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## PROPAGATION AND CULTIVATION OF VACCINIUM SPECIES AND LESS KNOWN SMALL FRUITS
## VACCINIUM ĢINTS SUGU UN MAZĀK ZINĀMO AUGĻAUGU PAVAIROŠANA UN AUDZĒŠANA

**Samir C. Debnath**
Atlantic Cool Climate Crop Research Centre, Agriculture and Agri-Food Canada,
P.O. Box 39088, 308 Brookfield Road, St. John's, NL A1E 5Y7, Canada,
e-mail: Samir.Debnath@AGR.GC.CA

**Abstract**
The production of *Vaccinium* species crops has recently been the subject of much interest globally because of an improved understanding of the important role of dietary fruit in maintaining human health. Cloudberry (*Rubus chamaemorus* L., family *Rosaceae*), a less known small fruit of medicinal importance, and the Vaccinium species are genetically heterozygous and do not reproduce progeny from seed that are similar to the seed parent. Tremendous progress in plant tissue culture, resulting in great advances in micropropagation, has occurred in these crops. Of particular significance has been the evolution of the technology permitting multiplication of these plants through bioreactor micropropagation. The in vitro morphogenesis seems to be highly dependent on the plant growth regulators and media used for the culture, which is again genotype specific. Although the automation of micropropagation in bioreactors has been advanced as a possible way of reducing propagation cost, optimal plant production depends upon a better understanding of the physiological and biochemical responses of plants to the signals of the culture microenvironment and an optimization of specific physical and chemical culture conditions to control the morphogenesis of berry plants in liquid culture systems. Clonal fidelity can be a serious problem and molecular strategies have been developed to reduce the variation to manageable levels. The paper focuses on conventional and bioreactor systems used for the in vitro culture of the *Vaccinium* species and cloudberry, cultivation of micropropagules and the employment of molecular markers in micropropagated plants for the assessment of genetic fidelity, uniformity, stability and true-to-typeness among donor plants and tissue culture regenerants.

**Kopsavilkums**
*Vaccinium* ģints kultūraugu audzēšanai pēdējā laikā visā pasaulē pievērsta pastiprināta uzmanība, jo arvien labāk tiek izprasta šo augļu diētiskā nozīme cilvēka veselības saglabāšanai. Lācenes (*Rubus chamaemorus* L., *Rosaceae* dzimta) – mazāk zināmi augļi ar medicīnisku nozīmi un *Vaccinium* ģints sugas ir ģenētiski heterozigoti un, pavairojot ar sēklām, nedod vecākiem līdzīgus pēcnācējus. Milzīgais progress augu šūnu kultūru izpētē ir izraisījis arī lielus uzlabojumus šo kultūraugu mikropavairošanā. Īpaši nozīmīga ir bijusi tādas tehnoloģijas attīstība, kas ļauj šo augu mikropavairošanu veikt bioreaktorā. Morfoģenēze *in vitro* apstākļos ir ļoti atkarīga no kultivēšanā izmantotajiem augu augšanas regulatoriem un barotnes, kas ir atkarīga no genotipa. Lai gan mikropavairošanas automatizācija bioreaktoros ir attīstīta kā iespējamais pavairošanas izmaksu samazināšanas ceļš, optimāla stādu ražošana ir atkarīga no labākas izpratnes par augu bioķīmisko un fizioloģisko reakciju uz mikrovides signāliem un specifisku fizikālu un ķīmisku kultivēšanas apstākļu optimizācijas, lai kontrolētu ogaugu morfoģenēzi šķidrās kultivēšanas sistēmās. Klonālā mainība var būt nopietna problēma, tāpēc ir izveidotas molekulāras metodes, lai samazinātu šo mainību līdz iespējami zemākam līmenim. Šajā rakstā lielākā uzmanība pievērsta konvencionālajām un bioreaktora sistēmām *Vaccinium* ģints kultūraugu un lāceņu *in vitro* pavairošanai, mikropavairoto augu kultivēšanai un molekulāro marķieru izmantošanai ģenētiskās atbilstības, viendabīguma, stabilitātes un autentitātes novērtēšanai starp donora augiem un audu kultūrā iegūtajiem stādiem.

