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A Shoot-Tip Culture Micropropagation System for Chokecherry[1]

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Abstract

A micropropagation system was developed to clonally propagate juvenile and mature chokecherry plants. Establishment of shoot tips from seedling plants was best on Murashige and Skoog (MS) medium with 5 µM benzyladenine (BA) and 5 µM indolebutyric acid (IBA). Significantly more shoots proliferated on the MS or Driver and Kuniyuki Walnut (DKW) medium than on Woody Plant medium. Rooting of microshoots could be induced on medium with either IBA or naphthaleneacetic acid (NAA). The highest rooting percentage and the greatest number of roots occurred when shoots were placed in a medium with 0.5 or 2.5 µM IBA. More than 20 chokecherry plants shown to have a high level of field tolerance to X-disease were micropropagated for further X-disease resistance testing using the technique described here.

Index words: micropropagation, chokecherry, cytokinin, in vitro, stone fruit, X-disease.

Significance to the Nursery Industry

X-disease, caused by X-disease phytoplasmas, is one of the most serious diseases in stone fruits in North America. It has been difficult to breed for X-disease resistant cultivars in susceptible species, such as peaches and cherries, because a species of stone fruit is either relatively resistant/tolerant or susceptible, and *Prunus* species are incompatible in hybridization. Genetic variation in X-disease resistance was recently found in chokecherry (*Prunus virginiana* L.). The micropropagation system developed in this study was used to clonally produce chokecherry plants shown to be putatively resistant to X-disease in earlier research. These clonal chokecherry trees will greatly facilitate confirmation of the putative X-disease resistance, and will be useful in studying host/phytoplasma interactions and in the eventual development of X-disease resistant cultivars in susceptible stone fruit species.

Introduction

X-disease, caused by X-disease phytoplasmas (formerly known as mycoplasmalike organisms), is one of the most serious diseases of stone fruit species (*Prunus* spp.) in North America. The disease is particularly devastating to peach (*P. persica* (L.) Batsch), sweet (*P. avium* (L.) L.) and sour (*P. cerasus* L.) cherries, nectarines (*P. persica* (L.) Batsch var. *nucipersica* (Suckow) K. C. Schneid.), and chokecherry (*P. virginiana* L.) (4, 10, 11, 12, 16). In *Prunus*, a species is generally either resistant, such as apricot (*P. armeniaca* L.) and plum (*P. domestica* L.), or susceptible, such as cherries, peach, and chokecherry. Since stone fruit species are incompatible in sexual hybridizations, it has been very difficult to transfer the resistance from resistant to susceptible species. The lack of genetic variation in X-disease resistance within the individual *Prunus* species has greatly hampered the understanding of host response to this devastating disease.

In 1983, the Bismarck Plant Materials Center of the U.S. Department of Agriculture established a chokecherry planting near Bismarck, ND, which consisted of over 3000 plants from 179 accessions from ND, SD, and MN. The major goal was to select X-disease resistant chokecherry seed sources and use them for wildlife, soil conservation, and urban landscape planting in the northern Great Plains, and for commercial and household fruit production to make jam, jelly, and wine. During the last 17 years, the planting has been heavily infested by X-disease. More than 70% of the trees are now dead, largely due to X-disease (17). In characterizing this germplasm collection, more than 30 trees have shown few or no X-disease symptoms (17), but they have all been confirmed to be infected by X-disease phytoplasmas, indicating these plants may be resistant or highly tolerant to X-disease (3). This variation of resistance/tolerance to X-disease within a single *Prunus* species, if confirmed, will be the first of its kind and will be very useful to studying the inheritance and the interactions of the host and the X-disease phytoplasma. However, confirming this putative resistance has been hampered by unavailability of genetically homogeneous chokecherry plants and by a lack of known pathogenic isolates of X-disease phytoplasmas.

