Antioxidant Properties of Lingonberry (*Vaccinium vitis-idaea* L.) Plants

By

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ST. JOHN'S NEWFOUNDLAND & LABRADOR CANADA

This work is dedicated to my parents

Abstract

Lingonberry (*Vaccinium vitis-idaea* L.) is an economically important fruit crop conventionally propagated by vegetative stem cutting. The present study is an investigation of the effects of different propagation methods as well as different geographical locations on the antioxidant properties of lingonberry plants. The study also aims to determine neuroprotective effects of the lingonberry fruits and leaves against glutamate-mediated excitotoxicity. In this study, it was observed that the leaves of *in vitro*- derived plants exhibited significantly higher antioxidant enzyme activities as compared to those obtained from *ex vitro* propagation. The total soluble phenolics, tannins, and flavonoids were enhanced in fruits of the *in vitro*-propagated plants, whereas in leaves, the levels of these metabolites (except flavonoids) were decreased in the *in vitro*-derived plants.

The study determined that the lingonberry clones collected from different geographical locations showed variability in terms of their antioxidant compounds. A positive correlation was observed between the levels of antioxidant compounds and latitude, altitude, reduced temperature and increased precipitation. Although the clones have been maintained in the same greenhouse environment under similar conditions for about 10 years since their collection date, the climatic conditions had an effect on their adaptation at the developmental stage influencing plant genotypes.

The effect of lingonberry extracts were determined on cells subjected to excitotoxicity by treating brain cell cultures of 1 to 2 day old rat pups with glutamate

(100 μM) for 24 hours in order to damage the cells by excitotoxicity. Glutamate treatment caused a ~20% cell loss when measured after 24 hours of exposure. While lingonberry fruit extract did not provide protection from glutamate toxicity, leaf extracts showed a significant neuroprotective effect. The greater protective effect of leaf extracts was in correlation with the levels of phenolics and antioxidant capacity.

In conclusion, the findings in this dissertation suggest that the tissue culture propagation technique has great advantages especially in enhancing antioxidant compounds as well as for increasing vegetative growth in lingonberry plants. Our findings also suggest that antioxidant levels increase with reduced temperature, increased precipitation, latitude and altitude. Overall, the antioxidant capacity of lingonberry leaves would be potentially beneficial for neuro-protection and slowdown of brain aging, and consumption of lingonberry products could have positive effect on human health.

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ABBREVIATIONS

AFR	Ascorbate Free radical
ATP	Adenosine Triphosphate
APX	Ascorbate Peroxidase
AMPA	2-Amino-3-(3-Hydroxy-5-Methyl-Isoxazol-4-yl) Propanoic Acid
AsA	Ascorbic Acid
Asc	Ascorbate
ANOVA	Analysis of Variance
BHT	Butylated Hydroxtoulene
BHA	Butylated Hydroxyanisole
BM	Basal Medium
Ca^{2+}	Calcium Ion
CCD	Charge Coupled Device
Cu^{2+}	Copper Ions
CE	Catechin Equivalent
DAPI	4',6-Diamidino-2-Phenylindole
DCMU	Dichlorophenyldimethylurea
DHA	Dehydroascorbate
DHAR	Dehydroascorbate Reductase
DNA	Deoxyribonucleic acid

DNTB	5-5'-Dithiobis (2-Nitrobenzoic Acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
DTT	Dithiothretol
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron Spin Spectroscopy
ESI	Electron Spray Ionization
Fe^{2+}	Iron Ions
GAE	Gallic Acid Equivalents
GSH	Glutathione
GSHPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSSG	Glutathione Disulphide
GPx	Glutathione Preoxidase
HBSS	Hank's Buffered Salt Solution
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
H_2O_2	Hydrogen peroxide
HNE	4-Hydroxy-2-Nonenal
HPLC	High Performance Liquid Chromatography
IP_3	Inositol Trisphosphate
LC	Leaf Culture
LC/MSD	Liquid Chromatography–Mass Spectrometer Detector

LOOH	Lipid Hydroperoxide
MDHAR	Monodehydroascorbate Reductase
MDHA	Monodehydroascorbate
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NC	Nodal Culture
NMDA	N-Methyl-D-Aspartate
$ONOO^-$	Peroxynitrite
*OH	Hydroxyl free radical
$O^{\bullet 2-}$	Superoxide
8-OHdG	8-Hydroxy-2′-Deoxyguanosine
PG	Propyl Gallate
PLO	Poly-L-Prnithine
PPF	Photosynthetic Photon Flux
PrI	Propidium Iodide
PBS	Phosphate Buffer Solution
PUFA	Poly Unsaturated Fatty Acids (PUFA)
RH	Relative Humidity
ROPs	Rho-like small GTPases from Plants

RNS	Reactive Nitrogen Species
SOD	Superoxide Dismutase
TBHQ	Tertiary Butyl Hydroquinone
SC	Stem Cutting
TNB	5-thionitrobenzoic acid
MES	2-(N-morpholino) Ethanesulfonic Acid
MDA	Malondialdehyde Equivalents
mGluRs	Metabotropic Glutamate Receptors
KOH	Potassium Hydroxide
TAC	Total Anthocyanin Content
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic acid
TFC	Total Flavonoid Content
TPC	Total Soluble Phenolic Content
TTC	Total Tannin Content

Chapter 1

Literature Review

The goal of this chapter is to provide a basic introduction to lingonberry plants and their propagation methods. The fundamental ideas and basic terms implemented throughout this dissertation i.e., oxidants, reactive oxygen species, antioxidants, etc are also described in this chapter. To further illustrate plant antioxidant systems, their components are presented briefly.

1.1. Introduction

Berries of *Vaccinium* species are known for their antioxidant activities and have been extensively studied in the past decade. Blueberry (*Vaccinium angustifolium* Aiton), cranberry (*Vaccinium oxycoccos* L.) and lingonberry (*Vaccinium vitis-idaea* L.) are the three commercially important berry crops of Vaccinium species. Other berries of Vaccinium species include bilberry (*Vaccinium myritillus* L.), red huckleberry (*Vaccinium paryifolium* Sm.), sparkleberry (*Vaccinium arboreum* M.) and creeping blueberry (*Vaccinium crassifolium* Andr.). Vaccinium berries are characterized by fleshy fruits with high ascorbate and anthocyanins levels (Yao & Vieira, 2007). Berries contain micronutrients that are essential for human health and are rich source of phenolic compounds, such as phenolic acids, flavonoids and anthocyanins (Zheng & Wang, 2003). Health benefits of berries have been reported by several research groups. It was reported that these fruits possess antioxidant, anti-tumor, anti-ulcer and anti-inflammatory activity (Zafra-Stone *et al.,* 2007). Berries have been shown to exhibit a wide range of biological effects such as

anticarcinogenic (Ames, 1983; Knekt *et al.,* 1997; Juranic *et al.,* 2005; Mertens-Talcott *et al.,* 2003) and protection against cardiovascular diseases (Hertog 1993; Keli *et al.,* 1996) and some other degenerative diseases caused by oxidative stress (Ames *et al.*, 1993). Many species from this family are utilized as commercially important fruit crops, medicinal plants and ornamental landscape ground cover (Jaakola *et al*., 2001). Cranberry proanthocyanidins have shown to maintain urinary tract health (Foo *et al.*, 2000a,b; Howell *et al.*, 2005) and inhibit acid-induced proliferation of human esophageal adenocarcinoma cells (Kresty *et al*., 2008). A plethora of literature is available on wild blueberries in animal model systems emphasizing their protective effects (Norton *et al.*, 2005; Kela *et al.*, 2009; Kristo, *et al*., 2010; Del Bo' *et al*., 2013). It has been reported recently that the consumption of wild blueberries for six weeks significantly decreases oxidized DNA and increases resistance to damage caused by oxidative stress (Riso *et al*., 2012).

The protective effects of these berries against the diseases and disorders mentioned above are attributed to antioxidant compounds especially polyphenolic compounds and some other low molecular weight phytochemicals. A correlation between antioxidant activities and the total phenolic content has been reported in raspberries (Anttonen & Karjalainen, 2005). Our studies on lingonberry support this correlation.

1.2. Lingonberry: Biology and Systematics

Lingonberry (*Vaccinium vitis-idaea* L.), also called partridgeberry in Newfoundland and redberry in Labrador, is a rhizomatous low creeping evergreen shrub which grows in Eurasia and North America (Luby *et al.,* 1991). There are as many as 25 regional names of lingonberry such as northern mountain cranberry, mountain bilberry, lingberry, foxberry and cowberry (Burt & Penhallegon, 2003). The morphological characteristics of lingonberry include fused petals, leathery leaves and terminal flower buds (Rabaey *et al.,* 2006). Lingonberry fruits are bright red to dark red in color and weigh 0.17 to 0.45 g and flowers are bell-shaped and white or pink in color (Penhallegon, 2006).

Newfoundland and Labrador is the largest lingonberry producing region in North America (Penny *et al.,* 1997), with about 140,000 kg harvested annually from native stands for processing, mostly for export (Jamieson, 2001). Lingonberry can withstand harsh environmental conditions and hence it is a widely distributed plant species. It has a circumpolar and circumboreal distribution (Trajkovski, 1987; Small *et al.,* 2003). Furthermore, acidic soils with pH range from 4.3 to 5.5 are favorable for lingonberry growth (Ailor & Penhallegon, 1999).

1.2.1. Lingonberry Taxonomy

Lingonberry (*Vaccinium vitis-idaea* L.) belongs to the family Ericaceae and genus Vaccinium. The Ericaceae is also known as the heather family and is geographically widespread. In Canada, 18 genera represent some of the most important native medicinal and

edible plants within this family include blueberry, bilberry, cranberry, Labrador tea and bearberry (Scoggan, 1979). This family is highly ornamental and includes mostly shrubs and small trees.

The Ericaceae family belongs to the order Ericales which consists of about 4500 species of plants which are divided into 8 families with 160 genera (Lens *et al.,* 2003). The eight families are: Cyrillaceae, Clethraceae, Grubbiaceae, Empetraceae, Epacridaceae, Pyrolaceae, Monotropaceae, and Ericaceae. The family Ericaceae consists of about 90% species of the order Ericales. It was reported that the Ericaceae family is amongst the top 10 families among plants used as traditional medicine (Saleem *et al.,* 2010).

Harborne and Williams (1998) have reported the distribution of flavonoids and simple phenolics in the leaves of 334 species of Ericaceae and found that these species are rich source of flavonoids and simple phenolics (Saleem *et al.,* 2010).

1.2.2. Lingonberry Types

There are two subspecies of lingonberry: (1) *Vaccinium vitis-idaea* ssp. L. *vitis-idaea* Britton, which is widespread in Europe and northern Asia (2) *Vaccinium vitis-idaea* L. ssp. *minus* Hult, which is abundant in Iceland, Greenland, North America, Northern Asia and Scandinavia (Luby *et al.*, 1991; Penney *et al.*, 1997). The subspecies *vitis-idaea* blooms twice each year, March to April and July to August while the subspecies *minus* blooms only once a year, in June or July (Penhallegon, 2006).

These two subspecies of lingonberry are distinguished mainly by their height and leaf size. The average plant height of ssp. *minus* is less than 20 cm and on the other hand the height of ssp. *vitis-idaea* is usually more than 30 cm (Fig. 1.1). The average leaf size of ssp. *minus is* 1 cm × 0.5 cm which is much smaller than that of ssp. *vitis-idaea* whose average leaf size is 2.5 cm × 1 cm (Fernald, 1970; Welsh, 1974). The lingonberry ssp. *vitis-idaea* comprise of the following 16 cultivars: Ammerland, Erntedank, Erntekrone, Erntesegen, European Red, Ida, Koralle, Koralle/German, Linnae, Masovia, Regal, Sanna, Scarlet, Splendor, Red Pearl and Sussi.

A

B

Figure 1.1. Pictures representing the two types of lingonberries (A) ssp. *vitis-idaea* (B) ssp. *minus,* showing differences in their morphology.

1.3. Propagation Methods

The conventional methods of lingonberry propagation, either from seeds or from stem cutting and rhizome division are very common (Penhallegon, 2006). Different propagation methods will be discussed briefly in the following sections.

1.3.1. Sexual Propagation

The propagation from seed is a sexual method wherein the genetic materials of the two plants are combined to produce a new plant. Vaccinium species are genetically heterozygous and therefore, do not produce progeny identical to the mother plant.

1.3.2. Vegetative Propagation

A vegetative method of propagation is a form of asexual reproduction in plants. In this method, a vegetative part or tissue from one plant is used to reproduce its clone (replica). The clone is a genetically identical plant propagated asexually from a single reproduction. The vegetative propagation methods include stem cutting, grafting, budding, and micropropagation, and are described as follows.

1.3.2.1. Vegetative Propagation by Stem Cutting

Traditionally, Vaccinium species are produced by vegetative means to retain the desired genetic characteristics and to allow the rapid production of plants. The most common

conventional method of vegetative propagation is by softwood cutting. The young, soft, first year branches with meristem are taken from the mother plant to produce its clones. Meristems are the tissues consisting of undifferentiated cells that produce new cells. These new cells then differentiates into several tissues and eventually form different organs of the plant (Castellano & Sablowski, 2005). The young stem cuttings of about four to five centimetres are then provided with potting soil pots which could be supplemented with growth hormones. These stem cuttings are allowed to grow for several weeks with proper soil maintenance, fertilization, weed removal and regular irrigation. The meristem tissues of growing stem differentiate into distinct parts to transform into a complete plant. Figure 1.2 represents the morphology of a fully-grown lingonberry cultivar "Splendor" propagated by softwood cutting.

Figure 1.2. Lingonberry cultivar "Splendor" propagated by conventional softwood cutting.

1.3.3. *In vitro* Propagation

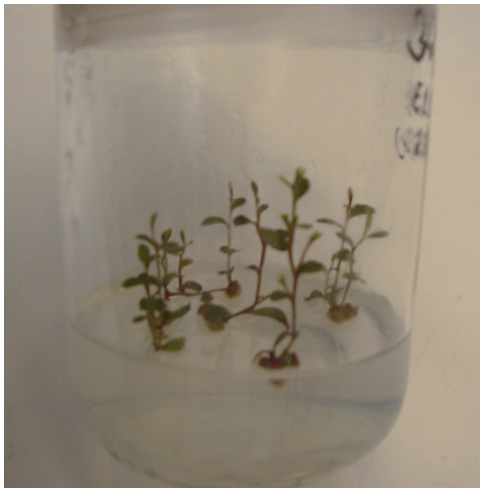
"*In vitro"* is a Latin term which means "in glass" or in an artificial environment created outside the living organism. Different tissue culture techniques are carried out *in vitro* for rapid production of new plants using tissues or tiny pieces from the mother plant. This is also called micropropagation since a small piece of plant is used to derive a new plant. A tissue from the mother plant is provided with growth medium mainly containing micro and macro nutrients, carbon source, growth hormones and vitamins. The entire procedure is carried out in a sterile environment and growth media are changed regularly. The tissue culture technique is a very efficient micropropagation method for economically important plants (Hosier, 1989).

Plant tissue culture and regeneration relies on two basic concepts: totipotency and plasticity. Plasticity is the ability of the plant tissues to alter their metabolism according to the environment which suits best for their growth and development. Haberlandt (1902) first explored plant cell culture to study morphogenesis and the concept of totipotency. Totipotency is the ability of a cell to develop into any cell type. As a result, when a plant is provided with a correct stimulus, it develops into a plant identical to the plant from which it originated. It is a characteristic of the cells in young tissues and meristems, and can be exhibited by some differentiated cells. However, it cannot be exhibited by those tissues that have developed into terminally differentiated structures like sieve tubes or tracheids. An *in vitro* environment causes plant cells to exhibit a high degree of plasticity, which results in development of different types of tissues from a single tissue by totipotency.

Tissue culture could be carried out in three different ways: i) from pre-existing buds through shoot proliferation (axillary shoot proliferation), ii) following shoot morphogenesis through adventitious shoot regeneration (adventitious shoot regeneration), and iii) through the formation of somatic embryos (Murashige, 1962).

1.3.3.1. Axillary Shoot Proliferation

In the axillary shoot proliferation method, the existing meristems grow and proliferate from the explants once removed from the parent plant. The apical and axillary buds or meristematic region of a stem tip are isolated from the mother plant and provided with the culture media in a controlled environment. Axillary shoot proliferation from the apical or axillary buds is initiated by providing them with a high cytokinin concentration (Fig. 1.3). For the commercial mass production of plants, propagation by axillary shoots proliferation is the most commonly used and reliable method to produce clones (Chawla, 2002).



A

B

Figure 1.3. Lingonberry plants propagated by axillary shoot proliferation (node tissue culture) (A) 6 weeks old and (B) 10 weeks old.

1.3.3.2. Adventitious Shoot Regeneration

Adventitious shoot formation is one of the pathways of *in vitro* plant regeneration, wherein the adventitious meristems may arise on stems, roots or leaf explants directly under the influence of growth hormones like cytokinins and auxins (Vookova & Gajdosova, 1992). Figure 1.4 represents a developing lingonberry plant propagated by adventitious shoot regeneration from excised leaves. The requirement of exogenous auxin and cytokinins for this process depends on the endogenous levels of hormones present in the tissue and hence varies with the tissue culture system (Davey & Anthony, 2010). Adventitious organs like buds and shoots may originate from calluses or near existing vascular tissues. This observation led to an idea of totipotency (Chawla, 2002). The generation of shoots from vegetative tissue of plants in reliable fashion allows the manipulation of genetic makeup in lingonberry to develop new cultivars with desired characteristics (Debnath & McRae, 2002).

In lingonberry, the regeneration of adventitious shoots from excised leaves was first reported by Debnath and McRae (2002). This technique has been very efficiently applied subsequently for many commercially important crops (Debnath, 2005) and is very frequently used in micropropagation.

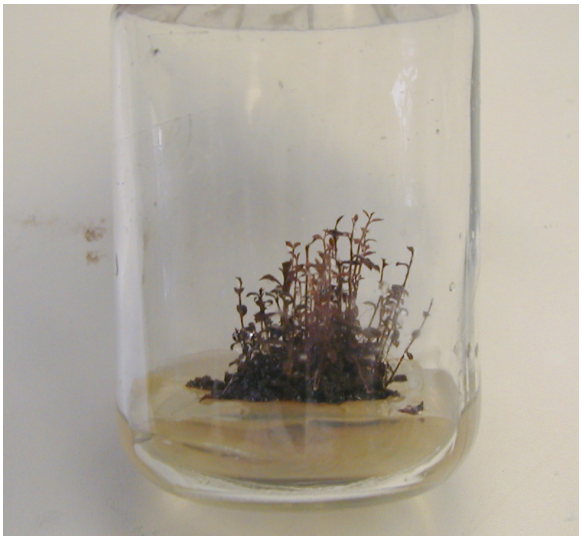


Figure 1.4. Eight weeks old lingonberry plant propagated by adventitious shoot regeneration (leaf tissue culture)

1.3.3.3. Somatic Embryogenesis

Somatic embryogenesis (SE) was defined by Thorpe (1988) as the development of diploid cells into differentiated plants through embryo stage without fusion of gametes. Somatic embryogenesis is the opposite of sexual or zygotic embryogenesis. Sharp *et al.* (1980) described two routes of somatic embryogenesis*:* direct embryogenesis and the indirect embryogenesis. In direct embryogenesis, embryos arise from a explant tissue without callus proliferation, whereas in indirect embryogenesis, proliferation occurs from the cell from which embryos are developed (Konar *et al.,* 1972).

1.4. Advantages of Micropropagation

The micropropagation method is becoming increasingly popular not only for commercial production of economically important plants, but also for crop improvement. A rapid production can be achieved from single mother plant with the desired traits. Conventional vegetative propagation methods are generally successful, however they are slow, labour intensive and only few propagules can be produced from a single plant (Debnath, 2005). Micropropagation of selected germplasm (genetic resources) can potentially multiply plants more rapidly than the traditional propagation methods. Commercial application of this technology is primarily for its enormous multiplicative capacity and the year round production of plants (Debnath, 2007).

1.5. Phytochemicals of Lingonberry Plants and Health Benefits

Lingonberries are rich in organic acids, phytochemicals, vitamin C, provitamin A (β-carotene), B vitamins (B_1, B_2, and B_3), and levels of potassium, calcium, magnesium, and phosphorus. The seeds contain significant quantities of omega-3 fatty acids (Oldemeyer & Seemel, 1976). A mature lingonberry fruit has approximately 5.5% protein and energy content of 509 Kcal per 100 g of fruit (Miller, 1976). A wide range of bioactive compounds are found in lingonberry plants which include mainly phenolic compounds such as phenolic acids, flavonoids and tannins. These compounds have antioxidant potential that attributes to the health promoting properties of lingonberry.

Lingonberries have been found to be one of the top competitors with regards to the highest antioxidant activity, compared to other berry fruits such as blackberries, blueberries, raspberries, strawberries, and cranberries (Wang & Jiao, 2000; Wang & Lin, 2000; Wang & Stretch, 2001), and have been used as a medicinal plant. Some reports have shown that lingonberry exhibits anticancer activity and that its extract can potentially induce apoptosis of human leukemia HL-60 cells (Bomser *et al*., 1996; Wang *et al.,* 2005). Lingonberry has demonstrated antimicrobial effect and has also been shown to inhibit urinary tract infection pertaining to its high arbutin levels (Larsson *et al*., 1993; Ho *et al*., 2001; Kontiokari *et al.,* 2002). Leaves of lingonberry can be used as an astringent and have diuretic properties (Lust, 1983; Chiej, 1984). Lingonberry has been used in treating gonorrhoea, a sexually transmitted disease (Duke & Ayensu, 1985). Lingonberry helps in curing gastric diseases and lowering cholesterol levels (Dierking & Dierking, 1993).

The biochemical properties of antioxidants and reactive oxygen species will be discussed in the following sections to provide a better understanding of their influence on living organisms and in particular, on human health.

1.6. Oxidants, Free Radicals and Reactive Oxygen Species (ROS)

Oxidants, such as free radicals and reactive oxygen species, are the oxidizing agents that can accept electrons from other chemical species. A free radical is an oxidant molecule that contain one or more unpaired electrons in its outermost shell and can exist independently (Halliwell, 2006). Reactive oxygen species (ROS) are those oxidants that are intermediates of

dioxygen formed when dioxygen (O_2) undergoes either physical or chemical activation (Perl-Treves & Perl, 2001). A reactive oxygen species may or may not be a free radical. Figure 1.5 illustrates the formation of ROS from triplet oxygen. When the ground state triplet oxygen is physically activated by transfer of energy, the oxygen molecule gains enough energy to change its spin and becomes the reactive oxygen species, singlet oxygen $^1O_2$. Chemical activation occurs when the oxygen molecule is reduced step by step. The activated intermediate products of oxygen are superoxide radical, hydrogen peroxide and hydroxyl radical (Yu, 1994) (Fig 1.5).

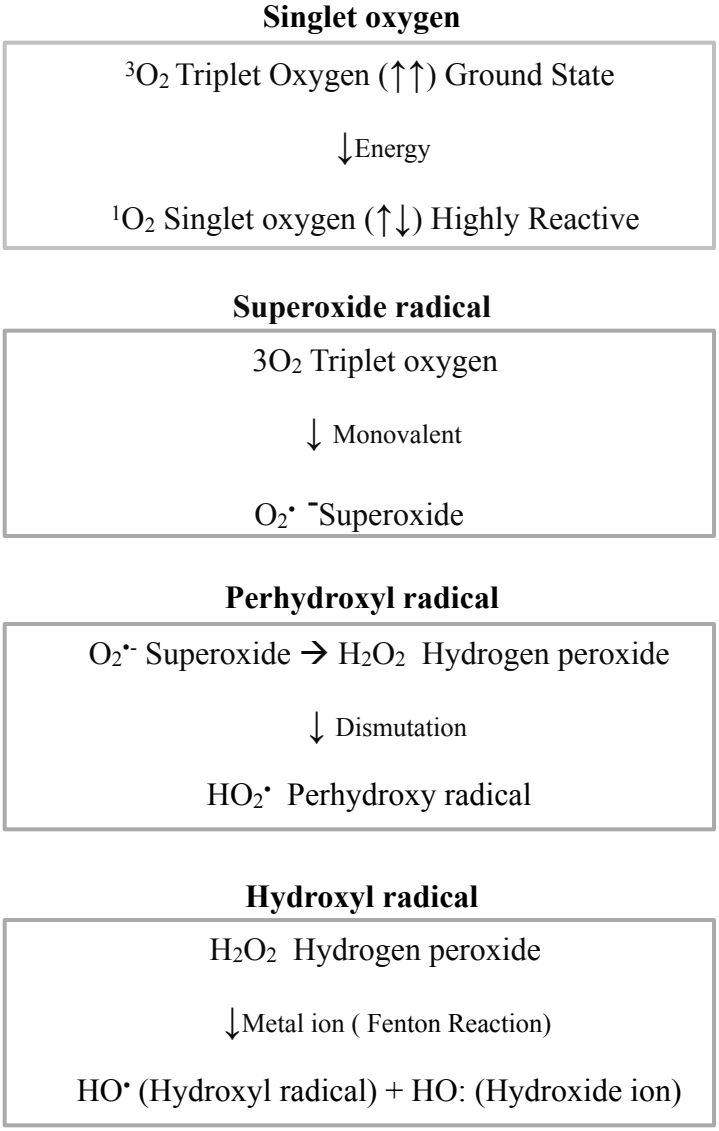


Figure 1.5. A figure illustrating the formation of reactive oxygen species in biological systems.

Reactive oxygen species and free radicals are formed in living cells as a part of normal metabolic processes contributing to regulatory roles in normal functioning of metabolic

pathways. These are also formed as a result of endogenous factors such as high temperature (Bruskov *et al.*, 2002), chilling (Einset *et al.*, 2007), pollution, exposure to industrial chemicals, ozone, ultraviolet radiation, X-ray, pesticides and certain drugs (Heck, 1968; Pell & Schlagnhaufer, 1997). Figure 1.6 shows different sources of ROS formation. Oxidative stress refers to the metabolic state of imbalance between ROS generation and detoxification (Heck, 1968). When ROS are accumulated in higher than normal concentration, oxidative stress and often apoptosis occurs. Apoptosis or programmed cell death is a mechanism which plants and other organisms use to eliminate damaged cells (Kroemer & Dallaporta 1998; Hoeberichts & Woltering, 2003).

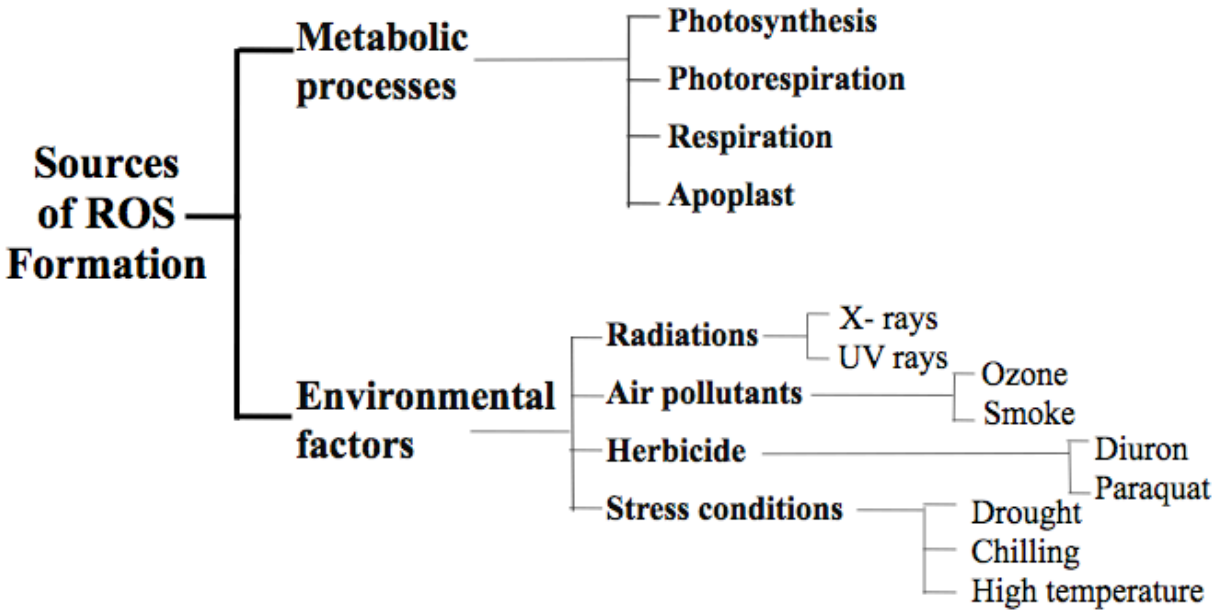


Figure 1.6. Sources of Reactive Oxygen Species (ROS) formation in biological systems.

ROS-mediated oxidative stress in biological systems is responsible for damaging cellular proteins, DNA and lipids. In human beings, ROS are partly responsible for aging (Sastre *et al.*, 2000) and several disorders and diseases like mutagenesis, cardiovascular diseases (Khan & Baseer, 2000), cancer (Kawanishi *et al.,* 2001), macular degradation and several other disorders. Overall, there are at least 70 disorders caused as a result of ROS (Ferrari & Torres, 2003).

1.7. Antioxidants

Antioxidants can be simply described as the substances which inhibit oxidation and prevent a cell from the damage caused by the oxidative stress. Antioxidants can be classified as primary or secondary, depending on their mechanism of action.

Primary antioxidants are the substances which can react with lipid radicals and convert them into a more stable form. Primary antioxidants donate a hydrogen atom to a lipid radical thus neutralising it. The product of a lipid radical after accepting a hydrogen atom does not initiate a new chain reaction by oxidising a free radical. This chain is also subject to termination when two free radicals come into contact (Fig. 1.7).

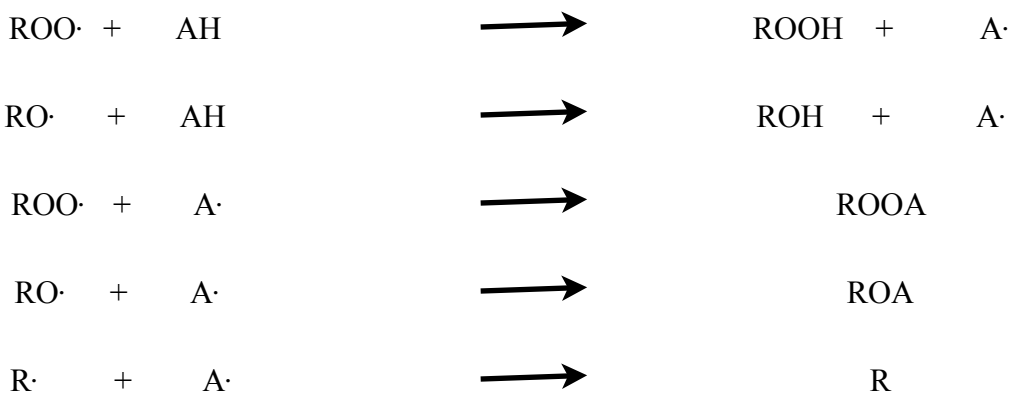


Figure 1.7. Action of primary antioxidants on lipid radical
(ROO· – Peroxyl radical, RO· –Alkoxy radical, AH- Primary antioxidant, A - Stable phenoxyl radical)

Secondary antioxidants are compounds which deactivate precursors of reactive oxygen species by acting as metal chelators, singlet oxygen quenchers, peroxide decomposers or inhibitors of lipoxygenase and other related enzymes (Shahidi, 1997).

1.7.1. Plant Antioxidant Systems

Plant systems have developed extensive protective mechanisms to combat harmful effects of free radicals. Plant cells are rich in antioxidants including a wide variety of metabolites and enzymes. Distributions and protective roles of different antioxidant components are discussed further in this chapter. Figure 1.8 summarizes the plant antioxidant systems.

