

# Plant regeneration from in vitro leaves of mature black cherry (*Prunus serotina*)

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**Abstract** A regeneration system was developed for *Prunus serotina* from a juvenile (F) and two mature genotypes (#3 and #4). Adventitious shoots regenerated from leaves of in vitro cultures on woody plant medium with thidiazuron (TDZ) and naphthaleneacetic acid (NAA). The best regeneration for genotype F (91.4%) was observed on medium with 9.08  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA. The highest mean number of shoots (8.2) was obtained on medium containing 9.08  $\mu\text{M}$  TDZ and 0.54  $\mu\text{M}$  NAA. Genotype #3 had the highest regeneration (41.7%) with a mean number of shoots (4.8) on 9.08  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA, whereas genotype #4 had a 38.8% regeneration with a mean of 3.3 shoots. Genotype #4 had the highest mean number of shoots (4.8) on 4.54  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA. Silver thiosulphate at 60 or 80  $\mu\text{M}$  increased the percent regeneration of the mature genotypes #3 (75%) and #4 (58%). Adventitious shoots were rooted (70–76%) and rooted plantlets survived after acclimatization to the greenhouse. The effect of kanamycin

concentration on adventitious shoot regeneration was also evaluated.

**Keywords** Adventitious shoots · Organogenesis · Rooting · Silver thiosulphate

## Abbreviations

BA	6-benzylaminopurine
GA <sub>3</sub>	Gibberellic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	Naphthaleneacetic acid
STS	Silver thiosulphate
TDZ	Thidiazuron
WPM	Woody plant medium

## Introduction

Black cherry (*Prunus serotina* Ehrh.) is a valuable hardwood in the eastern United States and Canada. The wood is valued for fine cabinets, furniture, architectural woodwork, and veneer. There is an increased demand for high quality black cherry wood and increased interest in establishing plantations with improved black cherry genotypes. Clonal propagation of elite, mature or genetically improved genotypes will help to increase the establishment of high-quality plantations. There are few reports of successful adventitious shoot regeneration from mature, elite

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hardwood trees. Adventitious shoot regeneration has been achieved from juvenile or seedling explants, but is difficult from mature tissue. Genetic transformation can provide a promising approach compared to conventional breeding of *Prunus* by facilitating the transfer of valuable genes. When a valuable trait of a mature genotype is to be preserved and another desired trait added, a reliable regeneration and transformation protocol for that mature genotype is necessary.

Adventitious shoot regeneration using leaf explants is established for several species of *Prunus*, such as *P. canescens* (Antonelli and Druart 1990), *P. padus* (Hammatt 1993), *P. domestica* (Bassi and Cossio 1991, 1994; Nowak et al. 2004), *P. persica* (Declerck and Korban 1996; Gentile et al. 2002; Scorza et al. 1995), *P. dulcis* (Ainsley et al. 2000; Miguel and Oliveira 1999; Tang et al. 2002), *P. armeniaca* (Perez-Tornero et al. 2000), *P. avium* (Bhagwat and Lane 2004; Matt and Jehle 2005), and *P. serotina* (Espinosa et al. 2006; Hammatt and Grant 1998).

Several factors influence the efficiency of adventitious shoot regeneration, such as culture medium, genotype, growth regulators (cytokinin and auxin), size, age, and position of the explants, photoperiod, or light intensity. Regeneration in *Prunus* occurred more frequently if leaves were cultured on woody plant medium (WPM) (Lloyd and McCown 1981) rather than Driver and Kuniyuki (1984) walnut (DKW) medium (Hammatt and Grant 1998). Young, expanding leaves respond better than mature leaves (Perez-Tornero et al. 2000). Wounded, immature, furled leaves from shoot tips failed to regenerate shoots (Bhagwat and Lane 2004). Shoot regeneration was higher in cultures incubated with a 16-h photoperiod as compared to those incubated in continuous darkness (Bhagwat and Lane 2004). Leaf surface orientation is also important during the regeneration process. In *P. avium*, placing the adaxial side of the leaf in contact with the medium was better than placing the abaxial side (Bhagwat and Lane 2004). Regeneration of shoots from *P. avium* leaf segments was much lower than from whole-leaf explants (Bhagwat and Lane 2004). A combination of cytokinin and auxin was also important for production of shoots from leaves. Thidiazuron (TDZ) produced a better response than 6-benzylaminopurine (BA) when inducing shoots from leaves of *P. avium*, *P. armeniaca*, and *P. serotina* (Espinosa et al. 2006; Hammatt

and Grant 1998; Perez-Tornero et al. 2000). In *P. avium*, regeneration was optimal when whole-leaf explants were wounded and cultured abaxial side up, on medium supplemented with TDZ and naphthalene-acetic acid (NAA) (Bhagwat and Lane 2004).