To produce homogeneous plants of these putatively resistant/tolerant chokecherry plants requires vegetative propagation. Rooting of cuttings is not suitable in this case because stone fruit plants are difficult to root. Grafting is not suitable either because the rootstock will interfere with scions in response to X-disease phytoplasmas. Micropropagation has been reported for a number of other *Prunus* species, but not for chokecherry. Hammerschlag (5) established 11 cultivars of peach *in vitro* and found that sterilizing shoots forced to break out of lateral buds significantly reduced contamination compared to sterilizing the buds themselves. Proliferation of peach was the greatest in a liquid MS medium with benzyladenine (BA) and indolebutyric acid (IBA) (5). The system was later extended to rooting and acclimation (6). The maximum rooting of microcuttings occurred when shoots were placed on ½-strength MS medium, stored in the dark at

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26C (79F) for 35–40 days, and then incubated on rooting medium in the dark at 26C (79F) for 14 days. Zimmerman and Scorza (18) showed BA was more effective than thidiazuron (TDZ) in promoting peach shoot proliferation. Snir (14, 15) propagated sweet and sour cherry cultivars, with best bud initiation on Knop's medium and optimal proliferation on MS medium. Morini et al. (8) examined photoperiod effect on *in vitro* growth of a plum rootstock. The 16- and 12-hr (light) photoperiod gave better growth than the 8-hr photoperiod. Cycles of 4-hr light/2-hr dark were found to promote *in vitro* growth and proliferation of plum (8) and peach (18). Therefore, the objective of this research was to develop a micropropagation system and use it to propagate genetically homogeneous chokecherry plants which have shown putative X-disease resistance/tolerance. Clonal materials eliminate genetic variation of host plants and reduce the number of plants to be inoculated in future research of host/pathogen interactions.

Materials and Methods

Chokecherry seeds collected from an open pollinated plant were germinated in the greenhouse. Actively growing shoot tips about 3 cm (~1 in) long were excised from 2-year-old seedlings, and were surface-sterilized in 70% ethanol for 2 min and in 15% commercial bleach solution for 15 min, followed by three rinses in sterile distilled water. The entire shoots were cultured on shoot initiation media in baby food jars [7 cm (~2.75 in) tall, 6 cm (~2.5 in) diameter, holding ~125 ml]. Each medium treatment consisted of five jars, with 4 shoots per jar. Three basic media used were: MS medium (9), Woody Plant medium (7), and Knop's medium (14). All media were supplemented with 3% (w/v) sucrose, 5.0 µM BA, and 5.0 µM IBA. The media were adjusted to pH 5.8 before addition of 7 g/liter agar (Sigma # A1296) and autoclaved at 121C (250F) for 20 min. Cultures were maintained at 24 ± 1C (75 ± 2F) under cool-white fluorescent lights (54 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at the shelf level with a 16-hr photoperiod. The average number of new shoots formed per jar was recorded after 3 and 5 weeks.

To determine the optimal medium for shoot proliferation, the newly proliferated shoots were cultured either on three basal media [MS, DKW (2), and WPM] with 4.4 µM BA, or on MS medium with different concentrations of BA or thidiazuron (TDZ). The numbers of new shoots formed per initial shoot were recorded after 4 weeks. In both experiments, each treatment had 4 replications (baby food jars) each with 5 shoots per replication. The experiment was repeated once.

The rooting of microshoots was tested in two experiments. In experiment 1, 15 microshoots each were cultured for 14 days on media with one of 7 concentrations of IBA. In experiment 2, 15 microshoots each were cultured on medium with either IBA or a-napthaleneacetic acid (NAA) at 5 µM or 10 µM. In regime A, shoots were cultured on the medium for 21 days without transfer. In regime B, shoots were cultured on medium with auxin for 5 days, then transferred to hormone-free medium for 16 days. Percentage of microshoots rooted and average number of roots per microshoot were recorded on day 21. Each treatment had 3 baby food jars, each with 5 shoots.