**Key words:** cloudberry, propagation, micropropagation, *in vitro*, genotype.

**Introduction**
The genus *Vaccinium* L. (family: *Ericaceae*) contains about 400 species, and one or more species are native to all continents except Antarctica and Australia (Vander Kloet, 1988; Ballington, 2001). It is typically characterized as having fleshy, more-or-less edible fruits with very high levels of vitamin C, cellulose, pectin and anthocyanins possessing antitumor, antiulcer, antioxidant and antiinflammatory activities (Wang *et al.*, 1999). The proanthocyanidins in cranberries have been shown to help prevent urinary tract infections through reduced adhesion of uropathogenic Escherichia coli (Howell *et al.*, 2005). Lingonberry fruits and leaves are used to lower cholesterol levels and treat stomach disorders, rheumatic diseases, and bladder and kidney infections (Novelli, 2003). Blueberry (*Vaccinium* spp.), cranberry (*V. macrocarpon* Ait.), and lingonberry (*V. vitis-idaea* L.) are three commercially cultivated *Vaccinium* fruit crops of economic importance. Although the majority of cultivated blueberry hectarage is in the United States and in Canada, they are also grown commercially in Europe, Asia, Africa, Australia, New Zealand and South America (Lehnert, 2008). While the leading countries in cranberry production are the United States, Canada, Latvia and Poland; its culture has also shown promise in Austria, Germany and Russia (http:////aesop.rutgers.edu/~bluecran/cranberrypage.htm).
Commercial lingonberry production primarily involves the harvesting of berries from wild populations in northern Europe, Asia and North America, with cultivated production still in its infancy compared with cranberries and blueberries (Ballington, 2001). The cloudberry (*Rubus chamaemorus* L., family *Rosaceae*), a less known small fruit crop, is a boreal circumpolar, rhizomatous dioecious perennial herb common to bogs. The berries and leaves of cloudberry are rich in vitamin C and tannins, and possess high ellagic acid content (Amakura *et al.*, 2000). Cloudberries are used medicinally to treat scurvey and diarrhea in traditional medicine (Thiem, 2003).
Although conventional vegetative propagation methods by cuttings or rhizome divisions are successful in these species, the micropropagation of selected germplasm can potentially multiply plants more rapidly than traditional propagation methods. Various culture conditions, basal media and growth regulators have been investigated for the micropropagation of these crops on semi-solid gelled media (for review, please see Debnath, 2003a, 2006a, 2007a).
However, these techniques are difficult to automate and the production cost is high. Automated bioreactors for large scale production of micropropagated plants are important for the micropropagation industry. Bioreactors are self-contained, sterile environments which capitalize on liquid nutrient or liquid/air inflow and outflow systems, designed for intensive culture and control

over microenvironmental conditions – aeration, agitation, dissolved oxygen, etc. (Paek *et al.*, 2005). This review provides an overview of in vitro culture and the production of micropropagated plants of blueberry, cranberry, lingonberry and cloudberry, and also highlights the research efforts of our programme at the Atlantic Cool Climate Crop Research Centre of Agriculture and Agri-Food Canada in St. John's, Newfoundland and Labrador.

Blueberry. There are five major groups of blueberry species which are commercially-grown: 1) lowbush (*V. angustifolium* Ait., *V. myrtilloides* Michx., V. boreale Hall and Aald.), 2) highbush (*V. corymbosum* L.), 3) half-high, which are hybrid or backcross derivatives of highbush-lowbush hybridizations; 4) southern highbush, which were developed from the hybridization of *V. corymbosum* with one or more species (mainly V. darrowi Camp and V. ashei Reade); and 5) rabbiteye (*V. ashei*). Micropropagation techniques using gelled media for axillary shoot production have been developed for lowbush (Debnath, 2004, 2007b), highbush (Gajdošová *et al.*, 2006; Litwińczuk and Wadas, 2008; Tetsumura *et al.*, 2008) and rabbiteye (Lyrene, 1980) blueberries.

