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Plant Regeneration of Chokecherry (*Prunus virginiana* L.) from *in vitro* Leaf Tissues[1]

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Abstract

An effective plant regeneration system was developed for chokecherry (*Prunus virginiana* L.) by using *in vitro* leaf tissues. Adventitious shoots regenerated from *in vitro* leaf tissues only when cultured on Woody Plant Medium (WPM), but not on Murashige and Skoog medium, supplemented with benzyladenine (BA) or thidiazuron (TDZ). Three chokecherry clones (NN, 10, and 17) responded differently to types and concentrations of cytokinins, ranging from 16.7 to 91.7% leaf explants regenerating shoots. A mean of four shoots was produced from each explant, with the most shoots (> 10) from clone NN on media with 5–10 µM BA. Higher concentrations of TDZ (> 8 µM) caused serious vitrification and eventual death of newly induced shoots. Regenerated shoots (> 1.5 cm) produced roots *in vitro* in half strength MS medium or *ex vitro* in Cellular Rooting Sponge (CRS) rooting plugs with or without auxin (NAA or IBA) treatments. Rooting was affected by auxin, genotypes, and the rooting methods.

Index words: *Prunus* species, X-disease, phytohormone, organogenesis.

Species used in this study: *Prunus virginiana* L.

Significance to the Nursery Industry

Plantings of chokecherry (*Prunus virginiana* L.), one of the most common native small tree or large shrub species for resource conservation and wildlife habitat in North America, have quickly declined because of X-disease. X-disease, caused by X-disease phytoplasmas, a group of cell-wall-less prokaryotes (formerly known as mycoplasmalike organism), is one of the most serious diseases of stone fruit species. So far, there are no methods available to effectively control this disease. Therefore, use of disease resistant cultivars is the first acceptable management method for X-disease. It is very difficult to breed X-disease resistant chokecherry cultivars conventionally due to hybridization incompatibility in the genus *Prunus* containing some resistant species, such as apricot and plum. Gene transfer makes it possible to directly transfer genes to the plant for enhancing disease resistance. An efficient regeneration system is a prerequisite for transferring foreign genes into plants. The regeneration system developed in this study will be used for gene transformation of chokecherry to develop X-disease resistant cultivars.

Introduction

Chokecherry (*Prunus virginiana* L.) is one of the most popular native small tree or large shrub species for resource conservation and wildlife habitat in North America due to its tolerance to harsh winters and alkaline soils (4, 8). Chokecherry has also been widely used for home processing of jellies, juices, sauces, and wine (4). Recently there has been increasing interest in chokecherry fruit production in the northern Great Plains because of its high antioxidant activity and relatively large fruit. Unfortunately, chokecherry is subject to severe damage from X-disease, one of the most serious diseases of stone fruit species (*Prunus spp.*) in North America (6). For example, in a planting at the USDA Plant Materials Center, more than 75% of the trees died within 15 years and remaining chokecherry trees are infected, primarily from X-disease. Such serious destruction hinders utilization of this unique species in this region.

Due to hybridization incompatibility in the genus *Prunus*, it is very difficult to transfer disease resistance from X-disease resistant species, such as apricot (*P. armeniaca* L.) and plum (*P. domestica* L.), to susceptible species, such as chokecherry, using traditional breeding methods. Genetic engineering techniques offer plant breeders a powerful tool to circumvent the restrictions of conventional plant breeding by directly introducing genes to existing cultivars without altering the existing genetic traits.

Successful genetic engineering depends on an efficient regeneration system. Regeneration protocols have been described for many *Prunus* species (1, 5, 9, 17), but only a few reports on chokecherry micropropagation are available (15, 18), and no research has been reported on chokecherry regeneration. Therefore, the objective of this research was to develop an efficient system to regenerate plants from vegetative plant tissues, which can be used for genetic improvement of chokecherry with other biotechnological techniques.