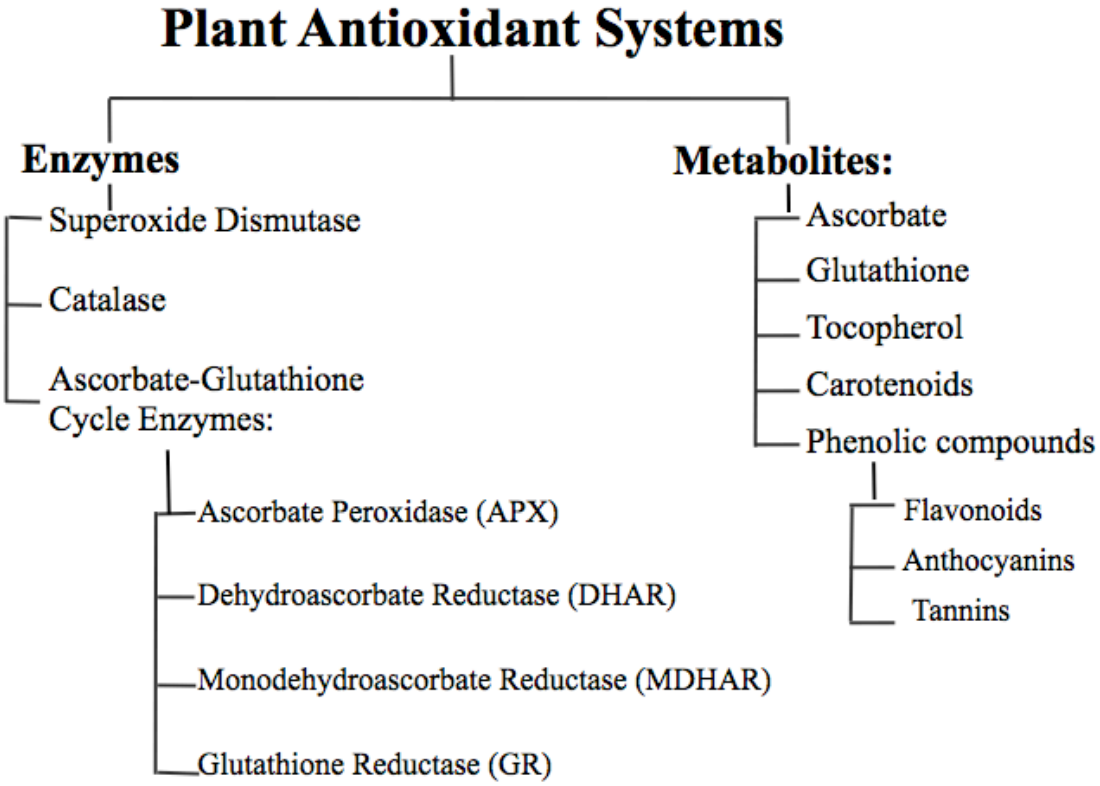


Figure 1.8. A flow chart representing plant antioxidant systems.

1.7.1.1. Antioxidant Enzymes

Antioxidant enzymes include superoxide dismutase, catalase, and the enzymes of the ascorbate-glutathione cycle, which include ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase.

1.7.1.1.1. Superoxide Dismutase (SOD)

Superoxide dismutase (EC 1.15.1.1) is a single representative of the subclass of metalloenzymes which catalyzes the dismutation of superoxide anion to hydrogen peroxide in aerobic and anaerobic organisms (Hassan, 1989). This enzyme was discovered by Irwin Fridovich and Joe McCord in 1965. SODs are distributed in almost all the cellular compartments in plant tissues including cytosol, mitochondria, chloroplasts, peroxisomes as well as in extracellular space (Blokhina *et al.*, 2003).

Superoxide dismutase (SOD) is considered to be the first line of defense against reactive oxygen species since the superoxide radical is the first reduction product of oxygen (Bannister *et al*., 1987). A catalytic dismutation of superoxide by SOD has 10,000 times faster rate than a spontaneous dismutation (Bowler *et al.*, 1992). Superoxide dismutases are classified into 3 types – Mn-SOD, Fe-SOD and Cu/Zn-SOD. Equations 1, 2 and 3 illustrates the reactions of superoxide radical with SOD.

SOD reaction: (where "M" stand for metal, based on the type of metal in the active site)

$$O_2^{\bullet -} + M^{2+} \longrightarrow O_2 + M^{+} \qquad [1]$$

$$O_2^{\bullet -} + M^{+} + 2H^{+} \longrightarrow H_2O_2 + M^{2+} \qquad [2]$$

Sum of 1 and 2:

$$2O_2^{\bullet -} + 2H^{+} \longrightarrow H_2O_2 + O_2 \qquad [3]$$

1.7.1.1.2. Catalase

Catalase (CAT) (EC 1.11.1.6) is one of the most important enzymes involved in regulation of H_2O_2 levels in the cell (Larson, 1988) and catalyze the reaction in equation 4.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \qquad [4]$$

In plants, catalase is localized in peroxisomes and scavenges H_2O_2 within the cells (Frederic & Newcomb, 1969). Catalase is not present in chloroplasts or other organelles except peroxisomes (Tolbert *et al.*, 1968). Barley mutants with low catalase exhibited injury under photorespiratory conditions (Kendall *et al.,* 1983).

1.7.1.1.3. Ascorbate-Glutathione Cycle

The ascorbate glutathione cycle, also known as Halliwell – Asada pathway (Fig. 1.9) operates in peroxisomes and mitochondria (Mittova *et al*., 2000). This cycle is comprised of following enzymes: ascorbate peroxidase (APX; EC 1.11.1.11), dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and glutathione reductase (GR; EC 1.8.1.7). The metabolites taking part in the cycle are ascorbate, dehydroascorbate, monodehydroascorbate (ascorbate free radical) and glutathione (Noctor & Foyer, 1998). This cycle scavenges H_2O_2 which is produced as a result of dismutation of superoxide (Jimenez, 1997). The cycle efficiently recycles the enzymes involved in it and maintains the homeostatic environment in the cell.

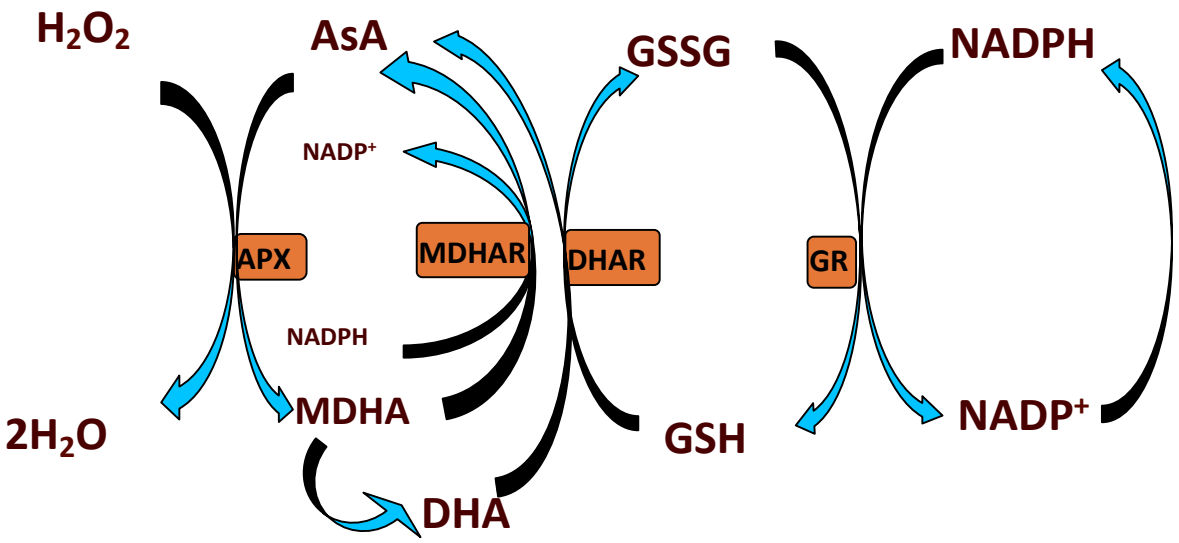


Figure 1.9: Ascorbate-Glutathione Cycle (Halliwell-Asada pathway) (modified form Noctor & Foyer, 1998). AsA - ascorbate; MDHA - monodehydroascorbate; DHA - dehydroascorbate; GSSG - oxidized glutathione; GSH - reduced glutathione; APX - ascorbate peroxidase, DHAR- dehydroascorbate reductase; MDHAR - monodehydroascorbate reductase; and GR - glutathione reductase.

Ascorbate (ASC) is the most important reducing substrate for H_2O_2 detoxification (Smirnoff & Wheeler, 2000). Ascorbate peroxidase (APX) is an important enzyme in ascorbate-glutathione pathway which uses two molecules of ascorbate to reduce H_2O_2 to water and generates two molecules of monodehydroascorbate (MDHA).

$$2 \text{ Ascorbate} + H_2O_2 \longrightarrow 2 \text{ MDHA} + 2H_2O \qquad [5]$$

Monodehydroascorbate (MDHA) disproportionates to ascorbate (ASC) and dehydroascorbate (DHA), if not reduced rapidly in the reaction as illustrated in equations 6 and 7. Thus, the regeneration of ascorbate is achieved enzymatically by monodehydroascorbate reductase (MDHAR) or spontaneously by the following reactions (equations 6 & 7). In a spontaneous

(non- enzymatic) reaction (equation 6), the electron donor for MDHA may be cytochrome *b* (Foyer & Halliwell, 1976) or reduced ferredoxin (Fd) (Foyer *et al.,* 1997).

Non enzymatic reduction:

$$MDHA + Fd_{red} \longrightarrow Ascorbate + Fd_{OX} \qquad [6]$$

Reduction by monodehydroascorbate reductase:

$$2MDHA + NADPH + H^{+} \xrightarrow{MDHAR} 2Ascorbate + NADP^{+} \qquad [7]$$

Dehydroascorbate (DHA) formed by the disproportionation of MDHA is reduced by dehydroascorbate reductase (DHAR) to regenerate ascorbate. This reaction uses glutathione as a reducing substrate (Foyer *et al.,* 1983) and generates glutathione disulphide (GSSG)

$$DHA + 2GSH \xrightarrow{DHAR} Ascorbate + GSSG \qquad [8]$$

Thus, the ascorbate pool is maintained by the above mentioned reactions. Glutathione reductase (GR) reduces GSSG to GSH, reduced glutathione, using NADPH as an electron donor (Equation 9).

$$GSSG + NADPH + H^{+} \xrightarrow{GR} GSH + NADP^{+} \qquad [9]$$

Ascorbate peroxidases are localized in chloroplasts, mitochondria, cytosol and peroxisomes (Foyer & Noctor, 2009). In chloroplast, ascorbate peroxidase is found both as soluble in stroma and bound to thylakoid (Miyake & Asada, 1992). Chloroplasts contain two isoforms of APX, stromal and thylakoid-bound APX. Chloroplastic APXs have several-folds higher catalytic turnover as compared to the cytosolic APX (Chew *et al.,* 2003) but can be easily inhibited by H_2O_2 (Hossain & Asada, 1984).

Glutathione reductase (GR) catalyses the reduction of glutathione disulphide (GSSG) to reduced glutathione using NADPH as an electron donor and thus completes the ascorbate glutathione cycle (Noctor & Foyer, 1998). The plants overexpressing GR showed high ascorbate levels whereas the mutants with no GR were found to be extremely sensitive to stress due to low ascorbate levels (Aono *et al.*, 1993).

Dehydroascorbate reductase (DHAR) is one of the important enzymes which contributes in maintaining ascorbate pool in leaves and catalyzes the reduction of DHA to ASC, in absence of which DHA would form oxalate and tartrate (Washko *et al.*, 1992).

The regeneration of monodehydroascorbate (MDHA) radicals to ascorbate in the ascorbate glutathione cycle is achieved enzymatically by MDHAR using NADPH as mentioned above. Monodehydroascorbate as a long lasting anion radical is an indicator of oxidative stress in plant tissues and can be detected by the electron spin spectroscopy (EPR) technique (Buettner & Jurkiewicz, 1993). Thus, the ascorbate-glutathione cycle involving the above mentioned enzymes and metabolites contributes to cellular homeostasis and scavenges ROS.

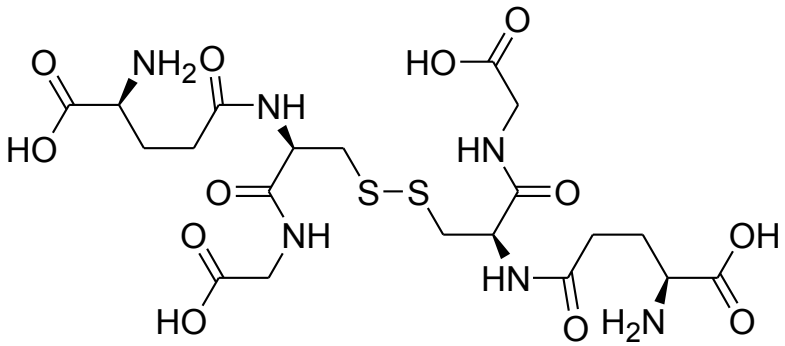
1.7.1.2. Antioxidant Metabolites

The plant antioxidant metabolites include ascorbate, glutathione, tocopherol, carotenoids, and phenolic compounds such as flavonoids, anthocyanins, proanthocyanidins (also known as tannins) and phenolic acids.

1.7.1.2.1. Ascorbate

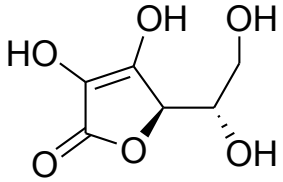
Ascorbate or ascorbic acid (ASC), also known as vitamin C, is the most important antioxidant in biological systems (Smirnoff, 2000). Figure 1.10 shows the structure of ascorbate. Ascorbate can be synthesized by all plants and animals, except primates and guinea pigs (Burns, 1957). Plants contain ascorbic acid in all cell types, organelles and apoplast wherein it can accumulate in millimolar quantities (Blokhina *et al.*, 2003). Ascorbate detoxifies ROS by directly reacting with hydroxyl radicals, superoxide, and singlet oxygen (Frei *et al.*, 1989). Ascorbate is very efficient in inhibiting peroxidation of human plasma lipids, and other plasma components, such as alpha-tocopherol and protein thiols (Padh, 1990), and hence protect membranes against damage caused by ROS. Ascorbate protects membranes by regenerating oxidized carotenes and tocopherols which are important antioxidants in the non-aqueous phase, thus also acting as a secondary antioxidant (Noctor & Foyer, 1998). Ascorbate plays a crucial role in the regulation of photosynthesis, in particular by providing protection against harmful effects of excess excitation energy and acting as a cofactor in the xanthophyll cycle (Noctor *et al.,* 1991). The mutants of Arabidopsis with low

ascorbate levels display slow growth rate and late flowering, and the mutant plants with no ascorbate die (Foyer & Halliwell, 1976).



glutathione

$C_{20}H_{32}N_6O_{12}S_2$
Mol. Wt.: 612.63



ascorbic acid

$C_6H_8O_6$
Mol. Wt.: 176.12

Figure 1.10. The structures of glutathione and ascorbic acid

1.7.1.2.2. Glutathione

Glutathione is a tripeptide (γ-Glu-Cys-Gly) (Fig 1.10) and is an abundant compound in plants found in almost all cell compartments (Foyer & Halliwell, 1976). Its role as a reducing substrate in the ascorbate-glutathione pathway was discussed in section 1.7.1.1.1.3. Glutathione is a very important antioxidant and maintains redox levels in both plants and animals (Law *et al.*, 1983). In addition, it is of great importance for the environment of a cell under normal and stress conditions (Rennerberg, 1982). Other than being a powerful antioxidant, glutathione in its reduced form is of significant importance in sulphur metabolism and regulates sulphur uptake (Kerk & Feldman, 1995). Glutathione is responsible for the regeneration of other crucial antioxidants of the ascorbate-glutathione cycle and is also important in recycling of tocopherol and carotenoid (Lamoureux & Rusness, 1993). Glutathione plays an important role in the detoxification of xenobiotics, regulation of the cell cycle (Marrs, 1996) and maintaining heavy metal concentrations in the cell (Rouhier *et al.*, 2008).

1.7.1.2.3. Carotenoids

Carotenoids were first isolated from carrots by Wackenroder in 1831. They are a class of natural pigments which are widespread in many fruits and vegetables. These lipid-soluble molecules are important antioxidants in both plants and animals (Perl-Treves & Perl, 2001). Carotenoids are accessory pigments in photosynthesis and protect photosynthetic cells against photosensitization in several ways. The best known property of carotenoids is their ability to

absorb light (Bartley & Scolnik, 1995). Carotenoids are present in reaction center; β-carotene and light harvesting complexes lutein and neoxanthin quench excited triplet state chlorophyll and protect chloroplast against photooxidative stress (Parker & Joyce, 1967; Strzalka *et al*., 2003). Carotenoids are involved in the xanthophyll cycle to protect photosynthetic cells by non-photochemical quenching through dissipation of excess energy within light harvesting antenna proteins (Edge & Truscott, 1999). Carotenoids can also quench singlet oxygen as shown below (equation 10) and protect photosynthetic cells from ROS damage (Foote & Dcuny, 1968) and lipid peroxidation.

$$^1O_2 + \text{carotenoid} \longrightarrow {}^3\text{carotenoid} + {}^3O_2 \qquad [10]$$

Some of the carotenoid compounds are the precursors of abscisic acid in plants which functions to modulate developmental and stress processes (Koornneef, 1986). Carotenoids are also of significant importance to animals and are an important constituent of human diet. It has been shown that carotenoids have anticancer activity and are effective in reducing the risk of several chronic diseases such as age-related macular degradation, and coronary heart disease (Hennekens, 1997).

1.7.1.2.4. Tocopherol

Tocopherol is a lipid soluble compound which is mainly localized in plastids and synthesized in envelopes of plastids. Tocopherol is a very important dietary nutrient in animals and humans since it is synthesized only in plants (Munne-Bosch & Alegre, 2002). α-

tocopherols are found in chloroplasts, while β, γ and δ tocopherols are found outside the organelle (Kamaleldin & Appelqvist, 1996).

1.7.1.2.5. Plant Phenolics

Phenolic compounds are a large class of secondary metabolites which are composed of an aromatic ring with a substitution of one or more hydroxyl groups and a number of other side groups (Shahidi & Naczk, 2004). This class of compounds is widely distributed in plants and includes simple phenolics, phenolic acids, coumarins, flavonoids, stilbenes, proanthocyanidins, lignans, and lignins (Naczk & Shahidi, 2006; Shahidi & Naczk, 2004). Phenolic compounds are responsible for fruit colour, odour, flavour, bitterness, astringency and stability against lipid oxidation. Table 1.1 shows different classes of phenolic compounds.

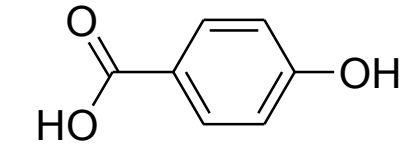
Table 1.1. Dietary sources of plant phenolics (Naczk & Shahidi, 2006)

Phenolic compounds	Dietary source
Phenolic acids	
Hydroxycinnamic acids	Blueberries, carrots, cereals, pears, cherries, tomatoes, citrus fruits, oilseeds, peaches, plums, eggplants, apricots
Hydroxybenzoic acids	Blueberries, Cereals, Cranberries, Oilseeds
Flavonoids	
Anthocyanins	Bilberries, black and red currants, blueberries, cherries, grapes, strawberries
Chalcones	Apples
Flavanols	Apples, blueberries, grapes, onions, lettuce
Flavanonols	Grapes
Flavanones	Citrus fruits
Flavonols	Apples, beans, blueberries, buckwheat, cranberries, lettuce, onions, olive, pepper
Flavones	Citrus fruits, celery, parsley, spinach, rutin
Isoflavones	Soybeans
Xanthones	Mango, mangosteen
Tannins	
Condensed	Apples, grapes, peaches, plums, mangosteens, pears
Hydrolyzable	Pomegranate, raspberries
Other phenolics	
Arbutin	Pears
Coumarins	Carrots, celery, citrus fruits, parsley, parsnips
Lignans	Buckwheat, flaxseed, sesame seed, rye, wheat
Stilbenes	Grapes

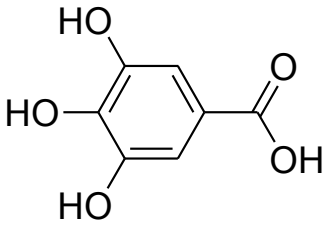
1.7.1.2.5.1. Flavonoids

Flavonoid is a term described for pigments in plants which are mostly derived from a benzo-gamma-pyran ring (Winkel-Shirley, 2001). Flavonoids are the large class of low molecular weight secondary metabolites found ubiquitously in higher plants. They are present in almost all parts of plants, from roots to flowers and fruits (Williams & Grayer, 2004). Red fruits, citrus fruits, apple, onion, coca, grapes, and tea are rich dietary sources of flavonoids (Mennen *et al.,* 2004). Flavonoids are divided into 14 different groups (Havsteen, 2002) including flavones, isoflavones, flavanones, flavonols, flavanols (catechins), and anthocyanidines which are well-characterized among the 14 groups (Table 1.1). Flavonoids have been reported to have widespread biological functions including plant pathogen interaction, pollination and seed development (Winkel-Shirley, 2001) but the most important property of flavonoids in biological systems is their antioxidant abilities. Figure 1.11 shows structures of some flavonoids.

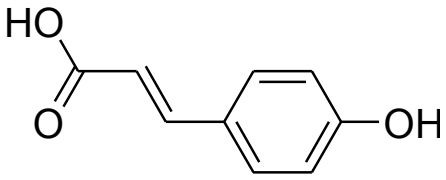
Flavonoids have the property of inhibiting auto-oxidation and scavenging free radicals (Bors *et al*., 1990). Flavonoids have an ability to suppress the Fenton reaction (Fig. 1.5) by their metal binding property. The functional group and region involved in metal chelation are β-ring and 4-keto and 5-hydroxy region of flavonoid (Cheng & Breen, 2000). During biotic and abiotic stress conditions such as drought, wounding and metal toxicity, many flavonoid biosynthetic genes are induced and flavonoid levels increase (Dixon & Paiva, 1995; Winkel-Shirley, 2002).



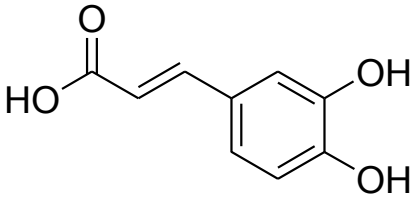
p-hydroxy benzoic acid



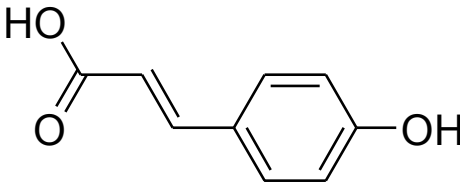
Gallic acid



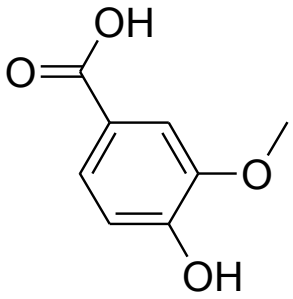
p-coumaric acid



Caffeic acid



p-coumaric acid



Vanillic acid

Figure 1.11. Structures of some flavonoid compounds.

1.7.1.2.5.2. Anthocyanins

Anthocyanins belong to a flavonoid class with strongly hydrophilic properties. They are responsible for the red and blue colour in fruits, vegetables and flowers and are widespread in nature. Anthocyanins are mostly localized in the epidermal layer of fruits but in some cases also found in the pulp of fruits (Shrikhande & Francis, 1976). In addition to being natural pigments, they are potent antioxidants (Kahkonen & Heinonen, 2003) and have the ability to prevent lipid oxidation (Satuegracia *et al.*, 1997) and scavenge free radicals (Wang *et al.*, 1997). Dietary intake of fruits and vegetables has been reported to have beneficial effects on human health (Hollman *et al.,* 1996; Knekt *et al*., 2002; Rissanen *et al*., 2003). Anthocyanins have been shown to have anticancer and anti-aging properties (Kong *et al*., 2003; Rossi *et al*., 2003). They are also important in improving the nutritional values of processed foods (Kahkonen *et al*., 2003; Viljanen *et al.*, 2004). Anthocyanins are distributed in lingonberries in a distinct pattern where cyanidin-3-galactoside accounts for 80%, cyanidin-3-glucoside is 5% and cyanidin-3-arabinoside is 11% of the total anthocyanins (Wang *et al*., 2005).

1.7.1.2.5.3. Proanthocyanidins

Proanthocyanidins are polyphenolic secondary metabolites present in higher plants. Proanthocyanidins are classified into two groups, namely, hydrolysable proanthocyanidins and complex proanthocyanidins (Khanbabae & Van Ree, 2001). Studies have shown that proanthocyanidins have high antioxidant potential as compared to ascorbate and tocopherol (Shi *et al*., 2003). They are present in the bark, fruits, leaves and seeds of many plants and provide protection for the plants. Grape seed proanthocyanidins were reported to have

chemoprevention of cellular damage (Joshi *et al.,* 2001). Proanthocyanidins are one of the major compounds present in grapes and wine that is responsible for cardioprotection (Bertelli & Das, 2009).

1.8. Conclusion

In summary, lingonberry (*Vaccinium vitis-idaea* L.) is an economically important plant with medicinal values and health benefits and is mainly propagated by vegetative means. Tissue culture techniques offer several benefits over traditional means of vegetative propagation method for the rapid and less laborious production of berries.

The available literature on lingonberry's antioxidant potential shows a wide gap in information available about leave's antioxidants. Therefore, this study aimed to determine the antioxidant enzyme activity of lingonberry leaves and determine the antioxidant metabolites of lingonberry fruits and leaves. Important antioxidant enzymes and metabolites were studied as well as ratio of reduced and oxidized ascorbate and glutathione were determined to correlate with the morphological features of differentially propagated lingonberry cultivars. *In vitro* experiments were done and biological models were used to determine the effect of propagation method on antioxidant activities of lingonberries.

Thus, the objective of this dissertation is: 1) to determine the effect of propagation methods on the levels of antioxidant enzymes like catalase and the ascorbate-glutathione cycle enzymes as well as phenolic compounds and their antioxidant activities of lingonberry fruits and leaves; 2) to determine the correlation between morphological

properties and ascorbate and glutathione metabolism in lingonberry leaves influenced by propagation methods; 3) to identify and quantify major phenolic compounds in lingonberry fruits and leaves; 4) to determine the effect of geographical locations on phenolic compounds and antioxidant activities of wild lingonberry clones; 5) to correlate the antioxidant efficiency of phenolics from lingonberry in inhibiting lipid peroxidation in food model systems and 6) to determine the efficiency of lingonberry fruit and leaf extracts in suppressing oxidative stress in brain.

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Chapter 2

Effects of *in vitro* and *ex vitro* Propagation Methods on Antioxidant Properties of Lingonberry (*Vaccinium vitis-idaea* L.) Cultivars

This chapter is an investigation of the effects of propagation methods on the antioxidant compounds of three lingonberry cultivars, Regal, Splendor, and Erntedank obtained from three different propagation methods: by ex vitro conventional softwood cuttings, by in vitro shoot proliferation of node explants and by in vitro adventitious shoot regeneration from excised leaves of micropropagated shoots.

2.1. Introduction

Lingonberry (*Vaccinium vitis-idaea* L.) is a commercially important fruit crop with a great medicinal value due to its high antioxidant properties (Jaakola *et al.* 2001; Wang *et al.* 2005; Lätti *et al.* 2011). Lingonberry plants are genetically heterozygous, so they are normally propagated by vegetative methods to achieve genetically identical offspring and to preserve advantageous characteristics. The conventional vegetative propagation method employed for lingonberry plants is by softwood cutting. The tissue culture technique is a more advanced method of micropropagation that offers a rapid production of numerous clones from the single mother plant. The tissue culture of lingonberry plants can be obtained either from node sections or from leaves (Debnath 2011).

A link between ascorbate metabolism and accumulation of phenolic compounds has been shown (Thomas *et al.* 1992) and may be connected, in particular with the role of ascorbate in metabolism of phenols in the apoplast and in scavenging phenoxyradicals (Horemans *et al.* 2000).

Studies have shown that the methods of propagation influence growth habits of plants (Debnath, 2011; Saez *et al.,* 2012). Significant morphological differences have been observed in plants obtained from softwood cuttings and tissue culture. It has been shown that the tissue culture-derived lingonberry plants are superior to the stem cutting method in terms of number of stems, branches, leaves, and rhizomes but produce less vigorous shoots and smaller berries (Debnath 2006). Similarly, some differences in morphology have been observed in plants derived from the *in vitro* micropropagation by node tissues and leaf tissue culture (Debnath 2005).

The controlled morphogenesis with initiation of new meristems in plants can be triggered by tissue culture techniques. By adding a cocktail of plant hormones, differentiated cells can be reset to start formation of new shoots or roots. Hormones act as triggers *via* signalling events, followed by a long chain of metabolic steps occurring after binding to receptors and leading to modulation of the redox state. The morphogenetic phenomena are directly controlled by metabolism and, in particular, by pairs of reduced and oxidized compounds, the most important of which are ascorbate and glutathione. This study explore the possibility that the differences in the morphogenetic process in tissue culture plants may be directly related to the redox state of glutathione and ascorbate. The modulation of redox state is linked to the

concentration of reactive species such as hydroxyl radicals, superoxide anion, hydrogen peroxide and monodehydroascorbate (ascorbate free radical, AFR).

The present study is performed on three contrasting lingonberry cultivars, Regal, Splendor, and Erntedank, each propagated by three methods: 1) stem cutting (SC) (which is taken as a control), 2) *in vitro* shoot proliferation from node tissue (node culture, NC), and 3) *in vitro* adventitious shoot regeneration from excised leaves of micropropagated shoots (leaf culture, LC). Leaves of each of the cultivars were analysed for levels of the antioxidant enzymes and of reduced and oxidized ascorbate and glutathione. Fruits and leaves of each of the three cultivars propagated by stem cutting and tissue culture were analysed for total soluble phenolic content, flavonoids, anthocyanin, tannin, and total radical scavenging capacity. The study shows important differences in the antioxidant metabolism of plants obtained by different methods of propagation which were also specific to each cultivar. The effects of propagation methods on the antioxidant metabolism are discussed in relation to the possible involvement of reduction levels of ascorbate and glutathione and of reactive oxygen species in morphogenetic processes in plants.

2.2. Material and Methods

2.2.1. Plant Material

The plants used in this study were greenhouse grown lingonberry cultivars Regal, Splendor, and Erntedank at Atlantic Cool Climate Crop Research Centre (ACCCRC), Agriculture and Agri-Food Canada, in St. John's, Newfoundland and Labrador. Figure 2.1 represents lingonberry cultivar Regal propagated by stem cutting, nodal culture and leaf culture.