Culture medium with silver thiosulfate (STS) enhanced the frequency of adventitious shoot regeneration in several species, such as *P. avium* (Escalaetes and Dosba 1993), apricot cultivars (*P. armeniaca*) (Burgos and Albuquerque 2003), *Brassica* spp. (Eapen and George 1997), and *Passiflora edulis* (Faria and Segura 1997). STS also improved plant regeneration from protoplasts of *Solanum hjertingii* and *S. capsicibaccatum* (Xu et al. 1991). STS reduced ethylene-induced culture abnormalities in seven potato (*S. tuberosum*) genotypes (Sarkar et al. 1999). However, in *P. avium*, the addition of STS did not show any positive effect on the regeneration efficiency (Matt and Jehle 2005).

Hammatt and Grant (1998) were the first to report adventitious shoot regeneration from juvenile leaf explants of *P. serotina*, however, a complete protocol including rooting and acclimatization of regenerated plantlets was not established. Espinosa et al. (2006) established a complete protocol for regeneration of plants of *P. serotina* from juvenile explants. The only report of *Agrobacterium* infection of black cherry was by Maynard and Fuernkranz (1989) using leaf pieces for regeneration and five *Agrobacterium* strains for gall formation tests. The objective of this study was to establish a regeneration protocol from both juvenile and mature tree explants. We also tested the ethylene inhibitor, STS, on regeneration of mature genotypes. We evaluated the effect of kanamycin concentration on adventitious shoot regeneration for use in genetic transformation studies.

## Materials and methods

### Plant material

Explants from two elite mature genotypes #3 and #4, and one 3-year-old seedling genotype F were obtained from stock plants growing in the greenhouse. Genotypes #3 and #4 were grafted stock plants obtained from phenotypically superior mature trees, 100-years-old, harvested in 2000, and sliced into veneer. The original scion wood was collected once the trees were on the ground. Both trees were selected and collected

by Lee Ekart of Danzer Forestland, Inc. and the trees originated near Bradford, Pennsylvania.

#### Micropropagation of black cherry

Nodal sections (2–3 cm in length) from the three genotypes were excised from actively growing greenhouse plants and surface disinfested in 70% (v/v) ethanol for 30 s, then in 15% (v/v) commercial bleach (5.25% sodium hypochlorite) for 20 min, followed by four rinses in sterile, deionized water. Nodal sections were placed vertically in 45 mm (d) × 97 mm (h) glass jars containing 40 ml of Murashige and Skoog (1962) (MS) medium with 3% (v/v) sucrose, 8.88 μM BA, 0.49 μM indole-3-butyric acid (IBA), 0.29 μM gibberellic acid (GA<sub>3</sub>), and 0.7% Difco-Bacto agar modified from Tricoli et al. (1985). The pH of the medium was adjusted to 5.7 before the addition of agar and autoclaved at 121°C for 20 min. Cultures were incubated at 25°C under a 16-h photoperiod provided by cool-white fluorescent lamps (100–140 μmol m<sup>-2</sup> s<sup>-1</sup>). Explants were transferred to fresh medium every 3 weeks for multiplication of shoots (see Espinosa et al. 2006 for details).

#### Adventitious shoot regeneration

Young, fully expanded leaves (1.5–2 cm<sup>2</sup>) that included the petiole from in vitro shoots were wounded by several cuts transversely along the midrib, and cultured abaxial side up on medium. Leaves used for regeneration were strictly selected using the following criteria. Shoot cultures were in an active state of growth and showing no signs of chlorosis. The youngest, fully expanded leaf at the shoot apex with a good vein pattern was selected. The underside of the leaf did not have a flat appearance, but rather one in which the veins were raised and inter-veinal tissue had a rough appearance. Usually, one or fewer leaves were harvested per shoot culture. The regeneration medium was WPM with 3% sucrose, 0.7% Difco-Bacto agar, and TDZ at 0, 2.27, 4.54, 6.81, or 9.08 μM in combination with NAA at 0, 0.27, 0.54, or 1.07 μM with a pH of 5.7. For each treatment, at least two Petri dishes (100 × 15 mm<sup>2</sup>) containing 30 ml medium were prepared with six leaf-petiole explants. Cultures were incubated in the dark at 25 ± 3°C for 3 weeks before exposure to light with a 16-h photoperiod (100–140 μmol m<sup>-2</sup> s<sup>-1</sup>). After 5 weeks on WPM,

leaf explants with callus were transferred to MS medium containing 8.88 μM BA, 0.49 μM IBA, and 0.29 μM GA<sub>3</sub> for shoot elongation. This experiment was conducted three times (total of 36 explants per treatment). Regeneration percentage and number of shoots per leaf explant were recorded after 8 weeks.