Materials and Methods

In vitro cultures were initiated by Zhang, et al. (18) using shoot tips from seed-propagated chokecherry plants grown at the USDA Plant Materials Center in Bismarck, ND. Shoots were subcultured in MS (12) medium supplemented with 2.5 µM benzyladenine (BA), 3% sucrose and 0.65% agar (Difco Co., Detroit, MI, #0140-01-0) every 4 weeks at 25C under cool-white light at approximately 36.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16-hr photoperiod. All other experiments were performed under these conditions unless otherwise noted.

Three *in vitro* clones (NN, 10, and 17), two media [MS and WPM (11)], and two cytokinins [BA and thidiazuron

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(TDZ)] at five concentrations (BA: 0, 5, 10, 20, and 40 µM; TDZ: 0, 2, 4, 8, and 16 µM) were evaluated for their effects on regeneration. Leaves of 4-week-old *in vitro* shoots were cut into three segments across the main vein. Six leaf segments were placed in each Petri plate (100 mm × 15 mm) containing 25 ml medium and cultured under conditions as described above. The experiment was conducted as a completely randomized design (CRD) consisting of two replications of a 3 × 2 × 5 factorial arrangement of genotype, hormone type, and hormone concentration, respectively. The data on percentage of leaf explants forming shoots and on the number of shoots per explant were collected after 4 weeks, and the data were subjected to analysis of Duncan's Multiple Range Test (DMRT) using the SAS program (16). Newly regenerated shoots were transferred to MS medium with 2.5 µM BA for proliferation.

Two rooting experiments (*in vitro* and *ex vitro*) were conducted to examine the rooting response of microcuttings to auxins and rooting media. In *in vitro* rooting experiment, microcuttings (> 1.5 cm) were pulsed in half strength MS basal medium containing 3% sucrose and 0.65% agar with five different auxin treatments (no auxin, 10 µM IBA (indolebutyric acid), 10 µM NAA (naphthalene acetic acid), 10 µM IBA + 5 µM NAA, or 5 µM IBA + 10 µM NAA). After pulsed for 2 weeks, microcuttings were transferred to the same medium without auxins for root formation. Rooting percentage and root number were recorded after 2 weeks in auxin-free medium. Each treatment had 15 microcuttings (10 microcuttings for clone NN) and replicated twice. Rooted plants were then transferred to flats filled with Jiffy Mix (Jiffy Mix. Shippagan, Canada) and covered with clear plastic tops for one week. The covers were gradually removed during the following one-week period. Surviving plants were potted into Sunshine Mix #1 (Fisons Western Corp., Vancouver, Canada) and grown in the greenhouse.

In *ex vitro* rooting experiment, the basal portion (approximately 3–5 mm) of microcuttings (> 1.5 cm) was quick-dipped (10 seconds) in H_2O, 10 or 100 µM IBA or NAA solution, and then inserted into Cellular Rooting Sponge (CRS, Grow-Tech Inc., Boothbay, ME) in plastic flats covered with clear plastic tops. Each treatment had at least 50 microshoots and was replicated twice. After four weeks, the covers were gradually removed during a one-week period. Rooting rate was recorded, and the rooted plants were replanted into Sunshine Mix #1 and grown in the greenhouse.

Results and Discussion

Effect of cytokinin and genotype on shoot regeneration. Significant effects of basal media on chokecherry regeneration were found. Adventitious shoots (Fig. 3A) regenerated from leaf explants only when cultured on WPM medium, but not on MS medium in the initial 4 weeks. New shoots were continually produced when leaf explants were subcultured on the same medium during the second 4-week period. BA and TDZ were effective for shoot regeneration from *in vitro* leaf tissues of chokecherry. Shoot regeneration was affected by the genotype and concentrations of BA (Fig. 1) or TDZ (Fig. 2). Overall, clones 17 and NN showed higher regeneration capabilities than clone 10 with several occasional exceptions. The highest regeneration rate for clone NN (91.7%) was obtained with 8 µM TDZ, and more than 80% of leaf explants produced shoots either with 4 µM TDZ or with 5 µM BA. For clone 17, 75% and 83.4% of leaf explants formed shoots on media with 8 µM TDZ and 10 µM BA, respectively.

