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 µM) but inhibited shoot elongation. However, usable shoots were obtained within 4 weeks by transferring shoot cluster to medium containing 1 µM 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 Stansys, 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 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; Liebler, 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-thidiazol-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\(^-\)[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 microsalts 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 (5µM) 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.](image-url)
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 explant orientation, medium salt formulation, to determine the effect of TDZ concentration, the same medium as used for rooting, and ac techniques (Debnath and McRae, 2001a).

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 (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 from the humidity chamber (6 weeks).

For the initiation of callus formation, TDZ concentrations up to 1 µM were used. The interaction, clone × TDZ concentration was significant 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 ≤ 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 (Tables 1 and 2). 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

| Treatment | Shoots ('no./explant') | Shoot ht (cm) | Leaves ('no./shoot') |
|-----------|------------------------|---------------|---------------------|
| Clone     |                        |               |                     |
| ‘NL1’     | 1.4 b                  | 2.2 b         | 3.7 b               |
| ‘EL1’     | 1.5 a                  | 3.0 a         | 4.5 a               |
| Medium1   |                        |               |                     |
| 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*  |               |               |                     |
| C, M, T, E | CxT, MxT, MxE,E  | C, M, T, E, CxM, | C, M, T, E, MxT, MxE,E, |

*Data were collected after 8 weeks in culture.

*Mean separation within columns and factors by Duncan’s multiple range test, P ≤ 0.05, whereby means associated with different letters signify significant differences.

*Please see text for detail.

*Significant effects (P ≤ 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.

| Clone | Medium | TDZ (µM) | Shoot vigor (scale 1–8) | Callus size (scale 0–8) |
|-------|--------|----------|-------------------------|-------------------------|
|       |        |          | Vertical                | Horizontal              |
| 'NL1' | BM     | 0.0      | 5.7                     | 1.7                     |
| 'NL1' | BM     | 0.1      | 5.1                     | 4.4                     |
| 'NL1' | BM     | 1.0      | 3.4                     | 6.1                     |
| 'NL1' | BM     | 5.0      | 2.2                     | 7.4                     |
| 'NL1' | MMS    | 0.0      | 6.1                     | 1.2                     |
| 'NL1' | MMS    | 0.1      | 5.3                     | 4.6                     |
| 'NL1' | MMS    | 1.0      | 3.4                     | 6.0                     |
| 'NL1' | MMS    | 5.0      | 2.2                     | 7.1                     |
| 'EL1' | BM     | 0.0      | 5.9                     | 1.6                     |
| 'EL1' | BM     | 0.1      | 5.2                     | 5.4                     |
| 'EL1' | BM     | 1.0      | 4.6                     | 6.8                     |
| 'EL1' | BM     | 5.0      | 3.8                     | 7.3                     |
| 'EL1' | MMS    | 0.0      | 5.1                     | 1.8                     |
| 'EL1' | MMS    | 0.1      | 4.5                     | 5.1                     |
| 'EL1' | MMS    | 5.0      | 3.4                     | 6.5                     |
| 'NL1' | BM     | 1.0      | 3.4                     | 7.2                     |
| 'NL1' | BM     | 0.1      | 4.6                     | 7.6                     |

Significant effects (P ≤ 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.