Shoot cultures can be initiated from nodal segments or from shoot tips. Media with low ionic concentrations are suitable for *Vaccinium* culture (Debnath and McRae, 2001a). While the woody plant medium (WPM) (Lloyd and McCown, 1980) was the best for highbush blueberry micropropagation (Sedlak and Paprstein 2009), Debnath (2004, 2007b) established in vitro lowbush blueberry cultures on a modified cranberry tissue culture medium (BM-C) (Debnath and McRae, 2001a).

Tetsumura *et al.* (2008) observed a mixture of equal parts of Murashige and Skoog (1962) (MS) and WPM containing 20 µM zeatin was the best for in vitro shoot proliferation of highbush blueberry cultivars. Zeatin was effective for shoot initiation and proliferation of lowbush blueberries (Debnath, 2004) although Gonzalez *et al.* (2000) observed the best shoot multiplication of highbush blueberry with 25 µM N6-[2-isopentenyl] adenine (2iP) in the culture medium.

A low concentration of an auxin [5.7 µM 3-indolyl-acetic acid (IAA)] is beneficial when added to the induction medium (Morrison *et al.*, 2000). However, using low levels of zeatin (2-4 µM) and sucrose (20 g $l^{-1}$), Debnath (2004) reported an increased the in vitro-shoot multiplication rate of the lowbush blueberry by about 50 to 100-fold over a 12-week interval when shoots were exposed to lower irradiance (15 µmol $m^{-2}$ $s^{-1}$). A major problem in blueberry micropropagation is the formation of unwanted callus at the base of the explants and the occurrence of spontaneous adventitious shoots (Litwińczuk and Wadas, 2008).

Cao *et al.* (2002) reported shoot regeneration in the highbush blueberry based on a two-step pre-treatment and regeneration on TDZ medium. Explants of 2-week-old shoot cultures were incubated the following regime: pretreatment medium # 1 containing 5 µM TDZ and 2.6 µM naphthalene acetic acid (NAA) for 4 days, pretreatment medium #2 containing 7 µM zeatin riboside and 2.6 µM NAA for 3 days, regeneration medium containing 1 µM TDZ for 6 weeks, and last on a medium without growth regulators for 10 days. Debnath (2009a) developed a two-step shoot regeneration protocol in lowbush blueberry where leaf cultures produced multiple buds and shoots on 2.3–4.5 µM TDZ within 6 wk of culture initiation. The greatest shoot regeneration came from young expanding basal leaf segments positioned with the adaxial side touching the culture medium and maintained for 2 weeks in darkness. TDZ-initiated cultures were transferred to a medium containing 2.3–4.6 µM zeatin and produced usable shoots after one additional subculture.

Application of bioreactor micropropagation in Vaccinium crops is still at the infancy stage. A protocol for Vaccinium micropropagation using a temporary immersion bioreactor (TIB) system in a liquid medium combined with a in vitro culture on a semi-solid gelled medium has been developed in the author's laboratory. Successful shoot regeneration and proliferation have been obtained in the lowbush blueberry (Figure 1.), cranberry and lingonberry (S. C. Debnath, unpublished).

*In vitro*-derived shoots are rooted either in vitro (Litwińczuk and Wadas, 2008; Tetsumura *et al.*, 2008) or, most frequently, in *ex vitro* conditions on an acidic substrate such as 1 peat : 1 perlite (v/v) (Gonzalez *et al.*, 2000) and 4 peat : 2 vermiculite : 1 perlite (v/v/v) (Morison *et al.*, 2000) without an auxin-pretreatment. An auxin-pretreatment was unnecessary for the *ex vitro* rooting of blueberries (Gonzalez *et al.*, 2000) although Debnath (2009a) found 80 % to 90 % rooting in lowbush blueberries when microshoots were dipped in 4.9 mM 3-indolebutyric acid (IBA) before planting in 3 peat : 2 perlite (v/v) medium. For *ex vitro* rooting, the microcuttings are generally

maintained in a mist chamber with very high relative humidity (95 %) and then transferred to a greenhouse (85 % relative humidity, RH) for acclimatization. In vitro rooting can be induced in the shoot proliferation medium containing 1-2 µM zeatin (S.C Debnath, personal communication) or without plant growth regulators (PGR) (Tetsumura *et al.*, 2008).

