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Micropropagation of 'Amethyst' Purple Raspberry (*Rubus occidentalis* L. x *R. idaeus* L. 'Amethyst')[1]

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Abstract

A micropropagation method was developed for a cold hardy purple raspberry cultivar (*Rubus occidentalis* × *R. idaeus* 'Amethyst'). *In vitro* shoot cultures were initiated from shoot tips of a 30-year old 'Amethyst' plant. The effects of basal medium, plant growth regulator, and temperature on shoot proliferation were investigated. Shoots were produced from explants in both Murashige and Skoog (MS) and Driver-Kuniyuki Walnut (DKW) media supplemented with different concentrations of thidiazuron (TDZ) and benzyladenine (BA), solely or combined. One micro molar TDZ gave rise to the maximum proliferation rate. Interactions between BA and medium or TDZ were significant. Shoots produced on media with 1.0 µM TDZ had thick stems and small, dark green leaves whether BA was absent or present. Shoots can be rooted both *in vitro* and *ex vitro* with or without IBA at 0 to 1.0 µM. However, combination of rooting and shoot multiplication by adding a low level of TDZ to rooting medium produced multi-cane plants resulting in shortening propagation time, increasing survival rate, and lowering the production cost.

Index words: propagation, combination of rooting and shoot multiplication, tissue culture.

Species used in this study: *Rubus occidentalis* x *R. idaeus* 'Amethyst'.

Significance to the Nursery Industry

In the upper Great Plains regions, harsh winter conditions, such as absence of insulating snow cover, low and fluctuating temperature, and desiccating winds, often result in considerable cold injury and winter kill of plants. Purple raspberry (*Rubus occidentalis* x *R. idaeus* L.), a cross between red and black raspberries, tends to be as cold tolerant as red raspberries. Although several purple raspberry cultivars have been grown in this region, only a few cultivars, such as 'Amethyst', 'Brandywine', 'Marion', 'Royalty', and 'Sodus' are recommended for commercial production in several state extension publications.

Purple raspberry growth is similar to one of its parents, black raspberry, in that primocanes are only produced from crown buds at the base of floricanes. Purple raspberry plants do not sucker, so clumps remain in their original planting location. Unlike red raspberries, which are easily propagated from suckers that develop from the roots, purple raspberries are generally propagated by tip layering which is relatively slow compared to other propagation methods, such as cuttings and division due to the limited propagation materials. Tip layering of 'Amethyst' has been attempted for several years, but the propagation efficiency is still remains very low (< 20%), which may have contributed to the decline in its availability to the public and production.

Micropropagation has proved to be a very efficient method to propagate many woody plant species. Compared to other clonal propagation methods (cuttings, grafting, and division), micropropagation has such advantages as high propagation rate, year-round operation, uniformity of propagated plants, and low costs, etc. This study demonstrated that 'Amethyst' can be easily propagated by micropropagation. The method developed in this study can be used to mass propagate this cultivar to quickly respond to the increasing demand and could be applied in propagation of other related species or cultivars by commercial tissue culture laboratories.

Introduction

Purple raspberry (*Rubus occidentalis* L. x *R. idaeus* L.), a hybrid cross between red and black raspberries, is more vigorous and productive than its parents and tends to be as cold tolerant as red raspberry (3, 4). Raspberries have been widely used either fresh or processed into jellies, juices, ice creams, and jams. Recently there has been increasing interest in raspberry fruit production in the northern Great Plains because of their high oxygen radical absorbance capacity (ORAC) (11, 16) and activity of carcinogenesis inhibition (11, 16). Similar to its black raspberry parent, purple raspberry does not sucker and only produces primocanes from the crown buds at the base of floricanes, which keeps clumps in their original planting location. Therefore, purple raspberries are mainly propagated by tip layering instead of suckering as other raspberries.