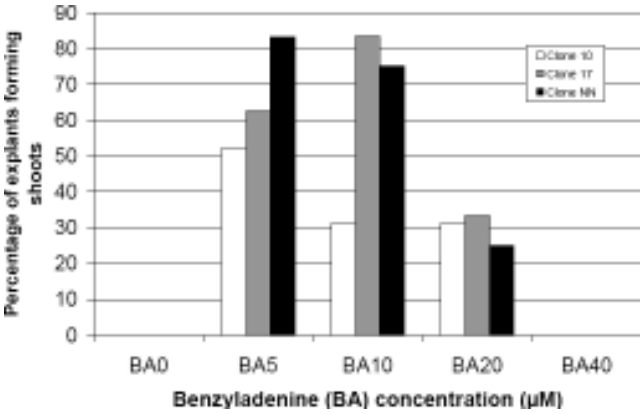

Fig. 1. Effect of genotype and BA on regeneration frequency of chokecherry from *in vitro* leaves. Different letters above the bars indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$).

No significant difference was found in shoot number produced from each clone (data not shown). Shoots regenerated from leaf segments of all tested clones on media with TDZ were smaller and tortile with light green, vitrified leaves. Newly-induced shoots were seriously vitrified and eventually died on medium with higher concentrations of TDZ (> 8 µM). Higher quality shoots were regenerated from media with BA, and no vitrification was found.

Although *in vitro* shoots of these chokecherry clones performed well on MS medium (18), no shoots were regenerated on the MS basal medium with two cytokinins for all three genotypes tested. High ammonium and other salts in the MS medium may inhibit shoot initiation. Some research found that a lower ammonium medium performed better than high ammonium media for *Prunus* tissue culture (14). Thus, determination of medium type should always be one of the first important steps in establishing a regeneration system for different genotypes.

TDZ has proven to be a powerful plant growth regulator to stimulate shoot regeneration for many species, especially for many recalcitrant woody species (13). However, TDZ generally causes shoot vitrification. Low concentrations of TDZ combined with other cytokinins may overcome the prob-

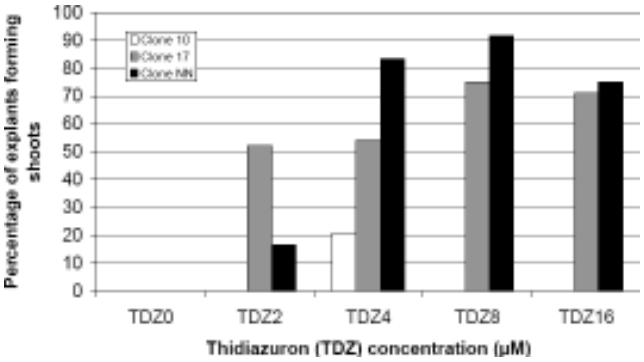

Fig. 2. Effect of genotype and TDZ on regeneration frequency of chokecherry from *in vitro* leaves. Different letters above the bars indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$).

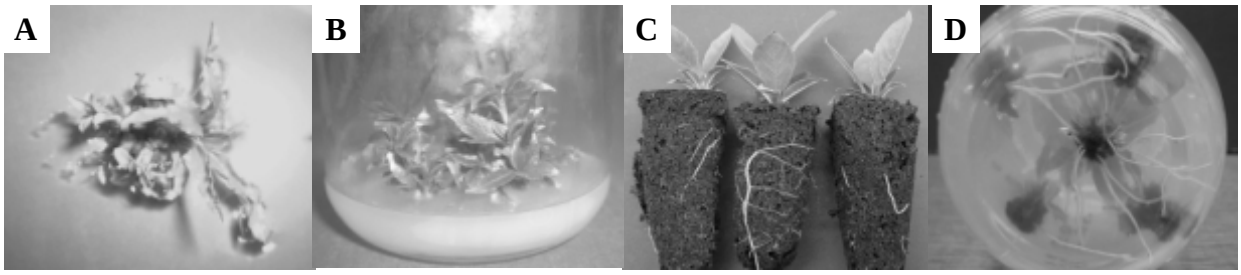

Fig. 3. Plant regeneration of chokecherry (*Prunus virginiana* L.). (A) *In vitro* shoot regenerated from leaf segment on WPM medium containing 10 µM BA, (B) Newly regenerated shoots were proliferated on MS medium with 2.5 µM BA, (C) Roots were induced *ex vitro* in the Cellular Rooting Sponge, and (D) Roots developed *in vitro* in the rooting medium.

lem and produce healthy shoots at a high regeneration frequency.