Figure 1. Shoot proliferation of wild lowbush blueberry 12 weeks after transfer to a bioreactor system containing liquid medium supplemented with 2 µM zeatin.

Cranberry. Marcotrigiano and McGlew (1991) and Smagula and Harker (1997) recommend a high 2iP concentration along with an auxin (IAA or IBA) in the culture media to increase cranberry shoot proliferation. Debnath and McRae (2001b) established *in vitro* cranberry cultures and maintained them in a medium containing low levels of cytokinin to avoid excessive callus formation at the base of explants and the formation of somaclonal variants.

Shoot organogenesis from cranberry explants has been reviewed by McCown and Zeldin (2005). A number of factors such as genotype, culture medium (including growth regulators and their combinations), the physical environment, the explant development stage, etc. can affect adventitious shoot regeneration. Qu *et al.* (2000) regenerated shoots from cranberry leaves by culturing on a basal medium supplemented with 10 µM TDZ + 5 µM 2iP. Elongation of adventitious shoots began 2 weeks after transfer to the basal medium without growth regulators.

Both *in vitro* and *ex vitro* methods have successfully been used to root and acclimatize micropropagated cranberry shoots (Qu *et al.*, 2000; Debnath and McRae, 2001b). For *in vitro* rooting, shoots are cut at the base and then placed onto an auxin-free medium (Qu *et al.*, 2000; Debnath and McRae, 2001b, 2005). *In vitro*-derived shoots (>1.5 cm long) can also be rooted *ex vitro* in shredded sphagnum moss in pots (Qu *et al.*, 2000).

Debnath and McRae (2005) developed a protocol that enables cranberry multiplication in one step, i.e. multiplying shoots and having them rooted in the same culture medium containing 2-4 µM zeatin. The main advantage of this protocol is that all the shoot tips of the *in vitro*-grown plantlets can be used for shoot proliferation and rooting, whereas basal rooted nodal segments can be transferred to the peat-perlite medium and acclimatized in the greenhouse (Debnath, 2008).

Lingonberry. Lingonberries grow wild in diverse habitats, ranging from lowland to upland and mountain areas, in largely acid soils to pure peat bogs (Gustavsson, 1997). 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).

Various culture conditions, basal media, and growth regulators have been investigated for axillary shoot proliferation of the lingonberry (Debnath and McRae, 2001a; Jaakola *et al.*, 2001; Debnath, 2005a, b). Debnath and McRae (2001a) compared four different media for the shoot proliferation of lingonberry cultivars: 'Regal', 'Splendor' and 'Erntedank', and found that a reasonable balance of shoot multiplication rate and desirable growth characteristics was attained in a new medium

(BM-C) formulated in the author's laboratory (Debnath and McRae, 2001a). Debnath (2005a) observed that TDZ supported shoot proliferation in lingonberries at low concentrations (0.1 to 1 µM) but inhibited shoot elongation. However, usable shoots were obtained within 4 weeks by transferring shoot clusters to the culture medium containing 1µM zeatin. In the lingonberry, shoot proliferation is greatly influenced by explant orientation, changing the orientation of explants from vertically upright to horizontal increases the axillary shoot number, but decreases shoot height and leaf number per shoot (Debnath, 2005a). Debnath (2005b) observed that the best response was afforded by sucrose at 20 g $l^{-1}$ both in terms of explant response and shoot development potential, although glucose supported shoot growth equally well, and in a wild clone at 10 g $l^{-1}$ it resulted in better in vitro growth than sucrose.

