, and #4 was 91%, 75% , and 58% respectively, obtained on WPM supplemented with 9.1 µM TDZ plus 1.1 µM NAA. The highest mean number of shoots was achieved on the same medium; 8.2 (F), 5.1 (# 3), and 4.7(# 4). The rooting efficiency of shoots was 87 % (F), 82% (# 3), and 65% (# 4) by treated with 2.5 mM IBA. In vitro leaves were transformed using *Agrobacterium tumefaciens* strain AGL1 carrying an RNAi construct containing an *AGAMOUS* gene. Selection and regeneration of transformed cells and shoots was carried out for 12 weeks on a medium containing kanamycin. Shoot regeneration was achieved using WPM supplemented with 9.1 µM TDZ, 1.1 µM NAA, plus 10 mg/L kanamycin. Timentin (300 mg/L) was used after three days of co-culture to kill the *Agrobacterium*. Late selection was carried out on the same medium except kanamycin was increased to 15 mg/L. Transgenic black cherry shoots were achieved which have been confirmed by PCR. Three out of 118 shoots of genotype F were kanamycin resistant, but only one was confirmed positive by PCR. Ninety one shoots of genotype # 3 were kanamycin resistant, and PCR is underway to confirm these putative transgenic shoots.

## Introduction

Genetically improved trees containing foreign genes are subject to government regulatory guidelines for field planting because of the potential for dispersal of transgenic pollen, and the environmental impact could be difficult to predict and control (Meilan et al., 2001). To reduce the dispersion of all genes, engineering reproductive sterility will help simplify the impact analysis and thus facilitate regulatory and public approval (Strauss et al., 1995). This will allow landowners to freely plant transgenic trees without concern for ecologically affecting an ecosystem. An efficient regeneration system is essential for genetic transformation. In this study, we optimized a method previously established (Espinosa et al., 2006) for black cherry regeneration in order to genetically transform and propagate elite mature black cherry with a reproductive sterility gene. With an efficient regeneration system (58-75%) that we optimized, we are using a RNAi construct which contains an *AGAMOUS* gene to impart reproductive sterility.

## Objectives

1. Establish a highly efficient regeneration system.
2. Establish a reliable transformation system .
3. Transform a reproductive sterility gene into black cherry via *Agrobacterium.*
4. Select transformants using kanamycin and confirm transformants by PCR.
5. Acclimatize the transgenic plantlets to the greenhouse.

## Materials and Methods

**Regeneration**

In vitro whole-leaf explants were cut transversely along the midrib and incubated on WPM ( Lloyd and McCown , 1980) supplemented with 9.08 µM TDZ plus1.07 µM NAA. Cultures were incubated for 3 weeks in the dark plus 1 week in the light ,then transferred to MS + 8.88 µM BA + 0.49 µM IBA + 0.58 µM $GA_3$ + 60 µM STS +30g•$L^{-1}$ sucrose for 2 months.

**Kanamycin Sensitivity**

Leaf explants with wounds were used.
Kan at 0, 5, 10, 15, 20, 25, or 30 mg/L was tested.
An avirulent *Agrobacterium* strain was co-cultured with leaves for 30 min. Timentin at 300 mg/L was added to kill the *Agrobacterium*.

**Transformation**

Two *Agrobacterium* strain EHA105 and ALG1 were grow in induction media (Gelvin, 2006) for 2 to 4 hours before use. Leaf explants were exposed to *Agrobacterium* for 30 to 40mins, co-cultured in the dark for 2 to 3days, washed, then cultured on regeneration media ( with or without kan selection). Kanamycin 15 mg/L was added at three days or 4 wk after co-culture.

**Confirmation of transgenic plants**

DNA was extracted from putative transgenic plants and from the plasmid to run PCR.