To propagate from the mature chokecherry trees, small branches from 20 putatively X-disease resistant trees in the Plant Materials Center planting near Bismarck, ND, were collected in early spring after the chilling requirement for bud dormancy was met. The branches from each tree were placed individually in beakers with distilled water under room temperature. The forced shoots, about 1–2 cm (0.4–0.8 in) long, were excised and sterilized like the seedling shoot tips. The sterilized shoots were cultured on MS media with either 2.0 or 5.0 µM BA. The other conditions were the same as those used to culture seedling shoots.

A total of 109 *in vitro* rooted plants and 140 non-rooted microshoots were transferred to non-sterile soilless medium (Jiffy Mix, Shippegan, Canada). Trays holding the plant material were covered with clear plastic tops to maintain a high level of humidity for 2 weeks. The covers were then gradually removed to allow plants to acclimate to ambient conditions for 2 weeks. Survival rates were recorded after an additional 2 weeks.

Data in Tables 1–2 and Figures 1–3 were subjected to analysis of variance (ANOVA) with mean separation by the Fisher's LSD at $P \leq 0.05$ (13).

Table 1. Chokecherry shoot initiation influenced by basal media containing 5.0 µM BA and 5.0 µM IBA.

	Average number of new shoots/explant[z]	
Basal medium	**After 3 weeks**	**After 5 weeks**
MS	2.4a[y]	4.8a
WPM	1.9a	2.2b
Knop's	0.0b	0.3c

[z]Only shoots longer than 1 cm (0.4 in) were counted.

[y]Means followed by different letters within a column were separated with Fisher's LSD after analysis of variance ($P \leq 0.05$).

Results and Discussion

Basal media had a significant effect on *in vitro* shoot initiation (Table 1). After 3 weeks in culture, no new shoots formed on Knop's medium, while 2.4 and 1.9 shoots formed on MS and WPM medium, respectively. After 5 weeks, an average of only 0.3 shoots formed on Knop's medium, while an average of 4.8 new shoots was produced on MS medium, which was significantly more than that on WPM (2.2 new shoots).

When these new shoots were cultured on three basal media supplemented with 4.4 µM BA, shoot proliferation occurred on all three media (Table 2). Medium with MS or DKW salts yielded more new shoots and shoots longer than 1 cm (0.4 in) than medium with WPM salts.

The BA and TDZ concentrations tested had significant effects on shoot proliferation (Fig. 1), with BA at 5.0 µM

Table 2. Effect of basal media supplemented with 4.4 µM BA on shoot proliferation of chokecherry cultured for 4 weeks.

Medium[z]	No. of new shoots[z]	No. of shoots > 1 cm (0.4 in)
MS	3.4a[y]	0.7a
DKW	3.0a	0.8a
WPM	1.4b	0.0b

[z]The data were the average of two experiments.

[y]Means followed by different letters within a column were separated with Fisher's LSD after analysis of variance ($P \leq 0.05$).

