

# Shoot regeneration from organogenic callus of sweet cherry (*Prunus avium* L.)

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**Abstract** A complete method to regenerate adventitious shoots and to produce field-ready trees from three commercial cultivars of sweet cherry (*Prunus avium* L.) is described. The effects of explant types, pre-treatments, basal media, and phloroglucinol on cultivars Bing, Sweetheart, and Lapins were investigated. Callus developed on four explant types: apical shoot tips isolated from orchard trees; and punctured shoot tips, stem sections, and shoot bases of in vitro shoot cultures. Callus formed on Bing (5%), Sweetheart (8%), and Lapins (20%) shoot tips from orchard trees after 4 months on Murashige and Skoog medium (MS) at half-strength with 3  $\mu$ M benzylaminopurine (BA). In vitro-derived explants formed callus after 3 months on Woody Plant Medium with 3  $\mu$ M BA (W3B): punctured shoot tips (Sweetheart and Lapins 67%), stem sections (Sweetheart 31%, Lapins 27%), and shoot bases (Sweetheart 10%, Lapins 17%). Pre-treatment of shoot cultures on MS with 3  $\mu$ M BA

and 1 mM phloroglucinol increased callus formation three-fold on shoot base explants. Callus was separated from parental explants and maintained on MS with 3  $\mu$ M BA. Shooting was induced by transferring callus to W3B. At 2 weeks, shoot development approached 100%. By 4 weeks, 7–17 shoots had formed on each explant. Callus was maintained for 1.5 years with no decrease in shoot production. Shoots were grafted onto Mazzard (*P. avium*) rootstocks with 54% (Sweetheart), 57% (Lapins), and 21% (Bing) success after 5 weeks.

**Keywords** Adventitious shoots · Basal medium · Fruit tree · Grafting · Phloroglucinol

## Abbreviations

BA	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	$\alpha$ -naphthaleneacetic acid
WPM	Woody Plant Medium

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## Introduction

Sweet cherry (*Prunus avium* L.) is an economically important temperate fruit tree. Cherries have important markets both as fresh fruit and when processed (Ivanicka and Pretova 1986). The sweet cherry

industry is interested in developing cultivars with enhanced pest and disease resistance, improved tolerance to abiotic stresses, and improved quality traits (Brown et al. 1996).

Conventional breeding has produced sweet cherry cultivars with novel traits that satisfy many of these interests (Brown et al. 1996) but is limited by a lack of appropriate germplasm for some breeding goals. Development of a transformation system to introduce desired genes into *P. avium* could therefore complement conventional breeding. For example, classical breeding has been unsuccessful in developing *Prunus* cultivars with sufficient resistance to the plum pox virus, which causes a serious disease of stone fruits. However, transgenic plum trees (*P. domestica*) highly resistant to the virus have been developed and are undergoing field trials (Hily et al. 2004).

Transformation of fruit trees is hindered by many factors. Important obstacles are: the establishment of adapted shoot cultures used as a source of explants (McCown 2000), poor regeneration and tissue culture response from many genotypes, and a low transformation efficiency of cultivars that regenerate well in tissue culture (Gentile et al. 2002; Petri and Burgos 2005; Gómez-Lim and Litz 2004). Therefore, to generate transgenic sweet cherry, an efficient regeneration method for a diversity of cultivars and explant types would be a great advantage.

During the past two decades, there has been considerable progress in tissue culture and regeneration of sweet cherry. Shoot regeneration has been demonstrated from both seed-derived tissues (Lane and Cossio 1986; Schmidt and Kardel-Meisner 1992) and mature tissues. Most reports of shoot regeneration from mature tissue sources used leaves as explants (Bhagwat and Lane 2004; Matt and Jehle 2005; Takashina et al. 2003; Tang et al. 2002; Yang and Schmidt 1992). Oh et al. (1991) obtained direct and indirect shoot regeneration from shoot tips. Recently, Matt and Jehle (2005) reported shoot regeneration from internode segments. To our knowledge, regeneration from basal explants of shoot cultures, and from apical shoot tips taken directly from orchard trees, have not been previously reported for sweet cherry.

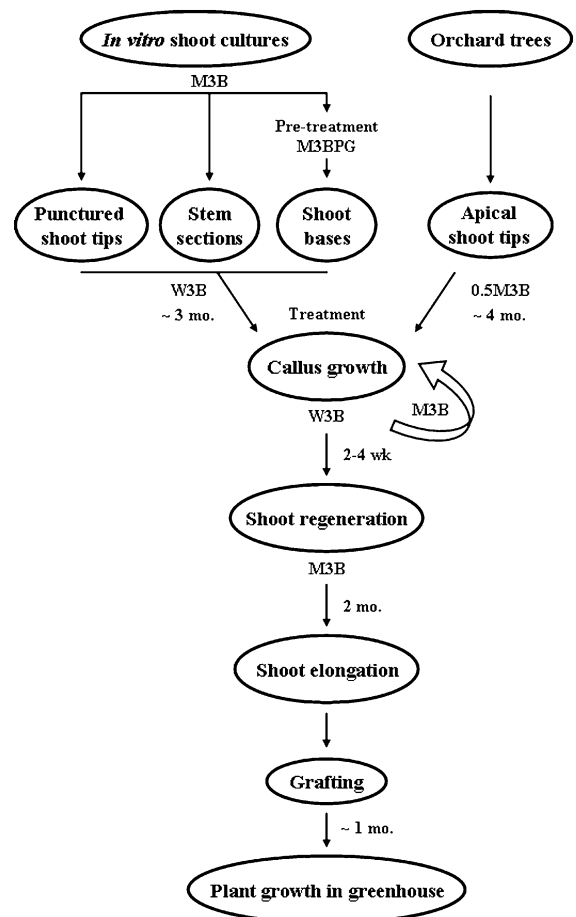
The aim of this study was to develop a procedure to regenerate shoots from ex vitro and in vitro explant sources and to produce plants ready for the field. The method is outlined in Fig. 1. Commercial sweet

cherry cultivars were used to evaluate the influences of explant types, basal media, phloroglucinol, and pre-treatments on callus induction and growth and on shoot regeneration. Regenerated shoots were grafted onto rootstock and grown in the greenhouse.