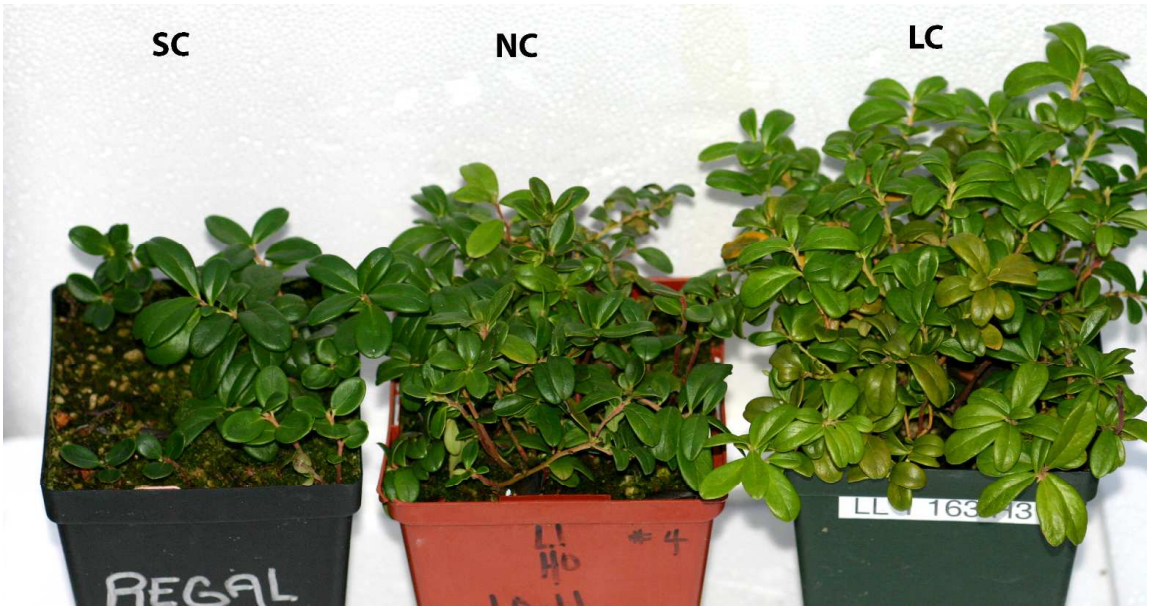

Figure 2.1. Lingonberry cultivar Regal propagated by different methods. SC - stem cutting-derived plant; NC - nodal culture-derived plant; LC - leaf culture-derived plant

2.2.2. Plant Propagation

2.2.2.1. Plant Growth and Development

Lingonberry cultivars Regal, Splendor, and Erntedank were obtained from Debnath (2005). The shoot proliferation medium D (Debnath & McRae's 2002) containing three-quarters of macro-salts and micro-salts (hereafter called basal medium, BM) was used for *in vitro* propagation. It was also supplemented with 25 mg/ml sucrose, 3.5 mg/ml Sigma A 1296 agar, and 1.25 mg/ml gelrite (Sigma Chemical Co., St. Louis). The medium was adjusted to pH 5.0 and then was autoclaved for 20 min at 121°C. Cultures were first maintained at 20° ± 2°C for 16 hours under the light of photosynthetic photon flux (PPF) density of 30 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps and then were subcultured every 8 weeks (Foley and Debnath 2007). Shoot regeneration was observed from the leaf and nodal explants (Debnath & McRae 2002; Debnath 2005). After eight weeks of culture initiation, buds and shoot clumps were transferred to 175 ml Sigma baby food glass vessels containing 35 ml BM with zeatin (1 μM). BM containing zeatin is needed for proper shoot proliferation of lingonberry culture *in vitro* (Reed & Abdelnour-Esquivel, 1991). Buds and shoot clumps were then cultured for further 8 weeks for shoot elongation at the photoperiod of PPF density of 30 μmol m^{-2} s^{-1} at 20° ± 2°C for 16 h similarly as provided during shoot proliferation by Foley & Debnath (2007a).

Shoots from leaf and nodal tissue cultures of Regal, Splendor, and Erntedank were obtained and grown in a greenhouse according to Debnath (2006). Eight-week old *in vitro* derived elongated shoots (4 to 5 cm long) were rooted in 45-cell plug trays (cell diameter 5.9

cm, cell depth 15.1 cm; Beaver Plastics, Edmonton, AB, Canada) containing peat: perlite [2:1 (v/v)] in a humidity chamber with a vaporizer (Conviron E15; Controlled Environments Ltd., Winnipeg, MB, Canada) at 22° ± 2°C and 95% relative humidity (RH), with a 16 hour photoperiod (PPF 55 μmol m^{-2} s^{-1} at culture level). No rooting compound was applied (Debnath 2006; Foley & Debnath 2007).

2.2.2.2. Establishment of Softwood Cutting Lingonberry Cultivars

Terminal softwood cuttings, 4 - 5 cm long, were taken from the greenhouse grown Regal, Splendor, and Erntedank cultivars that were used for tissue culture. These stem cuttings were rooted in 45-cell plug trays as described above (*vide supra*) for tissue-culture obtained shoots (Foley & Debnath 2007).

After 8-10 weeks, rooted plantlets of stem cuttings, nodal cultures and leaf cultures were transferred to plastic pots (10.5 × 10.5 ×12.5 cm) containing the same medium used for rooting and were maintained in humidity chamber and acclimatized by gradually lowering the humidity by 3 - 4% per week over a 3 week period. The plants that were well-developed and hardened were then grown in the greenhouse under natural light condition at 20°C, 85% RH and 16 hour photoperiod at maximum PPF of 90 μmol m^{-2} s^{-1} (Debnath 2006; Foley & Debnath 2007). Fertilization (20-8-20 N-P-K, Plant Products Co. Ltd., Brampton, ON) and irrigation was supplied to plants when required (Debnath 2006). All plants were treated and maintained in similar manner. The age of plants during the morphological data collection and chemical analysis was about 10 years.

2.2.2.3. Morphological Characteristics

The morphological data including plant height, number of rhizomes per plant, number of branches per rhizome, number of branches per plant, leaves per branch, leaves per plant, berry weight, and berry diameter were collected from 15 plants per treatment.

Fresh young leaves and mature ripe fruits from 5 plants per treatment were harvested for biochemical assays and immediately frozen into liquid nitrogen and transferred to -80 °C until extraction. For biological assays 5 plants per treatment were used and all the experiments were done in triplicate (total 15 samples).

2.2.2.4. Determination of Leaf Chlorophyll Content

The total chlorophyll (*a*+*b*) and chlorophyll *a* and *b* were determined by the procedures of Arnon (1949). Leaf tissue (0.5 g) was ground in a chilled mortar and pestle in 5 ml 100% acetone and centrifuged for 5 minutes at 3500 g. The supernatant was used to measure chlorophyll content. 50 µl of extract was added to 950 ml of 80% acetone and absorption was measured at 663 nm, 645 nm and 750 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*) and contents of chlorophyll *a*, *b* and total chlorophyll were determined by the following formulae.

Chlorophyll *a* (mg/g fresh weight) = $[(12.7\ A_{663}) - (2.69\ A_{645})] \times V$

Chlorophyll *b* (mg/g fresh weight) = $[(22.9\ A_{645}) - (\ 4.68\ A_{663})] \times V$

Chlorophyll *a*+*b* (mg/g fresh weight) = [(20.08 A_{645}) - (8.02 A_{663})] × V

where V is the volume of the chlorophyll extract.

2.2.2.5. Determination of Reduced and Oxidized Ascorbate and Glutathione

The leaves of lingonberry cultivars were ground to powder in pre-chilled mortar and pestle with liquid nitrogen and homogenized with 2% metaphosphoric acid. The homogenate was centrifuged at 2,100 g for 20 min at 4ºC. The supernatant was used for measurement of reduced and oxidized ascorbate and glutathione. Ascorbate (AsA) and dehydroascorbate (DHA) were determined according to the method developed by Kampfenkel *et al.* (1995). This assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA and the spectrophotometric detection of Fe^{2+} complexed with 2,2-dipyridyl. Determination of total ascorbate was performed after reduction of dehydroascorbate (DHA) to ascorbate (Asc) with dithiothretol (DTT). For total ascorbate determination, the reaction mixture contained 100 µl of aliquot of extract, 250 µl of 50 mM phosphate buffer solution (pH 7.5) containing 2.5 mM EDTA and 50 µl of 10 mM dithiothretol. It was incubated for 10 minutes at room temperature. Excess DTT was removed by adding 50 µl of 5% N-ethylmaleimide. Total ascorbate (ASA +DHA) was determined as shown below. Ascorbate was determined using similar reaction mixture in which 100 µl of H_2O was added instead of DTT and N-ethylmalemide.

In both the reaction mixtures, the following reagents were added: 0.2 ml of 10% trichloroacetic acid (TCA), 0.2 ml of 44% ortho-phosphoric acid, 0.2 ml of 4% α, α'-dipyridyl

in 70% ethanol, and 0.3% (w/v) $FeCl_3$. Colouring was observed after shaking and the mixture was incubated at 40 °C for 40 minutes. Absorbances were recorded at 525 nm using a UV/ Visible spectrophotometer (Ultrospec 4300 *pro*).

The reduced (GSH) and oxidized (GSSG) glutathione were determined according to Zaharieva and Abadía (2003). The method is based on the reaction of 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) with GSH forming 5-thionitrobenzoic acid (TNB) that absorbs at 412 nm. Total glutathione (GSH+GSSH) was determined in 125 mM potassium phosphate buffer (pH 7.5) containing 0.3 mM Nicotinamide adenine dinucleotide phosphate reduced (NADPH), 6.3 mM EDTA, 100 µM 5,5-dithio-bis (2-nitrobenzoic acid) and 200 µl of aliquots of leaf extract in total volume of 1 ml. Before the start of reaction, the pH of acid extract was brought to 7.7 by diluting it fivefold in 0.28 M K_2HPO_4. The reaction was started with 5 µl (0.5 U) glutathione reductase. The change in absorbance was monitored at 412 nm for 200 seconds using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*).

To measure GSSG, the aliquots of leaf extract were mixed with 2- vinylpyridine at a ratio of 25:1, vortexed for 1 minute and incubated for 1 hour at 25 °C. GSSG was then determined in same way as described above for total glutatione. The values were corrected by measuring control rates in the absence of extract, and total GSH was calculated from a standard curve. The GSH concentration was calculated as the difference between total GSH and GSSG (calculated as GSH equivalents).

2.2.2.6. Measurement of Ascorbate-Glutathione Cycle Enzymes and Catalase

The lingonberry leaves were ground to powder using mortar and pestle in liquid nitrogen. One hundred mg of material was homogenized on ice in 1 ml of 50 mM MES/KOH buffer (pH 6.6) containing 40 mM KCl, 2 mM $CaCl_2$ and 1 mM sodium ascorbate. The homogenate was centrifuged for 10 minute at 12,000 g at 4°C.

The activities of enzymes of the ascorbate-glutathione cycle were measured according to Murshed *et al.* (2008) with modifications.

a) Ascorbate Peroxidase (APX)

The assay medium for ascorbate peroxidase (EC 1.11.1.11) was 50 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM sodium ascorbate, and sample extract The reaction was started by adding H_2O_2 (final concentration 2.5 mM) and the decrease in reaction rate was determined spectrophotometrically by absorbance change at 290 nm (ε=2.8 mM/cm) using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*).

b) Dehydroascorbate Reductase (DHAR)

Dehydroascorbate reductase (EC 1.8.5.1) activity was measured at 265 nm (ε = 14 mM/ cm) using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). The assay buffer contained 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH 7.0),

0.1 mM EDTA, and 2.5 mM GSH and leaf extract. The reaction was initiated by adding freshly prepared DHA (final concentration 0.8 mM).

c) Monodehydroascorbate Reductase (MDHAR)

Monodehydroascorbate reductase (EC 1.6.5.4) activity was measured in 50 mM HEPES buffer (pH 7.6) containing 2.5 mM sodium ascorbate, 0.25 mM NADH and the extract. The assay was initiated by adding 0.4 Unit/ml of ascorbate oxidase (Sigma) and the reaction rate was monitored at 340 nm (ε = 6.22 mM/cm) using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*).

d) Glutathione Reductase (GR)

Glutathione reductase (EC 1.8.1.7) activity was measured at 340 nm (ε = 6.22 mM/cm) in 50 mM HEPES buffer (pH 8.0) containing 0.5 mM EDTA, 0.25 mM NADPH and leaf extract. The reaction was started by adding GSSG to final concentration 1 mM.

e) Catalase

Catalase (EC 1.11.1.6) activity was measured at 240 nm (ε = 43.1 M/cm) (Aebi, 1974) using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). Degradation of hydrogen peroxide by catalase can be measured by the decrease in absorbance at 240 nm.

2.2.2.7. Extraction and Estimation of Soluble Phenolics

For this study, the fruits of NC plants were not available and hence only the fruits of SC and LC plants were used to study the effects of propagation methods on phenolic compounds. Soluble phenolics and other compounds were extracted from fruits and leaves in 80% acetone with 0.2% formic acid in the ratio of 1:10 (w/v) with 8 hours of shaking at 4 ºC which was found to be the best extraction solvent (gave highest phenolic levels) among ethanol, methanol, acetonitrile at various aqueous mixtures with different shaking periods. Homogenous mixture of samples and solvent was then centrifuged at 20,000 *g* for 20 minutes. The residue was extracted twice under same conditions and the supernatants were mixed together and further diluted to make the working concentration of 25 mg/ml for fruits and 1 mg/ml for leaves.

a) Determination of the Total Soluble Phenolic Content (TPC)

The total soluble phenolic content in both leaves and fruits was determined using Folin-Ciocalteu reagent as described by Chandrasekara and Shahidi (2011a) with modifications. The Folin-Ciocalteu reagent (0.5 ml) was added to centrifuge tubes containing 0.5 ml of extracts and mixed well. One ml of saturated sodium carbonate solution was added to each tube to neutralize the reaction. The final volume was adjusted to 10 ml with water and vortexed for 1 minute. The tubes were kept in dark for 35 minutes at room temperature and then centrifuged at 4,000 *g* for 10 minutes. The absorbance was measured at 725 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). The total soluble phenolic content of each sample

was determined using a gallic acid standard curve and expressed as milligrams of gallic acid equivalents (GAE) per g of berry or leaf fresh weight.

b) Determination of the Total Anthocyanin Content (TAC)

The total anthocyanin content was measured by pH differential method according to Foley and Debnath (2007). The TAC was measured based on reversible conversion of anthocyanins from their oxonium form to the hemiketal form. Absorption at 510 nm and 700 nm was measured using UV/Visible spectrophotometer (Ultrospec 4300 *pro*) in buffers at pH 1.0 and pH 4.5 and the difference between the two values was used to determine total anthocyanin content. Results are expressed as catechin equivalents (CE).

c) Determination of the Total Flavonoid Content (TFC)

Total flavonoid content was measured by an aluminum chloride colorimetric assay (Zhishen *et al.* 1999). One ml of extract or standard solution of catechin (0.5 mg/ml) was mixed with 4 ml of water, followed by addition of 0.3 ml 5% $NaNO_2$, 0.3 ml of 10% $AlCl_3$ (after 5 minutes) and 2 ml of 1 M NaOH (one minute later), the volume was adjusted (with water) to 10 ml. The absorbance was measured at 510 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). The total flavonoid content was expressed as μmol of catechin equivalent (CE) per g of leaf or fruit fresh weight.

d) Determination of the Total Tannin (Proanthocyanidin) Content (TTC)

The total tannin (proanthocyanidin) content of the leaves and fruits samples was determined by the method developed by Chandrasekara and Shahidi (2011a). Five ml of 0.5% vanillin-HCl reagent were added to 1 ml of extract, mixed thoroughly and incubated at room temperature for 20 minutes. A separate blank for each sample was read with 4% HCl in methanol. The absorbance was read at 500 nm UV/Visible spectrophotometer (Ultrospec 4300 *pro*), and the content of proanthocyanidins was expressed as μmol of CE per g of leaf or fruit fresh weight.

e) Determination of the Total Radical Scavenging Activity

A DPPH assay was conducted according to the method of Brand-Williams *et al.* (1995) with modifications. The stock solution of 1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol was diluted to 60 μM, 1.9 ml of the latter was mixed with 0.1 ml of fruit or leaf extract, shaken vigorously and left in dark for 20 minutes. The absorbance was read at 515 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). The scavenging capacity was expressed as percentage of inhibition of DPPH consumption. The gallic acid standard curve was used to express the results as GAE equivalent.

2.2.2.8. Analysis of Phenolic Compounds by High - Performance Liquid Chromatography (HPLC)

For the analysis of phenolic compounds by high- performance liquid chromatography (HPLC), diluted supernatants of berries were evaporated at room temperature for 2 to 4 days in dark and lyophilized at -50 °C for 72 hours at -50 °C. The freeze-dried samples were extracted in aqueous methanol solution (10 g/l) and filtered through a 0.45 µm polytertafluoroethylene membrane syringe filter. The reversed phase HPLC analysis was carried out using an Agilent 1100 LC/MSD trap system (Agilent Technologies, Palo Alto, CA, USA). A C18 Column (4.6 mm × 150 mm) with 5 µm particle size (Chromatographic Specialities, Brockville, ON, Canada). The eluents were 0.5 % aqueous formic acid (A) and acetonitrile-methanol (95:5) (B) with an initial gradient of 85% solvent A at 0 minute to 0% solvent A and 100% solvent B at 30 minutes. The flow rate and the injection volume were 1.0 ml/min and 90 µl respectively. Compounds of interest were detected using a UV/Visible spectrum (spectral range from 250 to 550 nm) and retention times. Mass spectras were taken for confirmation of identity of compounds using LC/MSD (liquid chromatography / mass selective detector) ion trap system in electron spray ionization (ESI) negative ion mode. Authentic standards were used for identification and making calibration curves for quantification. HPLC was run in MS/MS mode for identification of sugar units attached to phenolics.

2.2.2.9. Inhibition of Lipid Oxidation by Lingonberry Extracts in Pork Model System

The thiobarbituric acid reactive substances (TBARS) were determined in cooked pork by the method described by Chandrasekara and Shahidi (2011b) with some modifications. The ground pork was bought from a local supermarket. The berries used in this study were from the three lingonberry cultivars Regal, Splendor and Erntedank obtained by stem cutting (SC) and leaf tissue culture (LC). Ground pork was mixed with 20% (w/w) deionized water in Mason jars. The extracts of berries (2 g in 72 ml of 80% aqueous acetone containing 0.2% formic acid) and extracts and butylated hydroxytoluene (BHT) were added to meat (100 g) separately and thoroughly homogenized. The control samples were prepared with no addition of the extract. All samples were cooked together in a water bath at 90 °C for 40 minutes with continuous stirring after which the cooked pork was cooled at room temperature. The samples from each jar, after they have been cooled at the room temperature, were divided into four separate 20 ml plastic tubes and stored in refrigerator at 4 °C for 14 d except for one set which was accounted for 0 d. Day 0 sample was analyzed the same day for TBARS. The other samples were drawn on days 3, 7 and 14 for analysis of TBARS. Five ml of a 10% (w/v) trichloroacetic acid (TCA) solution was added to 2.5 g of cooked meat in 50 ml centrifuge tubes and vortexed with high speed for one minute. Five ml of aqueous thiobarbituric acid (TBA) solution (20 mM) were added to each tube followed by vortexing and centrifugation at 3500 *g* for 8 minutes. Supernatants were collected after filtration using Whatman No. 3 filter paper and then heated for 40 minutes using water bath followed by cooling at room temperature. The color of supernatants changed to pink after boiling and the absorbance was

measured at 532 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). 1,1,3,3-tetramethoxypropane (precursor of malondialdehyde, MDA) was used to prepare a standard curve. Results were expressed as mg of MDA equivalents per kg of sample.

2.2.2.10. Statistical Analysis

All the experiments for biochemical analysis were repeated in triplicate with 5 plants per treatment. The data in the text, tables, and figures are expressed as means ±SD of three biological replicates (5 plants per each treatment). Data for all the characteristics were subjected to analysis of variance (ANOVA) using the SAS statistical software package (Release 8.2; SAS Institute, Inc., Cary, NC, USA). Significance of F-tests were evaluated at $P \leq 0.05$. Differences among treatments were further analysed using Duncan's multiple range test.

2.3. Results

2.3.1. Morphological Characteristics and Chlorophyll Content of Differentially Propagated Lingonberry Cultivars

All the cultivars obtained by *in vitro* culture (NC and LC) were superior to *ex vitro* SC plants in terms of the number of shoots, branching, and rhizomes whereas LC plants were characterized by a higher number of leaves per branch but less secondary branching as compared to NC plants (Figs 2.1 & 2.2). Figure 2.2 is a graphical representation of morphological differences in the differentially propagated lingonberry cultivars. The data

show variability in relation to cultivar, as well as to the method of propagation. The analysis of variance (Table 2.1) indicates significant inter-cultivar differences and effects of propagation methods on morphology. The cultivar Splendor was found to be superior among the three cultivars in terms of plant height, number of leaves per branch, berry mass and berry diameter. Regal had the highest of number of rhizomes, whereas Erntedank exhibited the highest branching. Notably, many vegetative characteristics (such as height, number of rhizomes, leaves per branch) increase in tissue-propagated plants (more in LC than in NC) as compared to SC plants. On the contrary, the average mass of berr and berry diameter were lower in tissue culture plants compared to SC plants, and lowest in NC plants. The same tendency was observed in the concentrations of chlorophylls *a* and *b*, which are lower in tissue culture plants than SC plants but there were no inter-cultivar differences in chlorophyll concentrations (Fig. 2.3).

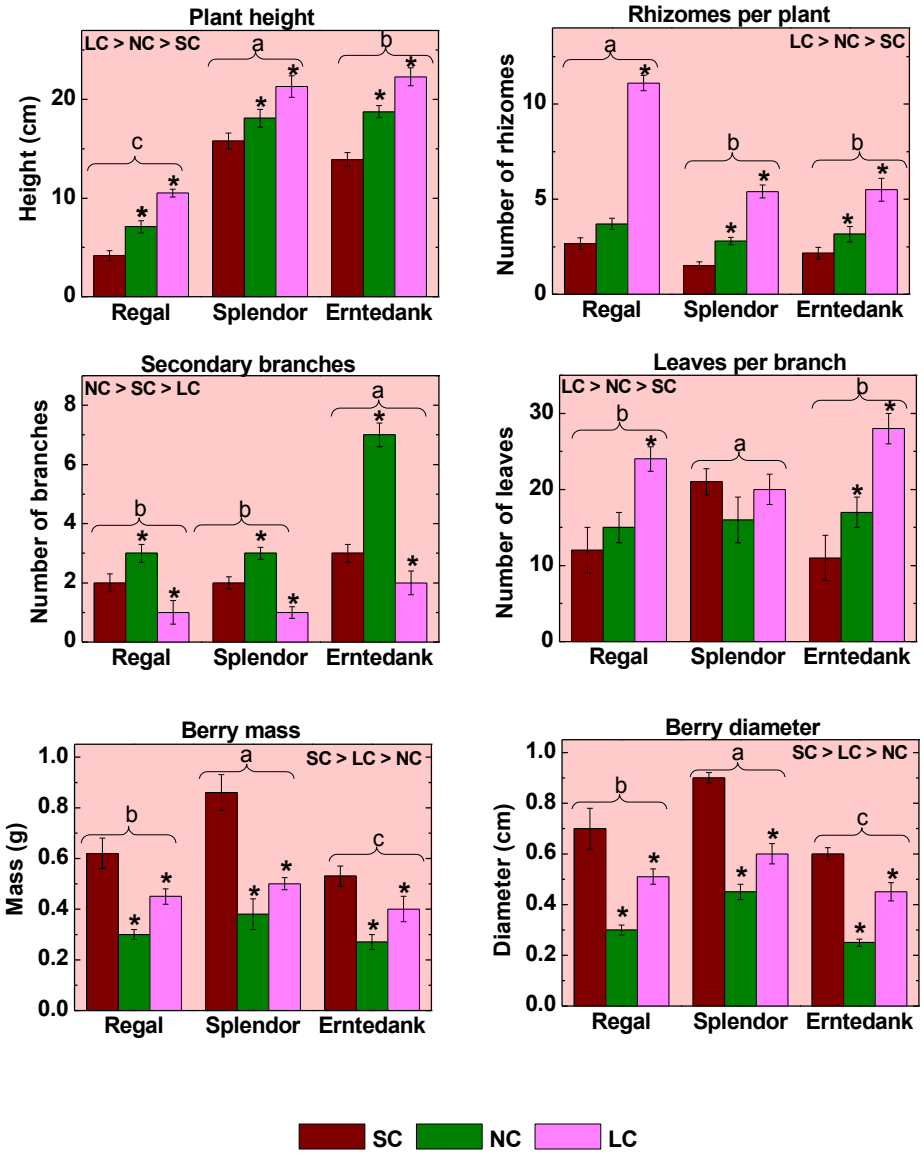

Figure 2.2. Morphological characteristics of lingonberry cultivars Regal, Splendor and Erntedank obtained by the three different propagation methods: stem cutting (wine bars), node cultures (green bars), leaf cultures (pink bars). SC - Stem cutting; NC - Nodal culture; LC - Leaf culture. Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard. Letters a, b and c indicate differences between the cultivars at $P < 0.05$ estimated by Duncan's multiple range test. The values with same letters are not significantly different

Table 2.1. Effects of genotype (cultivars) and the propagation methods on morphological characters in three lingonberry cultivars

Parameter	Plant height (cm)	# of rhizome	# of branches	Leaves per branch	Berry mass (g)	Berry diameter (cm)
Cultivar						
Regal	7.12 c[z]	5.11 a	6.61 a	17.67 b	0.54 b	0.52 b
Splendor	18.86 a	4.11 b	4.72 b	23.39 a	0.59 a	0.60 a
Erntedank	17.89 b	4.44 b	4.16 b	18.28 b	0.46 c	0.45 c
Propagation method (PM)						
SC	11.48 c	1.72 c	2.40 c	14.44 c	0.70 a	0.71 a
NC	14.78 b	4.28 b	5.00 b	18.00 b	0.35 c	0.35 c
LC	17.61 a	7.67 a	8.11 a	26.89 a	0.54 b	0.51 b
Analysis of variance (P values)						
Cv	<0.0001	0.0140	<0.0001	<0.0001	<0.0001	<0.0001
PM	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cv × PM	0.0562	0.2466	<0.0001	0.0029	0.0016	0.0075

[z] - Means within columns, and factors, followed by different lower-case letters indicate differences at $P < 0.05$ by Duncan's multiple range test. Propagation method: SC-stem cutting; NC, Nodal culture; LC - leaf culture; Cv - Cultivars; PM - Propagation methods; Cv × PM - Interaction between cultivars and propagation methods.

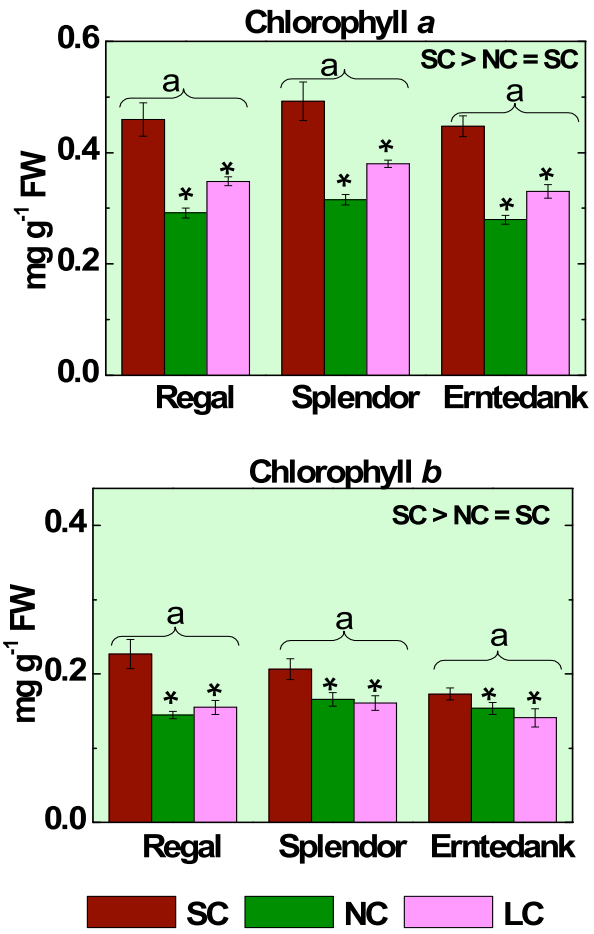

Figure 2.3. The content of chlorophyll *a* and chlorophyll *b* of lingonberry cultivars Regal, Splendor and Erntedank obtained by three different propagation methods: from stem cutting (wine bars), from node cultures (green bars), from leaf cultures (pink bars). SC - Stem cutting; NC - Nodal culture; LC - Leaf culture. Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard. Letters a, b and c indicate differences at $P < 0.05$ by Duncan's multiple range test. Values with same letters are not significantly different.

Table 2.2. The content of chlorophyll *a* and *b* (mg/g FW) in three lingonberry cultivar as affected by propagation method and genotype (cultivars)

Parameter	Chlorophyll *a*	Chlorophyll *b*
Regal	0.23[z] a	0.11 a
Splendor	0.25 a	0.11 a
Erntedank	0.22 a	0.10 a
Propagation method (PM)		
SC	0.47 a	0.20 a
NC	0.30 b	0.15 b
LC	0.235 b	0.15 b
Analysis of variance (P values)		
Cv	0.3106	0.1061
PM	<0.0023	0.0011
Cv × PM	0.9271	0.7132

[z] - Means within columns, and factors, followed by different lower-case letters indicate differences at $P < 0.05$ by Duncan's multiple range test. Propagation method: SC-stem cutting; NC, Nodal culture; LC - leaf culture; Cv - Cultivars; PM - Propagation methods; Cv × PM - Interaction between cultivars and propagation methods.

2.3.2. The Levels of Reduced and Oxidized Ascorbate and Glutathione

The content of total ascorbate (AsA + DHA) and total glutathione (GSH + GSSG) differed as a result of different methods of propagation (Fig. 2.4, Table 2.3). Although, the total ascorbate content was not significantly different among the three cultivars, there were some inter -propagation differences observed. Significantly high total ascorbate content was detected in Splendor and Erntedank propagated by Leaf tissue culture (LC). DHA levels increased significantly in all cultivars obtained from LC (but not from NC) as compared to SC (Fig. 2.4 & Table 2.3). The DHA levels were highest in Splendor compared to Regal and Erntedank.

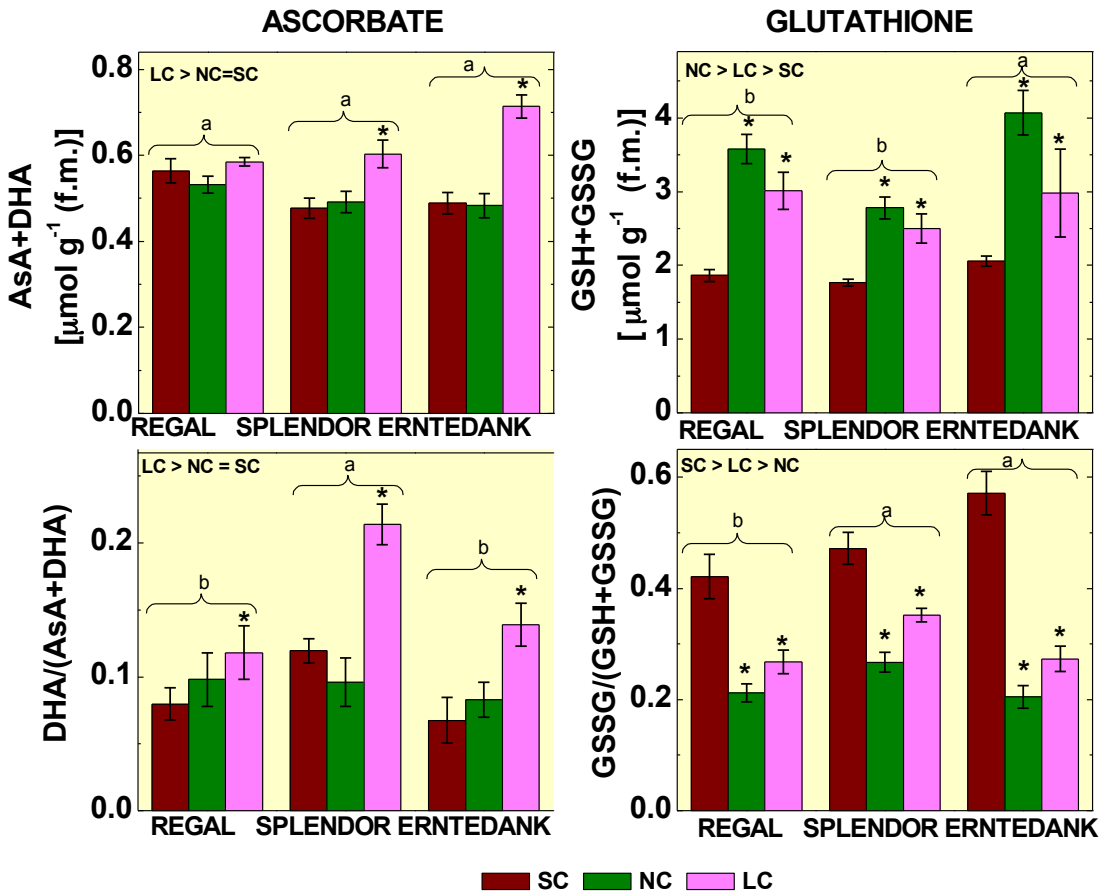

Figure 2.4. Levels of ascorbate and glutathione and the relative content of the oxidized species (DHA, GSSG) in leaves of three lingonberry cultivars (Regal, Splendor, Erntedank) propagated by three different methods: stem cutting (wine bars), from node cultures (green bars), from leaf cultures (pink bars). SC - Stem cutting; NC - Nodal culture; LC - Leaf culture. Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard.