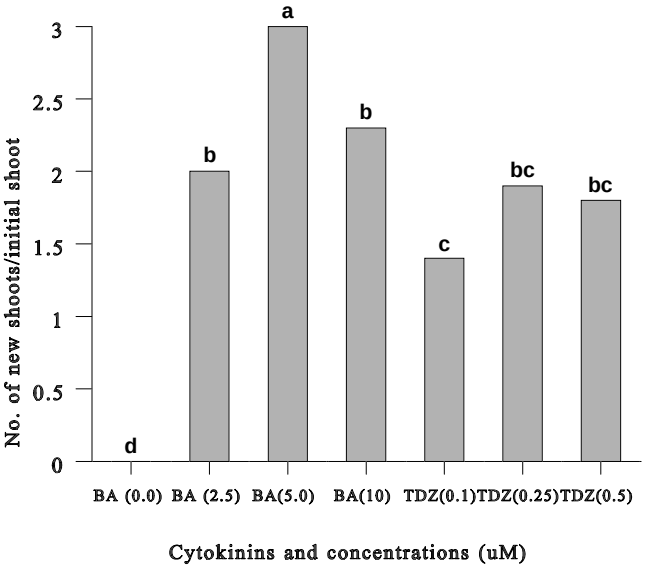

Fig. 1. Effect of cytokinins (BA and TDZ) on chokecherry shoot proliferation on MS medium. Shoots longer than 1 cm (0.4 in) were counted 4 weeks after culture. Means are separated, indicated by different letters above the bars, with Fisher's LSD after analysis of variance ($P \leq 0.05$).

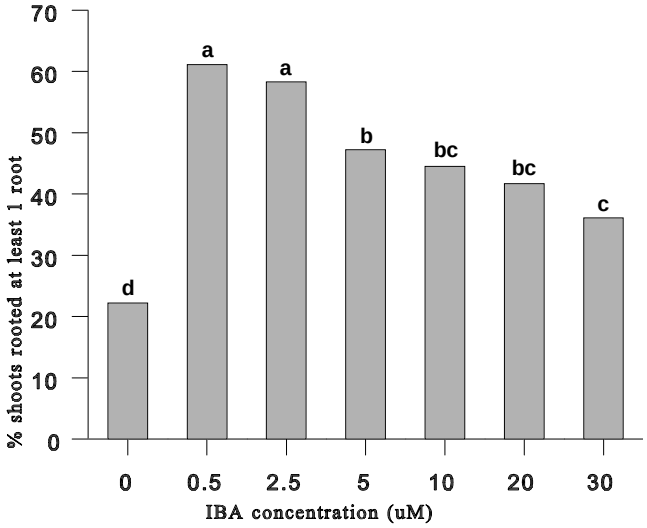

Fig 2. Effect of IBA on rooting from chokecherry microshoots on 1/2 MS medium. Percentage of microshoots rooted were recorded 2 weeks after culture. Means are separated, indicated by different letters above the bars, with Fisher's LSD after analysis of variance ($P \leq 0.05$).

producing the most shoots (average of 3.0 new shoots). Without BA, no shoots were produced. About 1.5–2.0 new shoots were produced on media with 0.1–0.5 µM of TDZ (Fig. 1).

Rooting of microcuttings was affected by the amount of IBA in the medium (Fig. 2). The highest percentage of microcuttings, about 60% after 14 days, rooted on media containing 0.5 or 2.5 µM of IBA (Fig. 2), with about 2 roots per shoot. In a second experiment which evaluated two auxins and two exposure regimes, IBA was shown to be more effective in inducing rooting in chokecherry than NAA (Fig. 3). There were no differences in rooting for both IBA concentrations and exposure regimes tested. When cuttings were exposed to NAA for 5 days and then transferred to NAA-free medium, a significantly higher percentage of cuttings rooted than those which were kept in the NAA-containing medium for 21 days (Fig. 3).

Chokecherry clones from mature stock plants showed differences in shoot proliferation (Table 3), ranging from 1.1 to 4.7 new shoots produced per initial shoot. Microshoots were more prolific on the medium containing 5.0 µM than those on the medium containing 2.0 µM BA.

When 109 rooted microplants were transplanted *ex vitro*, 94% survived. Of the 140 non-rooted cuttings transplanted, only 19% survived.

We report here the first standard 4-step *in vitro* procedure to micropropagate chokecherry. Using this system, more than 20 putatively X-disease-resistant chokecherry plants have been cloned and will be used for confirming X-disease resistance/tolerance and for studying X-disease phytoplasma/chokecherry interactions. Several reports showed that stone fruits can be initiated on Knop's medium (e.g., 14), but our results showed chokecherry initiated better on MS medium, which consists of more ingredients, similar to those used in peach (5, 6, 18). Basal medium greatly affects shoot proliferation, as reported in other woody species (1). MS and DKW were more effective than WPM, which was developed for micropropagation of woody plants (7). Cytokinin also influences chokecherry shoot proliferation, with BA being more effective than TDZ, as shown in peach (18), although TDZ has been reported in many other species for effective shoot proliferation. Rooting on medium can be induced by either IBA or NAA, with IBA being more effective. Treating with auxin for 5 days is enough to induce rooting. Exposure to auxin, especially with NAA, for 21 days can inhibit root formation. The higher rooting percentages in IBA media in Fig.