## Materials and methods

### Culture medium and conditions

The basal media consisted of Murashige and Skoog (MS; Murashige and Skoog 1962) supplemented with



**Fig. 1** Summary of sweet cherry (*Prunus avium*) adventitious shoot regeneration protocol. Media are MS or half-strength MS with 3  $\mu$ M BA (M3B or 0.5M3B, respectively), or M3B supplemented with 1 mM phloroglucinol (M3BPG), or WPM with 3  $\mu$ M BA (W3B)

0.4 mg/l thiamine-HCl, 100 mg/l myo-inositol, and 30 g/l sucrose, or half-strength MS (with half-strength salts and full-strength vitamins), or Woody Plant Medium (WPM; Lloyd and McCown 1980) with 20 g/l sucrose. Prior to autoclaving, the pH was adjusted to 5.6 with 1 M NaOH and 4.5 g/l (Petri dishes) or 5 g/l (shoot culture jars) Agargel (Sigma, St. Louis, MO) was added to media. Callus and shoot cultures were supplemented with 3  $\mu$ M 6-benzylaminopurine (BA). Petri dishes were 100  $\times$  15 mm in size and were wrapped in Parafilm, unless otherwise indicated. Shoot cultures were maintained in 500 ml glass jars containing  $\sim$ 80 ml medium, unless otherwise indicated. Jars were fitted with plastic lids having a 12 mm-diameter hole covered with two layers of medical tape (3M Micropore<sup>TM</sup>, 3M Canada, London, ON) for ventilation. Cultures were placed in growth chambers set at a temperature of 24°C, 44% humidity, a 16-h photoperiod, and a light intensity of  $\sim$ 26.7  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> (shoot cultures) and 18.5  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> (callus cultures), provided by cool-white fluorescent lights.

#### Shoot culture initiation, establishment, and maintenance

Sweet cherry (*Prunus avium* L.) cultivars Lapins, Sweetheart, and Bing were used in experiments. Trees were certified virus-free and were maintained in the budwood orchard collection at the Pacific Agri-Food Research Centre, Summerland, BC, Canada. Apical shoot tips and axillary buds were collected in July 2003, March 2004, and July 2004. Bing trees were 25 years old, Lapins were 15 years old, and Sweetheart were 5 years old. Tissues were pre-sterilized in a solution of 10% commercial bleach (Old Dutch<sup>TM</sup>, containing 5.25% NaOCl) for 5 min followed by a 5 min rinse under tap water. Tissues were trimmed to 1 cm in length and outer bud scales were removed. Explants were sterilized with 70% ethanol for 1 min, followed by 10% commercial bleach with 1 drop Tween-20/100 ml for 5 min, with occasional stirring. The sterilizing solution was decanted, replaced with a fresh solution, and explants were sterilized for an additional 5 min. Tissues were rinsed four times with sterile, distilled water. Explants were trimmed to  $\sim$ 0.5 cm in length and cut ends were pushed into culture initiation medium. Four explants were placed

in each dish. Culture initiation medium was half-strength MS with 3  $\mu$ M BA (0.5M3B). During the first month in culture, explants were transferred to fresh medium at weekly intervals. Explants were then subcultured at 4-week intervals in 100  $\times$  25 mm Petri dishes. Explants with shoot growth were consolidated into dishes, contaminated explants were discarded, and remaining explants that did not produce new growth were consolidated. After 4 months, shoots were transferred to glass jars containing MS with 3  $\mu$ M BA (M3B). Five to six shoots were placed in each jar. Shoot cultures were maintained by subculturing at 4-week intervals.

#### Callus initiation

##### *Ex vitro explants*

Consolidated apical shoot tips and axillary buds that did not form shoots were used. Tissues were collected and prepared as outlined in the preceding paragraph. The time to callus formation was recorded in three trials. In one trial, callus formation was quantified monthly by recording the number of apical shoot tips and axillary buds forming callus. Callus formation was calculated as the number of explants forming callus/total number of explants harvested. The statistical analysis treated explant, rather than dish, as the unit of replication.

##### *In-vitro explants*

Three year-old established shoot cultures of cultivars Lapins and Sweetheart were used. Shoot cultures were maintained on M3B medium, unless otherwise stated. Leaves and petioles were removed from shoots with a scalpel. Shoots were then divided into three explant types: apical tips, stems, and shoot bases. Explants of each type were collected and pooled. Treatments were composed of MS, half-strength MS, or WPM supplemented with 3  $\mu$ M BA, (M3B, 0.5M3B, and W3B, respectively). Explants were subcultured at 4-week intervals. The number of explants forming organogenic callus was recorded after 3 months. Explants were prepared as follows:

Punctured shoot tips: initial observations suggested that wounding shoots facilitated organogenic callus formation. (These observations are considered

further in the Discussion.) Therefore, explants were wounded before transfer to treatments. Apical shoot tips were cut to  $\sim 0.25$  cm in length. Under a dissecting microscope, small leaves were removed from the apex. Using a sterile 1 ml syringe, the apical tip was pierced 5–7 times. Explants were inoculated into treatment media straight up, with the cut base pushed into medium. There were three replicate dishes, each containing four explants.

Stem sections: using a dissecting microscope, longitudinal sections  $\sim 0.25$ – $0.5$  mm thick were cut with a scalpel over the entire stem. There were three replicate dishes, each containing 15 explants. For statistical analysis, culture dishes were categorized according to the number of explants forming callus (0, 1–2, 3–4, or 5–6 callus-forming explants).

Shoot bases: preliminary trials suggested that callus formation on shoot base explants occurred at a much lower frequency than on shoot tips and stem sections. Therefore the effects of pre-treatments were also considered. Shoot cultures were pre-treated for 2–3 subcultures (8–12 weeks). Pre-treatments were W3B, M3B with 1 mM phloroglucinol (M3BPG; 1, 3, 5-trihydroxybenzene; Sigma, St. Louis, MO), or M3B (control). Following the pre-treatment period, the base of the stem ( $\sim 1$  mm) and its associated callus was excised from shoots, divided into 3–5 mm-diameter pieces, and transferred to treatments to promote organogenic callus formation. There were 2–3 replicate dishes, each containing five explants. The experiment was repeated twice.