The total glutathione content was significantly higher in the *in vitro* derived plants (NC and LC), which corresponded also to its less oxidized level (less GSSH) (Fig. 2.4 & Table 2.3). The reduction potentials (E_{hc}) of glutathione (GSSG/2GSH half-cell) were calculated according to the formula of Schafer and Buettner (2001).

$$E_{hc} = -240 - (59.1/2) \log ([GSH]^2/[GSSG]) \text{ mV}$$

Table 2.3. Effects of genotype and propagation methods on total and reduced ascorbate and glutathione content in three lingonberry cultivars

Parameter	ASA+DHA	DHA/(ASA+DHA)	GSH+GSSG	GSSG/(GSH+GSSG)
Regal	0.56 a [z]	0.11 b	2.82 b	0.29 b
Splendor	0.52 b	0.14 a	2.35 b	0.36 a
Erntedank	0.56 a	0.10 b	3.04 a	0.35 a
Propagation method (PM)				
SC	0.51 b	0.09 b	1.89 c	0.49 a
NC	0.50 b	0.09 b	3.48 a	0.23 c
LC	0.63 a	0.16 a	2.83 b	0.29 b
Analysis of variance (P values)				
Cv	0.3106	0.1061	0.0016	0.0075
PM	<0.0001	<0.0001	<0.0001	<0.0001
Cv × PM	0.9271	0.7132	0.0562	0.2466

[z] - Means within columns, and factors, followed by different lower-case letters indicate differences at $P < 0.05$ by Duncan's multiple range test. Propagation method: SC-stem cutting; NC, Nodal culture; LC - leaf culture; Cv - Cultivars; PM - Propagation methods; Cv × PM - Interaction between cultivars and propagation methods.

The SC plants possessed the least negative reduction potential of glutathione (from -235 mV in Erntedank to -242-245 mV in Regal and Splendor, respectively), while NC had the most negative values (from -262 mV in Splendor -270 in Regal and -272 mV Erntedank), and slightly less negative values were in LC (from -254 mV to -265 mV)

2.3.3. Activities of the Ascorbate-Glutathione Cycle Enzymes and Catalase in Differentially Propagated Lingonberry Cultivars

Activities of the ascorbate-glutathione cycle enzymes and catalase in the leaves of studied cultivars were affected by the propagation method as well as the genotypes (inter cultivar differences) (Fig. 2.5, Table 2.4). Although, the APX activity in Erntedank remained similar for all three propagation methods, it was significantly higher in *in vitro* (NC & LC)

derived Regal and Splendor as compared to those derived from SC. Among the *in vitro* derived Regal and Splendor, APX levels were higher in LC derived plants then those derived from NC. In general, the increase in APX corresponds to a higher content of the oxidized ascorbate species (DHA) (Fig. 2.5 & Table 2.3). Glutathione reductase (GR) activity in leaves was affected by the propagation methods in a similar way as APX. The NC plants did not show a significant increase in GR activity except for Splendor, while the GR activity in LC plants was the highest, 5-10 times higher than in control plants. The increase in GR corresponds to a decrease of the portion of the oxidized glutathione (GSSG) in relation to the total glutathione content (Fig. 2.5). Monodehydroascorbate reductase (MDHAR) activity showed a similar pattern in Splendor and Erntedank as GR but the difference between LC and SC was most striking in Regal and small in Erntedank. GR activity in Splendor was not affected by propagation method. Dehydroascorbate reductase (DHAR) activity exhibited a completely different pattern as compared to MDHAR, APX and GR. In Erntedank, we observed very low DHAR activity. The low DHAR together with high APX, in fact shows a consistency with the DHA content in investigated plants (Fig. 2.5). According to Duncan's multiple range test, DHAR was significantly low in NC plants and highest in SC plants (Table 2.4). CAT activity exhibited a similar pattern as APX for all the cultivars. Both APX and CAT were highest in Regal and lowest in Erntedank. All the studied enzymes except DHAR showed the highest levels in LC and the lowest in SC (Fig. 2.5).

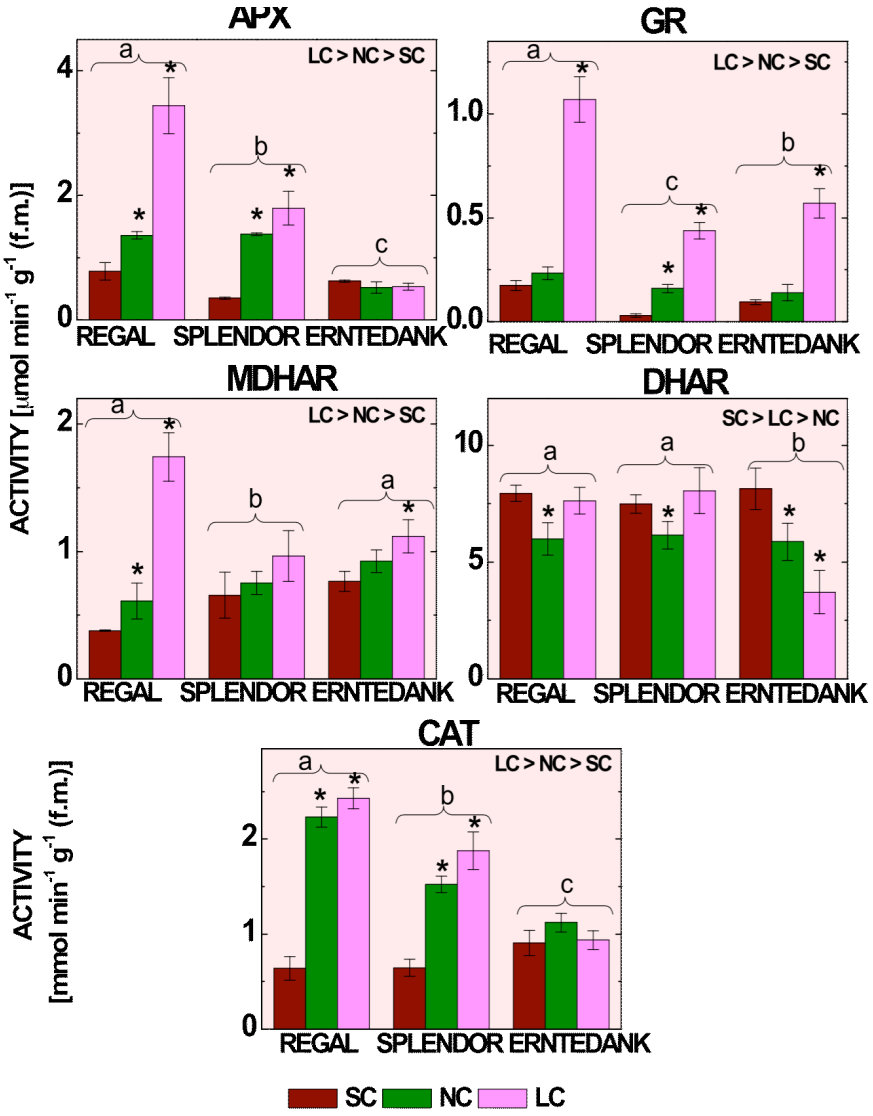

Figure 2.5. Activities of enzymes of the ascorbate-glutathione cycle (ascorbate peroxidase – APX, glutathione reductase – GR, monodehydroascorbate reductase – MDHAR, dehydroascorbate reductase -DHAR and of catalase (CAT) in leaves of three lingonberry cultivars (Regal, Splendor, Erntedank) propagated by stem cutting (wine bars), from node cultures (green bars) and from leaf cultures (pink bars). Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard.

Table 2.4. Effects of genotype and propagation method on antioxidant enzyme activities (in μmol min^{-1} g^{-1} FW) in three lingonberry cultivars (for catalase in mmol min^{-1} g^{-1} FW)

Parameter	APX	GR	DHAR	MDHR	CAT
Cultivar					
Regal	1.87 a [z]	0.49 a	7.18 a	0.90 a	1780.38 a
Splendor	1.17 b	0.21 c	7.23 a	0.79 b	1348.33 b
Erntedank	0.55 c	0.26 b	5.90 b	0.93 a	980.53 c
Propagation method (PM)					
SC	0.58 c	0.10 c	7.85 a	0.59 c	707.98 c
NC	1.08 b	0.17 b	6.00 c	0.76 b	1622.06 b
LC	1.91 a	0.69 a	6.46 b	1.27 a	1764.06 a
Analysis of variance (P values)					
Cv	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PM	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cv × PM	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[z] - Means within columns, and factors, followed by different lower-case letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Propagation method: SC-stem cutting; NC, Nodal culture; LC- leaf culture; Cv- Cultivars; PM- Propagation methods; Cv × PM- Interaction between cultivars and propagation methods

2.3.4. Phenolic Compounds and Radical Scavenging Capacity

The content of total soluble phenolics and other antioxidant compounds in lingonberry cultivars showed different (often opposite) patterns for fruits and leaves and was influenced by propagation methods. As evident from Figure 2.6, TPC was 5-10 times lower in fruits than in leaves (as calculated per g fresh weight). The leaves of NC and LC plants exhibited a significant decrease in phenolic content compared to SC plants. In fruits, the observed variations were smaller and the total phenolic content, in contrast with leaves, was shown to be enhanced by *in vitro* propagation method in agreement with previous data of Foley and Debnath (2007).

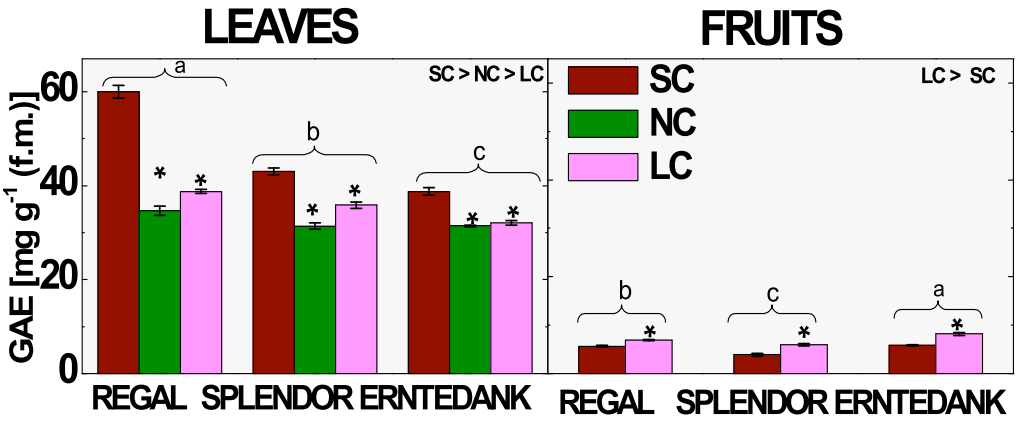

Figure 2.6. The total phenolic content in leaves and fruits of three lingonberry cultivars propagated by different methods: propagated by stem cutting (wine bars), from node cultures (green bars), from leaf cultures (pink bars). Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard.GAE – gallic acid equivalents.

Table 2.5. Effect of genotype and propagation method on levels of metabolites in leaves of three lingonberry cultivars

Parameter	Phenolics	Flavonoids	Anthocyanin	Tannin	DPPH
Cultivar					
Regal	44.476 a[z]	11.05 a	8.05 a	23.913 a	33.144 c
Splendor	36.794 b	10.95 b	7.46 c	21.404 b	33.344 a
Erntedank	33.777 c	8.813 c	8.05 a	16.560 c	33.221 b
Propagation method (PM)					
SC	46.934 a	10.791 c	9.043 a	22.749 a	33.560 a
NC	35.584 b	11.716 a	8.69 a	20.584 b	33.092 b
LC	32.528 c	10.216 b	8.65 a	18.545 c	33.058 c
Analysis of variance (P values)					
Cv	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PM	<0.0001	<0.0001	0.0098	<0.0001	<0.0001
Cv × PM	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[z] - Means within columns, and factors, followed by different lower-case letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Propagation method: SC-stem cutting; NC, Nodal culture; LC- leaf culture; Cv- Cultivars; PM- Propagation methods; Cv × PM- Interaction between cultivars and propagation methods

Table 2.6. Effect of genotype and propagation method on levels of metabolites in fruits of three lingonberry cultivars

Parameter	Phenolics	Flavonoids	Anthocyanin	Tannin	DPPH
Cultivar					
Regal	6.273 b[z]	8.870 b	4.645 b	10.244 c	13.930 a
Splendor	4.894 c	6.920 c	7.088 a	12.947 a	10.124 c
Erntedank	7.051 a	10.477 a	4.440 b	11.211 b	11.213 b
Propagation method (PM)					
SC	5.127 b	6.575 b	5.656 a	10.628 b	10.793 b
LC	7.018 a	10.937 a	5.126 b	12.306 a	12.717 a
Analysis of variance (P values)					
Cv	<0.0001	<0.0001	0.0011	<0.0001	<0.0001
PM	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cv × PM	0.0014	<0.0001	<0.0001	<0.0001	<0.0001

[z] - Means within columns, and factors, followed by different lower-case letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Propagation method: SC-stem cutting; NC, Nodal culture; LC- leaf culture; Cv- Cultivars; PM- Propagation methods; Cv × PM- Interaction between cultivars and propagation methods

The total anthocyanin content (expressed as catechin equivalents), was not influenced by the propagation method except the significant decrease in leaves of Erntedank plants obtained from NC and LC plants. The anthocyanin content was lower in fruits than in leaves (Fig. 2.7, Tables 2.5 & 2.6).

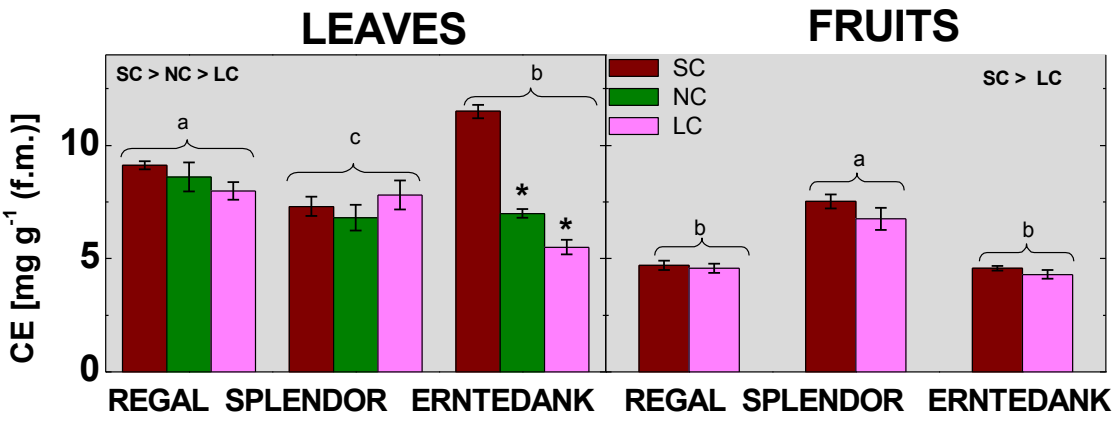

Figure 2.7. The total anthocyanin content in leaves and fruits of three lingonberry cultivars propagated by different methods: propagated by stem cutting (wine bars), from node cultures (green bars), from leaf cultures (pink bars). Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the control.CE – catechin equivalents.

The total flavonoid content (expressed as catechin equivalents) was significantly higher in the fruits and leaves of *in vitro*-derived plants then SC plants except for Regal LC plants where the level was significantly lower than in SC plants (Fig. 2.8, Tables 2.5 & 2.6).

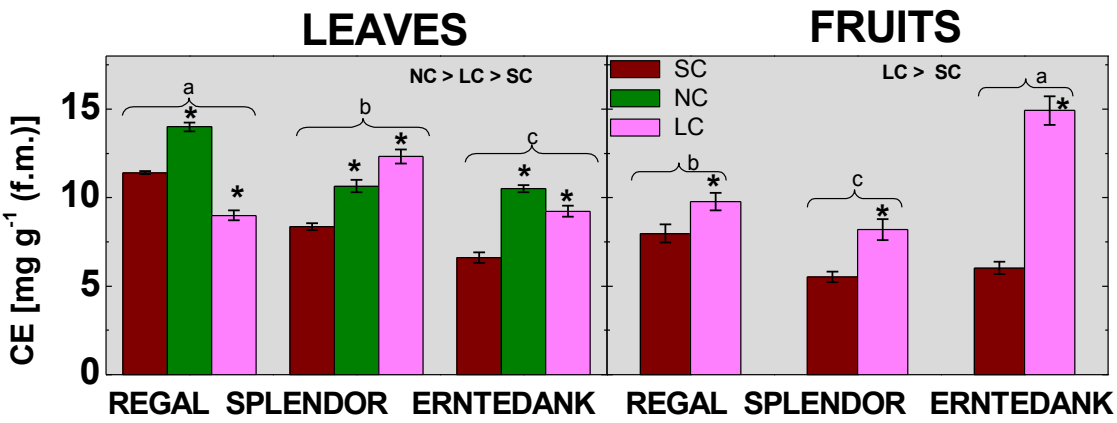

Figure 2.8. The total flavonoid content in leaves and fruits of three lingonberry cultivars propagated by different methods: propagated by stem cutting (wine bars), from node cultures (green bars), from leaf cultures (pink bars). Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard. CE – catechin equivalents

The total tannin content in leaves was not significantly different among the fruits of the three cultivar but were highest in leaves of Regal followed by Spelndor and was least in Erntedank. There was a tendency of a decrease of tannin content in the leaves of *in vitro*-derived plants except for Erntedank where the differences were insignificant. The total tannin content was significantly higher in fruits of *in vitro* derived plants compared to SC plants (Fig. 2.9, Tables 2.5 & 2.6).

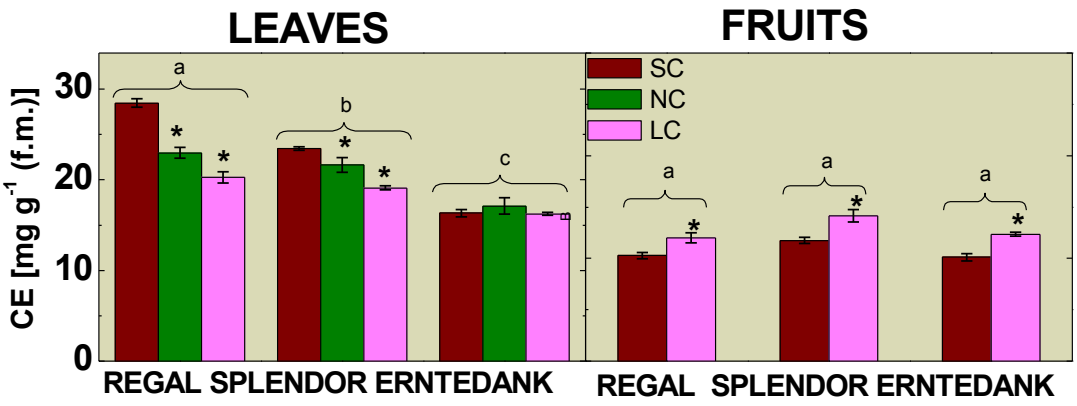

Figure 2.9. The total tannin content in leaves and fruits of three lingonberry cultivars propagated by different methods: propagated by stem cutting (wine bars), from node cultures (green bars), from leaf cultures (pink bars). Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard. CE – catechin equivalents.

The total DPPH radical scavenging capacity measured as gallic acid equivalents was twice as high in leaves than in fruits. In leaves it was not affected by the method of propagation while in fruits of plants obtained from tissue culture the radical scavenging capacity was ~10% higher as compared to fruits of control SC plants (Fig. 2.10, Tables 2.5 & 2.6).

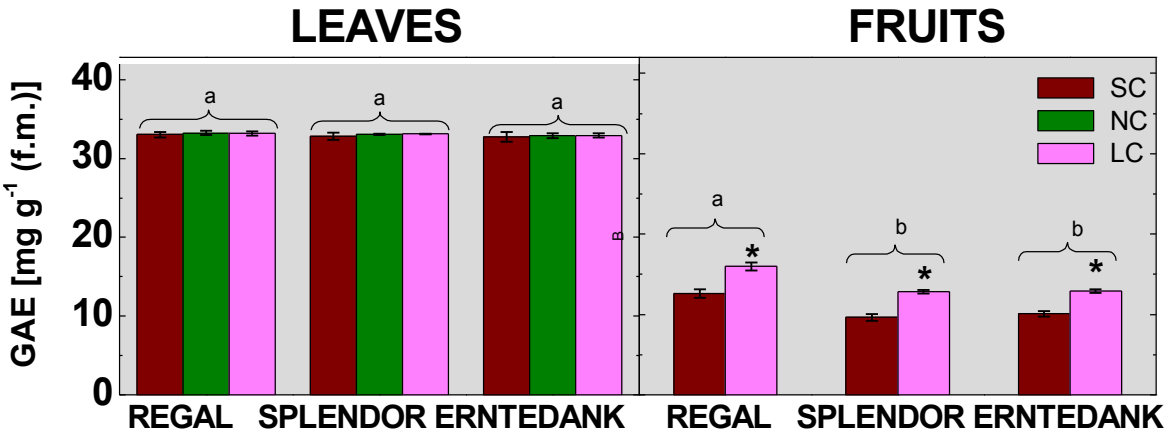

Figure 2.10. The total radical scavenging capacity in leaves and fruits of three lingonberry cultivars propagated by different methods: propagated by stem cutting (wine bars), from node cultures (green bars), from leaf cultures (pink bars). Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard. GAE – gallic acid equivalents.

2.3.5. Phenolic Compounds Identified in Leaves and Berry Extracts of Differentially Propagated Plants

The content of phenolic compounds identified in leaves and in berry extracts of differentially propagated lingonberry cultivars is shown in Table 2.7. A total of five phenolic compounds were identified in the extracts of both leaves and berries. Catechin, epicatechin, gallic acid, *p*-coumaric acid and quercetin were identified in the extracts according to their MS/MS spectra following the breakdown of sugar-phenolic esters. The weights of sugar moieties attached to quercetin were tentatively identified and confirmed with the literature (Anttonen *et al.* 2006; Ek *et al.,* 2006). At least four quercetin derivatives were tentatively identified in the extracts. Quercetin-3-O-galactoside, quercetin-3-O-glucoside and quercetin-3-O-arabinoside were unambiguously detected in fruit extracts while in leaves quercetin-3-O-rutinoside was also identified in addition to the other three derivatives. Quantification of the total quercetin and other identified compounds was done using standard calibration curve and peak areas. In fruits, the levels of identified phenolics showed significant increase in the *in vitro*-derived plants except for *p*-coumaric acid which was almost same in fruits of the *in vitro*- and *ex vitro*-derived plants. The trend was opposite in leaves and correlated with total phenolic assay. Significantly higher levels of identified compounds were observed in leaves of the *ex vitro*-derived plants except for *p*-coumaric acid.

Table 2.7. Effect of genotype and propagation method on phenolic compounds in lingonberry cultivars

Parameter	Gallic Acid		Catechin		Epicatechin		p-Coumaric acid		Quercetin	
	leaf	berry	leaf	berry	leaf	berry	leaf	berry	leaf	berry
Cultivar										
Regal	0.201 c[Z]	0.177 a	2.328 c	0.968 a	0.632 b	0.111 b	0.089 b	0.051 b	3.689 a	0.124 a
Splendor	0.331 b	0.163 a	2.153 b	0.857 b	0.576 c	0.134 a	0.124 a	0.047 b	2.613 c	0.107 b
Erntedank	0.225 a	0.168 a	3.316 a	0.961 a	0.729 a	0.082 c	0.083 b	0.102 a	3.508 b	0.120 a
Propagation method (PM)										
SC	0.282 a	0.154 b	3.149 a	0.782 b	0.706 a	0.072 b	0.099 a	0.063 b	3.505 a	0.095 b
LC	0.223 b	0.185 a	2.716 b	1.075 a	0.585 b	0.146 a	0.098 a	0.070 a	3.035 b	0.139 a
Analysis of variance (P values)										
Cv	<0.0001	0.3106	<0.0001	0.0011	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0004
PM	<0.0002	0.0005	<0.0001	<0.0001	<0.0001	<0.0001	0.8347	0.0031	<0.0001	<0.0001
Cv × PM	0.0077	0.9271	0.0108	<0.0001	<0.0001	<0.0001	0.4257	0.0104	0.0716	0.1140

[Z] – Means within columns, and factors, followed by different lower-case letters indicate differences at $P \leq 0.05$ by Duncan's multiple range test Data expressed in μg g^{-1} (FW) of leaf and berry sample. PM, Propagation method: SC, stem cutting (standard plants); LC, leaf culture; Cv – variance between cultivars (for all propagation methods); Cv × PM – variance between propagation methods (for all cultivars).

2.3.6. Inhibition by Lingonberry Extracts of Oxidation in Pork Model System

The extracts of berries did not decrease in the content of thiobarbituric acid reactive substances (TBARS) from the storage day 0 to the 14th day, but were clearly effective in inhibiting the level of oxidation for the entire storage period. This is shown in Table 2.8 where it is seen that the controls have high values of TBARS as malonic dialdehyde (MDA) equivalents in comparison to those of the samples with berry extracts. There were only small differences between the three cultivars, however more notable differences were observed between plants obtained by different methods of propagation. The berries of tissue culture-obtained plants were more efficient in terms of inhibiting the oxidation process: the TBARS values for meat with these extracts were much lower as compared to the extracts of stem cutting-obtained berries. After the 14th day of storage, berry extracts from leaf culture and stem cutting inhibited the formation of TBARS by 75-76% and 65-70% respectively (Fig. 2.11).

Table 2.8. Thiobarbituric acid reactive substances (TBARS) of lingonberry extracts expressed as µmol malondialdehyde equivalents (MDA) per kg of meat

Processing conditions	TBARS in MDA equivalents (µmol kg^{-1} meat)			
	Storage time			
	0 d	**3 d**	**7 d**	**14 d**
BHT	0.44 ± 0.10	1.29 ± 0.16	1.53 ± 0.28	1.77 ± 0.23
Control (no extract)	1.77 ± 0.23	16.17 ± 0.16	20.49 ± 0.55	19.31 ± 0.48
Regal, SC	4.73 ± 0.13	5.03 ± 0.24	5.35 ± 0.59	5.68 ± 0.60
Regal, LC	2.08 ± 0.03	2.88 ± 0.09	4.20 ± 0.11	4.12 ± 0.38
Splendor, SC	5.25 ± 0.29	6.32 ± 0.32	6.74 ± 1.03	6.72 ± 0.27
Splendor, LC	2.21 ± 0.03	3.75 ± 0.21	4.07 ± 0.09	4.58 ± .010
Erntedank, SC	4.98 ± 0.19	5.58 ± 0.45	6.02 ± 0.40	5.99 ± 0.44
Erntedank, LC	2.11 ± 0.10	3.75 ± 0.04	4.81 ± 0.29	4.46 ±0.21

Abbreviations: SC, Plants obtained from stem cutting; LC, plants obtained from leaf tissue culture; BHT, butylated hydroxytoluene (standard).

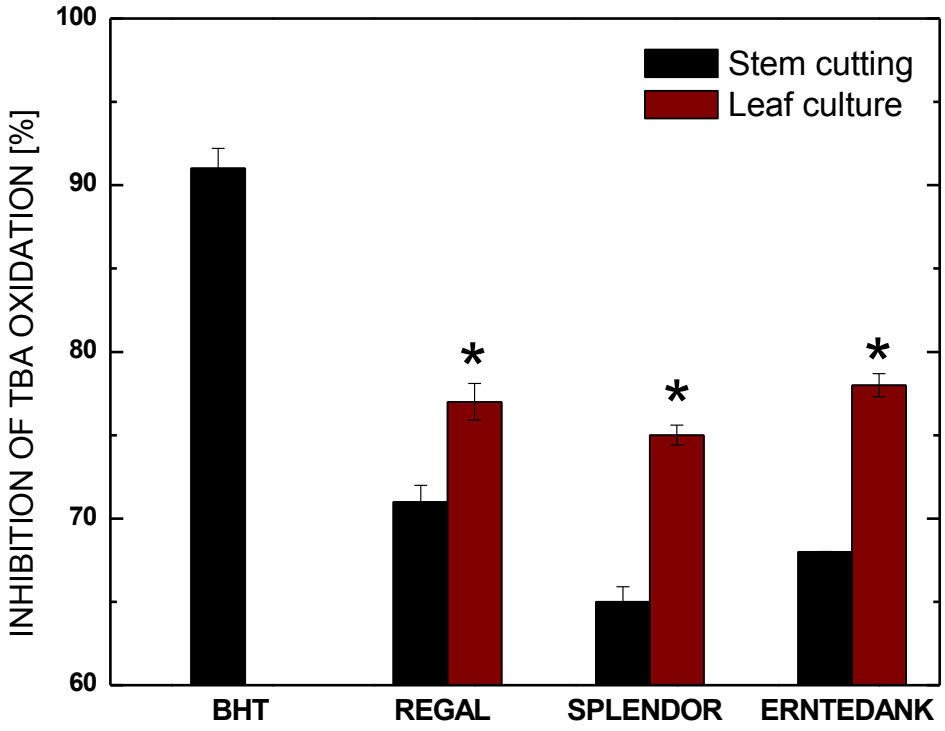

Figure 2.11. Inhibition of lipid oxidation by lingonberry extract and by butylated hydroxytholuene (BHT) after 14 d of storage in pork model system. Propagation by stem cutting (black bars) and from leaf cultures (brown bars). Means ± SE, n = 5, * – values significantly different at $P < 0.05$ from the standard.