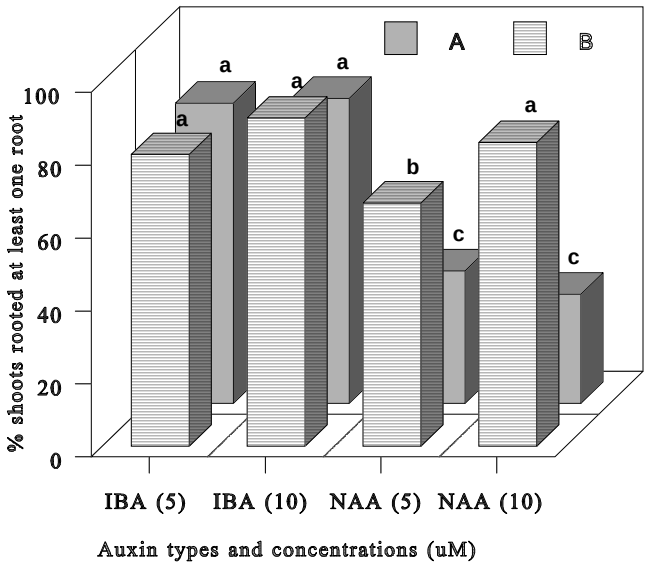

Fig 3. Effect of IBA and NAA on rooting from microshoots of chokecherry on 1/2 MS medium. A: Shoots were cultured on medium for 21 days. B: Shoots were cultured on medium with auxin for 5 days, then transferred to hormone-free medium for 16 days. Means are separated, indicated by different letters above bars, with Fisher's LSD after analysis of variance ($P \leq 0.05$).

Table 3. Shoot proliferation of putatively X-disease-resistant mature chokecherry clones.

Clones	No. of shoot tips initiated per medium	No. of shoots surviving	No. of new shoots/initial shoot[z]	
			Medium 1	Medium 2
R4-1935-3	15	11	1.6	4.2
RS-2002-3	15	15	1.5	3.9
R1-1970-2	15	15	1.7	4.1
R2-3671-3	15	12	1.3	2.8
R5-3644-4	12	12	dead	2.5
R4-1970-1	15	10	dead	2.0
R3-2013-3	15	14	1.5	1.9
R5-2015-4	15	12	1.3	2.3
R1-1982-1	15	11	dead	1.1
R2-2015-3	15	11	1.6	2.3
R1-2015-4	15	14	dead	2.5
R3-1900-4	15	7	dead	2.4
R5-1907-4	12	9	3.6	3.3
R2-2029-2	12	9	dead	3.4
R1-1964-1	12	5	dead	1.2
R1-2069-3	15	10	dead	3.0
R5-2015-2	15	11	2.5	4.7
R4-1933-2	15	15	dead	3.2
R4-1908-2	15	13	2.4	4.0
R4-1918-3	15	4	2.0	4.0

[z]Only shoots longer than 1 cm (0.4 in) were counted. Medium 1 and medium 2 were MS basal salts supplemented with 2.0 µM and 5.0 µM BA, respectively.

3 than in Fig. 2 probably is because rooting was assessed 7 days later. Rooted plants can be readily acclimated to the ambient environment. Using this system, we have established and proliferated 20 chokecherries which have shown putative resistance to X-disease. These genetically uniform host materials will facilitate the study of chokecherry/X-disease phytoplasma interactions.

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