Rooting and acclimatization. Rooting ability of chokecherry microcuttings was genotype dependent (Table 1). Clone NN is easier to root than the other two clones *in vitro*. Application of auxins induced *in vitro* rooting with an average of 73.8, 42.6, and 24.2% of microcuttings of clones NN, 17, and 10 produced roots, respectively. Pulsing with the mix of IBA and NAA, either high or low ratio of IBA/NAA, did not significantly increase *in vitro* rooting ability, which is not consistent with other reports (15). Differences in rooting rate among genotypes were also found in the *ex vitro* rooting experiment. Overall 49.6, 25.1, and 31.1% of microcuttings of clones 10, 17, and NN produced roots in CRS, respectively, in contrast to where clone 10 was relatively difficult to root *in vitro*. Unlike *in vitro* rooting, treatment with IBA or NAA did not significantly increase the rooting percentage. Results also showed that clone NN exhibited more consistent response to auxins in rooting in *in vitro* conditions. *Ex vitro* rooted plants had a stronger root system and more vigorous growth (Fig. 3C), and consequently had a higher survival rate than *in vitro* rooted plants (data not shown).

Table 1. Effects of IBA and NAA on *in vitro* and *ex vitro* rooting of chokecherry[z].

Auxin treatment (µM)	Clone 10	Clone 17	Clone NN
	***In vitro* rooting percentage**		
Control (H_2O)	0.0f	0.0f	25.0def
IBA 10	36.7cde	33.4cdef	90.0a
NAA 10	26.7def	63.4abc	80.0ab
IBA 10 + NAA 5	3.4ef	26.7def	60.0abcd
IBA 5 + NAA 10	30.0cdef	46.7bcd	65.0abc
Average[y]	24.2	42.6	73.8
	***In vitro* root number/microcutting**		
Control (H_2O)	0.0f	0.0f	1.0efg
IBA 10	2.4abc	2.1abcd	3.0a
NAA 10	1.4cdef	1.9bcde	2.9a
IBA 10 + NAA 5	1.0efg	1.3def	2.4ab
IBA 5 + NAA 10	1.6bcde	1.9bcde	2.5ab
Average[y]	1.60	1.80	2.95
	***Ex vitro* rooting percentage**		
Control (H_2O)	50.0a	30.0a	40.0a
IBA 10	55.0a	46.7a	13.3b
IBA 100	60.0a	22.3a	37.0a
NAA 10	40.0a	11.0a	43.0a
NAA 100	43.0a	15.7a	22.3a
Average[x]	49.6	25.1	31.1

[z]Table 1 includes 3 categories: *in vitro* rooting percentage, *in vitro* root number/microcutting, and *ex vitro* rooting percentage. Different letters in the same category indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$).

[y]Average excluding control (H_2O) treatment.

[x]Average including control (H_2O) treatment.

This study demonstrated the feasibility of organogenesis from leaf tissues of chokecherry. The difference in regeneration frequency and rooting ability of three genotypes indicated that regeneration capability in chokecherry is highly genotype dependent, which had been well documented in many other species (2, 10).

Genotype, developmental stage of cuttings, and environmental conditions all influence the formation of adventitious roots from cuttings (3). In this research, clone 10 which rooted poorly *in vitro* displayed a higher rooting response *ex vitro* in CRS even without auxin treatment. This indicates that rooting ability can be affected by environmental factors, such as salts in medium, air, light, etc. CRS is capable of holding moisture and air and preventing light, which are ideal for root induction and development (7). The direct *ex vitro* rooting method should be more practical and economical than the *in vitro* method because the *ex vitro* rooted plants can be transferred to ambient conditions without any additional acclimatization.

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