Currently, there are only a few purple raspberry cultivars commercially available. 'Amethyst', 'Brandywine', 'Marion', 'Royalty', and 'Sodus' are often mentioned by several state extension publications as good purple cultivars. 'Amethyst', introduced in 1968 at Iowa State University, has a better color, taste, and higher yield than most purple raspberry cultivars (8). 'Amethyst' also produces medium to large size fruits with a high sugar content and shiny appearance. Based on several years' field observations, purple raspberry 'Amethyst' is hardy enough for Fargo and other areas of North Dakota (unpublished results). Unfortunately, propagation of 'Amethyst' by tip layering has not been very successful.

Although micropropagation of raspberry species has been well documented (5, 14, 15), the majority of raspberry micropropagation research is focused on red raspberry (1, 7, 9, 10), black raspberry (1, 7), and dwarf raspberry (2). No research on *in vitro* propagation of purple raspberry has been reported. Data on effects of cultural conditions, such as temperature and light, on proliferation and *ex vitro* rooting and

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acclimation are still limited. Some previous results appear to be genotype-dependent and controversial (5, 7, 14, 15). The objective of this study was to develop a rapid vegetative propagation protocol for 'Amethyst' to meet the increasing demand for this elite, hardy purple raspberry cultivar.

Materials and Methods

Tissue culture establishment. Dormant shoots were collected in March 2004 from a 30-year-old *Rubus occidentalis* x *R. idaeus* 'Amethyst' plant growing in Jack Carter's backyard in Fargo, ND. Buds were forced to break in a container containing water under room temperature, and developing shoot tips (0.5–1.0 cm) were excised from shoots. Shoot tips were surface sterilized in 70% ethanol for 30 seconds followed by immersion in 10% Clorox (0.6% sodium hypochlorite) containing 3 drops of liquid soap per 100 ml solution for 15 min. Tips were rinsed three times in sterile distilled water, with a 5-min interval between each rinse, and blotted dry with sterile paper towels. Surface-sterilized shoot tips were cut 0.5–1.0 mm at the base and inserted into the initial medium [Murashige and Skoog (MS) medium (10) supplemented with 5 µM BA] in 100-ml baby food jars containing 25 ml medium. Starter cultures were transferred to fresh initial medium every 4 weeks. To control contamination, PPM (plant preservative mixture, PCT, Inc., Jefferson Place, Washington DC) was added to the medium in the first four subcultures. All media (initial and proliferation described below) were supplemented with MS vitamins, 3% sucrose and 0.7% agar (Difco Co., Detroit, MI, #0140-01-0) and adjusted to pH 5.8 before autoclaving. Each baby food jar contained four shoot explants. Shoot cultures were maintained at 25C (78F) under cool-white fluorescent light at approximately 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16-hr photoperiod. All experiments were performed under identical conditions unless otherwise noted.

Shoot proliferation. Several experiments were conducted to optimize proliferation conditions. Two basal media [MS and Driver-Kuniyuki Walnut (DKW) (6)] and two cytokinins (BA and TDZ) at three concentrations (BA at 0, 2.5, and 5.0 µM; TDZ at 0, 0.1, and 1.0 µM) were evaluated. The effect of culture temperature on proliferation was also determined. Shoot explants were cultured in DKW medium with 0.1 or 1.0 µM TDZ and placed in incubators at 22, 25, and 28C (70, 78, and 85F) during the 16-h photoperiod, respectively. The temperature was adjusted to 18C (65F) during the dark time. One set of cultures was placed in a culture room where temperature fluctuated from 25 to 30C (78 and 88F) frequently. After culturing for 4 weeks, the number of new shoots (>0.5 cm) produced per shoot explant was recorded. Each treatment consisted of three jars (sample replications) with three shoot explants in each jar.