#### Callus proliferation and adventitious shoot formation

After initiating callus, the effects of media on callus growth and shoot formation were examined. Callus from cultivars Lapins, Sweetheart, and Bing were used in the experiments. Callus emerging from punctured tip, stem section, and shoot base explants were separated from parental tissues, pooled, and transferred to treatments. Callus was maintained on treatments for at least two subcultures (8 weeks) before data were collected.

#### *Influence of media on adventitious shoot formation*

Shoot development was evaluated in response to three treatments: M3B, 0.5M3B, or W3B. Callus was

divided into 3–5 mm-diameter pieces and any shoots appearing on explants were removed before transfer to treatments. For each treatment, there were two dishes, each containing 10 explants. At 4 weeks, the number of explants forming shoots was recorded and, using a dissecting microscope, shoots were removed from each callus piece and counted. Statistical analyses used  $\ln$ -transformed counts to meet normality assumptions. Results indicated that shoot formation was affected by the choice of treatment. Based on these results, callus growth was investigated using M3B and 0.5M3B, and shoot formation was investigated using W3B medium.

#### *Callus growth*

Callus growth was measured in response to M3B or 0.5M3B media. Callus, with shoots removed, were divided into 3 mm-diameter pieces. For each treatment dish, 30 callus pieces were collected and massed. After 1 month on treatments, callus was massed to obtain fresh weight. There were six replicate dishes/treatment and the experiment was repeated twice. Callus growth (%) was calculated as the difference in final and initial mass/initial callus mass.

#### *Adventitious shoot formation*

Callus growing on M3B or 0.5M3B media, without visible shoot formation, were divided into 3–5 mm pieces and transferred to W3B medium in  $100 \times 25$  mm Petri dishes. There were three replicate dishes, each containing 10 callus pieces. The number of explants producing shoots was recorded weekly. At 4 weeks, the number of shoots growing on five randomly chosen explants on each dish was counted. Data from replicate dishes were averaged to obtain the number of shoots regenerating per callus explant. The experiment was repeated twice.

#### Shoot survival

After a 4 week treatment with W3B, shoots were separated from callus and transferred to M3B with 5 g/l Agargel for elongation. Medium was dispensed into clear, rectangular polypropylene containers fitted with XL-XXL vented lids (Combiness Full-Gas Microbox, Gent, Belgium). A range of 19–46 small

shoots were initially placed in each container. Shoots were subcultured at 4-week intervals. At first subculture, shoots had enlarged and were transferred to fresh medium at a lower density (20 shoots/container). At 2 months, 8–10 shoots were placed in each container; cultures were then maintained at that density. The initial number of shoots and the number of shoots surviving each month were recorded over a 3 month period. There were 2–3 trials for each cultivar. Shoot survival (%) at 3 months was calculated as the total number of surviving shoots/initial number of shoots.

### Grafting procedures

#### *Rootstocks*

Dormant rooted layers of the cherry rootstock cultivar Mazzard F12/1 (*P. avium*) were obtained in February 2004 (Trass Nursery Ltd., Langley, BC, Canada). One stick was planted per 20 l pot containing Premier Pro-Mix Bx growing medium (Premier Horticulture Ltd., Delta, BC, Canada) supplemented with Acer 13N-13P-13K Greenhouse and Nursery Controlled Release Fertilizer (Plant Products Co., Brampton, ON, Canada), according to manufacturer's directions. Rootstocks were fertilized biweekly, twice with 3 g/l 10N-52P-10K Plant Starter Fertilizer and subsequently with 3 g/l 15N-15P-18K Soilless Feed Fertilizer. Rootstocks broke dormancy 1 month after transfer to the greenhouse. Shoots were then maintained following the procedure of Lane et al. (2003).

#### *Scions*

Lapins, Sweetheart, and Bing shoots with vigorous growth were collected after 2–3 months on M3B in polypropylene containers. Large leaves and petioles were removed and shoots were trimmed to 10–15 mm in length. Scions were transferred to Petri dishes with the cut end submerged in M3B medium, and were then grafted to rootstocks within 24 h.

#### *Grafting*

Grafting was performed according to the detailed protocol of Lane et al. (2003). Briefly, scions were cut into a wedge shape and inserted into a slit made in the rootstock. The graft was held together with a

small strip of Parafilm. At 2–3 weeks, the Parafilm wrapping was gently slit open on the opposite side from the graft, but was not removed from the stem. Grafting was repeated four times over a 3 month period (March–May 2005). Two to three cultivars were used in each trial with 8–13 shoots grafted/cultivar. The number of successful graft unions (rootstocks with actively growing scions) was counted 5 weeks after grafting.

### Statistical analyses

Experiments evaluating shoot formation on callus explants were analyzed using ANOVA. All other analyses used logistic regressions. All analyses began with a saturated model. A reduced model was then generated by sequentially removing non-significant interactions. Post-hoc tests were used to clarify statistically significant main effects. Tukey's HSD was used following ANOVAs. Post-hoc tests following logistic regressions involved rerunning the analyses using different subsets of the data. Unless otherwise stated, the culture vessel was treated as the unit of replication. Analyses were carried out using JMP statistical software (version 4.0.3, SAS Institute, 2000).

## Results

### Callus initiation on ex vitro explants

Callus developed at a low frequency on both apical shoot tip and axillary bud explants from all cultivars. Apical shoot tips and, to a much lesser extent, axillary buds began to flush within 1 month after culture initiation. Explants that did not respond by developing leafy shoots turned brown-black in color. No callus grew from actively growing explants, only from necrotic explants. Callus grew from discrete areas on the surface of parental explants and was observed to form both in contact with, and above, the medium. For all cultivars, a pale yellow, friable callus with or without small green globules appeared on explants. Within 1 month after the appearance of callus, adventitious shoots began to appear growing on the callus. Among trials, time to callus formation ranged from 2–8 months. The likelihood of callus formation on 0.5M3B did not differ between cultivars

but was significantly higher using apical shoot tips than using axillary buds (Table 1). Each trial resulted in a small number of explants forming callus for all cultivars. For one representative trial, 20.3% Lapins (12/59), 5.1% Bing (2/39), and 7.7% Sweetheart (3/39) apical shoot tip explants callused after 4 months on 0.5M3B. Callus formation increased slightly by 6 months (Lapins 22.0%, Bing 12.8%, Sweetheart 10.3%). A smaller number of axillary bud explants formed callus at 6 months; 2.1% Lapins (2/94), 0% Bing (0/31), 3.4% Sweetheart (2/59).

