Micropropagation of Lingonberry: Influence of Genotype, Explant Orientation, and Overcoming TDZ-induced Inhibition of Shoot Elongation Using Zeatin

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Abstract. In an attempt to improve the micropropagation protocol for lingonberry (*Vaccinium vitis-idaea* L.), developed at the Centre, two lingonberry clones were compared for in vitro shoot proliferation on two different media supplemented with varying levels of thidiazuron (TDZ). TDZ supported proliferation at low concentrations (0.1 to 1 μ M) but inhibited shoot elongation. However, usable shoots were obtained within 4 weeks by transferring shoot cluster to medium containing 1 μ M zeatin. Genotypes differed significantly with respect to multiplication rate with 'EL1' producing the most shoots per explant. In both genotypes, shoot proliferation was greatly influenced by explant orientation. Changing the orientation of explants from vertically upright to horizontal increased axillary shoot number, but decreased shoot height and leaf number per shoot. Proliferated shoots were rooted on a 2 peat : 1 perlite (v/v) medium, and the plantlets were acclimatized and eventually established in the greenhouse.

Lingonberry (*Vaccinium vitis-idaea* L.), an important fruit crop in many northern latitude countries (Gustavsson and Stanys, 2000; Jaakola et al., 2001), is a medicinal plant rich in anthocyanin (Stark et al., 1978) antioxidants (Wang et al., 1997). The Newfoundland lingonberry, also called partridgeberry, ranks first among *Vaccinium* species in terms of oxygen radical absorbance capacity (ORAC) containing higher antioxidants than lowbush blueberry, cranberry, and bilberry (W. Kalt, personal communication). The extract arbutin, derived from lingonberry leaves, is used for stomach disorders (Racz et al., 1962). This circumboreal woody, rhizomatous, evergreen dwarf shrub (Luby et al., 1991; Vander Kloet, 1988) can also be used as a landscape ornamental ground cover (Dierking and Dierking, 1993).

Two subspecies of *V. vitis-idaea* have been recognized. The larger lowland race as *V. vitis-idaea* ssp. *vitis-idaea* (L.) Britton and the dwarf arctic-montane race as *V. vitis-idaea* ssp. *minus* (Lodd) Hult. (Hulten, 1949). The Canadian

province, Newfoundland and Labrador, is the largest North American lingonberry producing region (Penney et al., 1997) with about 140,000 kg harvested annually from native stands for processing, mostly for export (Jamieson, 2001). Other exporting countries are Sweden, Finland, and the former Soviet Union (Holloway, 1981; Liebster, 1977). Natural stands of lingonberries are harvested in Newfoundland, but due to the increased demand for the nutritious, natural fruit-based drinks, and other products that use processed lingonberries, demand now exceeds production. An increasing demand for berries of high quality has intensified the need to develop new cultivars for horticultural production. We initiated a program to develop improved lingonberry cultivars using biotechnology combined with classical breeding. Research on in vitro culture of the lingonberry which began at the Atlantic Cool Climate Crop Research Centre of Agriculture and Agri-Food Canada in St. John's, in 1999 under the Small

Fruit Development Program for Cool Climates has already yielded a considerable amount of information.

Thidiazuron, a substituted phenylurea (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea) with both cytokinin- and auxin-like effects (Mok et al., 1982; Visser et al., 1992), is a potent bioregulant of in vitro morphogenesis, and is now among the most active cytokinin-like substances for plant tissue culture. TDZ is used to induce shoot organogenesis in several species of recalcitrant woody plants (Murthy et al., 1998). The efficacy of TDZ for shoot proliferation has been demonstrated in apple (van Nieuwkerk et al., 1986), azaleas (Fellman et al., 1987), *Quercus* (Chalupa, 1988), carnation (Sankhla et al., 1995), *Hevea* (Seneviratne and Flagmann, 1996), cassava (Bhagwat et al., 1996), and rose (Singh and Syamal, 2001). While most of the early reports used the cytokinin *N*⁶-[2-isopentenyl]adenine (2iP) or zeatin for initiating new growth from lingonberry explants (Debnath and McRae, 2001a; Reed and Abdelnour-Esquivel, 1991; Serres et al., 1994), the effectiveness of TDZ on in vitro shoot proliferation has not been demonstrated with *Vaccinium* species. The objective of the current study was to determine the influence of TDZ in lingonberry for shoot proliferation and elongation testing of two native selections, one from Newfoundland and other from Estonia.