#### Effect of silver thiosulphate on regeneration

Leaves of the two mature genotypes #3 and #4 were prepared as previously described and placed on the best regeneration medium (WPM containing 9.08 μM TDZ and 1.07 μM NAA) with silver ion (Ag<sup>+</sup>), in the form of silver thiosulphate at 0, 40, 60, or 80 μM. Stock solutions (0.1 M) were prepared by dissolving 1.58 g of sodium thiosulfate (Sigma, S7026) in 100 ml of deionized water, and dissolving 1.7 g of silver nitrate (Sigma, S7276) into 100 ml of deionized water. STS was prepared with a molar ratio between silver and thiosulfate of 1:4. STS was filter-sterilized and added to the medium after autoclaving by adding 2 ml (0.02 M STS stock) per liter of medium to make 40 μM. Cultures were incubated in the dark and then transferred to the light as previously described for adventitious shoot regeneration. This experiment was conducted three times with 20 explants per treatment.

#### Kanamycin sensitivity

Kanamycin is commonly used to select for adventitious transformants. It is important to evaluate and minimize the effect that this antibiotic could have on adventitious shoot regeneration. An avirulent *Agrobacterium* strain At793 (obtained from Stanton Gelvin, Purdue University), which has no Ti plasmid was co-cultured with leaves. Leaf explants were placed on the best regeneration medium (WPM containing 9.08 μM TDZ and 1.07 μM NAA) and inoculated with a few drops of 0.8 OD<sub>600</sub> *Agrobacterium*. After 10 min, leaf explants were blotted dry on sterile filter paper and cultured again on regeneration medium for 2 days in the dark. Explants were then transferred to selection medium (WPM containing 9.08 μM TDZ, 1.07 μM NAA, 150 mg/l timentin, 60 μM STS, 0.7% Difco-Bacto agar, and kanamycin at 0, 5, 10, 15, 20, 25, or 30 mg/l) and cultured in the dark. Twelve explants were used for each treatment and replicated three times. Response of leaves was determined by counting the number of shoots initiated.

### Rooting and acclimatization of micropropagated shoots of mature genotypes

Shoots were cultured in MS micropropagation medium (described previously) for 5 weeks prior to rooting. Shoots (2–3 cm in length) were excised and the basal end dipped in 2.5, 5, or 10 mM IBA for 30 s or 3 min, then placed in Magenta GA-7 vessels containing ½-MS medium with 2% sucrose, 0.7% Difco-Bacto agar without plant growth regulators. Cultures were placed in the dark for 4 days prior to transfer to a 16-h photoperiod (60–80  $\mu\text{mol m}^{-2} \text{s}^{-1}$  m). There were 20 shoots for each replication, conducted three times for a total of 60 shoots. Number of roots, root length, and percent rooting were recorded 3 weeks after shoots were placed on root induction medium. Healthy rooted plantlets were transplanted into 5-inch plastic pots containing autoclaved medium (Pro-Mix BX, Premier Horticulture, Inc.) and maintained in one-gallon Ziploc plastic bags to maintain a high relative humidity. Plantlets were watered every other day or as needed and gradually acclimatized over a period of 6 weeks prior to transfer to the greenhouse. Survival rate in the greenhouse was recorded after 3 months. Microshoots from genotype F were not tested for this rooting method since shoots of this genotype could be rooted by using IBA in the medium (see Espinosa et al. 2006).

### Rooting and acclimatization of adventitious shoots

Shoots (2–3 cm in length) were excised and the basal end dipped in 2.5 mM IBA for 3 min (based on the results from rooting micropropagated shoots), then placed in Magenta GA-7 vessels containing ½-MS medium with 2% sucrose, 0.7% Difco-Bacto agar without plant growth regulators. Cultures were placed in the dark for 4 days prior to transfer to a 16-h photoperiod. There were 30 shoots for each replication, conducted three times for a total of 90 shoots. Number of roots, root length, and percent rooting were recorded 3 weeks after shoots were placed on root induction medium. Acclimatization was as described previously.

### Data analysis

Shoot numbers are presented as the mean  $\pm$  standard error of shoots regenerated from leaf explants. Regeneration percentage is expressed as the average