#### Callus initiation on in vitro explants

The appearance of explants changed over time on treatments; shoot base explants were composed of the base of the stem and its associated basal callus. This callus was firm, dense and became dark brown in color. It was easily distinguished from organogenic callus, which was a lighter color and had a friable texture (Fig. 2a). Many stem sections turned dark brown in color, and callus emerged from small, discrete locations (Fig. 2b). Remaining stem section explants were green, with callus forming over a large portion of the explant surface. Punctured shoot tip explants did not elongate and at 2 months, began to show signs of hyperhydricity, i.e., slightly swollen, lighter green, and translucent. Callus on punctured tips formed in the same manner as for ex vitro explants. In contrast, unwounded shoot tip explants continued to elongate and did not form callus (pers. obs). For all explant types, callus appeared as described for ex vitro explants.

Organogenic callus formed on nearly all cultivars, explant types, and treatments evaluated. Callus formation was first observed at 2 months on punctured tip and stem section explants. On punctured shoot tips, the frequency of callus formation for both cultivars was 33–67% after 3 months (Fig. 3a). Callus formation did not differ between cultivars but the treatment effect was marginally significant: explants on W3B were more likely to form callus than those on M3B or 0.5M3B (Table 1). Callusing on stem sections (20–31%) occurred at a much lower frequency than on punctured shoot tips at 3 months (Fig. 3b). Neither cultivar nor treatment affected the frequency of callus formation (Table 1). On shoot base explants, callus formation was first observed after 1 month on treatment media. To facilitate

comparison of callusing treatments on shoot base explants with other explant types, data from control shoot base explants not receiving a pre-treatment (M3B pre-treatment; Fig. 4a, b) are presented in Fig. 3c.

Organogenic callus formation on shoot base explants was affected by cultivar, treatment, and pre-treatment media (Fig. 4). Callus formation was consistently greater for Lapins explants than for Sweetheart (Table 1). For both cultivars, treatment with W3B encouraged significantly greater callus formation than treatment with 0.5M3B (Table 1). On average, callus formation using 0.5M3B was greater than that using M3B but the difference was not significant. The effect of pre-treatment differed significantly between trials (Table 1). In both trials, pre-treatment with W3B or M3BPG enhanced callus formation compared to M3B. In trial 2, explants pre-treated with W3B formed more callus than explants pre-treated with M3BPG. The same trend was evident in trial 1, but the difference, while suggestive, was not statistically significant. Given that the qualitative pattern was the same in both trials, data from both were pooled in Fig. 4.

Pre-treatments affected the appearance of Lapins and Sweetheart shoot cultures. For both cultivars, shoots on M3BPG produced many deep-green, shiny leaves with prominent venation and shoots readily multiplied. In contrast, M3B and W3B media did not support the same degree of enhanced growth, multiplication, and appearance of shoot cultures. Moreover, the appearance of shoot cultures on W3B worsened after the 3-month pre-treatment; most shoots showed signs of hyperhydricity, and longer time in culture resulted in severe symptoms of hyperhydricity and shoot death.

#### Influence of media on adventitious shoot formation

While all treatment media promoted callus growth, their effects on shoot development differed. Thus, a medium discouraging shoot formation was desirable for the callus propagation step (Fig. 1); whereas a medium encouraging shoot formation was necessary to induce adventitious shoot formation on callus at a later stage.

The number of callus explants forming shoots did not differ between cultivars but did differ between

**Table 1** Results of statistical analyses using logistic regression (LR), ordinal logistic regression (OLR), or analysis of variance (ANOVA)

Predictor	df	Test statistic	<i>p</i>
<i>Callus initiation on ex vitro explants (LR)</i>			
Cultivar × Tissue	2	$\chi^2 = 2.4$	0.31
Cultivar	2	$\chi^2 = 2.2$	0.33
Tissue	1	$\chi^2 = 21.5$	<0.0001
<i>Callus initiation on in vitro punctured shoot tip explants (OLR)</i>			
Cultivar × Treatment	2	$\chi^2 = 0.52$	0.77
Cultivar	1	$\chi^2 = 0.13$	0.72
Treatment	2	$\chi^2 = 6.17$	0.05
<i>Callus initiation on in vitro stem section explants (OLR)</i>			
Cultivar × Treatment	2	$\chi^2 = 0.002$	1
Cultivar	1	$\chi^2 = 1.8$	0.19
Treatment	2	$\chi^2 = 4.0$	0.13
<i>Effect of pre-treatments on callus formation on in vitro shoot base explants (OLR)<sup>a</sup></i>			
Trial × Pre-treatment	2	$\chi^2 = 9.5$	0.009
Cultivar	1	$\chi^2 = 28.4$	<0.0001
Treatment <sup>b</sup>	2	$\chi^2 = 9.3$	0.01
Trial 1: Pre-treatment <sup>c</sup>	2	$\chi^2 = 20.9$	<0.0001
Trial 2: Pre-treatment <sup>d</sup>	2	$\chi^2 = 29.6$	<0.0001
<i>Influence of media on number of callus explants forming shoots (LR)<sup>e</sup></i>			
Cultivar	2	$\chi^2 = 0$	1
Treatment <sup>f</sup>	2	$\chi^2 = 22.9$	<0.0001
<i>Influence of media on number of shoots forming per callus explant (ANOVA)</i>			
Cultivar × Treatment	4, 8	<i>F</i> = 0.8	0.53
Cultivar	2, 13	<i>F</i> = 0.8	0.49
Treatment <sup>g</sup>	2, 15	<i>F</i> = 49	<0.0001
<i>Adventitious shoot formation on callus explants (ANOVA)<sup>h</sup></i>			
Trial × Cultivar × Callusing medium	11, 24	<i>F</i> = 2.7	0.02
Trial 1: Cultivar × Callusing medium	2, 12	<i>F</i> = 1.6	0.23
Cultivar	2, 14	<i>F</i> = 0.4	0.7
Callusing medium	1, 16	<i>F</i> = 0.8	0.37
Trial 2: <sup>i</sup> Cultivar × Callusing medium	2, 12	<i>F</i> = 0.1	0.0008