Materials and Methods

Plant material and establishment of shoot cultures. Young, actively growing lingonberry shoots of the Newfoundland clone, 'NL1' and the Estonian clone, 'EL1' were obtained from plants being maintained in a greenhouse. While 'EL1' belong to *V. vitis-idaea* ssp. *vitis-idaea*, the clone 'NL1' is in the group of *V. vitis-idaea* ssp. *minus*. Shoot-tip cultures, following the method of Debnath and McRae (2001a) were established in 175-mL glass baby-food jars containing 35 mL BM medium [three-quarter macrosalts and macrosalts of Debnath and McRae's (2001a) shoot proliferation medium D] supplemented with (per liter) 25 g sucrose, 3.5 g Sigma A 1296 agar, and 1.25 g Gelrite (Sigma Chemical Co., St. Louis). The medium pH was adjusted to 5.0 before autoclaving at 121 °C for 20 min. Zeatin (5 μ M) was filter-sterilized and added to autoclaved

Fig. 1. Shoot vigor rating (1 to 8, from left to right) in lingonberry clone 'EL1' grown in vitro for 8 weeks on a culture medium.

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and cooled (40 to 50 °C) BM medium. This medium worked well with 'NL1' and 'EL1'. Culture jars were capped with clear permeable polypropylene caps (Sigma Chemical Co., St. Louis). After explant transfer, jars were sealed with Parafilm, placed upright and maintained at 20 ± 2 °C under a 16-h photoperiod with a photosynthetic photon flux (PPF) density of 30 µmol·m⁻²·s⁻¹ at the culture level provided by cool white fluorescent lamps.

Effect of TDZ concentration on shoot induction and proliferation from three-node explants. The purpose of this experiment was to determine the effect of TDZ concentration, explant orientation, medium salt formulation, and/or genotype influenced shoot induction and proliferation. A 2 × 2 × 4 × 2 factorial experiment (completely randomized) compared all combinations of two clones ('NL1' and 'EL1'), two media [BM and modified MS (Murashige and Skoog, 1962) medium containing half MS macrosalts and MS macrosalts and vitamins (MMS)], four TDZ concentrations (0, 0.1, 1, and 5 µM), and two explant positions (vertical and horizontal). Three-node stem sections with leaves intact from in vitro shoots maintained for 8 weeks in BM without PGRs were cultured in 175-mL glass baby-food jars (Sigma Chemical Co., St. Louis) containing 35 mL media. There were four jars per treatment for each clone and each jar contained five explants. The experiment was conducted three times.

Shoot elongation, rooting, and acclimatization. Shoots of the clones 'NL1' and 'EL1' initiated on TDZ-containing medium were transferred to 175-mL glass baby-food jars containing 35 mL BM supplemented with 1 µM zeatin after 8 weeks of culture initiation for elongation. Elongated shoots (3 to 4 cm long) were easily rooted using previously reported techniques (Debnath and McRae, 2001a). Briefly, shoots were excised just above the original explant, dipped in 39.4 mM indole-3-butyric acid (IBA) powder (Stim-Root#3, Plant Products Co. Ltd., Brampton, Ontario L6T 1G1, Canada) and planted in 45-cell plug trays (cell diameter = 5.9 cm, cell depth = 15.1 cm; Beaver Plastics, Edmonton, Alberta T5V 1H5, Canada) containing 2 peat: 1 perlite (v/v). Trays were placed in a humidity chamber with a cool mist vaporizer at 22 ± 2 °C, 95% RH at 16 h photoperiod at 55 µmol·m⁻²·s⁻¹ PPF for rooting. Plantlets were transferred to 10.5 (L) × 10.5 (W) × 12.5 (D) cm³ plastic pots containing the same medium as used for rooting, and acclimatized by gradually lowering the humidity over 2 to 3 weeks. Hardened-off plants were maintained in the greenhouse at 20 ± 2 °C, 85% RH, and 16-h photoperiod at a maximum PPF of 90 µmol·m⁻²·s⁻¹. The number of surviving plants was recorded when they were removed from the humidity chamber (6 weeks).

Data collection and statistical analysis. The following growth characteristics of surviving explants were measured for each treatment at 8 weeks: number of shoots (>1 cm long) per responding explant, shoot length (cm), number of leaves per shoot, shoot vigor, and callus size per explant. Vigor was determined by visual assessment (Fig. 1), on a scale of 1 (strongly vitrified, necrotic and/or malformed shoots)

to 8 (fully normal and healthy shoots with excellent vigor); 2 = less vitrified, necrotic and/or malformed shoots; 3 = no vitrification but with very poor vigor; 4 = possessing poor shoot vigor; 5 = with average shoot vigor; 6 = having good shoot vigor; and 7 = with very good shoot vigor. Callus size was rated as 0 (no callus formation) and 1 (<2 mm in diameter) to 8 (>14 mm in diameter).