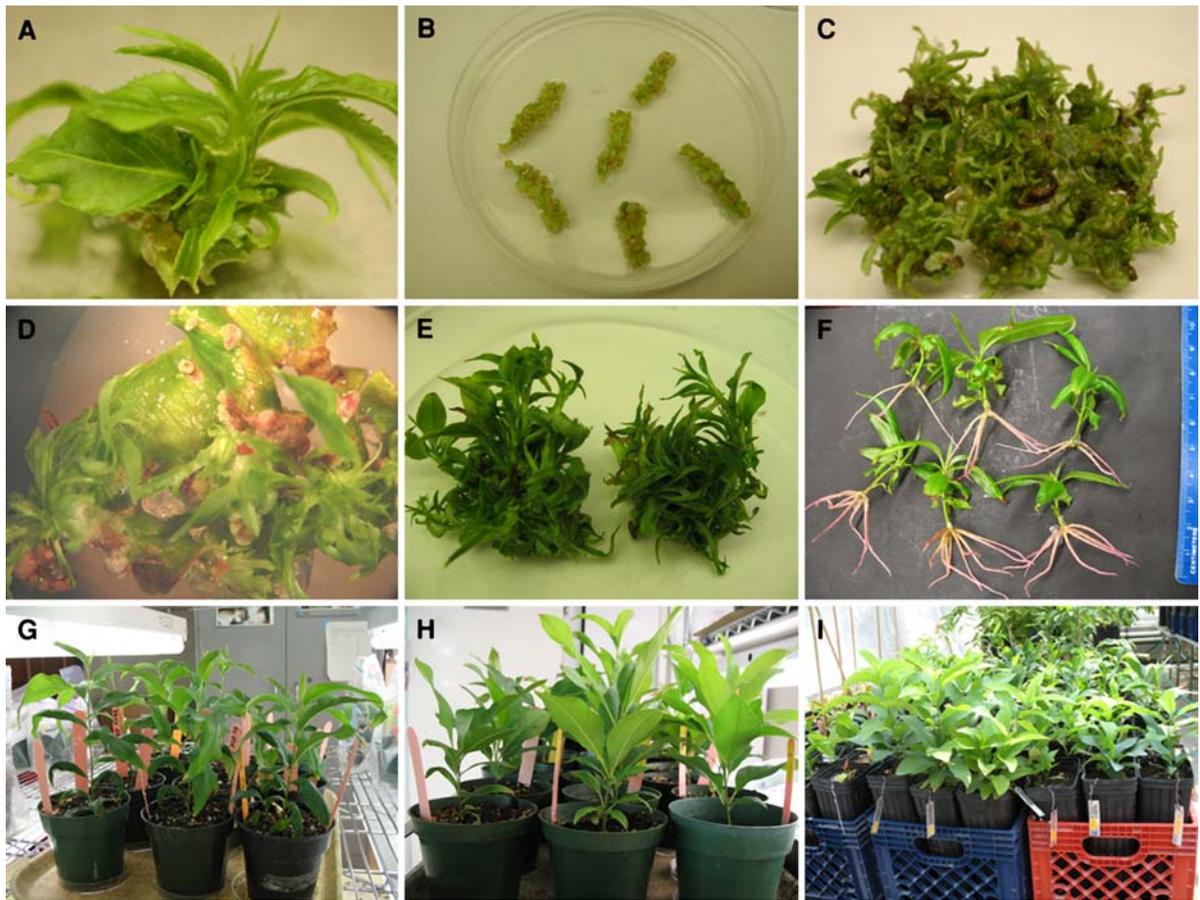
percentage of leaves that developed shoots divided by the number of total leaf explants. To understand the relation of factors, regeneration percentage data representing dependent variables were regressed upon the TDZ concentration as the independent variable. Linear and quadratic models were tested to fit the data at  $\alpha = 0.05$  level of significance. When the quadratic model was not significant, the linear model was used. If regression did not fit, the data were analyzed with an analysis of variance (ANOVA) using the GLM procedure of SAS (software version 9.1) and Tukey test was used to distinguish differences between treatment means at the  $\alpha = 0.05$  level.

## Results

### Adventitious shoot regeneration

Leaf explants doubled in size and produced callus after 2 weeks culture in darkness. Callus appeared mainly on the wounded edges and midribs, and became reddish when cultures were moved to the light. For genotype F, the first adventitious shoot (Fig. 1c) developed from leaves about 1 week after transfer to MS medium. Adventitious shoots began to develop after 2 weeks for genotypes #3 (Fig. 1d) and #4 and elongated to 2–3 cm within 8 weeks (Fig. 1e). Shoots developed much faster for the juvenile genotype F than from the mature genotypes. Genotype F was more regenerative (91.4%) than the two mature genotypes (41.7% for #3; 38.8% for #4) on 9.08  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA (Table 1). Genotype F also produced more adventitious shoots per explant and a cluster of shoots usually formed from the leaves. Most shoots developed along the length of the abaxial surface of the midrib. The highest mean number of shoots (8.2) for genotype F occurred on 9.08  $\mu\text{M}$  TDZ and 0.54  $\mu\text{M}$  NAA with 88.9% regeneration. The highest mean number of shoots (4.8) for genotype #3 was on 9.08  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA. Genotype #4 had only a 38.8% regeneration efficiency on 9.08  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA with the highest mean number of shoots (4.8) on 4.54  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA.

TDZ was the most important factor for adventitious shoot regeneration, as no adventitious shoots developed on explants exposed to media without TDZ.



**Fig. 1** Adventitious shoot regeneration from in vitro leaves of black cherry. (a) Mature genotype #3 stock shoot culture, (b) Leaf explants on adventitious shoot induction medium, (c) Adventitious shoots of F (seedling), (d) Two weeks of

Regeneration efficiency increased as TDZ concentration increased in the media (Table 1). The linear model fit well to all three genotypes and equations are listed in Table 1. For genotype F, the influence of TDZ on regeneration rate also showed a quadratic regression response. This can be explained as in a range of TDZ concentrations ( $<9.31 \mu\text{M}$ ), regeneration rate increased as TDZ increased. Higher concentrations of TDZ were tested for one replication, however, adventitious shoots formed as a cluster, and it took a much longer time for the cluster to develop into individual shoots (data not shown). NAA had no significant effect on regeneration rate (all  $P$  values greater than 0.05; data not shown). There was no interaction between TDZ and NAA for regeneration rate.