2.4. Discussion

This study explores the patterns of antioxidant metabolism among 1) the contrasting lingonberry cultivars and 2) the methods of their propagation. To reveal definitive correlations between morphological properties and antioxidant capacity of lingonberry plants, three cultivars were chosen that strongly differed in their basic features including height, number of rhizomes, branching, number of leaves per branch, berry mass and berry diameter. All the three cultivars adapted are to the cold and harsh climate of Newfoundland and exhibit a high degree of plasticity towards environmental conditions. Splendor is a moderately yielding cultivar and was found to be superior in height, berry mass and berry diameter and produced the highest number of leaves per branch compared to the other studied cultivars. In previous studies done on Vaccinium species, it was also reported that Splendor produces larger berries compared to other lingonberry cultivars (Morrison *et al.,* 2000; Debnath, 2006). Regal, a low-yielding cultivar, produced the highest number of rhizomes but was the shortest plant, and Erntedank, a high-yielding cultivar, produced the highest number of secondary branches but less rhizomes, less leaves per branch and the lowest berry mass and diameter. There is a strong difference in morphological properties of the cultivar Enterdank from other two studied cultivars. First, the difference in the height of Erntedank plants obtained from stem cutting and tissue culture is the highest among all cultivars. The percentage of change in the number of branches per rhizome (data not shown) is significantly higher in Erntedank. The propagation method had a profound effect on the morphological characteristics of lingonberry cultivars. Lingonberry cultivars propagated *in vitro* (LC and NC) were superior over the *ex vitro* (SC) propagated cultivars in all the studied morphological characters except for berry

size and mass. LC plants exhibited the highest plant height, number of rhizomes, and number of leaves per branch. NC plants exhibited highest number of secondary branches, lowest berry yield and berry size whereas SC plants showed highest berry yield, berry mass and berry diameter. Plants require considerable amount of nutrients and energy to produce fruits. The obtained results suggest that SC plants showed energy conservation by producing fewer rhizomes, leaves and branches. Similar results were observed in the study done by Debnath (2006). In case of *in vitro* propagation, substantial amounts of energy was directed into the production on new axillary shoots and rhizomes. With the commitment to vegetative production, the *in vitro* derived plants restrict berry yield, mass and diameter (Debnath 2006). The *in vitro* derived plants spread rhizomatously and produce larger number of rhizomes as compared to SC plants. Similar results were reported in previous studies done by Holloway (1985) and Debnath (2006). The increased rhizome production in SC and LC plants could be the result of hormones initially supplied in the culture medium. In the present study, zeatin hormone was used which is a cytokinin and mainly responsible for root initiation and stem development. Environmental variations initiates a plastic response that could lead to increased branching, increased leaf production or rhizome formation (Donohue *et al.*, 2001).

The levels of chlorophyll *a* and *b* were significantly affected by the propagation method but these levels were not affected by the cultivars. The content of chlorophyll *a* and *b* contents were higher in SC plants as compared to *in vitro* plants, indicating that SC plants absorb light in violet-blue and orange-red wavelengths better than the *in vitro* plants. It has been previously reported that plants grown in shade had higher chlorophyll content then those grown in the sun (Lance & Guy, 1992; Reyes *et al*., 1996). A reduction in chlorophyll level

was observed in *Schefflera arboricola* when provided with high light intensity (Kubatsch *et al*., 2007). In the present study, plants in the greenhouse were maintained in trays containing 15 4 inches pots side by side. Plants were so crowded that they provided shade to one another and from other nearby plants throughout the day. In the present study, unlike LC and NC, SC plants were placed in the greenhouse since the beginning and hence they were in the greenhouse for much longer period as compared to the NC and LC plants which could have lead to increased chlorophyll content in the SC plants as compared to LC and NC.

There are several stress factors involved with tissue culture plants such as mechanical injuries, wounding, osmotic shock due to sucrose content in the medium, nitrogen toxicity, hormonal imbalances, gas toxicity, and more. (Desjardins *et al*., 2007). In addition to the stresses caused in the *in vitro* environment, the tissue stress could be caused when *in vitro* plants are transferred to the greenhouse. There are some major differences between the environments of the plants growing *in vitro* and in the greenhouse environment, such as difference in lightening quality and quantity, nutrients, relative humidity, gaseous composition, relative humidity and growth substrate. Therefore, when plants are transferred from tissue culture environment to the greenhouse environment, it causes plant tissues to stress (Seelye *et al*., 2003). In response to abiotic or biotic stress, plants generate ROS. Mitochondria are a major source of ROS where molecular oxygen may undergo a univalent reduction in complexes I and III of the respiratory chain and lead to formation of superoxide which subsequently dismutates to hydrogen peroxide (Braidot *et al*., 1999).

The direct involvement of radical species in morphogenetic process has been shown (Jana & Shekhawat, 2012), *e.g.* in relation to loosening of cell walls (Cárdenas, 2009; Mülle *et al.*, 2009). Morphogenesis is a dynamic biological process that helps an organism develop its shape. Reactive oxygen species participate in cell expansion during morphogenesis in particular by affecting the activity of calcium channels required for polar growth (Carol & Dolan 2006). Earnshaw & Johnson (1985) reported the correlation between glutathione levels with morphogenetic competence in carrot suspension cultures and concluded that development of plant tissues occurs in a more oxidising environment. As an example, pro- and antioxidant enzymes can control development by establishing necessary concentrations of superoxide and H_2O_2 for regulation of plant cell expansion through the activation of Ca^{2+} channels (Foreman *et al.,* 2003). A concrete mechanism of directing morphogenesis may involve the control of polarized cell growth in plants by Rho-like small GTPases (ROPs) through plant-specific pathways involving the regulated release and scavenging of reactive oxygen species (Uhrig & Hülskamp 2006). The enzymes modulating concentration of reactive oxygen species such as the alternative oxidase of mitochondria are directly involved in determination of the rate of growth, branching and other morphogenetic events (McNulty *et al.,* 1988; Hilal *et al.,* 1997; Fiorani *et al.,* 2005; Frederico *et al.,* 2009). However, the most important in regulating morphogenesis are the enzymes of the ascorbate glutathione cycle (plus catalase) (Gupta & Datta, 2003; Vatankhah *et al.,* 2010) that directly determine the redox states of ascorbate and glutathione and the concentrations of AFR and H_2O_2.

Despite certain differences between the cultivars, general tendencies reflecting changes in the antioxidant metabolism were revealed. Generally, the leaves of plants obtained from the

in vitro condition showed significantly higher antioxidant enzyme activities (except for DHAR). This is in line with the observation that the total radical scavenging capacity was enhanced in both berries and leaves of the *in vitro*-propagated plants. The total soluble phenolics, tannins and flavonoids were found to be enhanced in fruits of plants obtained by the *in vitro*-propagation method whereas in leaves these metabolites were higher in SC plants except for flavonoids which was in higher concentrations in leaves of the *in vitro*-derived plants. The total anthocyanin content was not observed to be significantly different in fruits of differentially propagated plants.

Lower levels of total phenolics, anthocyanins and tannins were observed in leaves and higher levels in fruits. Reverse changes in antioxidant metabolites in fruits as compared to vegetative parts in relation to the method of propagation also correspond to lower number of berries, their lower weight, and size (Fig. 2.2). In general, this could be inferred to mean that tissue culture propagation enhances growth and metabolism in vegetative parts of plants, while the increased antioxidant properties of smaller berries can be explained by a higher proportion of berry coat which is enriched in antioxidant compounds.

The total ascorbate pool remained at similar levels in the three cultivars but was affected by the propagation method. The total ascorbate pool increased in the LC plants as compared to NC and SC. The total glutathione was significantly higher in NC and LC plants compared to SC plants. It has been reported that high light and low temperature caused increase in ascorbate and glutathione pool in *Dunaliella salina* (Haghjou *et al*., 2009).

The cellular redox state is considered as an important determinant for plant growth and development. Schafer and Buettner (2001) suggested that the resulting action of several redox couples which are able to interconvert between oxidized and reduced forms can trigger major metabolic events and determine morphogenetic processes in plants. The displacement of equilibrium between the reduced and oxidized forms of such compounds as ascorbate and glutathione can essentially affect tissue patterning and morphogenesis (Mitrovic *et al.,* 2012; Talukdar, 2012). The synergistically interacting redox couples of glutathione (GSH/GSSG) and of ascorbate (AsA/DHA + AFR) are defined as best indicators of the overall cellular environment (Schafer & Buettner, 2001). The involvement of GSH in cell division processes is proven by its stimulation of Arabidopsis root growth (Sanchez-Fernandez *et al.,* 1997). While GSH favours cell division and proliferation through a direct involvement with the cell cycle machinery, GSSG levels are related to differentiation processes (Schafer & Buettner, 2001; Stasolla *et al.,* 2004). It can be seen from the obtained data that more reduced glutathione pool in the *in vitro*-propagated plants results in their increased height and increased number of rhizomes and leaves per branch but decreased number of branches per rhizome.

Ascorbate and glutathione reduction pairs may have different roles in regulation of metabolism as they have different redox potentials. This was shown, for example, in that they both are needed to keep iron in the reduced form in plant hemeproteins (Igamberdiev *et al.,* 2006, 2011). While glutathione has a direct influence on morphogenetic phenomena, ascorbate also has effects on elongation via influence on cell walls. It is shown that more reduced ascorbate environment (high AsA/DHA ratio observed in stem cutting plants) ensures

the reactivation of meristematic cells (Stasolla & Yeung, 2006). Glutathione has high symplastic and low apoplastic pool while ascorbate pools are high both in symplast and in apoplast. Apoplastic ascorbate (being reduced from the cytosolic side) participates in reactions of phenolic compounds, e.g. in scavenging phenoxy radicals that participate in lignin formation (Horemans *et al.*, 2000). The higher level of total ascorbate and its lower reduction level correlate with increased height, higher number of branches and rhizomes per plant and higher leaf number per branch (Fig. 2.2). Also, more reduced ascorbate pool in stem cutting-derived plants corresponds to higher phenol, anthocyanin, and tannin contents which may be related to the reactions in apoplast in which ascorbate participates (Horemans *et al.,* 2000).

Glutathione can be considered as a major factor in keeping the redox state steady and determining morphogenetic processes. The actual glutathione redox potential is related to $[GSH]^2/GSSG$. Thus, unlike many other redox couples (e.g. $NADP^+/NADPH$), the glutathione redox potential depends on and can be influenced by absolute concentration as well as by changes in GSSG relative to GSH (Mullinieaux & Rausch 2005; Meyer, 2008). Even if the GSH/GSSG ratio remains unchanged, decrease in glutathione concentration alone will lead to an increase in redox potential, that is, the potential will become more positive and thus less reducing. More negative glutathione potentials in leaves of tissue culture plants correspond to their increased height, highly increased number of branches and rhizomes per plant, and smaller mass and diameter of berry. Less evident is the correlation with number of leaves per branch (not statistically different in Splendor) and with the number of secondary branches, which in all cultivars is higher in NC and lower in LC as compared to SC plants. It

may be related to more negative glutathione potentials in NC plants as compared to LC plants. Generally, more negative glutathione potentials stimulate cell proliferation (which corresponds in this case to increased height, number of primary branches and rhizomes, and, because of increased growth of vegetative parts, to smaller berries). Less negative potentials lead to differentiation processes (Schafer & Buettner, 2001), which corresponds to deposition of more biomass in berries in stem cutting-derived plants, their lower height and a higher number of secondary branches as compared to leaf culture-derived plants (Fig. 2.2).

The importance of glutathione pool size and its redox potential for determination of cell division, growth and even apoptosis has been mentioned in many studies (reviewed in Noctor *et al.,* 2012). The quiescent parts of plants, such as root quiescent centre and cells in organs such as seeds, maintain a highly oxidized intracellular state, in particular reflected in low level of reduction of the glutathione pool (Kranner *et al.,* 2006). Auxin accumulation in the root stem cells is dependent on the oxidized status of the cells (Jiang & Feldman, 2010). The reduction of glutathione content results in a non-functional root meristem while the shoot meristem is largely unaffected (Vernoux *et al.* 2000). A subsequent increase in the total cellular GSH pool is essential for the cells to progress to cell division (Diaz-Vivancos *et al.,* 2010). Glutathione synthesis is also required for pollen germination and pollen tube growth (Zechmann *et al.*,2011). Certainly, the reduction potential of glutathione is determined both by its reduction level and concentration (Schafer & Buettner, 2001) can give only an indirect indication of its influence because its direct effect should be observed in relation to its potential in meristematic cells.

2.5. Conclusion

In conclusion, the *in vitro* propagation method is a great tool for obtaining superior vegetative growth and enhanced levels of antioxidant compounds in lingonberry plants. Although, *in vitro* derived lingonberry plants produced less berries with reduced berry mass and diameter, the levels of phenolic compounds and total antioxidants were enhanced. From this study, it can be suggested that the active morphogenetic process is characterized by intensive formation and scavenging of reactive oxygen species which is reflected in the activities of antioxidant enzymes and metabolites as well as in the total radical scavenging capacity. Possible correlations between the growth and morphological characteristics of the studied cultivars may involve direct regulation of cell division and differentiation by the redox state of the ascorbate and glutathione. The data presented in this study show an important connection between the pool size and reduction levels of ascorbate and glutathione, activities of enzymes of the ascorbate-glutathione cycle, and the parameters of growth and differentiation of lingonberry plants.

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Chapter 3

Antioxidant Properties of Lingonberry (*Vaccinium vitis-idaea* L.) Plants as Affected by Different Geographical Locations

This chapter aims to determine the effects of different geographical locations on the antioxidant compounds in lingonberry cultivars and wild clones from four Canadian provinces. Differences in the genotypes and environmental factors such as latitude, altitude, precipitation and temperature at different geographical regions affecting antioxidant metabolism are discussed.

3.1. Introduction

Lingonberry plants can survive freezing temperatures and the cultivated subspecies (ssp. *vitis-idaea*) has been found to survive temperature as low as -17 ºC. In this instance, if extremely low temperatures or frost coverage occurs, up to half of the berry yield may be lost (Penhallegon, 2006).

The development of the two lingonberry subspecies, ssp. *vitis-idaea* (Eurasian) and ssp. *minus* (North American) is variable. The levels of antioxidants and their corresponding activity is expected to be different at different geographical locations with different climatic conditions. The changes in the levels of antioxidants in the plants are caused by

stressors, such as drought, temperature, ultraviolet radiation, pollutants, pathogens, and seasonal changes (Paliyath *et al*., 1997; Sývacý and Sökmen, 2004).

The climate of Western Europe is generally milder with cool summers and cold winters and is depicted as being a more favourable for plant growth compared to both North America or Asia (Seager, 2006). Mild climate is the result of northward oriented winds, directing subtropical air with slight warming from the Gulf stream, across Western Europe. Eastern North America encounters much cooler winters due to the deflection of westward winds by the Rocky Mountains which are replaced by prevailing Arctic winds. This gives Western European climate an additional 15-20 degree in temperature, in comparison to North American locations at similar latitudes (Seager, 2006).

Lingonberry plants that occur in North America are subject to wide variations in the climate. Lingonberry of Eastern North America, in this case encompassing plants from the regions of Québec, New Brunswick, Nova Scotia, and the island of Newfoundland, are influenced by climates from the Arctic, subarctic, humid continental, and Atlantic Canada Maritime regions. The Arctic region of Canada is located in the most northerly section of the country and classified as tundra with very cold winters and low precipitation. The subarctic climate occurs below the Arctic, between the 50^{th} and 58^{th} parallels, with low precipitation, cold long winters and fairly short cool summers. Both the Arctic and subarctic regions are found in the upper portion of Québec. The humid continental climate, present in both the largest portion of Québec and Newfoundland, is found below the subarctic and has hot and humid summers with fairly cold winters. Newfoundland differs slightly, as it is a subtype of the humid continental climate with cooler summers. The

regional climate of the Canadian Maritimes, encompassing New Brunswick, Nova Scotia and part of Québec is dependent on the elevation, proximity to the ocean, and direction of wind. The waters of the Atlantic carry warm moist air, which is indicate long cool winters and short warm summers, both with increased levels of precipitation (Saucier *et al*., 2003).

According to McManus and Wood (1991) Newfoundland is divided into four climatic regions, apart from its governing humid continental climate. These regions consist of the 1) West Coast, 2)Western Mountains and Central Uplands 3) Northeast Coast and Central Lowlands, and 4) the South Coast and Avalon. The West Coast is influenced by winds from the Gulf of St. Lawrence, resulting in reduced temperatures and increased precipitation. The Western Mountains and Central Uplands are influenced by the increase in elevation resulting in very harsh conditions, including increased precipitation, reduced temperatures, increased duration of cloud coverage, and heavy snow fall due to increased precipitation. The Northeast Coast and Central Lowlands have decreased levels of precipitation resulting in warm summers and cold winters due to the persisting ice patches. The South Coast and Avalon have highly variable winters and cool summers due to low cloud coverage and fog. Late fall and early winter are commonly subjected to increased precipitation leading to heavy rainfall.

This study focuses on how geography and climatic conditions influence the levels of antioxidants in lingonberries.

3.2. Materials and Methods

3.2.1. Plant Materials

The plant materials used in this study were the lingonberry cultivars Regal, Splendor and Erntedank of subspecies *vitis-idaea,* as well as the wild lingonberry clones of subspecies *minus* collected from 4 Canadian provinces: Quebec, Nova Scotia, and New Brunswick and from 10 locations in Newfoundland: St. Anthony, Cow Head, Brookfield, Hant's Harbour, Bell Island West, Pleasantville, Quidi Vidi, Cape Spear, Soldiers Pond, and Witless Bay. These plant were collected and propagated by Debnath (2005). Figure 3.1 represents the geographical distribution of lingonberry subspecies *minus* in the map of Eastern Canada and Figure 3.2 represents regions of Newfoundland from where the plants were collected. Table 3.1 records the locations with their longitudes, latitudes and altitudes. The wild lingonberry plants were collected individually from different locations in the fall season of 2001. The collection of plants was based on their superior berry production and apparently free from disease and insect pests. After the collection of wild plants from different locations, they were propagated by a vegetative stem cutting method and their clones were maintained in a greenhouse of at Atlantic Cool Climate Crop Research Centre (ACCCRC) in St. John’s, Newfoundland from the date of collection.

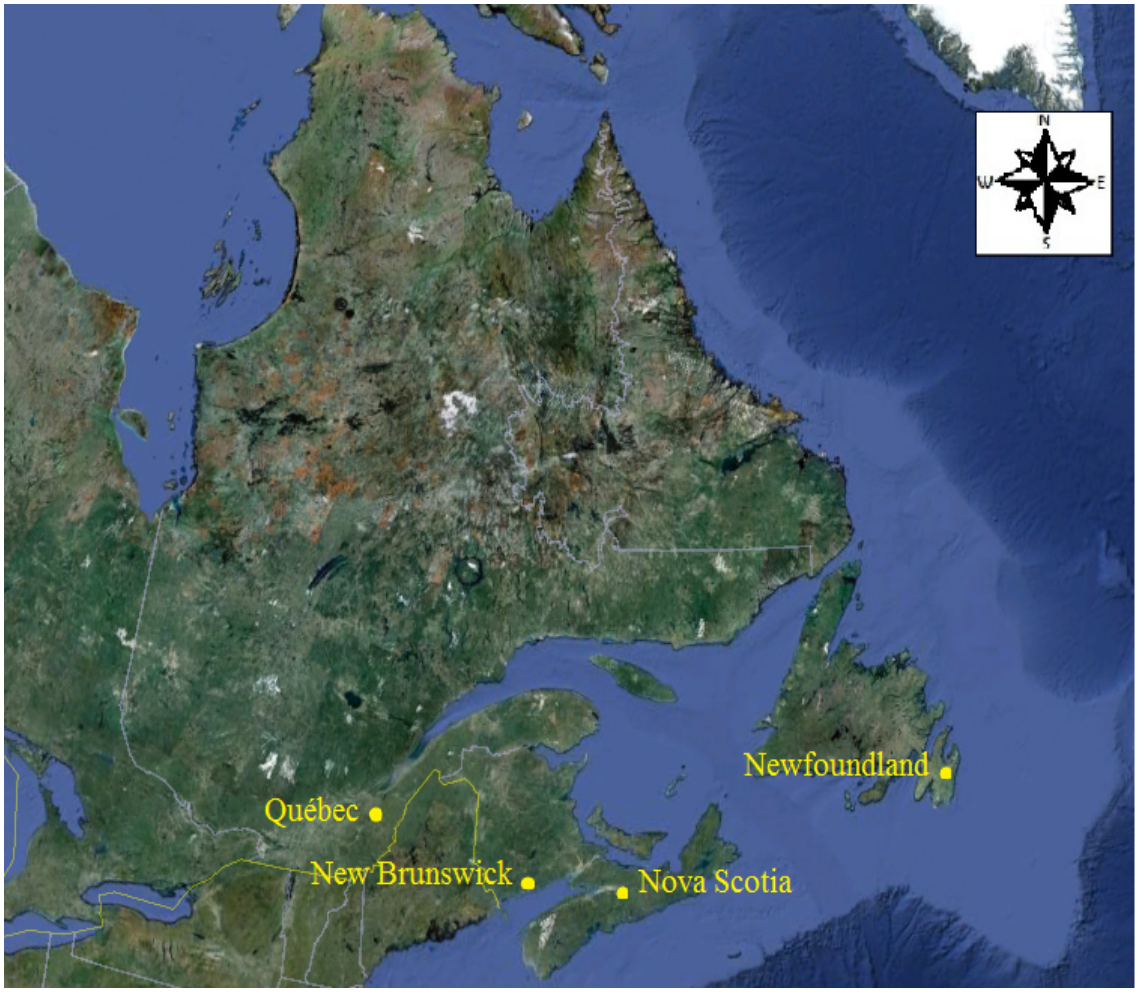

Figure 3.1. Map of eastern Canada showing the distribution of lingonberry subspecies *minus* (Sourced from Google Maps).

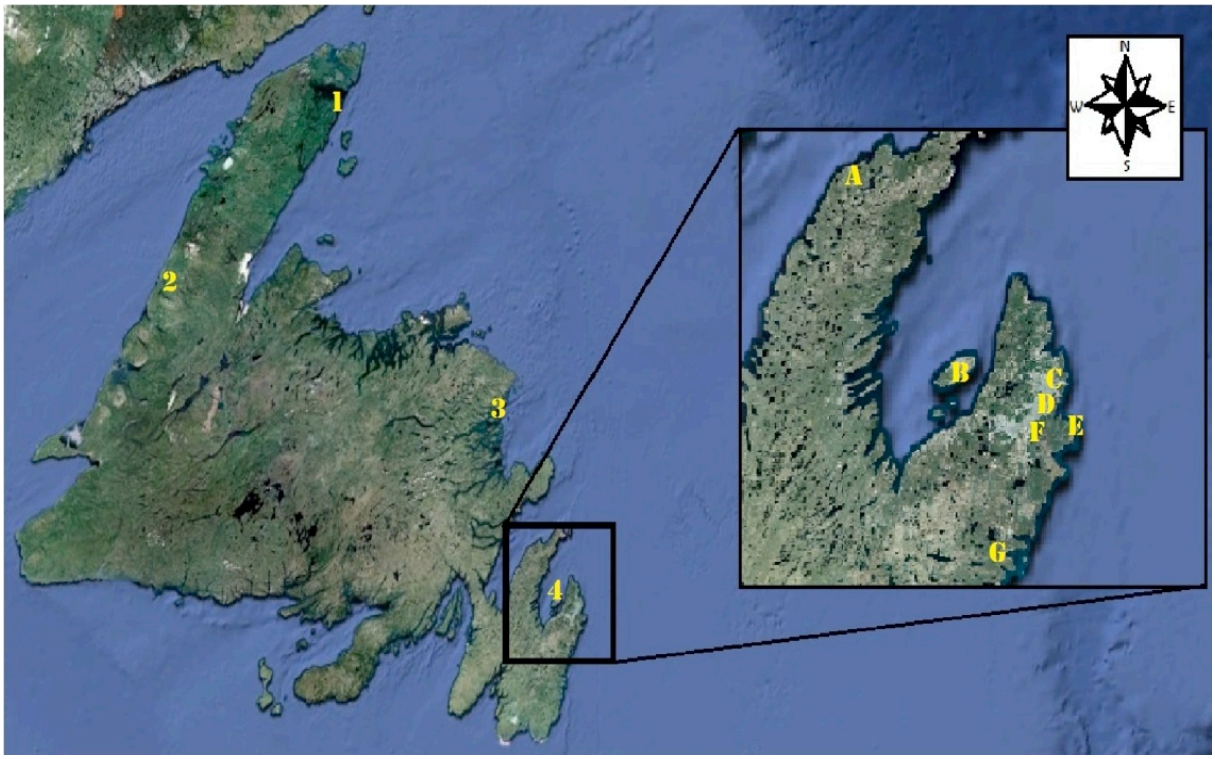

Figure 3.2. Map of Newfoundland showing the distribution of lingonberry subspecies *minus* in various regions: Western region (1.St. Anthony; 2. Cow Head); Central region (3. Brookfield); 4. Eastern and Avalon region (A. Hant's Harbour; B. Bell Island; C. Pleasantville; D. Quidi Vidi; E. Cape Spear; F. Soldiers Pond; G. Witless Bay)(Sourced from Google Maps).

Table 3.1. Geographical locations of collected lingonberry plants with their latitudes and longitudes and altitudes

Geographical Locations	Latitude (°)	Longitude (°)	Altitude (m)
Sorbet, Quebec	46.0	-68.4	15
Moncton, New Brunswick	46.1	-64.8	12.2
North Sydney, Nova Scotia	46.3	-60.3	11.7
St. Anthony, NL	51.4	-56.1	32.9
Cow Head, NL	49.9	-57.8	15. 2
Brookfield/N.W.V., NL	48.6	-54.0	106.7
Hant's Harbour, NL	48.0	53.16	10
Bell Island West, NL	47.36	52.58	140
Pleasantville, NL	47.35	52.41	114
Quidi Vidi, NL	47.34	52.40	114
Cape Spear, NL	47.31	52.37	114
Soldiers Pond, NL	47.20	53.04	114
Witless Bay, NL	47.20	53.04	114

3.2.2. Preparation of the Clones from Wild by Vegetative Stem Cutting

Terminal softwood stem cuttings, 4 to 5 cm long, were taken from the wild lingonberry plants and were rooted in 45-cell plug trays (cell diameter 5.9 cm, cell depth 15.1 cm; Beaver Plastics, Edmonton, AB, Canada) containing peat: perlite [2:1 (v/v)] in a humidity chamber with a vaporizer (Conviron E15; Controlled Environments Ltd., Winnipeg, MB, Canada) at 22° ± 2°C and 95% relative humidity (RH), with a 16 hour photoperiod (PPFD 55 μmoles m^{-2} s^{-1} at culture level). No rooting compound was applied (Debnath 2006, Foley and Debnath 2007). After 6-8 weeks, rooted plantlets were transferred to plastic pots (10.5 × 10.5 ×12.5 cm) containing the same medium used for rooting and were maintained in humidity chamber and acclimatized by gradually lowering the humidity by 3 to 4% per week over 3 weeks period. When the plants were developed well and hardened, they were grown in the greenhouse under natural light condition at about 20°C, 85% RH and 16 hour photoperiod at maximum PPF of 90 $umol.m^{-2}$ S^{-1} (Debnath, 2006; Foley & Debnath 2007). Fertilization (20-8-20 N-P-K, Plant Products Co. Ltd., Brampton, ON) and irrigation was supplied to the plants periodically in approximately 2 weeks (Debnath 2006). All the plants were treated in similar manner and were maintained in the greenhouse.

3.2.3. Extraction of Soluble Phenolics

Young leaves from lingonberry clones were harvested and frozen in liquid nitrogen immediately. Several extraction solvents were tested, among which 80% acetone with 0.2% formic acid gave the highest extraction of antioxidants and phenolic content. Leaves were ground to fine powder using liquid nitrogen and mortar/pestle.. Powdered leaves were mixed with solvent and shaken for 4 hours at 4ºC and then centrifuged at 19000 ×g at 4ºC. The ratio of solvent and leaves was 1:80 w/vol. The supernatant was transferred and further diluted to form a final concentration of 1 mg/ml.

3.2.4. Determination of the Total Soluble Phenolic Content

This colorimetric method is adapted from Chandrasekara and Shahidi (2011) with some modifications. It is based on the principle of reduction of a reagent which is a mixture of tungsten and molybdenum oxides (Singleton, 1974). 0.5 ml of each of the leaf extracts were transferred in separate centrifuge tubes. To each tube, 0.5 ml of Folin Ciocalteu reagent was added. The reaction was neutralized by adding 1 ml of saturated solution of sodium carbonate, the volume was adjusted to 10 ml by adding distilled water and vortexed for 60 seconds .

All reaction tubes were placed in the dark for 35 min at room temperature. After 35 minutes, the tubes were taken out of the dark to centrifuge at 4000 g for 10 minutes. Supernatants were transferred in glass tubes and their absorbance was recorded at 725 nm against the blank for each sample using a UV/Vis spectrophotometer (Ultrospec 4300 *pro*).

A gallic acid standard curve was prepared and the total phenolic content was measured as gallic acid equivalents (GAE) per g of leaf fresh weight.

3.2.5. Determination of the Total Anthocyanin Content

The total anthocyanin content was measured by the pH differential method according to Foley and Debnath (2007). The method is based on reversible conversion of anthocyanins from their oxonium form to their hemiketal form. Absorption at 510 nm and 700 nm was measured using UV/Vis spectrophotometer (Ultrospec 4300 *pro*) in buffers at pH 1.0 and pH 4.5 and the difference between the two values was used to determine total anthocyanin content. Results are expressed as catechin equivalent (CE) per g of leaf fresh weight.

3.2.6. Determination of the Total Flavonoid Content

The total flavonoid content was measured by an aluminum chloride colorimetric assay (Zhishen *et al*. 1999). One ml of extract or standard solution of catechin (0.5 mg/ml) was mixed with 4 ml of water, followed by addition of 0.3 ml 5% $NaNO_2$, of 0.3 ml of 10% $AlCl_3$ (after 5 min) and 2 ml of 1 M NaOH (one minute later), volume was adjusted (with water) to 10 ml. The absorbance was measured at 510 nm using UV/Vis spectrophotometer (Ultrospec 4300 *pro*). Total flavonoid content was expressed as μmol of catechin equivalent (CE) per g of leaf.

3.2.7. Determination of the Total Tannin (proanthocyanidin) Content

The total tannin (proanthocyanidin) contents of leaves of lingonberry cultivars and clones were determined by the method developed by Chandrasekara and Shahidi (2011). Five ml of 0.5% vanillin-HCl reagent were added to 1 ml of extract, mixed thoroughly and incubated at room temperature for 20 minutes. A separate blank for each sample was read with 4% HCl in methanol. The absorbance was read at 500 nm using UV/Vis spectrophotometer (Ultrospec 4300 *pro*), and the content of proanthocyanidins was expressed as μmol of CE per g of leaf tissue.

3.2.8. Measurement of the Antioxidant Activity

Antioxidant capacity of lingonberry leaves was determined by two methods as detailed below.

3.2.8.1. The Total Antioxidant Capacity by DPPH Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was conducted according to the method of Brand-Williams *et al*. (1995) with some modifications. The stock solution of 1 mM DPPH in methanol was diluted to 60 μmol, 1.9 ml of the latter was mixed with 0.1 ml leaf extract, shaken vigorously and left in dark for 20 minutes. The absorbance was read at 515 nm using a UV/Vis spectrophotometer (Ultrospec 4300 *pro*). The scavenging capacity was expressed as % of inhibition of DPPH consumption. The gallic acid standard curve was used to express the results as GAE per gram fresh weight of leaf.

3.2.8.2. The Total Reducing Power

The reducing power of lingonberry leaf extracts was determined by the method described by Chandrasekara and Shahidi (2011). The medium of assay was 200 mM phosphate buffer (pH 6.6) with 1% potassium ferricyanide. A 2.5 ml aliquot of diluted extract was added to the assay buffer and incubated for 20 minutes at 50ºC and then 2.5 ml of 10% trichloroacetic acid (TCA) was added to the assay, mixed and then centrifuged for 10 minutes at 1750 g. Supernatant (2.5 ml) was transferred to empty tubes and combined with 2.5 ml of deionized water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm using a UV/Vis spectrophotometer (Ultrospec 4300 *pro*), and the results were expressed as ascorbic acid equivalents using appropriate standard curves.

3.2.9. Statistical Analysis

All the experiments were repeated at six times. Data in the text, the table and figures are expressed as means ±SD of three biological replicates. Data for all characteristics were subjected to t-test at $P \leq 0.05$ to differentiate between the two groups. Duncan's multiple range test was conducted to observe the significant difference within the groups within the genotypes.

3.3. Results

3.3.1. Comparing the Two Subspecies of Lingonberry

Significant differences were observed between the two lingonberry subspecies in terms of the total phenolic content wherein ssp. *vitis-idaea* showed significantly low total phenolic levels (~40 to 60 mg/g GAE) as compared to clones from ssp. *minus* (~65 to 128 mg/g GAE) (Fig. 3.3).