Data were subjected to analysis of variance with the SAS statistical software package (Release 8.2, SAS Institute, Inc., Cary, N.C.). The shoot number per explant was transformed to the square root scale, before the ANOVA to stabilize the variance. Statistical F tests were evaluated for shoot number, shoot height, and leaf number per shoot at $P \leq 0.05$. Shoot vigor and callus size were tested by using a categorical data modelling procedure (PROC CATMOD in SAS). This method is appropriate for the analysis of categorical data (Compton, 1994; Grizzle et al., 1969; Koch et al., 1982; Stanek et al., 1987), and allows assessment of both main effects and interaction terms (as in analysis of variance).

Results and Discussion

Effect of TDZ concentration on shoot induction and proliferation. All cuttings placed horizontally on the culture media responded by internal swelling and developed callus around the cut ends at 0 to 5 µM TDZ from day 6 to 8 of culture. Vertical placement induced callus formation at the basal end of the explants only. Axillary buds with multiple shoots developed at all TDZ concentrations.

Usually, calli were creamy white to pale brown in color, nodular and compact and sometimes exhibited patches of red pigmentation. TDZ concentration significantly ($P \leq 0.05$) affected shoot proliferation and callus formation (Tables 1 and 2). Callus size increased with increasing dose of TDZ while shoot height, leaf number per shoot, and shoot vigor

diminished for both 'NL1' and 'EL1' explants (Tables 1 and 2). All of the TDZ treatments induced basal callusing which was particularly severe at the highest concentration (Table 2). The formation of basal callus was observed frequently in shoot cultures with strong apical dominance (Preece et al., 1991). In previous cases, basal callus turned brown and reduced shoot proliferation (George and Sherrington, 1984). Basal callusing has been attributed to the action of accumulated auxin at the basal ends (Marks and Simpson, 1994), which initiates cell proliferation, especially in the presence of cytokinins (Tao and Verbelen, 1996). The number of shoots per explant improved at TDZ concentrations up to 1 µM, but declined when >1 µM was used for both genotypes (Table 1). A concentration of 0.1 µM TDZ was sufficient to induce shoot proliferation, although the number of shoots per explant was significantly less than at 1 µM. Across all treatments, an average of 3 shoots per explant was obtained when 1 µM TDZ was used. The interaction, clone × TDZ concentration was important for shoot proliferation as the F values for shoot number, shoot height, and leaf number per shoot, and the Chi-square value for shoot vigor were significant (Tables 1 and 2). The medium × TDZ interaction was significant ($P \leq 0.05$) for shoot number, shoot height, and leaf number per shoot, but not for shoot vigor and callus size. TDZ concentration appeared to interact with explant positioning on the medium for number of shoots per explant and leaves per shoot (Table 1). Positioning of explants on the culture media significantly affected the shoot number; changing the orientation of explants from vertically upright to horizontal increased axillary shoot number, but decreased shoot height and leaf number per shoot in both clones across both media in all TDZ concentrations (Table 1). This experiment was repeated by subculturing nodal segments of random axillary shoots from each replication of each treatment. The results were similar to those presented

Table 1. Effects of TDZ concentration and explant orientation on shoot number, shoot height, and leaf number per shoot for lingonberry clones, 'NL1' and 'EL1' grown in vitro on two culture media^a.

Treatment	Shoots (√no./explant)	Shoot ht (cm)	Leaves (no./shoot)
Clone			
'NL1'	1.4 b ^a	2.2 b	3.7 b
'EL1'	1.5 a	3.0 a	4.5 a
Medium ^x			
BM	1.4 b	2.7 a	4.4 a
MMS	1.5 a	2.5 b	3.8 b
TDZ concentration (µM)			
0.0	1.0 d	3.7 a	5.4 a
0.1	1.6 b	2.8 b	4.8 b
1.0	1.8 a	2.4 c	3.7 c
5.0	1.4 c	1.7 d	2.6 d
Explant orientation			
Vertical	1.4 b	2.7 a	4.2 a
Horizontal	1.5 a	2.5 b	4.0 b
Significant effects ^w	C, M, T, E, C×T, M×T, T×E	C, M, T, E, C×M, C×T, M×T, C×M×E, M×T×E, C×M×T×E	C, M, T, E, C×T, M×T, M×E, T×E, C×M×T, C×T×E, M×T×E, C×M×T×E

^aData were collected after 8 weeks in culture.