Both TDZ and NAA had no significant effect on mean number of shoots. ANOVA analysis showed all

adventitious shoots of mature genotype #3, (e) Adventitious shoots of mature genotype #4, (f) Rooting of genotype #3, (g) Acclimatized plants of F, (h) Acclimatized plants of #4, and (i) Acclimatized plants of #3, #4, and F in the greenhouse

$F$  values are greater than 0.05. There were large differences among replications for the same treatment. Young, thick, large leaves responded better than older, small, thin, leaves. After 3 weeks on regeneration medium, phenolic exudates began to appear in the medium. Therefore, explants needed to be transferred to fresh medium every 2–3 weeks. Once shoot clusters formed, shoots needed to be separated and transferred to fresh medium in order to elongate, or shoots would deteriorate.

#### Effect of silver thiosulphate on regeneration

STS increased the regeneration rate for both mature genotypes when concentrations of  $60 \mu\text{M}$  (#3) or  $80 \mu\text{M}$  (#4) were used (Table 2). For genotype #4, regeneration rate regressed upon STS concentration

**Table 1** Adventitious shoot regeneration for three genotypes of black cherry<sup>a</sup>

Plant growth regulator ( $\mu\text{M}$ )		Genotype F		Genotype #3		Genotype #4	
TDZ	NAA	Regeneration (%)	Mean no. shoots	Regeneration (%)	Mean no. shoots	Regeneration (%)	Mean no. shoots
0	0	0	0	0	0	0	0
0	0.27	0	0	0	0	0	0
0	0.54	0	0	0	0	0	0
0	1.07	0	0	0	0	0	0
2.27	0	33.3	5.2 $\pm$ 3.5	0	0	0	0
2.27	0.27	22.2	5.5 $\pm$ 2.4	11.1	1 $\pm$ 0	0	0
2.27	0.54	38.9	6.4 $\pm$ 3.1	5.5	3.5 $\pm$ 3.3	0	0
2.27	1.07	44.4	6.3 $\pm$ 3.4	16.7	2.8 $\pm$ 0.7	0	0
4.54	0	63.9	5.8 $\pm$ 3.4	22.2	2.5 $\pm$ 1.5	0	0
4.54	0.27	47.2	4.6 $\pm$ 3.2	11.1	1.3 $\pm$ 1.5	0	0
4.54	0.54	63.9	6.4 $\pm$ 3.3	11.1	1.8 $\pm$ 1.4	16.6	3.7 $\pm$ 2.0
4.54	1.07	72.2	6.0 $\pm$ 3.9	13.8	2.4 $\pm$ 1.1	11.1	4.8 $\pm$ 2.2
6.81	0	69.4	6.1 $\pm$ 4.4	13.8	1.8 $\pm$ 0.8	11.1	3.8 $\pm$ 1.9
6.81	0.27	72.2	6.2 $\pm$ 4.5	19.4	3.7 $\pm$ 3.9	8.3	1.3 $\pm$ 0.5
6.81	0.54	61.1	7.6 $\pm$ 9.0	25	3.4 $\pm$ 2.3	5.6	3.5 $\pm$ 2.1
6.81	1.07	50	7.8 $\pm$ 4.6	11.1	2.0 $\pm$ 0.8	8.3	2.7 $\pm$ 1.5
9.08	0	58.3	6.2 $\pm$ 3.7	33.3	2.7 $\pm$ 1.8	8.3	3.7 $\pm$ 1.1
9.08	0.27	63.9	6.8 $\pm$ 3.7	13.8	1.4 $\pm$ 0.5	2.6	1.0 $\pm$ 0.6
9.08	0.54	88.9	8.2 $\pm$ 4.8	30.5	3.5 $\pm$ 3.1	13.8	2.8 $\pm$ 1.9
9.08	1.07	91.4	6.6 $\pm$ 4.2	41.7	4.8 $\pm$ 3.3	38.8	3.3 $\pm$ 2.1

Regeneration rate regressed upon TDZ concentration

Genotype	Model	R square	F value
F	$Y = 27.79 + 5.47X$	0.5785	0.0006
	$Y = -0.97X^2 + 16.76X + 1.08$	0.87	<0.0001
#3	$Y = 3.02X + 0.275$	0.6924	<0.0001
#4	$Y = 1.765X - 1.79$	0.3807	0.0038

Y represents percentage of regeneration rate. Variable X represents TDZ concentration