<sup>a</sup> Other higher-order interactions were all non-significant ( $p > 0.15$ ) and have not been presented

<sup>b</sup> Posthoc tests: W3B > 0.5M3B (df = 1;  $\chi^2 = 4.1$ ;  $p = 0.04$ ); 0.5M3B = M3B (df = 1;  $\chi^2 = 1.5$ ;  $p = 0.25$ ); W3B > M3B (df = 1;  $\chi^2 = 7.7$ ;  $p = 0.006$ )

<sup>c</sup> Posthoc tests: W3B ≈ M3BPG (df = 1;  $\chi^2 = 3.6$ ;  $p = 0.06$ ); W3B > M3B (df = 1;  $\chi^2 = 27$ ;  $p < 0.0001$ ); M3BPG > M3B (df = 1;  $\chi^2 = 30$ ;  $p < 0.0001$ )

<sup>d</sup> Posthoc tests: W3B > M3BPG (df = 1;  $\chi^2 = 5.8$ ;  $p = 0.02$ ); W3B > M3B (df = 1;  $\chi^2 = 63$ ;  $p < 0.0001$ ); M3BPG > M3B (df = 1;  $\chi^2 = 30$ ;  $p < 0.0001$ )

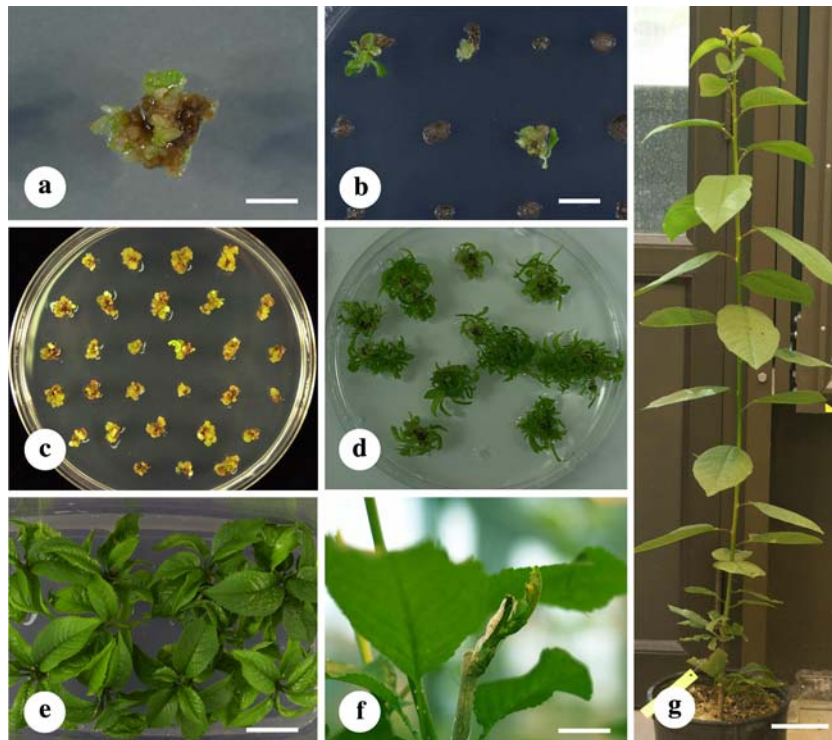
<sup>e</sup> The interaction between treatment and cultivar could not be assessed

<sup>f</sup> Posthoc tests: M3B < 0.5M3B (df = 1;  $\chi^2 = 16.6$ ;  $p < 0.0001$ ); M3B < W3B (df = 1;  $\chi^2 = 16.6$ ;  $p < 0.0001$ ); 0.5M3B = W3B (df = 1;  $\chi^2 = 0$ ;  $p = 1$ )

<sup>g</sup> Posthoc tests (Tukey HSD,  $p < 0.05$ ): W3B > 0.5M3B; 0.5M3B > M3B; W3B > M3B

<sup>h</sup> Trial × Cultivar × Callusing medium interactions were statistically significant, so results from each trial were analyzed separately

<sup>i</sup> The whole model test was statistically significant (df = 5, 12; *F* = 5.8;  $p = 0.006$ ). The sample size was not large enough to analyze results for each cultivar separately



**Fig. 2** Callus formation, shoot regeneration, and grafting in sweet cherry. (a) Callus development on a shoot base explant after 2 months on WPM with 3  $\mu\text{M}$  BA. Bar: 3.5 mm. (b) Callus and adventitious shoot formation on stem section explants after 2 months on WPM with 3  $\mu\text{M}$  BA. Bar: 5 mm. (c) Callus growth on MS with 3  $\mu\text{M}$  BA, 3 weeks after subculture. (d) Shoot development on callus explants after

4 weeks on WPM with 3  $\mu\text{M}$  BA. Photos (c–d) are of 10 cm-diameter dishes. (e) Regenerated shoots of cultivar Lapins after 3 months on MS with 3  $\mu\text{M}$  BA elongation medium. Bar: 2 cm. (f) Lapins scion pushing Parafilm wrapping aside 6 weeks after grafting onto Mazzard (*P. avium*) rootstock in greenhouse. Bar: 2 cm. (g) Growth of Sweetheart scion on Mazzard rootstock 3 months after grafting. Bar: 10 cm

treatments (Table 1). For each cultivar, shoot formation on M3B was much lower than on either 0.5M3B or W3B media (Fig. 5a). The number of callus explants forming shoots was consistently high ( $\geq 8$  explants forming shoots) or low ( $\leq 4$  explants forming shoots); intermediate values were not observed. Differences in shoot formation between treatments and cultivars were therefore assessed using a logistic regression with “high” and “low” as a binary response (Table 1).