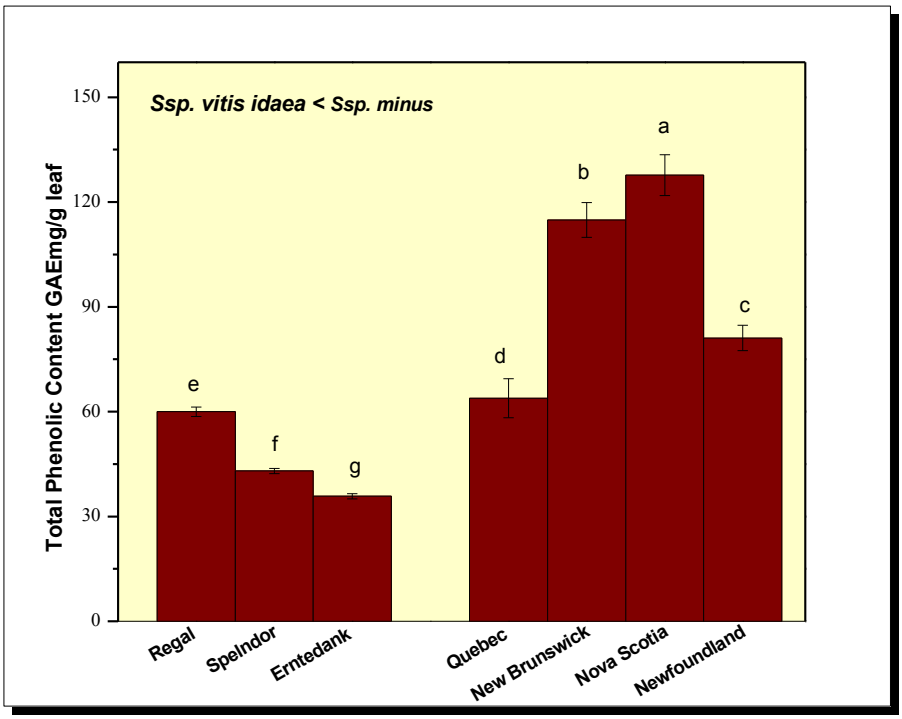

Figure 3.3. The total phenolic content of leaves for two lingonberry subspecies, ssp. *vitis-idaea and* ssp. *minus* from different geographical locations. Means ± SE, n = 6, * – values significantly different at P < 0.05 from the standard. Letters (a to f) represents the differences between the cultivars at $P \leq 0.05$ by Duncan's multiple range test. Bars with same letters are not significantly different.

The total anthocyanin content showed similar pattern as the total phenolics. The level of total anthocyanin content was significantly higher in lingonberry ssp. *minus* (~12 to 20 mg/g leaf CE) as compared to ssp. *vitis-idaea* (~7 to 11 mg/g leaf CE) (Fig. 3.4).

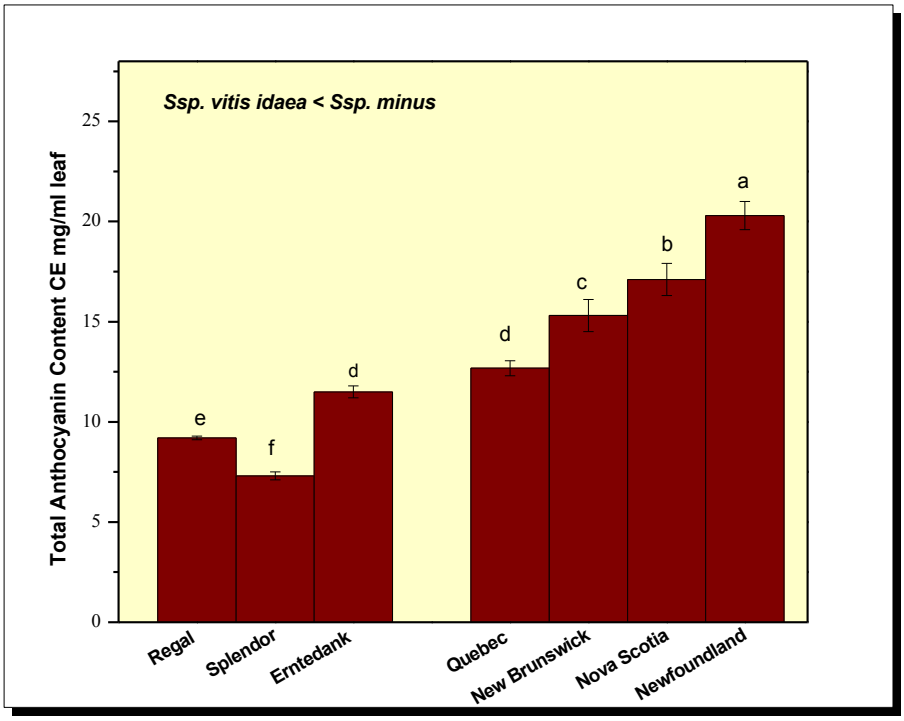

Figure 3.4. The total anthocyanin content of leaves for two lingonberry subspecies, ssp. *vitis-idaea and* ssp. *minus* from different geographical locations. Means ± SE, n = 6, * – values significantly different at P < 0.05 from the standard. Letters (a to f) represents the differences between the cultivars at $P \leq 0.05$ by Duncan's multiple range test. Bars with same letters are not significantly different.

The total flavonoid content of ssp. *vitis-idaea* was found to be very low (~ 7 to 11 mg/g leaf CE) compared to *ssp. minus* which was 50 to 115 mg/g leaf CE (Fig. 3.5).

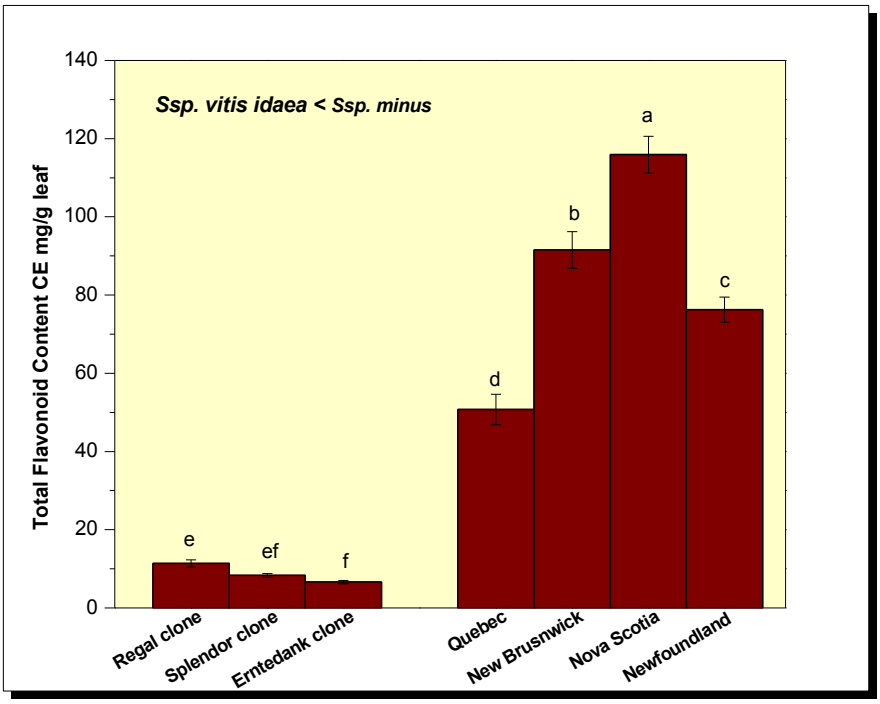

Figure 3.5. Total flavonoid content of leaves of two lingonberry subspecies, ssp. *vitis-idaea and* ssp. *minus* from different geographical locations. Means ± SE, n = 6, * – values significantly different at P < 0.05 from the standard. Letters (a to f) represents the differences between the cultivars at $P \leq 0.05$ by Duncan's multiple range test. Bars with same letters are not significantly different and those with more then one letter show overlap in their values.

The total proanthocyanidin content was observed to be significantly higher in cultivar Regal of ssp. *vitis-idaea* compared to ssp. *minus*. Splendor and Erntedank showed similar or lower levels of proanthocyanidin respectively compared to lingonberry clones from Newfoundland belonging to ssp. *minus* (Fig. 3.6).

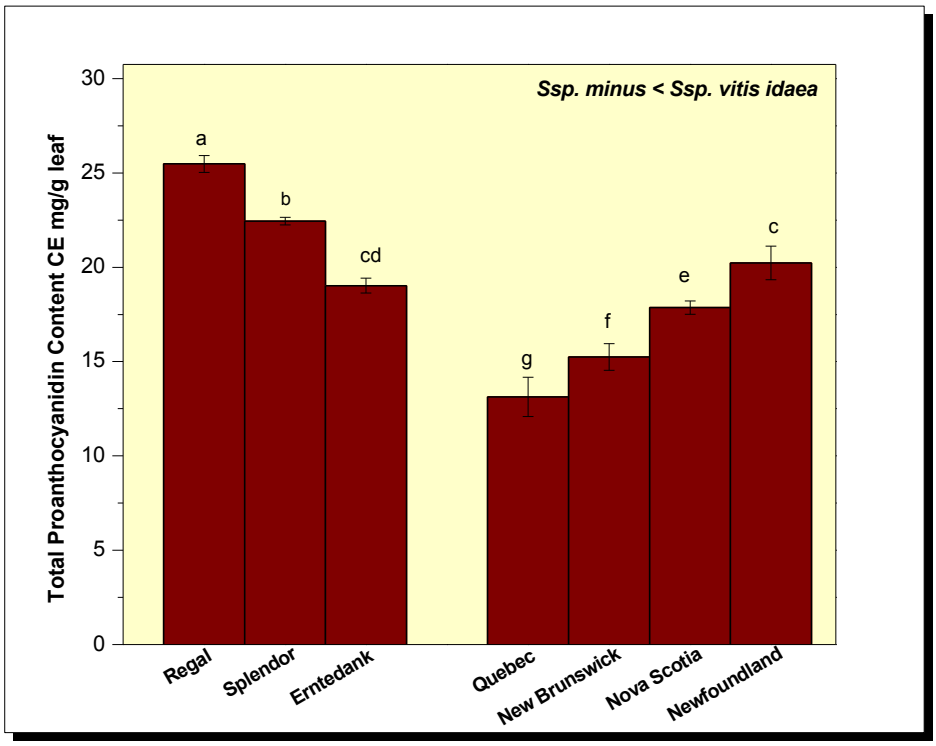

Figure 3.6. The total proanthocyanidin content of leaves for two lingonberry subspecies, ssp. *vitis-idaea and* ssp. *minus* from different geographical locations. Means ± SE, n = 6, * – values significantly different at P < 0.05 from the standard. Letters (a to f) represents the differences between the cultivars at $P \leq 0.05$ by Duncan's multiple range test. Bars with same letters are not significantly different and those with more then one letter show the overlap in their values.

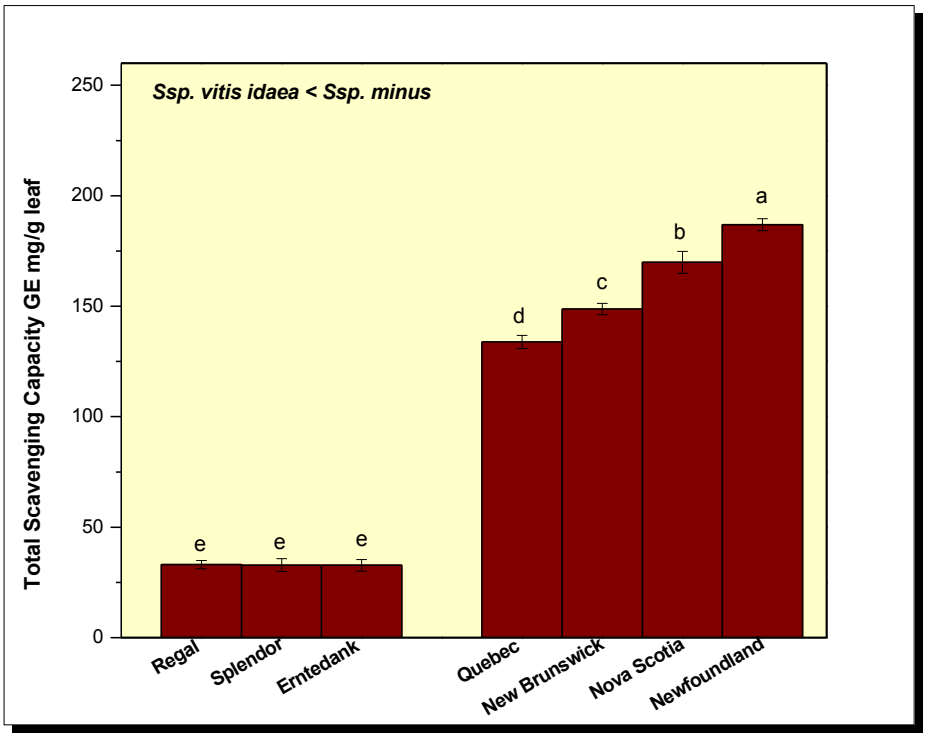

Figure 3.7. The total radical scavenging capacity of leaves for two lingonberry subspecies, ssp. *vitis-idaea and* ssp. *minus* from different geographical locations. Means ± SE, n = 6, * – values significantly different at P < 0.05 from the standard. Letters a,b,c, d and e represents the differences between the cultivars at $P \leq 0.05$ by Duncan's multiple range test. Bars with same letters are not significantly different.

The total radical scavenging activity (Fig. 3.7) and the total reducing power (Fig. 3.8) showed very similar patterns which were in line with the total phenolic content. Lingonberry ssp. *minus* exhibited significantly high antioxidant activities as compared to lingonberry ssp. *vitis-idaea.*

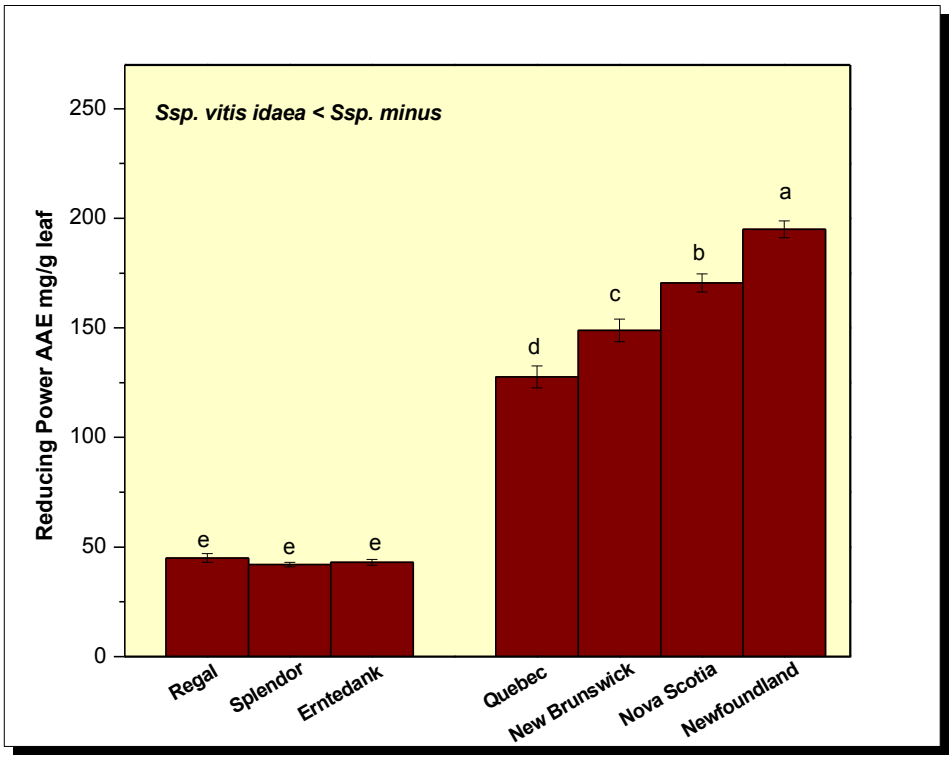

Figure 3.8. The reducing power of leaves for two lingonberry subspecies, ssp. *vitis- idaea and* ssp. *minus* from different geographical locations. Means ± SE, n = 6, * – values significantly different at P < 0.05 from the standard. Letters a,b,c, d and e represents the differences between the cultivars at $P \leq 0.05$ by Duncan's multiple range test. Bars with same letters are not significantly different.

3.3.2. Comparing North American Lingonberry Subspecies *minus* from Different Provinces of Eastern Canada and Atlantic Canada

The total phenolic content and the total antioxidant levels of lingonberry clones from different provinces of Eastern and Atlantic Canada: Québec, New Brunswick, Nova Scotia and the island of Newfoundland were acquired and compared. The values of all the studied antioxidant components for the Newfoundland samples were obtained by taking the average of 10 clones from different regions of this province.

The total phenolic and flavonoid content were found to be the lowest in the lingonberry clones from Québec followed by those from Newfoundland. Both the total phenolic and flavonoid content were significantly higher in New Brunswick compared to those from Newfoundland and highest in clones from Nova Scotia (Fig. 3.3 and Fig. 3.5).

The total anthocyanin and proanthocyanidin levels were the lowest in leaves of lingonberry clones from Québec followed by those from New Brunswick which was followed by the clones from Nova Scotia. Both the total anthocyanin and proanthocyanidin levels were highest in Newfoundland clones (Fig. 3.4 and Fig. 3.6).

The total antioxidant capacity measured by the radical scavenging capacity and the reducing power, showed similar results. The total antioxidant capacity was found to be the lowest in leaves of Québec clones, followed by New Brunswick clones and then Nova Scotia clones. The lingonberry clones from Newfoundland showed the highest antioxidant capacity (Fig. 3.7 and Fig. 3.8).

3.3.3. Comparing the Antioxidant Levels of of Lingonberry Clones From Different Regions in Newfoundland

The levels of total phenolic and anthocyanin content were signifiicantly high in leaves of the St. Anthony clones compared to the clones from other regions. As seen in figure (3.9) the content of total phenolic content in the leaves of wild lingonberry clones from all the other studied regions were not significantly different and the values were overlapping each other.

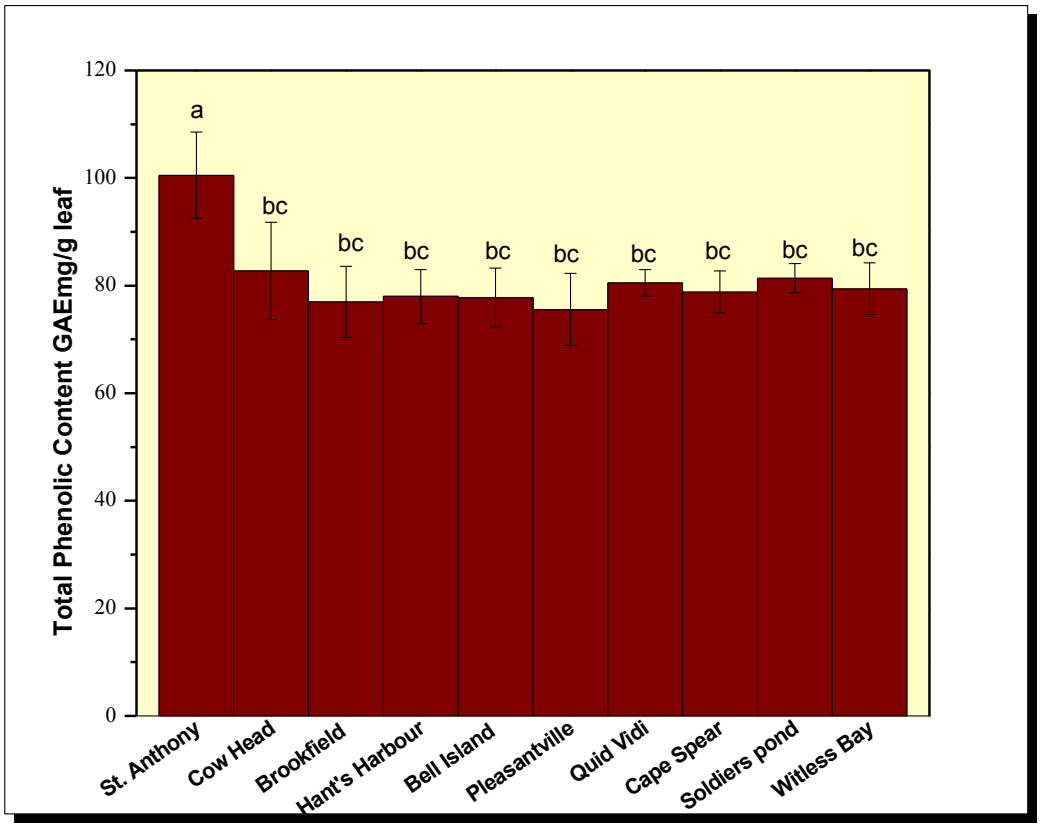

Figure 3.9. The total phenolic content in leaves of lingonberry subspecies ssp. *minus* from different regions of Newfoundland. Means ± SE, n = 6. Letters a, b, c, d, and e indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Values with same letters are not significantly different and values with 2 or 3 letters indicate the overlap between the components.

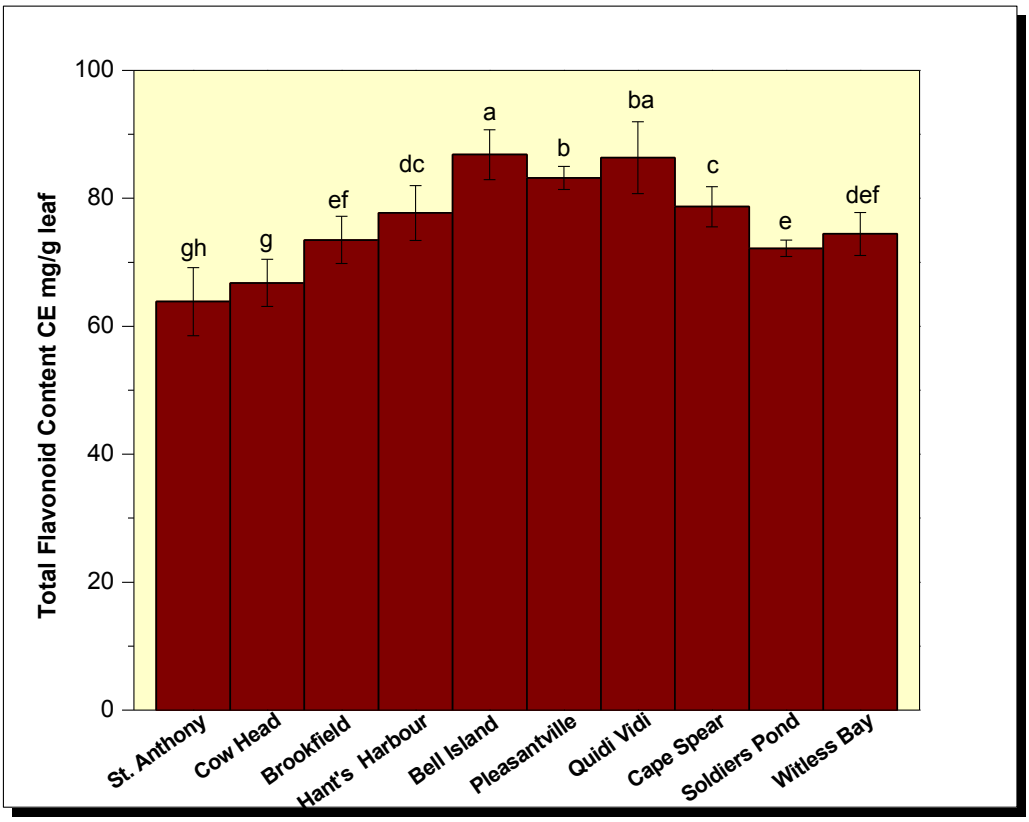

Figure 3.10. The total flavonoid content in leaves of lingonberry subspecies ssp. *minus* from different regions of Newfoundland. Means ± SE, n = 6. Letters a, b, c, d, and e indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Values with same letters are not significantly different and values with 2 or 3 letters indicate the overlap between the components.

The total flavonoid content was influenced by the different geographical locations within the province in quite a different way. Overall, an increase in the levels of flavonoids was observed when going from St. Anthony, to Cow Head and further increases from Brookfield to Bell Island through Hant's Harbour. The content of total flavonoids in leaves of wild lingonberry was not significantly different from Hant's Harbour, Bell Island, and Quidi Vidi. Flavonoid levels showed decreasing trends when tracing down from Quidi Vidi to Soldiers

Pond and Witless Bay, both of which showed similar levels of total flavonoid content (Fig. 3.10).

The content of total anthocyanins showed similar pattern as the totla phenolic content. The TAC were signifiicantly high in leaves of the St. Anthony clones and Cow Head clones compared to the clones from other regions. The TAC levels were not significantly different in the leaves of the clones from the other studied regions (Fig. 3.11).

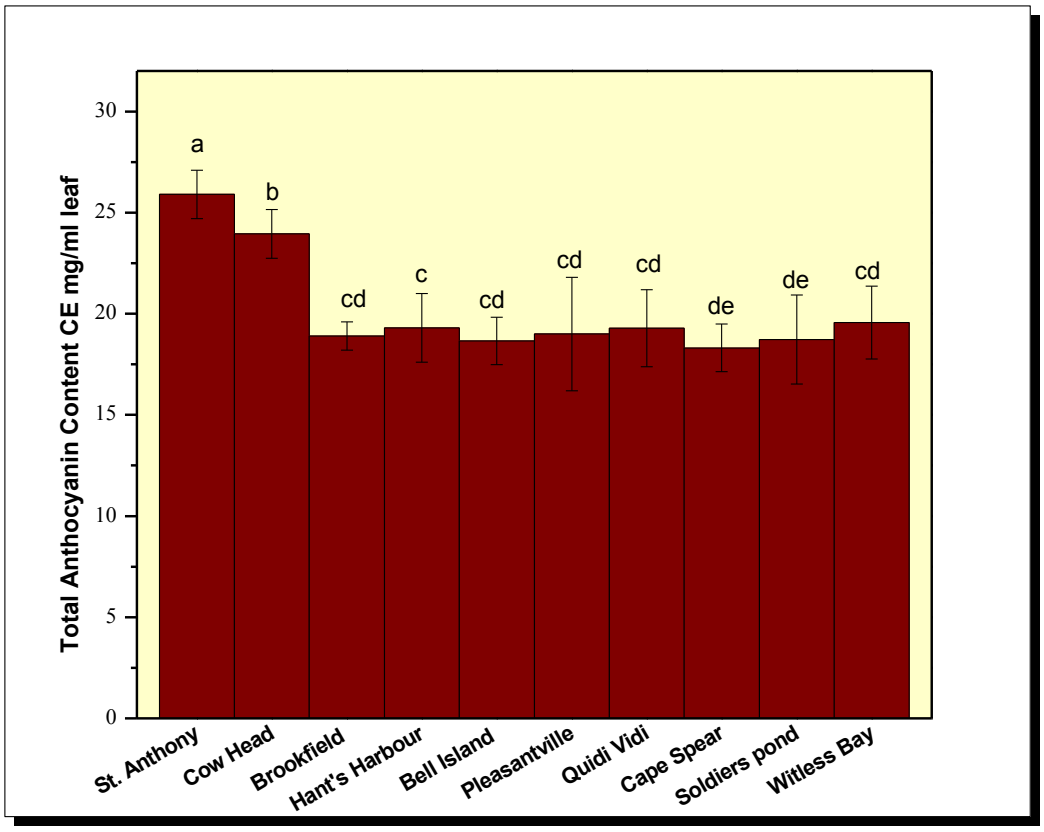

Figure 3.11. The total anthocyanin content in leaves of lingonberry subspecies ssp. *minus* from different regions of Newfoundland. Means ± SE, n = 6. Letters a, b, c, d, and e indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Values with same letters are not significantly different and values with 2 or 3 letters indicate the overlap between the components.

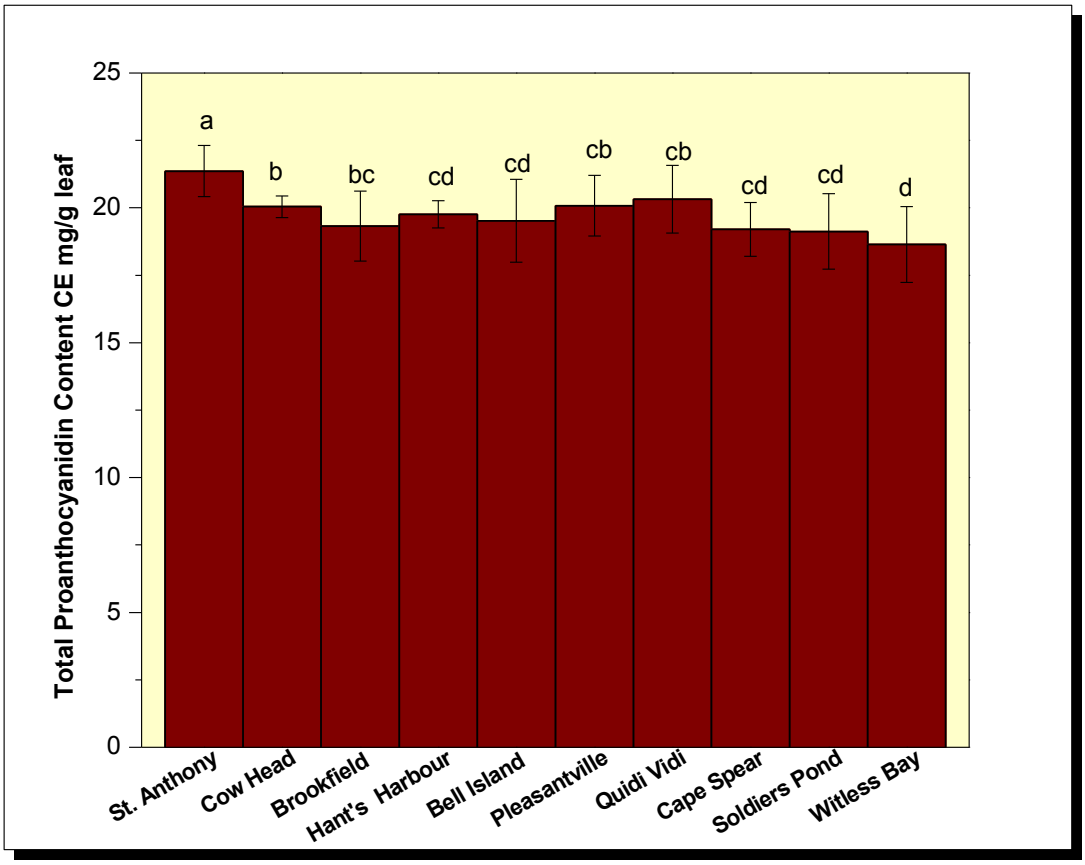

Figure 3.12. The total proanthocyanidin content in leaves of lingonberry subspecies ssp. *minus* from different regions of Newfoundland. Means ± SE, n = 6. Letters a, b, c, d, and e indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Values with same letters are not significantly different and values with 2 or 3 letters indicate the overlap between the components.

The levels of total proanthocyanidin content also exhibited similar trend as the total phenolic and anthocyanin content. St. Anthony clones showed highest levels followed by Cow Head clones. The differences were not significant in different regions of Newfoundland (Fig. 3.12).

Total antioxidant activities measured as the total radical scavenging capacity and the reducing power, exhibited similar patterns as that of phenolic, anthocyanin and ptoanthocyanidin content (Fig. 3.13 and Fig. 3.14). These levels were highest in St. Anthony clones followed by the Cow Head clones. The levels were not significantly different in the leaves of clones from other studied regions.