^bMean separation within columns and factors by Duncan's multiple range test, $P \leq 0.05$, whereby means associated with different letters signify significant differences.

^cPlease see text for detail.

^wSignificant effects ($P \leq 0.05$): C = clone, M = medium, T = TDZ concentration, E = explant orientation.

Table 2. Effects of TDZ concentration and explant orientation on shoot vigor and callus size for lingonberry clones, 'NL1' and 'EL1' grown in vitro on two culture media^a.

Clone	Medium ^y	TDZ (μM)	Shoot vigor (scale 1–8) ^x		Callus size (scale 0–8) ^w	
			Vertical	Horizontal	Vertical	Horizontal
'NL1'	BM	0.0	5.7	5.8	1.7	2.1
'NL1'	BM	0.1	5.1	5.1	4.4	5.1
'NL1'	BM	1.0	3.4	3.7	6.1	6.6
'NL1'	BM	5.0	2.2	2.0	7.4	7.7
'NL1'	MMS	0.0	6.1	5.7	1.2	1.6
'NL1'	MMS	0.1	5.3	4.8	4.6	5.1
'NL1'	MMS	1.0	3.4	3.1	6.0	6.3
'NL1'	MMS	5.0	2.2	2.0	7.1	7.4
'EL1'	BM	0.0	5.9	5.9	1.6	1.9
'EL1'	BM	0.1	5.2	5.1	5.4	5.7
'EL1'	BM	1.0	4.6	4.2	6.8	6.9
'EL1'	BM	5.0	3.8	3.6	7.3	7.7
'EL1'	MMS	0.0	5.1	4.7	1.8	2.1
'EL1'	MMS	0.1	4.5	4.3	5.1	5.6
'EL1'	MMS	1.0	4.1	3.9	6.5	7.0
'EL1'	MMS	5.0	3.4	3.1	7.2	7.6
Significant effects ^v			C, T, C×T		T	
Significant contrasts [TDZ (μM)]			0.0 vs. 0.1 ($P = 0.0330$), 0.0 vs. 1.0 ($P = 0.0001$), 0.0 vs. 5.0 ($P < 0.0001$), 0.1 vs. 1.0 ($P < 0.0002$), 0.1 vs. 5.0 ($P < 0.0001$), 1.0 vs. 5.0 ($P = 0.0001$)		0.0 vs. 1.0 ($P < 0.0001$), 0.0 vs. 5.0 ($P < 0.0001$), 0.1 vs. 1.0 ($P < 0.0001$), 0.1 vs. 5.0 ($P < 0.0001$), 1.0 vs. 5.0 ($P < 0.0189$)	

^aData were collected after 8 weeks in culture.

^yPlease see text for detail.

^xShoot vigor was scored on a scale of 1 (strongly vitrified, necrotic and/or malformed shoots) to 8 (fully normal and healthy shoots with excellent vigor); 2 = less vitrified, necrotic and/or malformed shoots; 3 = no vitrification but with very poor vigor; 4 = possessing poor shoot vigor; 5 = with average shoot vigor; 6 = having good shoot vigor; and 7 = with very good shoot vigor.

^wCallus size was rated as 0 (no callus formation) and 1 (<2 mm in diameter) to 8 (>14 mm in diameter).

^vSignificant effects ($P \leq 0.05$): C = clone, T = TDZ concentration.

Fig. 2. Elongated shoots on 1 μM zeatin-containing medium 4 weeks after transfer of 8-week-old thidiazuron (1 μM)-induced 'EL1' shoots from nodal explant.

in Table 1. The interactions among the four factors had major effects on shoot height and leaf number as they exhibited significant F values (Table 1).

Explants on cytokinin-free medium produced one unbranched shoot each, indicating strong apical dominance. Persistence of strong apical dominance is a major constraint in the

development of efficient in vitro procedures for clonal propagation of some plant species (George and Sherrington, 1984). Indeed, axillary branching in nodal explants occurred only when TDZ was applied exogenously in the present study. TDZ has an apical dominance release that accelerates shoot proliferation (Huetteman and Preece, 1993), which was observed in this study.