The first adventitious shoot regeneration from lingonberry leaves was described by Debnath and McRae (2002). Later, the regeneration efficiency has been much improved by Debnath (2005c) where leaf explants were cultured on the 1-5 µM TDZ-containing a nutrient medium for 8 weeks for bud and shoot regeneration followed by transferring on to the medium containing 1-2 µM zeatin for shoot elongation. Adventitious shoots have also been regenerated from hypocotyl segments of seedlings from open-pollinated seeds of lingonberry cultivars and a wild clone (Debnath, 2003b). Multiple bud and shoot regeneration can be obtained using apical segments of the hypocotyl from in vitro-grown lingonberry seedlings by incorporating 5-10 µM TDZ in the regeneration medium. Such TDZ-induced buds can be proliferated and elongated on a shoot proliferation medium containing 1-2 µM zeatin and 20 g $l^{-1}$ sucrose. Callus, bud, and shoot regeneration frequency, callus growth, and the number of buds and shoots per regenerating explant depend not only on the specific segment of the hypocotyl, but also on the parental genotype (Debnath, 2003b).

For rooting, 3 to 4 cm long *in vitro*-derived shoots are excised just above the original explant, dipped in 39.4 mM IBA powder, planted in a 2 peat : 1 perlite (v/v) medium and maintained in a humidity chamber [(22 ∀ 2EC, 95 % RH, 16 h photoperiod, 55 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux (PPF)]. *In vitro* proliferated shoots root easily within 4 weeks. Plantlets can be acclimatized by gradually lowering the humidity over 2 to 3 weeks and hardened-off plants can be maintained in the greenhouse at 20 ∀ 2EC, 85 % RH, and 16 h photoperiod at a maximum PPF of 90 µmol $m^{-2}$ $s^{-1}$ (Debnath and McRae, 2001a, 2002; Debnath, 2003a, b; 2005a, b, c).

Cloudberry. *In vitro* propagation of cloudberry has been reported in a gelled medium through axillary shoot initiation from seedling explants (Thiem, 2001) and through meristem cultures (Martinussen *et al.*, 2004). When meristem cultures were sub-cultured from clusters of 3 – 5 shoots, approximately 70 and 50 shoots were produced per cluster within 6 weeks at 8.9 µM BAP for the female cv. 'Fjellgull' and the male cv. 'Apollen', respectively. The addition of 5.5 µM gibberellic acid (GA3) reduced the number of shoots. Auxins (IBA, NAA) promoted root development *in vitro*, but inhibited the formation of new shoots (Martinussen *et al.*, 2004).

Debnath (2007c) established a protocol for the *in vitro* culture of wild cloudberry clones using a bioreactor system combined with a gelled medium. Cultures were established on a modified cranberry (*V. macrocarpon* Ait.) tissue culture medium containing 8.9 µM BAP. The addition of 5.8 µM GA3 in 8.9 µM BAP-contained medium improved shoot proliferation. TDZ supported rapid shoot proliferation at low concentration (1.1 µM) but induced a 20 to 30 % hyperhydricity in a plastic airlift bioreactor system containing a liquid medium. The bioreactor-multiplied hyperhydric shoots were transferred to a gelled medium containing 8.9 µM BAP and 5.8 µM GA3 and produced normal shoots within 4 weeks of culture. Proliferated shoots were rooted on a potting medium with a 65 % to 75 % survivability rate of rooted plants. Growth and morphology of micropropagated plants. Increased branching and vigorous vegetative growth are often noted in plants produced through *in vitro* culture. Morrison *et al.* (2000) observed that micropropagated lowbush blueberry plants from shoots that passed through several subcultures produced ten-fold more rhizomes than those of stem cuttings. The micropropagated lowbush blueberry plants produced longer and more stems with more leaves per stem than the conventional cuttings (Debnath, 2007b).