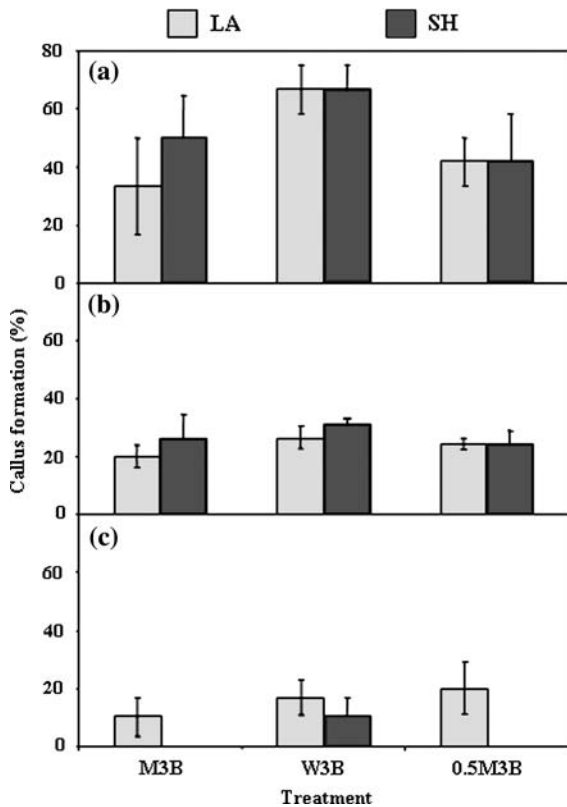
Likewise, the number of shoots forming on each callus explant did not differ between cultivars but did differ between treatments (Table 1). Callus growing on W3B produced significantly more shoots (7–12 shoots/explant) than callus on 0.5M3B (4–5 shoots/explant), which, in turn, produced more shoots than callus on M3B (2 shoots/explant) (Fig. 5b). Owing to these differences in shoot formation, callus growth

experiments were pursued using M3B and 0.5M3B media, while shoot formation experiments were carried out using W3B medium.

#### Callus growth

Many callus explants more than doubled in mass over a 1 month period on both M3B and 0.5M3B media (Fig. 2c). Callus fresh weight increased from 82–115% for Sweetheart, 91–133% for Lapins, and from 118–144% for Bing. Analyses of growth differences between cultivars and treatments have not been presented. Those analyses indicated that growth differences between cultivars and treatments were sensitive to the initial sizes of the callus pieces used, i.e., statistical interactions between initial callus size and the effects of interest (cultivar and treatment) were significant or suggestive. While such patterns



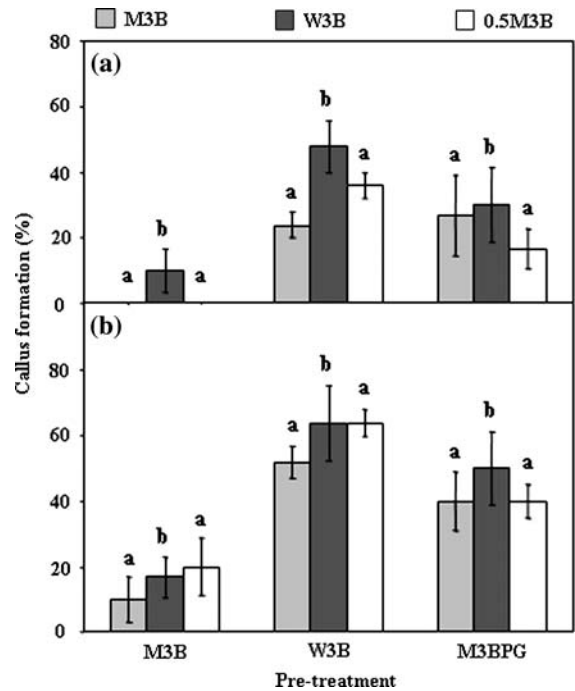


**Fig. 3** Callus formation on explants from in vitro shoot cultures of cultivars Lapins (LA) and Sweetheart (SH) after 3 months on treatment media. **(a)** Punctured shoot tips. Data are the means of three replicate dishes, each containing four explants. **(b)** Stem sections. Data are the means of three replicate dishes, each containing 15 explants. **(c)** Shoot bases. Data are the means of two trials, both with three replicate dishes, each containing five explants. Treatment media consist of 3  $\mu$ M BA supplemented to WPM (W3B), MS (M3B), or half-strength MS (0.5M3B). Vertical bars: standard error

may prove to be important as this protocol is refined, the qualitative result is of more immediate importance: callus from all three cultivars grew appreciably in both trials and using either treatment medium.

#### Adventitious shoot formation

Shoot regeneration on callus explants was almost 100% after 2 weeks on W3B medium. By 4 weeks, all explants had formed shoots in both trials, with 7–17 shoots forming per callus explant (Fig. 2d). The effects of callusing medium and cultivar on the number of shoots forming per explant differed significantly between trials (Table 1). In the first

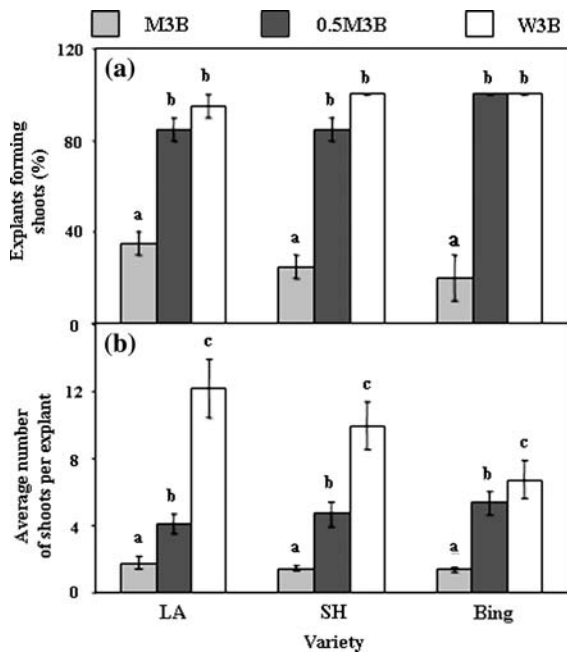


**Fig. 4** Callus formation on shoot base explants. Shoot cultures were pre-treated for 2 months, shoot base explants were isolated, and transferred to treatments. Callus formation was recorded after 3 months on treatment media. Pre-treatments are on the X-axis. Treatments are distinguished by bar shading. **(a)** Sweetheart. **(b)** Lapins. Results of two trials have been pooled for presentation. Each trial consists of 2–3 replicate dishes, each dish containing 5 explants. Means showing the same letter are not significantly different ( $p > 0.05$ ). Media consist of 3  $\mu$ M BA supplemented to WPM (W3B), MS (M3B), or half-strength MS (0.5M3B), or M3B with 1 mM phloroglucinol (M3BPG). Vertical bars: standard error

trial, regeneration was independent of both cultivar and callusing medium (Table 1). In the second trial, the whole model (i.e., including effects of both callusing medium and cultivar) was significant. However, the separate effects of each predictor could not be assessed (Table 1). Qualitatively, shoot formation on W3B medium was similar for all three cultivars and was similar for explants that had previously been maintained on either M3B or 0.5M3B callusing media.