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 macropropagate 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...
Grizzle, J.E., C.F. Starmer, and G.G. Koch. 1969. Analysis of categorical data by linear models. Biometrics 25:489–504.
Gustavsson, B.A. and V. Stanys. 2000. Field performance of ‘Sanna’ lingonberry derived by micropropagation vs. stem cuttings. HortScience 35:742–744.
Holloway, P.S. 1981. Studies on vegetative and reproductive growth of lingonberry (Vaccinium vitis-idaea L.). PhD diss. Univ. Minn., St. Paul.
Huetteman, C.A. and J.E. Preece. 1993. Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. 33:105-119.
Hulten, E. 1949. On the races in the Scandinavian flora. Svensk Botanik Tidskrift Bd. 43:383–406.
Jaakola, L., A. Tolvonen, K. Laine, and A. Hoholta. 2001. Effect of N-isopentenyladenine concentration on growth initiation in vitro and rooting of bilberry and lingonberry microshoots. Plant Cell Tiss. Org. Cult. 66:73–77.
Jamieson, A.R. 2001. Horticulture in Canada—Spotlight on the Atlantic provinces. Chronica Hort. 42:8–11.
Kim, M.K., H.E. Sommer, B.C. Bongarten, and S.A. Merkle. 1997. High frequency induction of adventitious shoots from hypocotyl segments of Liquidambar styraciflua L. by thidiazuron. Plant Cell Rpt. 16:536–540.
Koch, G.G., I.A. Amara, G.G. Davis, and D.B. Gillings. 1982. A review of some statistical methods for covariance analysis of categorical data. Biometrics 38:563–595.
Liebster, G. 1977. Experimental and research work on fruit species of the genus Vaccinium in Germany. Acta Hort. 61:19–24.
Luby J.J., J.R. Ballington, A.D. Draper, K. Pliszka, M.J. Hulten, E. 1949. On the races in the Scandinavian flora. Svensk Botanik Tidskrift Bd. 43:383–406.
Murthy, B.N.S., S.J. Murch, and P.K. Saxena. 1998. Analysis of parameters determining the efficiency of in vitro cytokinin treatments on flowering time in tobacco. J. Amer. Soc. Hort. Sci. 116:142–148.
Racz, G., J. Fuzi, and L. Fulop. 1962. A method for determination of the arbutin content of cowberry leaves (Folium vitis-idaea). Rumanian Med. Rev. 6:88–90 (abstr.).
Reed, B.M. and A. Abdelnour-Esquivel. 1991. The use of zeatin to initiate in vitro cultures of Vaccinium species and cultivars. HortScience 6:1320–1322.
Russel J.A. and B.H. McCown. 1986. Thidiazuron stimulated shoot differentiation from protoplast derived calli of Populus, p. 49. In: Abstracts of 6th Intl. Cong. on Plant Tissue and Cell Cultures, Univ. Minn., Minneapolis.
Sankhla, D., T.D. Davis, N. Sankhla, and A. Upadhyaya. 1995. In vitro regeneration of heat tolerant ‘German Red’ carnation through organogenesis and somatic embryogenesis. Gartenbauwissenschaft 60:220–232.
Seneviratne, P. and A. Flagmann. 1996. The effect of thidiazuron on axillary shoot proliferation of Hevea brasiliensis in vitro. J. Rubber Res. Inst., Sri Lanka 77:1–14.
Serres, R.A., S. Pan, B.H. McCown, and E.J. Stang. 1994. Micropropagation of several lingonberry cultivars. Fruit Var. 46:7–14.
Singh S.K. and M.M. Syamal. 2001. A short pre-culture soak in thidiazuron or forchlorfenuron improves axillary shoot proliferation in rose micropropagation. Sci. Hort. 91:169–177.
Smagula, J.M. and J. Harker. 1997. Cranberry micropropagation using a lowbush blueberry medium. Acta Hort. 446:343–347.
Stanek III, E.J., S.R. Diehl, M. Dgetluck, M.E. Stokes, and R.J. Prokopy. 1987. Statistical methods for analyzing discrete responses of insects tested repeatedly. Environ. Entomol. 16:319–326.
Stark, R., I.V. Hall, and P.A. Hendrickson. 1978. The arbutus industry in Newfoundland. Canadex (Hort. Crops) 230.
Tao, W. and J. Verbelen. 1996. Switching on and off cell division in cultured mesophyll protoplasts of tobacco. Plant Sci. 116:107–115.
van Nieuwkerk, J.P., R.H. Zimmerman, and I. Fordham. 1986. Thidiazuron stimulation of apple shoot proliferation in vitro. HortScience 21:516–518.
Vander Kloet, S.P. 1988. The genus Vaccinium in North America. Agr. Can. Publ. 1828.
Visser, J.A., J.A. Qureshi, R. Gill, and P.K. Saxena. 1992. Morphoregulatory role of thidiazuron: Substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in geranium hypocotyl cultures. Plant Physiol. 99:1704–1707.
Wang, H., G. Cao, and R.L. Prior. 1997. Oxygen radical absorbing capacity of anthocyanins. J. Agr. Food Chem. 45:304–309.

In Newfoundland and Labrador and the potential for expansion utilizing European cultivars. Acta Hort. 446:139–142.