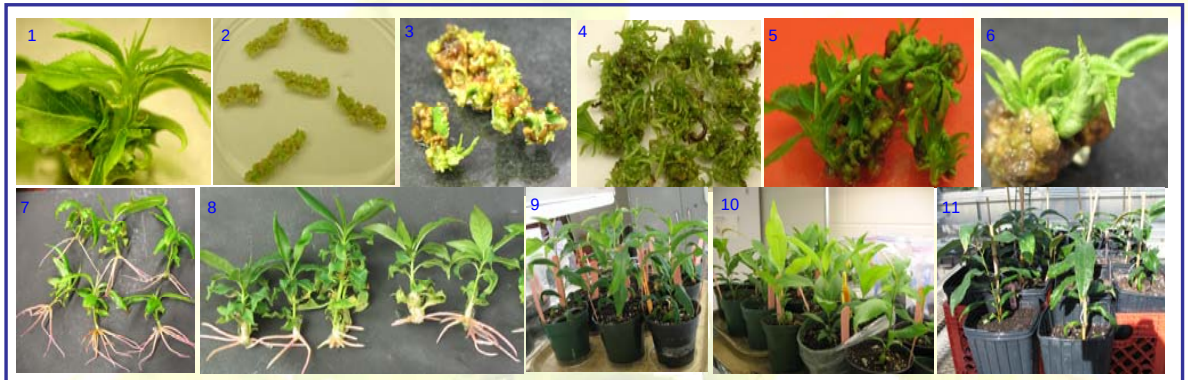



Fig1-11 Adventitious shoots regeneration of black cherry. (1) # 3 shoot in vitro (2) Leaf explants on WPM (3) Enlarged leaf explants on WPM (4 )Adventitious shoots F (5) Adventitious shoots # 3 (6 )Adventitious shoots # 4 ( 7) Rooted adventitious # 3 ( 8) Rooted # 4 ( 9) Acclimatized plant F after 4 wks (10 ) Acclimatized plantlets # 3 (11) Acclimatized F in the greenhouse

## Results

***1) GUS* assay**

Leaves show *GUS* positive after three days co-culture.

**2) Kanamycin sensitivity**

Table 1. Kanamycin sensitivity of black cherry seedling and mature genotypes

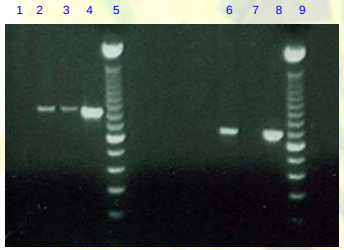
| Kan(mg/L) | F | | 3 | |
|---|---|---|---|---|
| | Regeneration (%) | Mean No. shoots | Regeneration (%) | Mean No. shoots |
| 0 | 91 | 4.0 | 37.3 | 1.8 |
| 5 | 72.2 | 2.7 | 19.4 | 1.4 |
| 10 | 33.3 | 1.4 | 11.1 | 1.0 |
| 15 | 22.2 | 1 | 0 | 0 |
| 20 | 16.7 | 1 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 |

**3) Transformation**

Table2. Summary of transformation for two genotypes of black cherry

| Genotype | Selection time | Kan resistant shoots | PCR-positive |
|---|---|---|---|
| F | 3 days | 10 | 1 |
| F | 28 days | 13 | To be done |
| 3 | 3 days | 27 | 0 |
| 3 | 28 days | 91 | To be done |

**4) PCR results**




Lane1 Water control,
Lane 2 BCF(10µl DNA)
Lane 3 BCF (5µlDNA)
Lane 4 Plasmid DNA
Lane 5 100bp ladder
Lane 6 BCF
Lane 7 BC3
Lane 8 Plasmid
Lane 9 100bp ladder


PCR1 : a portion of 35S promoter +MdAG2+a portion of PDK intron =756bp
PCR2: a portion of Intron +MdAG2+Portion of OCS terminator =684bp

## Conclusions

1. Black cherry was transformed and it show *GUS* transient expression.
2. Black cherry is very sensitive to kanamycin. When 15 mg/L was used for selection, 28 days late selection produced more regenerated shoots than 3 days early selection. To improve efficiency of transformation, late selection is highly recommended.
3. Black cherry can be transformed via *Agrobacterium*-mediated transformation and *Vir* gene induction is necessary. We obtained 104 kanamycin resistant putative, transgenic plants.
4. Black cherry transformation and regeneration is genotype dependent. For black cherry genotype F, only one shoot regenerated from one leaf explants. For genotype # 3, several shoots regenerated from the leaf explants. For genotype # 4, no shoots regenerated.
5. Rooting of black cherry adventitious shoots from mature genotypes can be achieved and plants acclimatized to the greenhouse.

## Abbreviations

BA-6-benzylaminopurine; $GA_3$—gibberellic acid ; IBA- indole-3-butyric acid; Kan-kanamycin; MS- Murashige and Skoog medium; NAA-napthaleneacetic acid; PCR-polymerase chain reaction; STS- silver thiosulphate; TDZ-thidiazuron; Tim-timentin; WPM-woody plant medium.

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