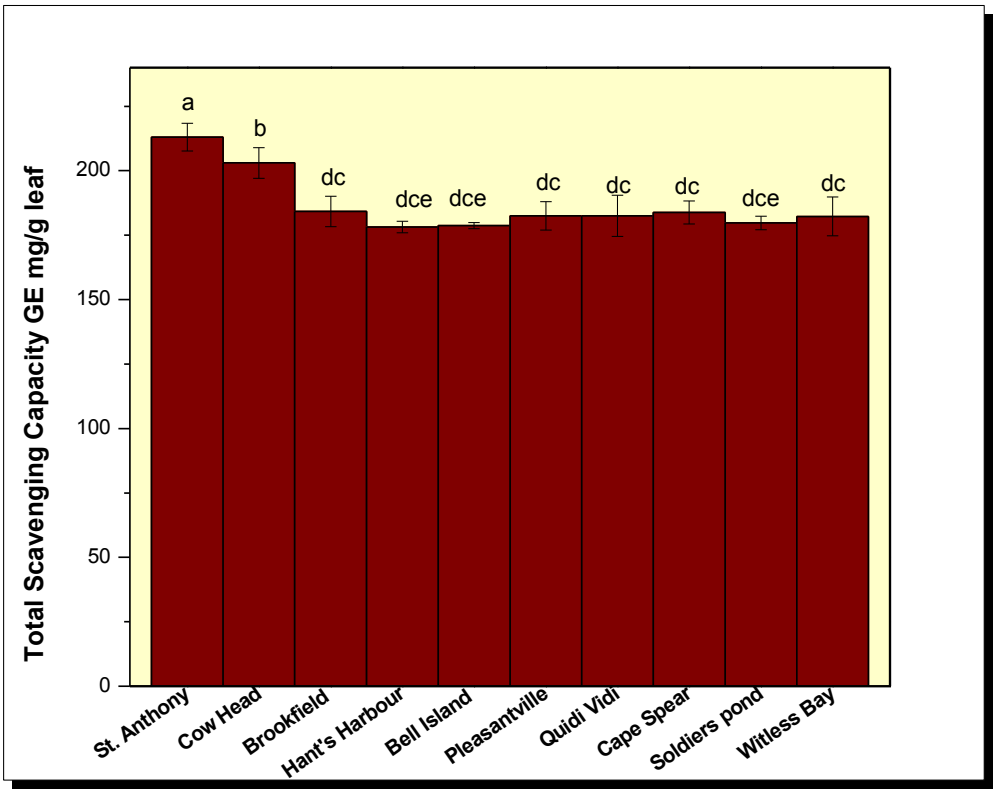

Figure 3.13. The total radical scavenging capacity in leaves of lingonberry subspecies ssp. *minus* from different regions of Newfoundland. Means ± SE, n = 6. Letters a, b, c, d, and e indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Values with same letters are not significantly different and values with 2 or 3 letters indicate the overlap between the components.

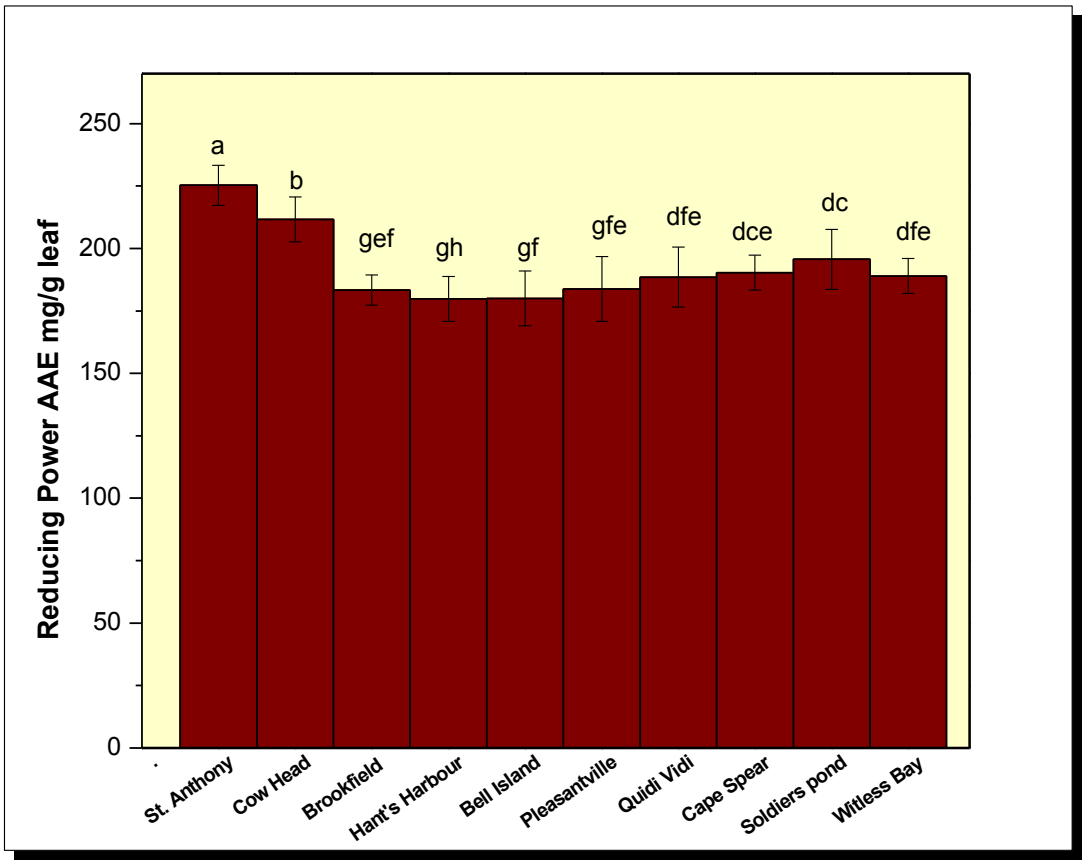

Figure 3.14. The reducing power in leaves of lingonberry subspecies ssp. *minus* from different regions of Newfoundland. Means ± SE, n = 6. Letters a, b, c, d, and e indicate differences at $P \leq 0.05$ by Duncan's multiple range test. Values with same letters are not significantly different and values with 2 or 3 letters indicate the overlap between the components.

3.4. Discussion

The two lingonberry subspecies, *V. vitis-idaea* L. ssp. *vitis-idaea* Britton and *V. vitis-idaea* L. ssp. *minus* Hult differed significantly in all the parameters of antioxidant quantification performed in the present study. Lingonberry cultivars of ssp. *vitis-idaea* (Regal, Splendor and Erntedank) which are originally from Europe, were found to have significantly lower total phenolic, anthocyanin and flavonoid contents, and total antioxidant capacity, as compared to wild North American lingonberry clones of ssp. *minus*. Only the level of total proanthocyanidin content showed the opposite trend. This observed difference in overall antioxidant capacity may be attributed to differences in their genotypes. Both subspecies exhibit several phenotypic differences including plant height, branching and blooming period. Figure 1.1 in Chapter 1 clearly represents morphological difference which is attributed due to their genotypic difference.

As mentioned earlier, all the wild clones were collected from different locations of four Canadian provinces in 2001 where they adapted to their specific environmental conditions. These clones were maintained under greenhouse conditions for about 10 years at the time of study. All the plants were treated equally under the same greenhouse environment. They were provided with the same irrigation, fertilisation, and temperature since they were in the same greenhouse. Climatic conditions might have an impact on of the genotype of the plants during their initial developmental stage when they were adapting to that particular climatic condition.

The environmental conditions of Western Europe are milder than that of Eastern North America (Seager 2006), and this could result in lower levels of the described antioxidants

being synthesized without necessarily utilizing them to their fullest extent. The ssp. *vitis-idaea*'s energy expenditure utilized for biosynthesis of antioxidant metabolites is reduced allowing alternative spending of substantial amounts of free energy. This energy can be utilized in other processes such as fruit development through subsequent blooming, not presented in ssp. *minus* which only have single bloom occurrences (Penhallegon, 2006).

A comparison of lingonberry ssp. *minus* among the regions of Eastern and Atlantic Canada, including Québec, New Brunswick, Nova Scotia, and the island of Newfoundland (Fig. 3.2) showed general trends of an increase in total anthocyanin content, total proanthocyanidin content, and total antioxidant capacity, from least to greatest in the order of the provinces listed above. The results indicate a genetic basis for the variations in the levels of the antioxidant components. Table 3.2 represents average annual temperature of different locations of plant collection.

Newfoundland's vastness and remote position adds to the relative diversity in regional climate and therefore the variety of testing results. Overall the levels of antioxidants and scavenging capacity are higher in most cases, except total flavonoid content and total phenolic content, which are similar to Québec. Although both Newfoundland and Québec are found in similar humid continental climate regions, Newfoundland is highly influenced by variation caused by its proximity to the ocean thus placing it into a subtype of the Humid Continental determination of climate. The colder temperatures and variety of climatic regions make it difficult to accurately compare Newfoundland to the rest of eastern Canada, without first describing the general trend across the entire province. A series of ten locations across the province of Newfoundland were examined for relative antioxidant levels and scavenging

activity of lingonberry clones based on the four climatic regions as described above by McManus and Wood (1991). These areas are representative of the three of the four regions excluding the Western Mountains and Central Uplands. St. Anthony and Cow Head represents the Western coast, Brookfield represents the central region of Newfoundland, Hant's Harbour, Bell Island, Pleasantville, Quidi Vidi, Cape Spear, Soldiers Pond and Witless Bay are from the Avalon or the East Coast of Newfoundland. In correlation analysis, a representative from each region was used *i.e.,* Cow Head (Western, NL), Brookfield (Central, NL) and Quidi Vidi (Avalon region).

The Québec clone showed the lowest trend of antioxidant quantification and activity in every assay but not as low as found in ssp. *vitis-idaea*, with the exception of the total proanthocyanidin content. Although each are occurring in Humid Continental regions, influences from proximity to Arctic and Subarctic regions have effect on relative intensity and duration of weather extremes.

There are several factors that could influence the yield of secondary metabolites in plants growing in specific environments such as genotype, culture conditions, latitude, altitude, temperature, precipitation and photoperiod.

A correlation analysis of all studied components was done with environmental factors such as latitude, altitude, average annual temperature and average annual precipitation. The critical values of Pearson's correlation coefficient (R) were strongly significant with (R> 6215) (R values given in correlation graphs, Fig. 3.15 to 3.18). The data for average annual temperature and precipitation was obtained from weather network statistics where the

sampling period was 30 years for all the regions under study. (Table 3.2). The annual precipitation data for Brookfield was not available and hence central Newfoundland was excluded for the correlation analysis between biochemical components and annual precipitation.

Table 3.2. The average annual temperature and precipitation of different regions of studied plant collection sites

Locations	Avg. Annual Precipitation (mm)	Avg. Annual Temperature (ºC)
Quebec	976	6.4
New Brunswick	1143	6.2
Nova Scotia	1396	6.6
Western Newfoundland	1210	3.9
Central Newfoundland	N/A	6.8
Eastern Newfoundland	1513	4.7

Some reports have indicated that reduced temperatures increase the yield of anthocyanins in *V. myrtillus* (Martinussen *et al*., 2009) and in other species (Chalker-Scott, 1999; Winkel-Shirley, 2002; Choi *et al.,* 2009). Similar results were observed in our study where a negative correlation between anthocyanin levels and average annual temperature was detected (Fig. 3.15, Table 3.3). The total proanthocyanidin content and total antioxidant activity also increased with reduced temperature. Figure 3.15 represents a correlation analysis of interaction between average annual temperature and studied biochemical components. The total phenolic and flavonoid content did not show any correlation with temperature. There are several other factors that influence the accumulation phenolic compounds. Light plays an

important role in biosynthesis of anthocyanin in cold temperatures (Jaakola & Hohtola, 2001). Plants in cold climates are able to maintain high rates of photosynthesis at lower temperatures compared to those growing under warmer climate. This feature enables them to increase the amount of fixed carbon available for secondary metabolites (Jaakola & Hohtola, 2001)

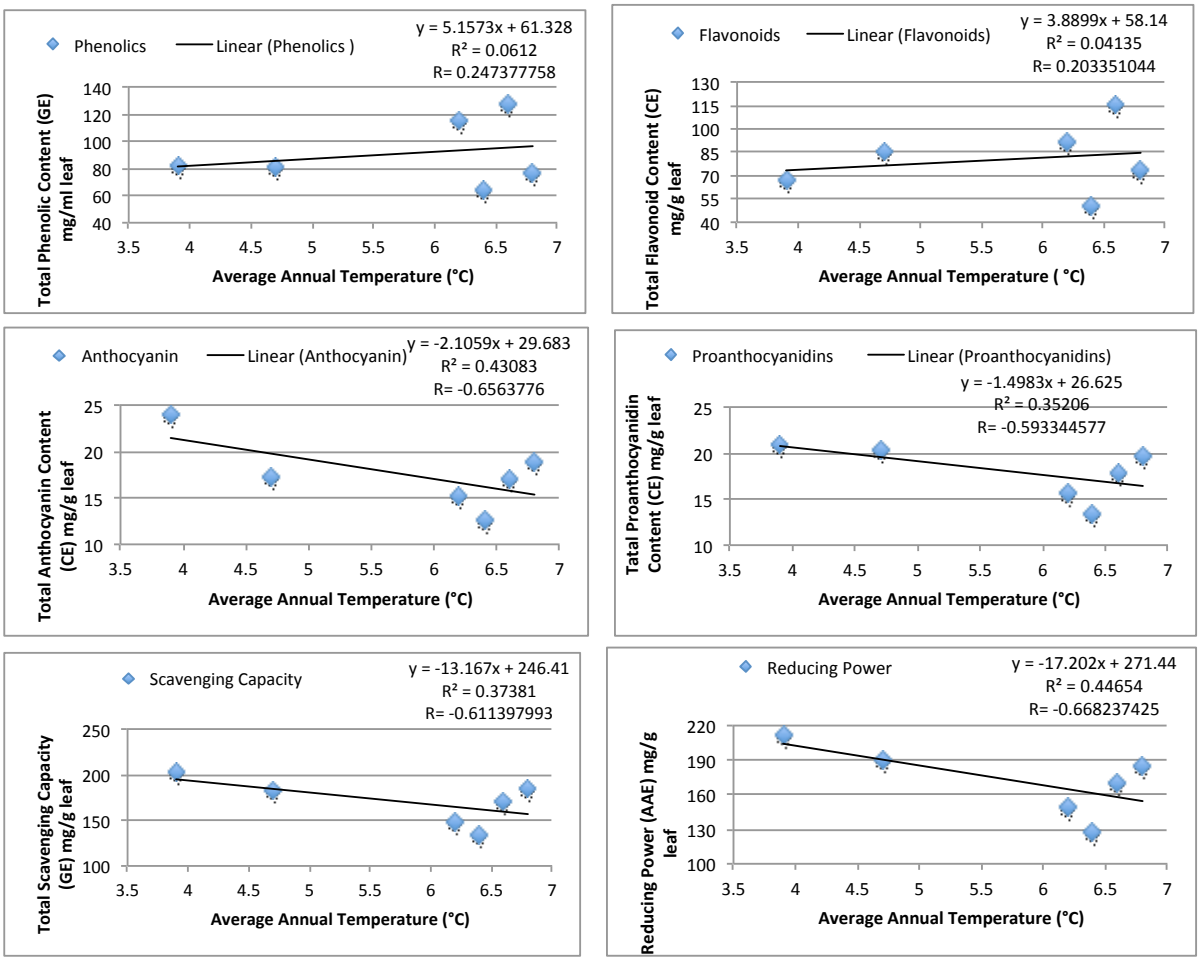

Figure 3.15. Graphs representing a correlation between average annual temperature and the contents of phenolics and antioxidants in the leaves of lingonberry clones from different regions (Quebec, New Brunswick, Nova Scotia, Western Newfoundland, Central Newfoundland and Eastern Newfoundland/Avalon region)

Latitude is another factor to be considered. At different latitudes there is wide variation in plant growth conditions in different seasons due to light, temperature, sunlight and other

factors. Several reports have demonstrated that the plants growing in northern latitudes have higher contents of phenolic compounds compared with their southern counterparts. (Hårdh & Hårdh 1977; Lätti *et al.* 2008, 2010). It was detected by Lätti *et al.* (2008) that southern populations of billberry exhibited a significantly lower content of total anthocyanins, although there was extensive variation among the populations. Martz *et al.* (2009) detected a positive correlation between increasing latitude and the content of phenolic compounds. In our study, increasing latitude exhibited a positive correlation between anthocyanin, proanthocyanin and total antioxidant activities and latitude (Figure 3.16). The content of total phenolics and flavonoids did not show any such correlation. The critical values for Pearson's correlation coefficient for total phenolic and flavonoids were 0.13414059 and -0.409874761, respectively which were not significant.

In a study done by Oleszek *et al.* (2002), contrasting results were observed. The needles of *Pinus sylvestris* trees from higher latitudes showed the lowest levels of total flavonoids indicating their local adaptations based on genetic differences in the regulation of flavonoid biosynthesis among populations of *P. sylvestris* (Jaakola & Hohtola, 2010).

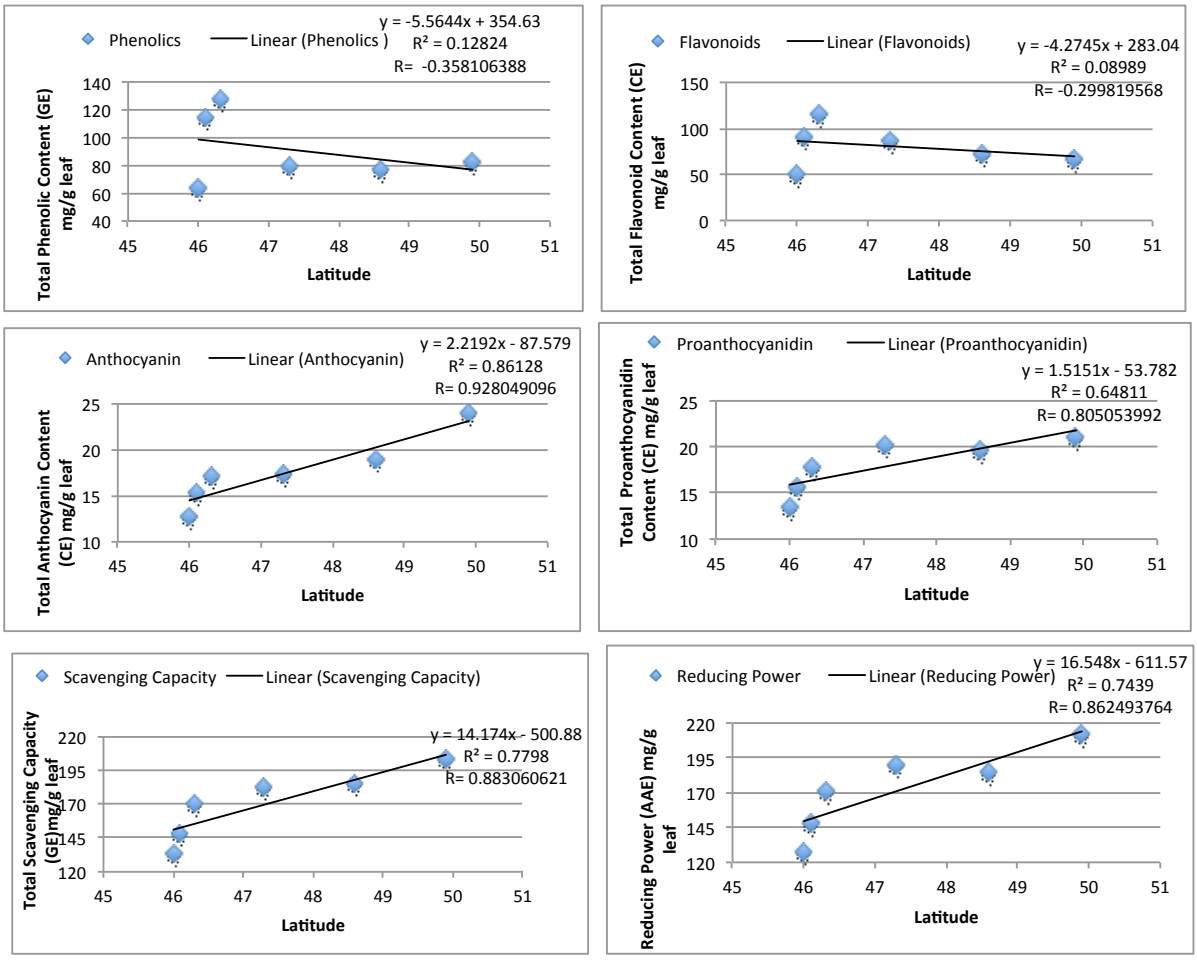

Figure 3.16. Graphs representing a correlation between latitude and the contents of phenolics and antioxidants in the leaves of lingonberry clones from different regions (Quebec, New Brunswick, Nova Scotia, Western Newfoundland, Central Newfoundland and Eastern Newfoundland/Avalon region)

Altitude also affected the contents of secondary metabolites in plants. At high altitudes, higher solar radiations have an impact on biosynthesis of secondary metabolites leading to an increase as a response to UV radiation (Jaakola & Hohtla, 2010). Increasing altitude showed increased levels of the total anthocyanins, proanthocyanidins and total antioxidant activities

(Fig. 3.17, Table 3.3). The total phenolics and flavonoid content had no correlation with altitude.

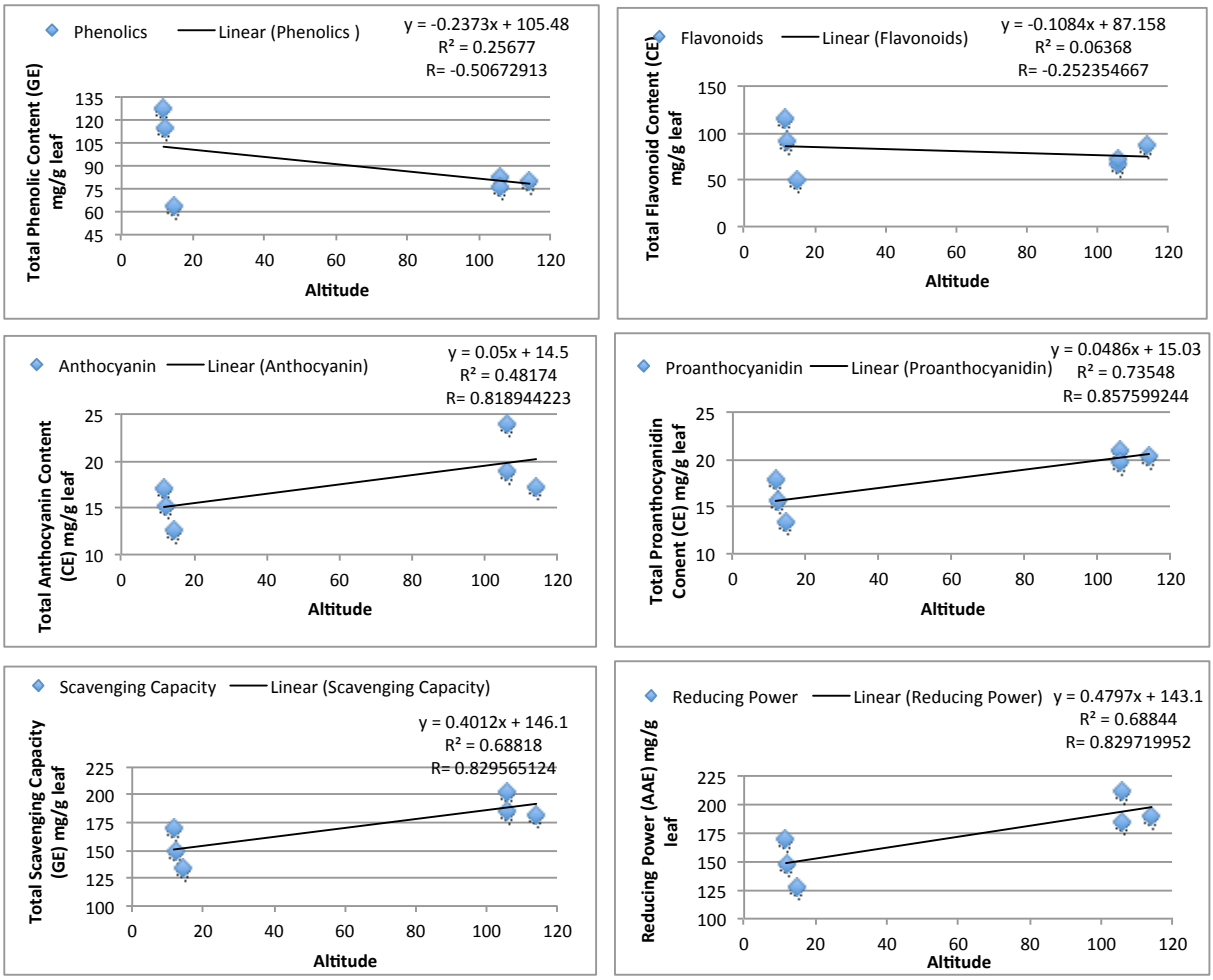

Figure 3.17. Graphs representing a correlation between altitude and the contents of phenolics and antioxidants in the leaves of lingonberry clones from different regions (Quebec, New Brunswick, Nova Scotia, Western Newfoundland, Central Newfoundland and Eastern Newfoundland/Avalon region)

Nova Scotia and New Brunswick were relatively relatively similar in all aspects, including the difference in total flavonoid content and total phenolic content, both exhibiting high levels.

Both provinces are located in the Atlantic Canada Maritime region and are heavily influenced by both high precipitation leading to extended winters and cool summers and coastal winds which increase the rates of convection, increasing plant heat loss.

Precipitation is another important environmental component that could have impact on antioxidant compounds. In a study conducted by Neeser (2006), antioxidant activity was observed to be higher in orchards that had more precipitation. In the present study, positive correlation was observed between the average annual precipitation and all the studied parameters except for total phenolic compounds and anthocyanin levels where the correlation was not significant (Fig. 3. 18, Table 3.3).

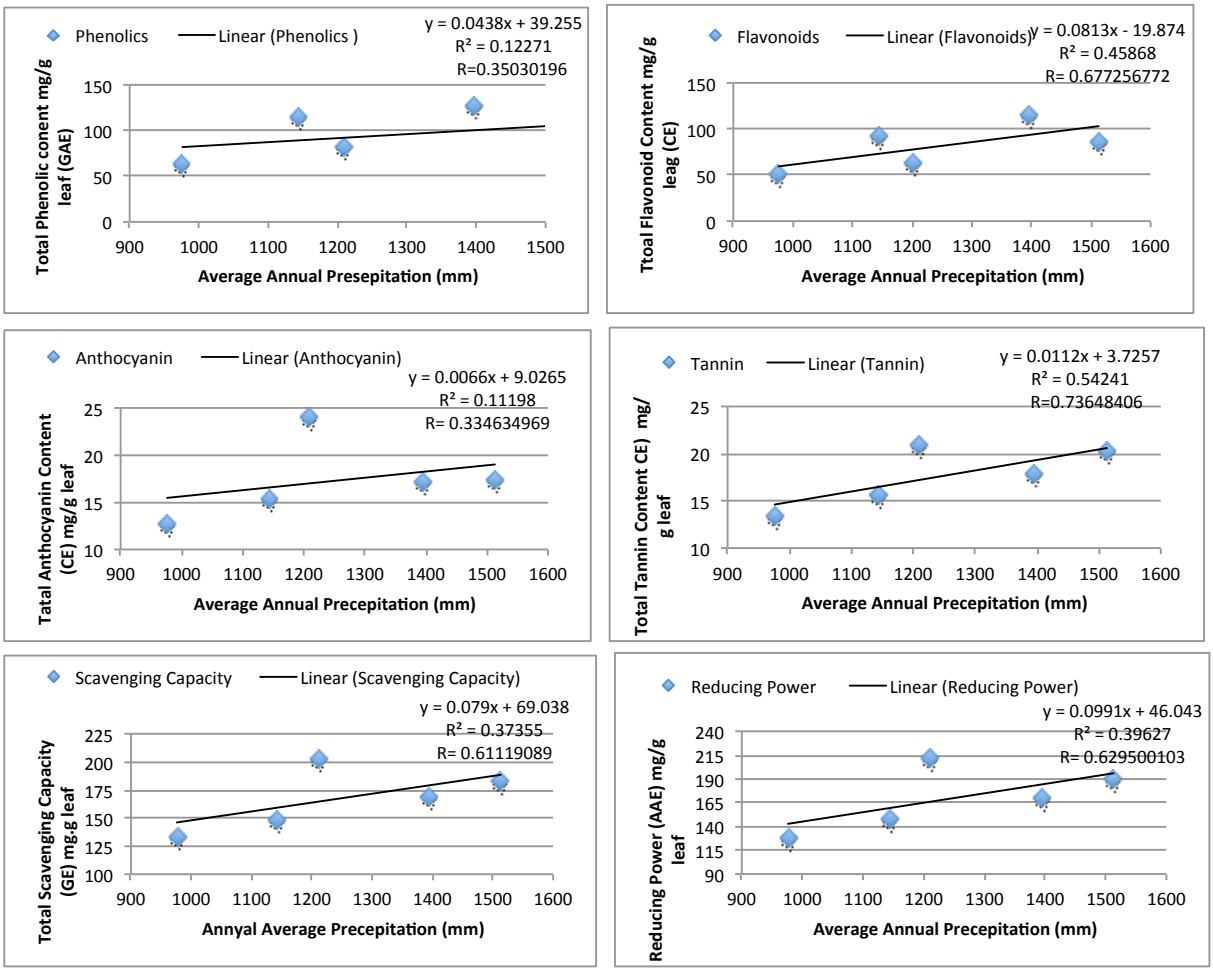

Figure 3.18. Graphs representing a correlation between average annual precipitation and the contents of phenolics and antioxidants in the leaves of lingonberry clones from different regions (Quebec, New Brunswick, Nova Scotia, Western Newfoundland, Central Newfoundland and Eastern Newfoundland/Avalon region)

There is scarce information about gene-environment interaction in terms of biosynthesis of secondary metabolites and not much is available information about the variation in phenolic content in relation to the genome. It seems that there is genetic control of biosynthesis of secondary metabolites. There are several reports indicating differences in anthocyanin and

proanthocyanidin concentration of plants influenced by differences in their genotypes (Howard *et al*. 2003; Cho *et al*., 2005; Ortega-Regules *et al.*, 2006). However, it is not very clear if the differences in the phenolic components result from the climate conditions or genetic adaptation to the growth environment at their early developmental stage.

3.5. Conclusion

The levels of phenolic compounds and antioxidants in the leaves of lingonberry clones are highly variable and are affected by environmental factors such as temperature, precipitation, latitude and altitude as well as by genotype. Lingonberry plants growing at different latitudes are primarily the plants with different genotypes, although the biosynthesis of secondary metabolites and antioxidant compounds in plants at specific climate have been the result of their adaptation. The climatic conditions had an effect in adaptation of lingonberry clones with different antioxidant components although all plants were maintained in the same greenhouse environment under similar conditions for about 10 years. This indicate a complex relationship between antioxidant gene and environment.

There is a great variation between lingonberry genotypes. The significant differences between the two subspecies, *ssp. vitis-idaea* and *ssp. minus,* make a clear understanding of difference in levels of their phenolic compounds.

It could be concluded from the results that differences in climatic conditions along with different geographical locations have an impact on biochemical properties of lingonberry plants and that this difference is responsible, to a significant degree, for phenolic contents and

antioxidant activities. Lingonberry plants occurring at higher latitudes and being subjected to harsh weather conditions were shown to occur with higher antioxidant activities and levels.

It was observed that with the difference in the subspecies the variation in morphology and possibly the climatic region of the plants origin correlate with the levels of phenolic compounds and the antioxidant activities. This is could be due to the multiple blooming periods in ssp. *vitis-idaea* resulting in a lower production of antioxidants, whereas ssp. *minus* exhibits a single blooming period allowing for substantial antioxidant production.