<sup>a</sup> Leaf explants cultured on woody plant medium with thidiazuron (TDZ) and naphthaleneacetic acid (NAA) for 3 weeks in the dark and then transferred to a 16-h photoperiod. Means  $\pm$  standard error

fit the linear model ( $Y = 7.85X + 18.25$  with  $R^2 = 0.559$ ). The linear or quadratic model did not fit for genotype #3. Some leaf cultures on medium with STS appeared necrotic, but adventitious buds still formed from the callus. Separation of adventitious buds and transfer to elongation medium allowed healthy shoots to develop. The effect of STS was slightly different for each genotype. Genotype #3 produced the highest regeneration rate of 75% on 60  $\mu\text{M}$  STS. There was no significant difference for mean number of regenerated shoots among STS concentrations tested. Genotype #4 had the best

adventitious shoot regeneration (58%) and a mean number of shoots of 2.4 on 80  $\mu\text{M}$  STS.

#### Kanamycin sensitivity

The mature genotype #3 was more sensitive to kanamycin than the seedling genotype F (Table 3). There was no adventitious shoot regeneration for #3 when leaf explants were grown on medium with kanamycin at or greater than 15 mg/l. Both regeneration rate and the mean number of shoots regressed upon concentrations of kanamycin showed that the

**Table 2** Effect of silver thiosulphate (STS) on adventitious shoot regeneration of mature black cherry

STS ( $\mu\text{M}$ )	Genotype #3		Genotype #4	
	Regeneration (%)	Mean no shoots	Regeneration (%)	Mean no. shoots
0	40.3	$3.1 \pm 1.6$	33	$2.6 \pm 0.9$
40	30.5	$3.0 \pm 1.7$	28.5	$2.5 \pm 0.6$
60	75	$3.6 \pm 1.4$	32	$2.0 \pm 1.4$
80	51	$3.4 \pm 1.6$	58	$2.4 \pm 0.9$

Regeneration rate regressed upon STS concentration

Genotype	Model	R square	F value
#3	Does not fit any model		
#4	$Y = 7.85X + 18.25$	0.559	0.05

Y represents percentage of regeneration rate. Variable X represents STS concentration

n = 60; The regeneration percentage is the number of explants that produced adventitious shoots. Means  $\pm$  standard error

quadratic model fit well, as indicated by the equations in Table 3. Tukey test showed that there was a significant difference among different kanamycin treatments (Table 3). For genotype F, a few shoots ( $1.0 \pm 0.5$ ) regenerated on medium with 20 mg/l kanamycin and these shoots became chlorotic and did not elongate.

Rooting and acclimatization of micropropagated shoots of mature genotypes

Roots appeared as early as 4 days after placing the microshoots in the light. Most roots developed 7–9 days after being placed in the light. Rooting rate was regressed upon IBA concentration and dipping

**Table 3** Kanamycin sensitivity of black cherry leaf explants from juvenile (F) and mature (#3)

Kanamycin (mg/L)	Genotype F		Genotype #3	
	Regeneration (%)	Mean no. shoots	Regeneration (%)	Mean no. shoots
0	91	$4.0 \pm 1.6^a$	37.3	$1.8 \pm 0.7^a$
5	72.2	$2.7 \pm 1.7^{ab}$	19.4	$1.4 \pm 0.7^{ab}$
10	33.3	$1.4 \pm 0.9^b$	11.1	$1.0 \pm 1.4^b$
15	22.2	$1.5 \pm 0.5^b$	0	0
20	16.7	$1 \pm 0.5^b$	0	0
25	0	0	0	0
30	0	0	0	0

Regeneration rate and mean number shoots are regressed upon kanamycin concentration

Source	Model	R square	F value
Genotype F	Regeneration rate	$Y = 0.103X^2 - 6.18X + 92.9$	0.9766
	Mean no. shoots	$Y = 0.003X^2 - 0.224X + 3.82$	0.9532
Genotype #3	Regeneration rate	$Y = 0.073X^2 - 3.34X + 36.14$	0.9840
	Mean no. shoots	$Y = 0.003X^2 - 0.15X + 1.94$	0.9379

Y represents percentage of regeneration rate or mean number shoots. Variable X represents kanamycin concentration

n = 36; The regeneration percentage is the number of explants that produced adventitious shoots. Means ( $\pm$ standard error) within a column followed by the same letter are not significantly different by Tukey test and  $p = 0.05$

time, however, it did not fit the linear or quadratic model. Therefore, ANOVA analysis and Tukey test were applied to test for significant differences among treatments. IBA concentration had a significant effect on rooting efficiency ( $P < 0.0001$ ) for both genotypes (Table 4). Rooting percentage, mean root number, and mean root length decreased with increasing IBA, when used as a 3 min dip. Treatment 2.5 mM IBA (3 min dip) was optimal for both mature genotypes producing 82% and 71% rooting, respectively for genotypes #3 and #4. With this treatment for genotype #3, the maximum root number for a shoot was 16 and the mean number of roots was  $5.4 \pm 3.0$  (Table 4). For genotype #4, the mean number of roots was  $6.1 \pm 3.2$  with a maximum root number of 12. IBA at 1.25 mM was tested for one replication; however, most rooted shoots produced only one root (data not shown). High concentrations of IBA inhibited root elongation. Acclimatization rate was also affected by the concentration of IBA with a survival rate of 68% for #3 and 84% for #4, when using a 2.5 mM IBA dip for 3 min. Genotype #3 had

a better rooting percentage than #4; however, genotype #4 acclimatized better than #3 and grew much faster in the greenhouse.