Rooting and acclimatization. Shoots from proliferation medium were rooted either *in vitro* or *ex vitro*. Based on results of shoot proliferation study, i*n vitro* rooting was combined with shoot elongation and multiplication by subculturing shoots in MS medium with or without IBA at 0, 0.5, and 1.0 µM and TDZ at 0, 0.1, 0.5, and 1.0 µM. *In vitro* plants were then transferred to flats filled with Jiffy Mix (Jiffy Mix; Shippegan, NB, Canada) and covered with clear plastic tops. The covers were replaced with 6-hole (2 cm hole) and 18-hole covers at second and third week, respectively. The covers were eventually removed at the fourth week. Surviving plants were transplanted into 3 × 3 × 3 in. pots with Sunshine Mix #1(Fisons Western Corp., Vancouver, BC, Canada) and grown in a Controlled Environment Agricultural (CEA) greenhouse.

For *ex vitro* rooting, an entire clump of shoots was removed from proliferation medium (DKW with 1.0 µM TDZ) after 4 weeks proliferation culture and washed with tap water. After being inserted into Jiffy Mix medium in flats, cuttings were watered with either distilled water or 10 µM IBA solution. Flats were covered with non-hole clear plastic tops for 1 week and then replaced with 6-hole and 18-hole tops for 1 week each. The covers were eventually removed during 3-week period and rooting percentage and survival rate were recorded after another addition week. Each treatment included at least 50 microcuttings. The rooted plants were transferred to Sunshine Mix #1 and grown in the same greenhouse above.

The shoot proliferation experiment was conducted as a completely randomized design (CRD) consisting of two replications of a factorial arrangement with a 2 × 3 × 3 of basal medium, PGR types, and PGR concentration, respectively. The i*n vitro* rooting experiment was conducted as a CRD with two PGR types (IBA and TDZ) at 3 and 4 concentrations, respectively. CRD design was also used to conduct the temperature experiment with four temperatures and one PRG type (TDZ) at 2 concentrations. The *ex vitro* rooting experiment was also conducted as a CRD. All experiments were replicated twice at a one or two-month interval. Data were subject to analysis of variance and mean comparison using the GLM procedure of SAS software Version 9.1(13).

Results and Discussion

Effects of basal medium and cytokinin on proliferation. Shoot tips started growing within 1 week and the lateral buds broke out from initial explants in 2–3 weeks in MS medium with 5 µM BA. More than 90 % of initial explants survived in initial medium. Tissue cultures appeared to be stable after four subcultures at 4-week intervals.

Shoots proliferated *in vitro* in BA and/or TDZ containing medium (Fig. 1A). Without cytokinin, no shoots were produced in MS medium, and only 0.5 new shoots per explant were produced in DKW medium (Table 1). Significant interactions in BA × medium and BA × TDZ were observed (Table 1). There was no significant difference in shoot proliferation between BA concentrations when BA was used as the only cytokinin source. However, raising the TDZ concentration resulted in significant increase of shoot proliferation rate in all treatments indicating that this raspberry is more sensitive to TDZ than BA. Overall, TDZ at 1 µM in DKW medium

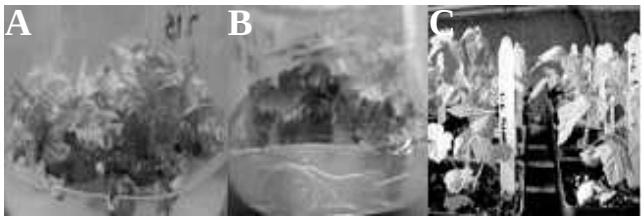

Fig. 1. Micropropagation of *Rubus occidentalis* L. x *R. idaeus* L. (A) *In vitro* shoot proliferation on DKW medium containing 1.0 µM TDZ; (B) Roots were induced and shoots were proliferated *in vitro*; (C) Acclimated plants were potted and grown in the greenhouse.

Table 1. Effect of basal medium and cytokinin on shoot proliferation of *Rubus occidentalis* L. x *R. idaeus* L[z].