Softwood cutting-derived 'Herbert' highbush plants grew more slowly and produced less and shorter shoots than micropropagated ones, although the majority of cutting-propagated plants developed flowers earlier, flowered more abundantly and bore larger berries than those of tissue culture plants (Litwiczuk *et al.*, 2005). Micropropagated cranberry plants have an excellent juvenile

period and produce vigorous vegetative growth (mostly runners) during their first season but do not produce flowers until their third growing season (Serres and McCown, 1994). Micropropagated 'Bergman', 'Pilgrim' and 'Stevens' plants produced more runners and uprights with more leaves per upright than the conventional cuttings (Debnath, 2008).

Debnath (2005d, 2006b) observed that the *in vitro*-derived lingonberry plants produced more stems, leaves and rhizomes than the conventional cuttings. Under field condition, rhizome production and total plant weight were greater for tissue culture plants than for stem cuttings in the lingonberry cultivar, 'Sanna' (Gustavsson and Stanys, 2000). After 4 years of growth, the tissue culture plants of 'Splendor' and 'Erntedank' lingonberries produced berries with more antioxidant activity, although the berry diameter, number and yield per plant were higher in the stem cutting plants (Foley and Debnath, 2007).

## Conclusions

The commercial propagation of the *Vaccinium* species and cloudberry by tissue culture is becoming increasingly common as it is a reliable and efficient method, especially for the rapid introduction of new cultivars. In breeding programs, the technique can provide advantages in: (i) the mass production of elite selections and for analysis in a replicated trial of new releases, (ii) germplasm conservation, (iii) accelerating the breeding process by in vitro selection.

Large-scale liquid cultures combined with automated bioreactors can eliminate most manual handling in micropropagation and decrease production costs significantly. Cultures in liquid medium are advantageous for several plant species but may limit the gas exchange of the plant materials and often cause asphyxia and hyperhydricity, resulting in malformed plants and loss of material.

True-to-type propagules and genetic stability are prerequisites for the application of micropropagation. Molecular markers are powerful tools in the genetic identification of clonal fidelity. Special classes of markers including restriction fragment length polymorphism (RFLP), random-amplified polymorphic DNA (RAPD), arbitrary primed PCR (AP-PCR), DNA amplified fingerprinting (DAF), simple (short) sequence repeat (SSR), short tandem repeat (STR), sequence characterized amplified region (SCAR), sequence-tagged sites (STSs), amplified fragment length polymorphism (AFLP) and inter simple sequence repeat (ISSR) are appropriate for genetic analysis of tissue culture-raised plants. RAPD and ISSR marker analyses have been developed in the author's laboratory to identify genetic diversity in the Vaccinium species (Debnath, 2007d, 2009b) and in cloudberry germplasm (Debnath, 2007e), and can be used to study the clonal fidelity of the micropropagated plants of these species.

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## CLIMATICALLY DETERMINATE PROJECTIONS OF RESOURCES OF VACCINIUM SPECIES IN BELARUS TO 2050
## KLIMATISKI NOTEIKTA PROGNOZE VACCINIUM ĢINTS SUGĀM BALTKRIEVIJĀ LĪDZ 2050. GADAM

**Valery Grimashevich**
Forest Institute of the National Academy of Sciences of Belarus,
Gomel, 220012 Minsk str. Surganova, 2v, Belarus, e-mail: grimashevich@gmail.com

### Abstract
Based on the predicted dynamics of the forest fund in terms of global climate change we have made a climatically determinate projection of the resources of major species of wild berry plants, namely, cowberry (*Vaccinium vitis-idaea* L.), bog blueberry (*Vaccinium uliginosum* L.), European cranberry (*Oxycoccus palustris* Pers.) and bilberry (*Vaccinium myrtillus* L.), in Belarus to the year 2050 at 5-year intervals. The amounts of European cranberries and bilberries may increase, while those of cowberries and bog blueberries may decrease towards the end of the forecast period. On the whole the amounts of all the principal wild berry plants may increase by 11 percent.