#### Rooting and acclimatization of adventitious shoots

All genotypes could be rooted (70–76%) (Table 5). Roots grew relatively fast with a mean length of from 2.6 cm to 3.5 cm within 3 weeks after root induction. There was no significant difference for root length among the three genotypes. Rooted plantlets were acclimatized after 6 weeks of growth in the lab and 76–84% of rooted plantlets survived after acclimatization to the greenhouse.

#### Discussion

Adventitious shoots were regenerated, rooted, and acclimatized from one immature and two mature genotypes of *P. serotina*. Successful regeneration is a

**Table 4** Rooting of in vitro culture shoots for two mature genotypes of black cherry

IBA (mM)	Dip time (s)	Rooting (%) <sup>a</sup>		Mean root no.		Mean root length (cm)		Survival rate (%) <sup>c</sup>	
		#3	#4	#3	#4	#3	#4	#3	#4
2.5	30	38.4 ± 1.7 <sup>bc</sup>	39.7 ± 6.1 <sup>b</sup>	4.1 ± 2.4 <sup>b</sup>	3.9 ± 2.9 <sup>b</sup>	2.4 ± 0.7 <sup>b</sup>	1.9 ± 0.6 <sup>a</sup>	74.1	68
2.5	180	81.7 ± 3.4 <sup>a</sup>	70.5 ± 3.4 <sup>a</sup>	5.4 ± 3.0 <sup>a</sup>	6.1 ± 3.2 <sup>a</sup>	2.9 ± 0.4 <sup>a</sup>	2.0 ± 0.5 <sup>a</sup>	68.2	84.2
5	30	47.0 ± 8.3 <sup>b</sup>	26 ± 4 <sup>c</sup>	2.5 ± 1.4 <sup>b</sup>	3.6 ± 2.1 <sup>b</sup>	2.2 ± 0.8 <sup>b</sup>	1.4 ± 0.5 <sup>b</sup>	47.0	41.7
5	180	39.9 ± 8.0 <sup>bc</sup>	16.4 ± 6.7 <sup>d</sup>	3.3 ± 1.6 <sup>b</sup>	2.6 ± 1.3 <sup>b</sup>	1.1 ± 0.4 <sup>d</sup>	1.4 ± 1.2 <sup>b</sup>	28.6	50
10	30	19.5 ± 2.3 <sup>d</sup>	25.7 ± 4.1 <sup>c</sup>	4.9 ± 3.6 <sup>b</sup>	4.3 ± 2.4 <sup>b</sup>	1.3 ± 0.8 <sup>c</sup>	1.0 ± 0.7 <sup>c</sup>	15.7	21.4
10	180	27.8 ± 10.3 <sup>cd</sup>	9.5 ± 4 <sup>d</sup>	3.4 ± 4.8 <sup>b</sup>	2.4 ± 0.9 <sup>b</sup>	1.0 ± 0.6 <sup>d</sup>	1.1 ± 0.7 <sup>c</sup>	11.1	10.0

Analysis of variance of rooting percentage

Genotype	Total no. plantlets rooted (and potted)	Total no. plantlets surviving in the greenhouse <sup>b</sup>	Source of variation	DF	Mean square	F value	Pr > F
#3	151(114)	59	IBA	2	1991.5	46.2	<0.0001
			TIME	1	986.2	22.9	0.0004
			IBA*TIME	2	998.9	23.2	<0.0001
#4	115(104)	60	IBA	2	2568.1	107.2	<0.0001
			TIME	1	12.8	0.5	0.4785
			IBA*TIME	2	972.7	40.6	<0.001

<sup>a</sup> Data taken 3 weeks after induction of roots. Means ( $\pm$ standard error) within a column followed by the same letter are not significantly different by Tukey test and  $p = 0.05$

<sup>b</sup> Only healthy plantlets with healthy roots were potted and acclimatized

<sup>c</sup> Data taken 6 weeks after acclimatization in the growth room

**Table 5** Rooting of adventitious shoots for three genotypes of black cherry

Genotype	Rooting <sup>a</sup> (%)	Mean no. roots	Root length (cm)	No. healthy plantlets potted <sup>b</sup>	Survival rate (%) <sup>c</sup>
F	75.5	3.4 ± 2.2 <sup>a</sup>	3.5 ± 1.4 <sup>a</sup>	46	82.6
#3	72.2	4.9 ± 3.1 <sup>a</sup>	2.6 ± 0.8 <sup>a</sup>	29	75.8
#4	70.0	2.4 ± 1.2 <sup>b</sup>	2.9 ± 1.0 <sup>a</sup>	25	84.0

n = 90

<sup>a</sup> Data taken 3 weeks after induction of roots. Means (±standard error) within a column followed by the same letter are not significantly different by Tukey test and  $p = 0.05$