Cytokinin (µM)		Proliferation rate[y]		Analysis of variance			
TDZ	BA	MS	DKW[x]	Source of variation	DF	Mean square	F
0	0	0h	0.5gh	BA	2	1.0869	2.14[NS]
0	2.5	2.2def	1.4efgh	TDZ	2	20.6443	40.61[***]
0	5.0	2.5def	1.1fgh	Medium (MED)	1	0.2916	0.57[NS]
0.1	0	1.3efgh	2.0defg	BA × MED	2	5.9705	11.75[***]
0.1	2.5	1.9defg	1.3efgh	BA × TDZ	4	3.7607	7.40[***]
0.1	5.0	2.9cd	1.8defg	TDZ × MED	2	0.7286	1.43[NS]
1.0	0	2.8de	5.8a	TDZ × BA × MED	4	0.6951	1.37[NS]
1.0	2.5	4.8ab	4.3bc				
1.0	5.0	3.2cd	1.8defg				

[z]Values followed by the same letter within each column are not significantly different according to the Fisher Least Significant Difference (LSD) test at $p \leq 0.05$.
[y]The proliferation rate is expressed as the number of shoots produced per explant after 4 weeks *in vitro* culture.
[x]MS: Murashige and Skoog medium; DKW: Driver-Kuniyuki Walnut medium.
[***]Significant at $p \leq 0.001$.
[NS]Nonsignificant.

and a combination of TDZ and BA at 1 and 5 µM, respectively, in MS medium gave rise to a higher shoot proliferation rate. Shoot proliferation rate was significantly lower in TDZ-containing DKW medium when BA was added, whereas the shoot proliferation rate was significantly increased when BA was added to TDZ-containing MS medium. Shoots produced on media with 1.0 µM TDZ had thick stems and small, dark green leaves. Plants produced from those healthy shoots may perform well in soil survival and field establishment.

Effect of culture temperature on proliferation. Culture temperature significantly affected shoot proliferation (Table 2). More shoots were generated under 25 and 28C (78 and 85F) than under 22C (70F) conditions. Temperatures and TDZ concentrations significantly affected proliferation rate. Significant difference in proliferation was found among temperatures when higher TDZ concentration was used. Shoots cultured in 1.0 µM TDZ under 28C (85F) produced most shoots with 4.3 proliferated shoots per explant. Fluctuating temperature significantly lowered shoot proliferation. Only 0.9 and 1.8 shoots were produced per explant when culture temperatures were not constant.

Rooting and acclimatization. In order to develop a rapid micropropagation method for purple raspberry, *in vitro* rooting was combined with shoot multiplication by adding TDZ into rooting medium. About 39% of microcuttings rooted in auxin-free medium (Table 3; Fig. 1B). Application of IBA significantly increased *in vitro* rooting percentage, but not the root number. TDZ appeared to be a main factor influencing both rooting percentage and root number per cutting. In IBA-free medium, TDZ at a low level (≤ 0.1 µM) stimulated *in vitro* rooting; however, high concentration of TDZ (≥ 0.5 µM) inhibited root production, with fewer roots induced per cutting. The IBA × TDZ interaction was not significant. An average of 4.3 new shoots were proliferated in TDZ containing medium, whereas, only 0.8 new shoots were proliferated in TDZ-free medium (data not shown), indicating that application of TDZ, especially at a low level, in the rooting medium, stimulated formation of both roots and shoots. Table 3 also showed that both IBA and TDZ significantly affected the proliferation rate. High concentration of IBA showed the inhibition of shoot proliferation. There was a significant interaction between IBA and TDZ on 'Amethyst' shoot proliferation. More than 90% of rooted plants survived after being transplanted into Sunshine Mix #1 grown in the greenhouse. In addition, plants growing in TDZ-containing medium had green and thick leaves. Rooted plants with healthy leaves and multiple shoots (which will become canes) will perform well upon soil transplanting and field establishment.