The clone often profoundly affects explant performance. Using shoot explants cultured on medium containing TDZ, Preece et al. (1991) and Kim et al. (1997) reported differences in axillary shoot proliferation among woody plant species. The clones in this study, which belong to two different subspecies, differed in their shoot multiplication and development potential (Tables 1 and 2). This result is reported in other studies on *Vaccinium* species including lingonberry (Debnath and McRae, 2001a; Serres et al., 1994) and cranberry (Debnath and McRae, 2001b; Marcotrigiano and McGlew, 1991; Smagula and Harker, 1997). Because cells within the same plant can have different endogenous levels of plant growth regulators and additional variation in receptor affinity or cellular sensitivity to plant growth regulators (Minocha, 1987), it is reasonable to expect that in vitro response will vary with clone. More studies on diverse genotypes, particularly within each subspecies, are required to further characterize genotypic variation of lingonberry responses to in vitro conditions.

In this study, clone 'EL1' exhibited better in vitro response than 'NL1' for axillary shoot proliferation (Tables 1 and 2). This trend continued with subsequent root formation and

plantlet establishment (data not shown). These results suggest that clone 'EL1' has further potential to serve as clonal plant material for in vitro studies of lingonberry.

In this study, shoots arising from node-associated callus in the medium at the explant base were termed 'short shoots' (basal shoots <1 cm long) and were not counted as axillary shoots because it was difficult to distinguish whether short shoots were of axillary or adventitious origin. Nevertheless, short-shoot numbers appeared to increase with increasing TDZ concentration for both clones (data not shown). Adventitious bud formation in lingonberries was reported by Serres et al. (1994) for shoots grown on medium with high cytokinin concentrations. One objective of this study was to establish clonal material of specific genotypes. Axillary shoot material, which is easier to handle, was more useful than short shoots for subsequent rooting and plantlet establishment. Because adventitious shoots may have an increased frequency of somaclonal variation (Huetteman and Preece, 1993) and the clonal fidelity of short shoots is more questionable than that of axillary shoots, TDZ concentrations of 5 μM or more should be avoided if nodal explants of lingonberries are to be cultured for clonal propagation. However, if the objective is to generate variability in a crop, a short callus step prior to adventitious shoot induction may prove effective.

Shoot elongation, rooting and acclimatization. Shoots of the clones 'NL1' and 'EL1' did not elongate >2 to 3 cm during the 8 weeks following culture initiation in BM containing 0.1 to 5 μM TDZ. To encourage shoot elongation, cultures were then transferred to BM containing 1 μM zeatin. Previous studies with adventitious shoots demonstrated that transferring clumps of adventitious shoots to BM with 1 μM zeatin stimulated shoot development and elongation (Debnath, 2004). Applying a similar treatment to clumps of axillary shoots to medium containing 1 μM zeatin improved shoot elongation within 4 weeks (Fig. 2).

The inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity (Huetteman and Preece, 1993). This has been reported in several fruit tree species including *Malus* (van Nieuwkerk et al., 1986) and *Populus* (Russel and McCown, 1986). In the present investigation, attempts to micropropagate shoots using the proliferation medium supplemented with TDZ were not successful due to excessive inhibition of shoot elongation. However, the TDZ effect could be overcome within 4 weeks by transferring TDZ-initiated cultures to medium containing 1 μM zeatin. Zeatin was also found to be effective for shoot initiation in *Vaccinium* species (Reed and Abdelnour-Esquivel, 1991), and for shoot proliferation of lingonberry (Debnath and McRae, 2001a) and highbush blueberry (Chandler and Draper, 1986; Eccher and Noe, 1989).

In vitro proliferated shoots for two clones rooted easily within 4 weeks, corroborating previous reports on lingonberry axillary shoots (Debnath and McRae, 2001a; Serres et al., 1994). Lingonberry microcuttings performed well in the greenhouse and plants acclimatized

readily to the greenhouse with survival rates of 80% to 90%.

Results achieved in this study demonstrate that 0.1 to 1 μM TDZ can induce maximum shoot proliferation in lingonberry but must be eliminated for shoot elongation. Zeatin (1 μM) must be used for shoot elongation before rooting. Compared to most other compounds with cytokinin activity, lower concentrations of TDZ can stimulate axillary shoot proliferation in many woody plants, whereas higher TDZ concentrations may result in the formation of both axillary and adventitious shoots (Chalupa, 1988; van Nieuwkerk et al., 1986). Shoot cultures of nodal explants became indistinguishable morphologically from the stock culture used as explant source, showing similar in vitro performance in terms of proliferation rate and rooting capacity.

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