#### Shoot survival

Shoot survival on M3B elongation medium varied among cultivars. At 3 months, the number of surviving shoots increased for Lapins (226/159;



**Fig. 5** Shoot development on callus of cultivars Lapins (LA), Sweetheart (SH), and Bing after 4 weeks on treatment media. (a) Percent shoot formation. (b) Number of shoots forming on each shoot-producing explant. Data are the means of 2 replicate dishes, each containing 10 explants. Means showing the same letter are not significantly different ( $p > 0.05$ ). Treatment media consist of 3  $\mu$ M BA supplemented to WPM (W3B), MS (M3B), or half-strength MS (0.5M3B). Vertical bars: standard error

**Table 2** Ex vitro grafting of regenerated shoots onto Mazzard (*P. avium*) rootstock

Scion cultivar	Trial number <sup>a</sup>	Number of graft unions/grafted scions <sup>b</sup>	Survival (%)
Sweetheart	1	8/13	61.5
	2	2/10	20
	3	5/8	62.5
	4	7/10	70
Lapins	1	8/13	61.5
	3	6/10	60
	4	5/10	50
Bing	2	1/8	12.5
	4	3/10	30

<sup>a</sup> Trials were repeated over a 3 month period

<sup>b</sup> Graft unions are defined as rootstocks with actively growing scions. The number of graft unions was recorded 5 weeks after grafting

142%) and Sweetheart (186/89; 209%) but decreased for Bing (31/94; 33%). For cultivars Lapins and Sweetheart, a small number of shoots died at each subculture. However, the surviving shoots multiplied from axillary buds, increasing the total number of shoots over time. Conversely, Bing experienced greater shoot losses and shoot multiplication was not as frequent, resulting in a decrease in the total number of shoots at each subculture. At 3 months, regenerated shoots of all cultivars were morphologically similar to established shoot cultures from field material (Fig. 2e).

### Grafting

Regenerated shoots from all cultivars were successfully grafted onto rootstock in the greenhouse (Table 2). Growing scions began to bulge under their Parafilm covering 2–3 weeks after grafting. Within 1 week after cutting the Parafilm, scions grew large enough to push aside the wrapping (Fig. 2f). Scions that did not flush within 4–5 weeks after grafting appeared desiccated and necrotic under the Parafilm covering. When results of the four trials were pooled, the grafting success of Bing (21%) was lower than that of Sweetheart (54%) or Lapins (57%). It was not possible to determine whether this variation in grafting success was due to differences between cultivars or to differences between trials because two of the three cultivars were not represented in all trials. Grafted trees were grown in the greenhouse for 6 months (Fig. 2g).

### Discussion

This study describes a new procedure for shoot regeneration in sweet cherry. Callus formation was demonstrated on explants taken from orchard trees and in vitro shoot cultures. Conditions encouraging callus proliferation and recovery of large numbers of adventitious shoots were identified. Regenerated shoots were grafted onto rootstock and grown in the greenhouse. An outline of the method is presented in Fig. 1.

The diversity of tissue sources that can be used successfully with this approach results in a more flexible regeneration method for sweet cherry. Organogenic callus development directly from apical

shoot tips taken from orchard trees avoids the lengthy time and resources required for initiation, establishment, and maintenance of large stocks of shoot cultures for providing tissues. Explants can be taken directly from elite trees during the growing season and the resulting callus can be induced to regenerate shoots or maintained in culture for longer periods of time. Alternatively, obtaining explants from in vitro shoot cultures can be advantageous because tissues are available year-round, shoots are maintained in a controlled environment, and use of in vitro material avoids losses due to contamination and higher phenolic levels from field material (McCown 2000). For the latter reasons, in vitro explants were used in the majority of experiments in this study.

Callus and adventitious shoot formation were first observed on necrotic explants isolated from orchard trees. Based on these observations, it was speculated that the process of isolating and sterilizing buds caused wounding, which stimulated callus growth and adventitious shoot regeneration (Bhatia et al. 2005). The results were then reproduced using in vitro shoot tips; organogenic callus formation occurred on punctured shoot tips and not on unwounded tips. Induction of organogenic callus on stem section and shoot base explants may also have been a consequence of wounding meristematic regions that were present within these tissues while isolating explants. The specific origin of organogenic callus formation was not investigated further in this study, as the objective was to develop a procedure to obtain adventitious shoots.

The type of explant influenced the rate and quantity of callus production. The availability of tissues differed among explant types, so direct comparisons of callus formation among explant types were not possible. However, trends in the frequency of callus formation were evaluated qualitatively. When treated with 0.5M3B, ex vitro explants did not form as much callus as did in vitro explants. Callus also developed more quickly on in vitro explants. One interpretation of this difference is that decontamination of field-grown tissues and transfer to in vitro culture imposes a stress that may be disruptive to explanted tissues, and subsequently reduces or slows callus formation compared to in vitro-derived explants. Teng et al. (2002) demonstrated this effect with ginseng species. Among ex vitro explant sources, apical shoot tips tended to form

more callus than did axillary buds, which suggests that callusing may also be influenced by the physiology of the donor trees (Benson 2000). Finally, among in vitro-derived explants, the frequency of callus formation declined basipetally: on all treatments, callus formation was higher for shoot tip explants than for stem sections and, without pre-treatments, was further reduced when shoot bases were used. This pattern suggests the presence of a physiological gradient along the shoot affecting callus formation. Cuenca et al. (2000) described similar differences in regeneration frequency with the position of explants along the stem of beech (*Fagus sylvatica*) shoots.