<sup>b</sup> Only healthy plantlets with healthy roots were potted and acclimatized

<sup>c</sup> Data taken 6 weeks after acclimatization in the growth room

key step for *Agrobacterium*-mediated transformation of this species. Hammatt and Grant (1998) and Espinosa et al. (2006) reported that *P. serotina* can be regenerated from juvenile source leaf explants by using TDZ and NAA with a regeneration efficiency of 62% and 38.3% depending on the study. We report a higher regeneration efficiency of 91.4% for a juvenile genotype and 39–42% for mature genotypes. Explants from seedlings are superior to mature genotypes for percent regeneration of *P. serotina* as well as for most plant species. Mature genotypes usually respond much slower than seedling sources for regeneration, elongation, rooting, and acclimatization.

The reproducibility and consistency of results among replications was variable. This is a common problem in regeneration studies (Burgos and Albuquerque 2003; Escalettes and Dosba 1993). A possible explanation is because of the physiological state of the leaf explants. We found that cultures maintained under higher light intensity ( $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) produced thicker, larger leaves, and regenerated more shoots at a higher efficiency, than leaves growing under lower light intensity ( $\leq 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) (preliminary data not shown). Our results are similar to a study of micropropagation of ornamental *Prunus* species which reported that Nanking cherry, choke cherry, and Sargent cherry multiply better under high light intensity ( $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) (Kalinina and Brown 2007). Leaf growth stage is very important for adventitious shoot regeneration. Only strictly selected leaves with good characteristics, as described previously, regenerated adventitious shoots with a high efficiency.

In our study, adventitious shoot regeneration for the mature genotypes improved with the addition of the ethylene inhibitor STS, a finding consistent with previous reports on regeneration of other *Prunus*

species (Burgos and Albuquerque 2003; Escalettes and Dosba 1993). It was reported that auxin stimulates ethylene production, which causes leaf senescence and prevents regeneration. STS is an ethylene inhibitor which can inhibit ethylene-induced leaf abscission. STS improved plant regeneration from protoplasts in *Solanum hjertingii* and *S. capsicibaccatum* (Xu et al. 1991). The effect of STS was more obvious during the transformation process than the regeneration process because it reduces necrosis of the leaves from overgrowth of *Agrobacterium tumefaciens* and preventing leaf senescence, as observed in our study.

Black cherry was very sensitive to kanamycin, with mature lines not regenerating shoots when kanamycin was 15 mg/l or greater. Almond (*P. dulcis*) is similarly sensitive, so 15 mg/l kanamycin was used for selection of almond transformants (Miguel and Oliveira 1999). Our seedling line was more resistant to kanamycin; however, regenerated shoots on higher kanamycin (20 mg/l) medium did not elongate and develop into a healthy shoot for rooting. This result was confirmed by other studies that most *Prunus* species are very sensitive to kanamycin. Delayed selection (incorporating antibiotics into the culture medium, such as kanamycin, a few days after the start of regeneration) is a successful strategy for transformation of apple (Yao et al. 1995; Yepes and Aldwinckle 1994) and apricot (Machado et al. 1992). Ramesh et al. (2006) used delayed selection strategy for transformation of *P. dulcis* because of the adverse effect of kanamycin which inhibits organogenesis.

We successfully rooted adventitious shoots using an IBA dip. Compared to an earlier study (Espinosa et al. 2006) the percentage of rooting for adventitious

shoots was higher (70–76% compared to 27%) and the mean number of roots was higher (2.4–4.9 compared to 1–2.5). Acclimatization survival rate in our study was higher (76–84%) than that reported by Bhagwat and Lane (2004) (20–40%) and Tricoli et al. (1985) (10%). However, we also found that some shoot tips deteriorated after rooting. Only healthy plantlets with a healthy root system were acclimatized to the greenhouse.

The complete protocol for adventitious shoot regeneration of mature black cherry was to culture the whole leaf on WPM with 9.08  $\mu\text{M}$  TDZ and 1.07  $\mu\text{M}$  NAA in darkness for 3 weeks, followed by transfer to the light for 2 weeks before transfer to MS medium. Elongated shoots could be rooted by dipping in 2.5 mM IBA 3 min. Rooted plantlets could be acclimatized to the greenhouse within 6 weeks after root induction. To our knowledge, this is the first report of a complete protocol for adventitious shoot regeneration and rooting from mature black cherry. This reliable regeneration system will be used to transform these mature, elite genotypes of black cherry by *Agrobacterium*-mediated transformation.

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