In the *ex vitro* experiment, an average of 70.8% of the cuttings rooted (data not shown). No significant difference

Table 2. Effect of culture temperature and TDZ concentration on shoot proliferation of *Rubus occidentalis* L. x *R. idaeus* L[z].

			Analysis of variance			
Temperature (C)	TDZ (µM)	Proliferation rate[y]	Source of variation	DF	Mean square	F
22	0.1	1.4cd	Temperature	3	3.1774	24.00[***]
22	1.0	1.9c	TDZ	1	9.2873	70.14[***]
25	0.1	2.0c	Temperature × TDZ	3	1.1369	8.59[**]
25	1.0	3.8b				
28	0.1	1.7cd				
28	1.0	4.6a				
25–30	0.1	0.9d				
25–30	1.0	1.8c				

[z]Values followed by the same letter within each column are not significantly different according to the Fisher Least Significant Difference (LSD) test at $p \leq 0.05$.
[y]The proliferation rate is expressed as the number of shoots produced per explant after 4 weeks *in vitro* culture.
[**, ***]Significant at $p \leq 0.01$, and 0.001, respectively.
[NS]Nonsignificant.

Table 3. Effect of plant growth regulators (PGRs) on shoot proliferation and *in vitro* rooting of *Rubus occidentalis* L. x *R. idaeus* L[z].

PGR (µM)					Analysis of variance							
							Mean of square			F		
IBA	TDZ	Prol. rate[y]	Rooting (%)	Roots/ cutting	SOV	DF	PRO	RPT	RNM	PRO	RPT	RNM
0	0	0.7e	38.9bc	3.6a	IBA (I)	2	5.327	445.829	0.032	8.59**	1.21^{NS}	0.06^{NS}
0	0.1	1.0e	83.4a	2.5abc	TDZ (T)	3	41.458	7070.761	7.978	66.83***	19.21***	16.39***
0	0.5	6.1bc	11.1c	1.0c	I × T	6	3.026	357.366	0.570	4.88**	0.97^{NS}	1.17^{NS}
0	1.0	7.2ab	11.1c	1.0c								
0.5	0	0.7e	88.9a	2.6abc								
0.5	0.1	1.8e	77.8ab	3.2a								
0.5	0.5	3.7d	22.2c	1.3c								
0.5	1.0	3.7d	5.6c	1.0c								
1.0	0	0.9e	76.4ab	2.3abc								
1.0	0.1	2.1de	88.9a	3.0ab								
1.0	0.5	4.9cd	18.1c	1.5bc								
1.0	1.0	8.1a	16.7c	1.3c								

[z]Values followed by the same letter within each column are not significantly different according to the Fisher Least Significant Difference (LSD) test at $p \leq 0.05$.

[y]The proliferation rate is expressed as the number of shoots produced per explant after 4 weeks *in vitro* culture.

[x]SOV: Source of Variance; PRO: proliferation rate; RPT: rooting percentage; RNM: Root number per cutting.

[**]Significant at $p \leq 0.005$

[***]Significant at $p \leq 0.001$.

[NS] Nonsignificant.

in rooting percentage was found between IBA and water treatments. Approximately 200 cuttings rooted *ex vitro* and more than 95% *ex vitro* rooted plants survived after they were potted and grown in the greenhouse for two months (Fig. 1C). Micropropagated 'Amethyst' plants will be planted in the field for further morphological and horticultural evaluation.

The research results showed that purple raspberry 'Amethyst' can be efficiently micropropagated. MS or DKW media supplemented with BA and TDZ, solely or combined can be used for *in vitro* proliferation. In this study, we combined *in vitro* rooting and shoot multiplication in one step resulting production of multi-cane plants, which simplified the propagation process and lowered production cost. Furthermore, multi-cane plants will be easier to acclimatize and field establish. This protocol may also be applicable to other raspberry cultivars or related species.

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