The choice of basal medium influenced callus formation. Callus development on punctured shoot tip and shoot base explants was more likely to occur on W3B, while stem section explants were not affected by treatments. Overall, these results suggest that W3B is the most suitable treatment to encourage callus formation among in vitro-derived explants. However, callus growth did occur in the presence of both low and high macronutrient-containing basal media (WPM, half-strength MS, and MS, respectively) and on all explant types, indicating that all treatments can promote callus formation. Oh et al. (1991) also evaluated the effect of MS and half-strength MS basal media on organogenic callus formation on shoot tip explants and concluded that the lower-salt medium was best for callusing.

Pre-treatment of shoot cultures significantly improved the number of shoot base explants developing organogenic callus. However, pre-treatments also produced visible differences in overall shoot culture morphology and growth. Shoot cultures pre-treated with W3B became hyperhydric. After the experiments had concluded, these shoots began to exhibit a considerable reduction in quality and substantial shoot loss. Conversely, high-quality shoot cultures could be continuously maintained and multiplied on M3BPG, and provided a constant source of explants for callus initiation. For these reasons, M3BPG is the preferred pre-treatment medium. Following pre-treatment of shoot cultures with M3BPG, callus formation on shoot base explants treated with W3B was three-fold greater than callusing on W3B without pre-treatment (Fig. 4). Similarly in peach (*Prunus persica*), pre-conditioning shoot apices improved callus formation on leaf explants (Gentile et al.

2002). The addition of phloroglucinol to pre-treatment medium (M3BPG) enhanced shoot growth and appearance as well as axillary shoot proliferation for all cherry cultivars. Phloroglucinol is a phenolic compound used to stimulate shoot and root growth in shoot cultures (Sarkar and Naik 2000). It has been incorporated into shoot culture media of many *Prunus* species, including sweet cherry (Takashina et al. 2003), wild cherry (Hammatt and Grant 1997), and almond (Ainsley et al. 2001).

In choosing a medium to promote callus growth, three criteria needed to be met to facilitate the maintenance and multiplication of cultures; reduced shoot formation, prolific callus growth, and the ability to regenerate shoots when removed from the callusing medium. During maintenance of callus cultures, reduced shoot formation greatly facilitates monthly subculture. Shoot development on callus was affected by the choice of basal medium; therefore, subsequent treatments to promote callus growth were limited to those encouraging the least shoot formation, i.e., M3B and 0.5M3B.

As only a small amount of callus is initially produced by each explant, identifying a treatment that encourages prolific callus growth is important, because it minimizes the time in culture needed to generate enough tissue for subsequent experiments. Callus growth was qualitatively similar between M3B and 0.5M3B treatments. In many cases, callus more than doubled in mass over the subculture period; for the three cultivars tested, callus growth increased 91–144% after 1 month on M3B medium. Matsuta and Yamaki (1988) achieved even greater callus growth on leaf disks after 35 days on MS supplemented with BA and 2,4-D or NAA; there was an increase of more than 50 times the initial mass using sweet cherry cultivars Compact Stella, NY 1193, and Satonishiki.

Finally, shoot formation on W3B medium was qualitatively similar whether callus was maintained on M3B or 0.5M3B, suggesting that the choice of callus maintenance medium did not affect shoot formation. Callus has been maintained on both media for almost 1.5 years without any observed decline in callus growth or loss in regeneration potential. Despite the suitability of both media for callus growth and maintenance, M3B is preferred because it more closely adheres to the outlined criteria; callus formed less shoots on M3B than 0.5M3B, which is an

advantage during subculture of large numbers of callus cultures.

In this study, 100% of callus explants formed shoots on W3B at 4 weeks. In contrast, Oh et al. (1991) achieved a much lower frequency of shoot formation on callus explants after 4 weeks on half-strength MS supplemented with either 4.44  $\mu\text{M}$  BA (25%) or 0.44  $\mu\text{M}$  BA (30%). The differences in shoot formation could be due to the choice of basal medium. Woody Plant Medium, a low macronutrient-containing medium, has been previously used to encourage shoot regeneration in leaf explants of sweet cherry (Bhagwat and Lane 2004; Takashina et al. 2003; Tang et al. 2002). Matt and Jehle (2005) describe additional combinations of low macronutrient-containing basal media for shoot regeneration in leaf and internode sections.

Regenerated shoots were elongated on M3B medium. Shoots of Lapins and Sweetheart cultivars grew well on this medium with a high frequency of shoot survival and adventitious multiplication. In contrast, Bing shoots grew poorly. This cultivar effect was also observed in shoot cultures initiated from orchard trees (pers. obs.). In many cases, optimum culture conditions are genotype-dependant for *Prunus* species (Pérez-Tornero and Burgos 2000). These results emphasize the importance of identifying suitable culture conditions for shoot cultures of each cultivar.

Valuable sweet cherry cultivars are grafted onto rootstock for planting in commercial orchards. For this reason, regenerated shoots were grafted rather than rooted. Advantages of grafting shoots are discussed in more detail by Lane et al. (2003). Once grafted, scion growth occurs after the union of cambium tissue between the scion and rootstock, or graft union, is established (Raharjo and Litz 2005). Acclimatization of the scion to greenhouse conditions is achieved as it emerges from the Parafilm covering (Lane et al. 2003). Bhagwat and Lane (2004) achieved a high frequency of rooted shoots (85%) of the cultivar Lapins, but plantlet survival decreased during acclimatization to greenhouse conditions (20%). In this study, a higher percentage of Lapins shoots survived grafting (50–61.5%), possibly due to fewer losses during acclimatization to in vivo conditions. These results suggest that grafting is a useful means of generating trees from regenerated cherry

shoots. Ultimately, trees produced by this shoot regeneration method should be established in the field and tree performance evaluated and compared to commercially propagated trees under field conditions.

Results of this study demonstrate high-frequency shoot regeneration for commercially important cultivars. The method is flexible, allowing incorporation of many explant types derived from in vitro shoots (shoot tips, stem sections, and shoot bases) as well as apical shoot tips taken from orchard trees. Callus has been maintained and multiplied in culture for up to 1.5 years with no apparent loss of regeneration potential. Callus generates large numbers of adventitious shoots that can be grafted onto rootstock in the greenhouse, producing field-ready trees. This protocol should prove useful in future efforts to develop transgenic sweet cherry cultivars expressing desirable traits.

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