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Chapter 4

Effects of Lingonberry Extracts on Oxidative Stress in Rat Brain Cultures

This chapter aims to determine the neuroprotective effect of extracts from fruits and leaves of wild lingonberry clones from Newfoundland against glutamate-mediated excitotoxicity.

4.1. Introduction

Overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide anion, hydrogen peroxide, peroxyl radicals, nitric oxide and peroxynitrile radicals could lead to oxidative stress. These reactive species can damage proteins, lipids and DNA, leading to lipid peroxidation, altered signal transduction pathways and destruction of membranes and organelles that could be responsible for the development of neurodegenerative diseases (Sastre *et al.*, 2000). The brain is particularly susceptible to oxidative stress due to its high oxygen demand, as well as due to the fact that it is enriched with polyunsaturated fatty acids. Moreover, a high iron concentration and low levels of antioxidants are also factors responsible for overproduction of ROS and RNS in brain cells (Lau *et al.,* 2005; Slemmer *et al.,* 2008). It has been reported by several researchers that excessive production of oxidative products of protein, lipid and DNA oxidation are associated with neurodegenerative disorders (Halliwell, 2006). The balance between ROS and antioxidants in biological systems is referred to as redox homeostasis and is essential for normal cell function (Droge, 2002). In order to

combat oxidative stress, there are several types of endogenous enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione preoxidase (GPx). There are also several non-enzymatic antioxidants that are obtained primarily in the diet, including tocopherol, ascorbate, carotenoids and various polyphenolic compounds (Valko *et al.*, 2007; Slemmer *et al.,* 2008). Figure 4.1 represents causes and effects of oxidative damage in the brain.

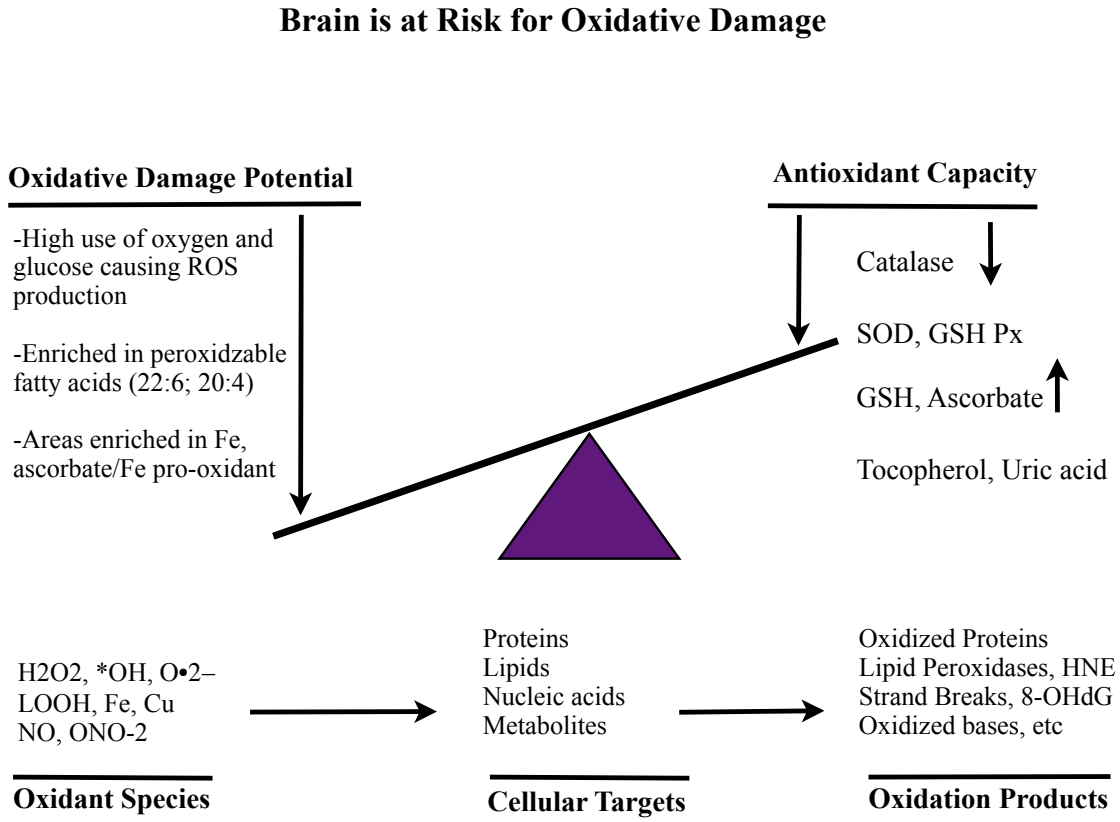

Figure 4.1. Representation of oxidative damage in brain illustrating its causes and effects. (Modified from Floyd and Hensley, 2002) (H_2O_2 - hydrogen peroxide, ·OH - hydroxyl free radical; $O^{\bullet 2-}$ – superoxide, LOOH - lipid hydroperoxide, Fe -iron ions, Cu - copper ions, NO - nitric oxide, $ONOO^-$ - peroxynitrite, SOD - superoxide dismutase, GSHPx - glutathione peroxidase, GSH - glutathione, HNE - 4-hydroxy-2-nonenal, and 8-OHdG, 8-hydroxy-2′-deoxyguanosine)

Polyphenols are a large class of natural compounds that have high antioxidants and potential beneficial effects such as anti-inflammatory, anticancer, anti-fungal, anti-microbial, and anti-ulcer properties (Rahman *et al.,* 2007). These classes of compounds also appear to have positive effects on the cardiovascular system, which may be due to their ability to act as free radical scavengers or by other mechanisms (Slemmer *et al.,* 2008). Polyphenols are abundant in plants, especially vegetables and fruits. Berry crops are very rich sources of polyphenolic antioxidants, particularly flavonoid compounds (Zheng & Wang, 2003). Since plant derived supplements are considered as natural and hence potentially safer than synthetic drugs, there has been an increasing demand for 'nutraceuticals' (Raskin *et al.,*2002). The term nutraceutical was originally defined by Dr. Stephen DeFelice as a daily nutritional supplements like food or a part of food, which have beneficial effects in treating or preventing diseases (Kalra, 2003). Berries have been reported to have diverse health promoting phytochemicals and are very rich sources of polyphenolics especially flavonoids, anthocyanins, and proanthocyanidins. An enormous body of research has been published suggesting that the dietary consumption of berries has positive effects on human health and diseases (Seeram, 2008; Battino *et al.,* 2009). The health promoting properties of berries are gaining continued interest in the berry market not only as antioxidants, but also because of their bioactive properties *in vivo* (Seeram, 2008; Seeram & Heber, 2006).

In particular, the potential of berries and their constituents to protect the brain from aging and neurodegenerative disease has gained increased attention in recent years. For example, dietary supplementation with polyphenol containing fruits can decrease age-related behavioural deficits in rats (Shukitt-Hale *et al*., 2005). In a recent study conducted with a

mouse model of Alzheimer's disease, treatment with berries rich in polyphenols decreased the extent of behavioural abnormalities associated with the disease (Vepsäläinen *et al.*, 2013). Other experimental studies have shown that rats fed with a diet enriched with blueberries can protect the brain against oxidative stress and associated learning deficits (Duffy *et al.,* 2008). Surprisingly, a diet enriched with blueberries has been demonstrated to later protect animals from the damage induced by ischemic stroke (Sweeney *et al*., 2002; Wang *et al.,* 2005).

The present work aims to study the polyphenolic content, antioxidant capacity and potential neuroprotective effects of fruit and leaf extracts from lingonberries that grow natively in Newfoundland, Canada. Following initial chemical analysis, the cell cultures derived from rodent brains were subjected to high levels of glutamate, the most prominent endogenous excitatory neurotransmitter present in the mammalian central nervous system. Glutamate plays an important role in long-term potentiation, a cellular mechanism of learning and memory, under physiological conditions and contributes to other cognitive functions (Chen *et al*., 2009; Suzuki *et al*., 2007). Increased glutamate levels could result in glutamate excitotoxicity (Goto *et al*., 2009). Glutamate-induced 'excitotoxicity' is a pathological process by which cells are damaged and killed by excessive stimulation from neurotransmitters such as glutamate and similar substances (Fig. 4.2). This abnormal process produces oxidative and nitrosative stress, and likely contributes to the pathology of traumatic brain injury, stroke, neurodegenerative disorders, and normal brain aging (Slemmer *et al.,* 2008; Mehta *et al*., 2012; Weber, 2012).

The neuroprotective effects of lingonberries have not yet been studied, and hence this will be the first report in this field. In the present study, the effects of lingonberry leaf and fruit

extracts were determined on cell cultures subjected to glutamate excitotoxicity. Possible correlations between bio-activities and phenolics compounds as well as antioxidant capacities have been made.

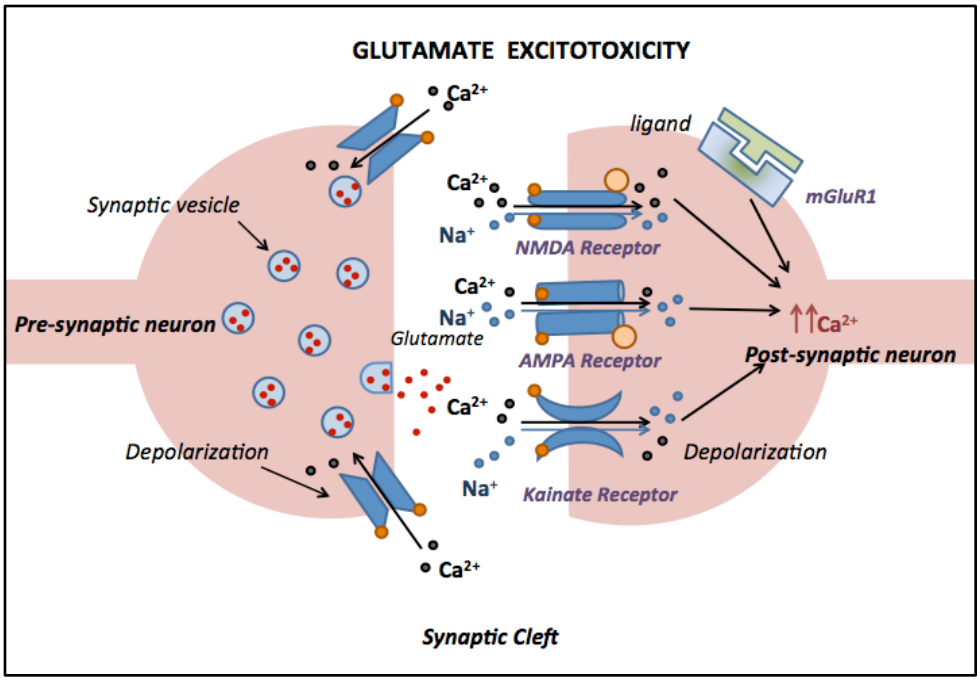

Figure 4.2. Glutamate excitotoxicity: Glutamate-mediated increases in Ca^{2+}.(Modified from Weber 2004). Glutamate activates ionotropic receptors (i.e. AMPA and NMDA receptors) on neurons that leads to an increase in Ca^{2+}. Glutamate also activates metabotropic receptors (group I mGluRs) which produce IP_3.

4.2. Methods

Plant materials used in the present study were fruits and leaves of clones of wild Newfoundland lingonberry plants developed by Debnath (2005). The plants were collected in the fall of 2001 and propagated by a vegetative stem cutting method and their clones maintained in a greenhouse of at Atlantic Cool Climate Crop Research Centre (ACCCRC) in St. John's, Newfoundland from the date of collection.

4.2.1. Preparation of Clones from Wild by Vegetative Stem Cutting

Terminal softwood stem cuttings 4 to 5 cm long were taken from wild lingonberry plants and rooted in 45-cell plug trays (cell diameter 5.9 cm, cell depth 15.1 cm; Beaver Plastics, Edmonton, AB, Canada) containing peat: perlite [2:1 (v/v)] in a humidity chamber with a vaporizer (Conviron E15; Controlled Environments Ltd., Winnipeg, MB, Canada) at 22° ± 2°C and 95% RH, with a 16 hour photoperiod (PPF 55 μmoles m^{-2} s^{-1} at culture level). No rooting compound was applied (Debnath, 2006; Foley & Debnath 2007). After 6-8 weeks, rooted plantlets were transferred to plastic pots (10.5 L, 10.5 W and 12.5 D) containing the same medium used for rooting and were maintained in humidity chamber and acclimatized by gradually lowering the humidity by 3 to 4% per week over 3 weeks period. Plant well developed and hardened were then grown in the greenhouse under natural light condition at about 20°C, 85% RH and 16 hour photoperiod at maximum PPF of 90 $umol.m^{-2}S^{-1}$ (Debnath 2006; Foley & Debnath 2006). All the plants were treated equally. Fertilization (20-8-20 N-P-

K, Plant Products Co. Ltd., Brampton, ON) and irrigation was supplied to plants when required (Debnath 2006).

Young green leaves and ripe fruits of *Vaccinium vitis-idaea* L. were harvested and immediately stored at -20 ºC. Sample extraction was carried out from the collected leaf and fruit samples in 80% (v/v) acetone with 0.2% formic acid in the ratio 1:2 and was subjected to 30 min shaking on ice. The sample mixture was then centrifuged at 20,000 *g* for 20 minutes at 4 ºC. Supernatants were collected and the procedure was repeated with the residues. Both supernatants were mixed together and the final concentrations for leaf and fruit samples were 25 mg/ml and 166 mg/ml, respectively.

4.2.2. Biochemical Assays

Lingonberry leaf and fruit extracts were further diluted ten times with the extraction solvent for biochemical assays.

4.2.2.1. Determination of the Total Soluble Phenolics

The total soluble phenolic content in both leaves and fruits were determined using the Folin-Ciocalteu reagent as described by Chandrasekara and Shahidi (2011) with some modifications. 0.5 ml of Folin-Ciocalteu reagent was added to centrifuge tubes containing 0.5 ml of extracts and vortexed. One ml of saturated sodium carbonate solution was added to each tube to neutralize the reaction. The final volume was adjusted to 10 ml with water and vortexed for 30 seconds. The reaction mixtures were kept in the dark for 35 min at room

temperature and then centrifuged at 4,000 *g* for 10 min. The absorbance was measured at 725 nm using Ultrospec 4300 *pro* UV/Vis spectrophotometer. The total soluble phenolic content of each sample was determined using gallic acid standard curve and expressed as milligrams of a gallic acid equivalents (GAE) per g of berry or leaf fresh weight.

4.2.2.2. Determination of Total Anthocyanin Content

The total anthocyanin content was measured by a pH differential method described by Foley and Debnath (2007). Absorption at 510 nm and 700 nm was measured using a UV/ Visible spectrophotometer (Ultrospec 4300 *pro*) in buffers at pH 1.0 and pH 4.5 and the difference between the two values was used to determine total anthocyanin content. Results are expressed as catechin equivalents (CE).

4.2.2.3. Determination of the Total Flavonoid Content

The total flavonoid content was measured by an aluminum chloride colorimetric assay (Zhishen *et al*. 1999). One ml of extract or standard solution of catechin (0.5 mg/ml) was mixed with 4 ml of water, followed by addition of 0.3 ml 5% $NaNO_2$, of 0.3 ml of 10% $AlCl_3$ (after 5 min) and 2 ml of 1 M NaOH (one minute later), the volume was adjusted (with water) to 10 ml. The absorbance was measured at 510 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). The total flavonoid content was expressed as μmol of catechin equivalent (CE) per g of leaf or fruit.

4.2.2.4. Determination of the Total Proanthocyanidin (Tannin) Content

The total proanthocyanidin contents of sample extracts were determined by the method developed by Chandrasekara and Shahidi (2011). Five ml of 0.5% vanillin-HCl reagent were added to 1 ml of extract, mixed thoroughly and incubated at room temperature for 20 min. A separate blank for each sample was read with 4% hydrochloric acid (HCl) in methanol. The absorbance was read at 500 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*), and the content of proanthocyanidins was expressed as μmol of chatechin equivalent (CE) per g of leaf or fruit.

4.2.2.5. Determination of the Total Antioxidant Activity

The total antioxidant capacity of samples was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which was conducted according to the method of Brand-Williams *et al*. (1995) with some modifications. The stock solution of 1 mM DPPH in methanol was diluted to 60 μmol, 1.9 ml of the latter was mixed with 0.1 ml leaf extract, shaken vigorously and left in the dark for 20 minutes. The absorbance was read at 515 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*). The scavenging capacity was expressed as percentage of inhibition of DPPH consumption. The gallic acid standard curve was used to express the results as GAE.

4.2.2.6. Determination of Reducing Power

The reducing power of extracts was determined by the method described by Chandrasekara and Shahidi (2011). The medium of assay was 200 mM phosphate buffer (pH 6.6) with 1% potassium ferricyanide. 2.5 ml of the diluted extract was added to the assay buffer and incubated for 20 min at 50 ºC and then 2.5 ml of 10% trichloroacetic acid (TCA) was added to the assay, mixed and then centrifuged for 10 min at 1750 g. 2.5 ml of supernatant was transferred to empty tubes and combined with 2.5 ml of deionized water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm using a UV/Visible spectrophotometer (Ultrospec 4300 *pro*), and the results were expressed as ascorbic acid equivalents using appropriate standard curves.

4.2.3. Cell Culture Experiments

The cell culture experiments utilized one-to-three day old Sprague-Dawley rat pups and were conducted in the School of Pharmacy at the Health Science Centre, Memorial University. All procedures using rat pups were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (protocol number 12-20-JW).

4.2.3.1. Dissection of Rat Pup Brains

The brains of 1-3 day old Sprague-Dawley rat pups were dissected and cortical cultures were prepared as described previously (Weber *et al*., 2012). The rat pups were euthanized and the brains were removed from the skull and placed in 15 ml of cold Hank's buffered salt

solution (HBSS) supplemented with 10 mg/ml PenStrep in a small petri dish which was placed on ice. The cerebellum was cut and discarded. The right and left cortical hemispheres were separated by a scalpel. Then, the corpus callosum was removed. The cerebellum and callosum were removed because they give a low yield of cell and the cells are generally very small. In addition, there are many neurons and glia in the cortex that use glutamate. Next, the blood vessels and membranes surrounding the brain were removed with the help of tweezers under microscopic light.

4.2.3.2. Poly-L-ornithine Plate Coating

Poly-L-ornithine (PLO) promotes the adhesion of cells to the culture wells. Culture plates were coated with PLO solution one day prior to cell culture preparation and plates were incubated in a humidified incubator at 37 ºC with 5% CO_2.

4.2.3.3. Preparation of Cell Cultures

The hemispheres, free of blood vessels, were placed in a centrifuge tube containing 10 ml of cold HBSS. Before centrifuging, the cell aggregates were triturated to smaller pieces by gently using a serological pipette (by pipetting -10 times in and out). The tissue was then washed by centrifuging at 1000 rpm at 4 ºC for 3 min. The supernatant was removed and 10 ml of fresh HBSS was added. The tissue was again washed at 1000 rpm at 4 ºC for 3 min. and the supernatant was discarded. 0.8 ml of 0.25% Trypsin-EDTA in HBSS supplemented with 9.2 ml of fresh HBSS were then added. The tubes were incubated for 15 min at 37 ºC and were then centrifuged at 1000 rpm at 4 ºC for 3 minutes. After discarding the supernatant,

fresh growth media (10 ml) was added to the tubes and they were centrifuged at 1200 rpm at 4 ºC for 5 min. Four ml of fresh growth media was added to the tubes after discarding the supernatants and cells were triturated using a 5 ml serological pipette until all large aggregates were dissociated. The suspension was filtered through a 70 µm nylon strainer into a 50 ml plastic tube and diluted with growth media up to 12 ml if using two 24 well plates (1 ml for one 24 well plate). Table 4.1 and 4.2 represents detailed chemical composition of the culture medias and growth medias used in the study respectively.

The PLO (Poly-L-ornithine) coated plates were taken out of the incubator and the PLO was removed and the wells were washed twice with distilled water (300µL per well for 24 well plate). 250 µl was transferred to each well in a 24 well plate. All the cultures were maintained in a humidified incubator (5% CO_2, 37ºC). Half of the media in cultures was replaced two days after plating, and then twice per week, with serum-free media containing 2% B27 supplements (GIBCO). In general, glia formed a confluent monolayer that adhered to the membrane substrate, whereas neurons adhered to the underlying glia. It has been previously found that these cultures contain approximately 12% neurons as determined by NeuN, which is a neuronal marker expressed strongly in nuclei and perikarya (Weber *et al.,* 2012), with the remaining cells representing the glial population. Approximately 95% of NeuN-negative cells stained positively for glial fibrillary acidic protein (Invitrogen, Camarillo, CA), suggesting that the majority of glial cells in these cultures are composed of astrocytes. These cultures were used for experiments at 9-16 days in vitro (DIV).

4.2.3.4. Glutamate Exposure and Extract Treatment

The cell culture experiments were carried out after the extracts were filtered through a sterile filter of 40 µm pore size. Before treating cells with the extract, the solvent used in the extraction of lingonberry fruits and leaves was first tested for its effect on cell cultures. The appropriate amount of solvent that did not affect the cultures was determined. Glutamate was dissolved in sterile distilled water (dH_2O). The cell cultures were exposed to glutamate (100 µM) in a volume of 3 µl per 0.3 ml of cell culture media, and control cultures received an equivalent volume of sterile dH_2O. The cell cultures were treated with 1 µl lingonberry fruit extract (166 mg/ml) and leaf extract (25 mg/ml) at the time of glutamate exposure, and were treated with the leaf and fruit extracts for 24 hours. It was found that cultures treated with 1 µl of solvent alone had no significant change in cell number after 24 hr. For each treatment plate, at least two control treatments were performed using dH_2O as well as two glutamate treatments. Experiments were performed in at least three separate culture preparations and each condition was represented by at least six samples.

4.2.3.5. Fixing and Staining of Cell Cultures

After 24 hours of treatment, cell cultures were fixed for 20 minutes with 4% paraformaldehyde, according to Engel *et al*. (2005). Then 250 µl of culture media from each of the treated well was replaced with 250 µl of propidium iodide (PrI) solution in the dark. After 5 minutes, the PrI solution was removed and wells were washed with phosphate-buffered saline (PBS; pH 7.4). Next, 4% Paraformaldehyde fixative solution (250 µl) was

added to each well and was allowed to stand for 20 minutes. Wells were washed twice with PBS solution and dehydrated with 100% ethanol. 10 µl of DAPI (4',6-diamidino-2-phenylindole) was added and then the cells were covered with cover slip. DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy. DAPI can pass through an intact cell membrane, therefore it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower.

4.2.3.6. Capturing Images

Images were captured using a Zeiss Observer A1 microscope and a Pixelfly qe CCD camera (PCO., Kelheim, Germany). Five sets of images (5 DAPI and 5 PrI images) with magnification of 200x were captured from different regions of each well. Images of DAPI-positive cells were captured with a DAPI optical filter set, while PrI images were captured using a Texas red filter set using the software IPlab.

4.2.3.7. Cell Counts

At least two investigators who were blind to the treatment conditions were used to count the amount of cells in each image. The mean of two readings for each well was generated and calculated accordingly. Data on the number of DAPI-positive cells is expressed as a percentage of control values for each given experimental day. Condensed nuclei data is expressed as the percentage of the total amount of DAPI-positive cells that contained

condensed nuclei within each condition. The percentage of condensed nuclei was calculated using the program Image J (Rasband, & Image). Representative images of cellular morphology in control and glutamate-treated cultures were captured using differential interference contrast microscopy.

Table 4.1. Preparation of chemicals for cell culture experiments

Chemicals	Preparation
Poly-L-ornithine	To prepare the PLO solution, 100 mg of PLO was mixed with 10 ml of distilled water to achieve 10mg/ml solution. 300 µl of this solution was then transferred to each centrifuge tube (2 ml) and then these tubes are stored in -80ºC.
Hank's buffered salt solution (HBSS)	Hank's buffered salt solution is without Calcium chloride, magnesium chloride and Magnesium sulphate. First different solutions were made and stored at 4 ºC and then the final solution was made. Stock #1 was made. 8.0g of NaCl and 0.4g of KCl was dissolved in 90 ml of distilled water and the volume was made up to 100 ml. Stock #2 was made. Anhydrous Na_2HPO_4 (0.358 g) and KH_2PO_4 (0.60 g) was dissolved in 90 ml distilled H_2O and then the solution was made up to 100 ml with distilled water. Stock #5 was made. 0.35g of $NaHCO_3$ was added to 10 ml of distilled water.
Trypsin-EDTA 0.1%	Trypsin-EDTA was dissolved in HBSS containing Penstrep to obtain 10µg/ml. 100 ml of Trypsin-EDTA was taken and added to 100 ml of HBSS, which has 1ml of Penstrep to make a solution of 10µg/ml and stored in -18 ºC.
Propidium iodide solution	Propidium iodide solution was made by diluting 3µl of propidium iodide in 10 ml of PBS (pH 7.4) ...solution in dark.
Paraformaldehyde Fixative	The 400 ml of distilled water was taken in a 1000 ml beaker under a fume hood and was heated to 70 ºC and then 40 g paraformaldehyde was added to it and stirred well. This solution appeared cloudy. Cold 10N NaOH was added slowly drop wise to the solution until it cleared. 500 ml of 0.2M cold Phosphate buffer was added to it. Then distilled water was added to the solution to make up the volume to 1000 ml. This solution was filtered using a bottle filter and stored in the fridge.

Table 4.2. Preparation of Growth and Culture Media

Media	Preparation
Growth Media Preparation	First, growth media free of Horse serum (HS) was prepared by adding sterile 5 ml PenStrep, 8 ml glucose solution, sodium pyruvate solution, 5 ml N2 supplements into a 500 ml bottle of sterile BME and was stored at 4ºC. Then, to make growth media containing 10% HS, 4.5 ml HS (portions in the freezer) was added to 45 ml growth media. A 50 ml labeled plastic tube was used and the media was stored at 4ºC. The solution was filtered when needed.
Culture media Preparation	Serum free culture media was prepared using Basel medium algae (BME) (500ml) with 5 ml PenStrep, 8 ml glucose solution, 5 ml Sodium pyruvate Sln, 5 ml N2 supplements and 10 ml B27 supplements. Media was then sterilized through a bottle filter and stored at 4ºC.

4.2.4. Statistical Analyses

All of the biochemical experiments were repeated at least three times. Data in the text and the figures are expressed as means ±SE of three replicates. Statistically significant differences were determined by the non-parametric unpaired t-test using the statistical program SPSS (IBM Inc.). In all cases the confidence coefficient was set at 0.05. The data for the bioactivity (cell culture) experiments were analyzed with one-way ANOVA ($p < 0.05$) followed by Tukey's multiple comparisons test using the statistical program GraphPad Prism (La Jolla, CA, USA). Data represented in figures are expressed as means ± SE of at least 6 wells per condition. Significance was also set at $p < 0.05$ for these experiments.

4.3. Results

4.3.1. Biochemical assays

The content of total soluble phenolics, flavonoids, anthocyanins and tannins was significantly higher in the lingonberry leaves versus the fruits ($p<0.05$) (Fig. 4.3 - Fig 4.6). These finding correlated well with total radical scavenging capacity and reducing power, in which the leaves had much higher activity compared to fruits (Fig. 4.7 & Fig. 4.8).

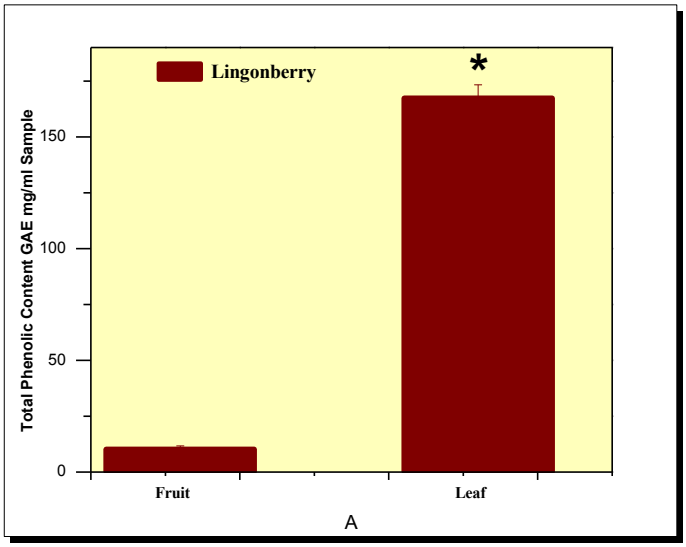

Figure 4.3. The total soluble phenolic content in lingonberry fruits and leaves. Means ± SE, n = 3, * – values significantly different at $P < 0.05$ from the control. GAE – gallic acid equivalents.

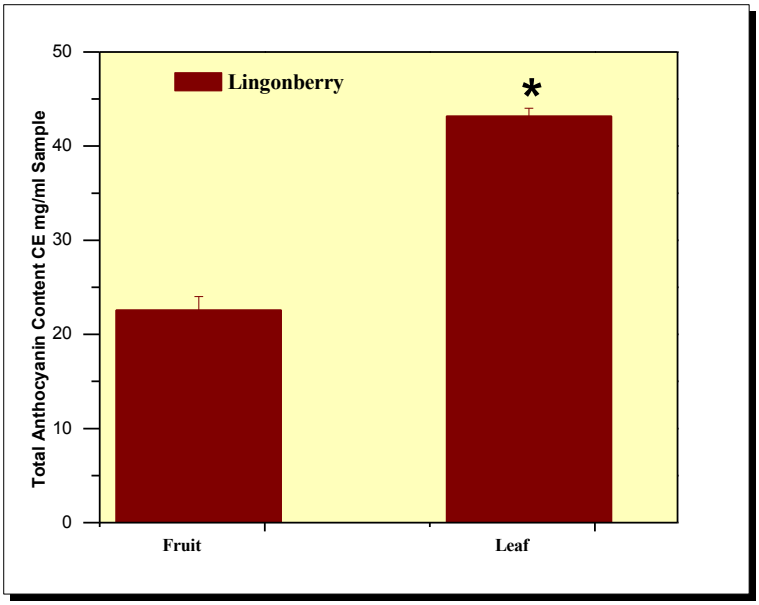

Figure 4.4. The total anthocyanin content in lingonberry fruits and leaves. Means ± SE, n = 3, * – values significantly different at P < 0.05 from the control. CE – catechin equivalents.

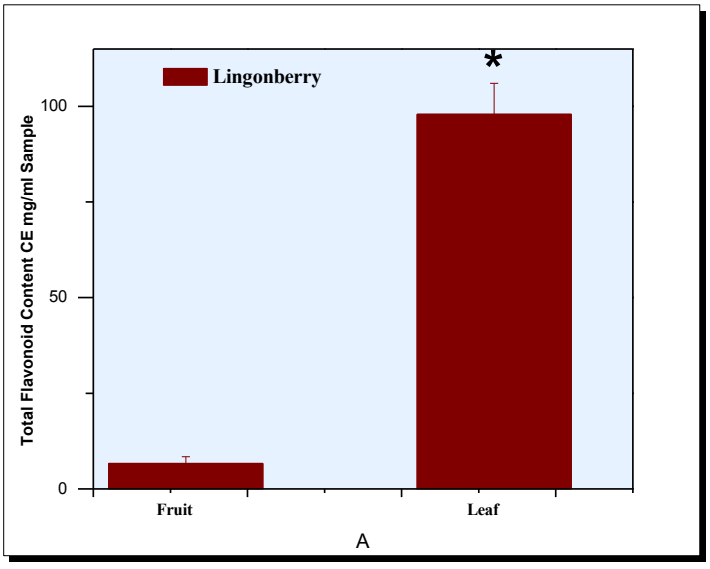

Figure 4.5. The total flavonoid content in lingonberry fruits and leaves. Means ± SE, n = 3, * – values significantly different at P < 0.05 from the control. CE – Catechin equivalents.

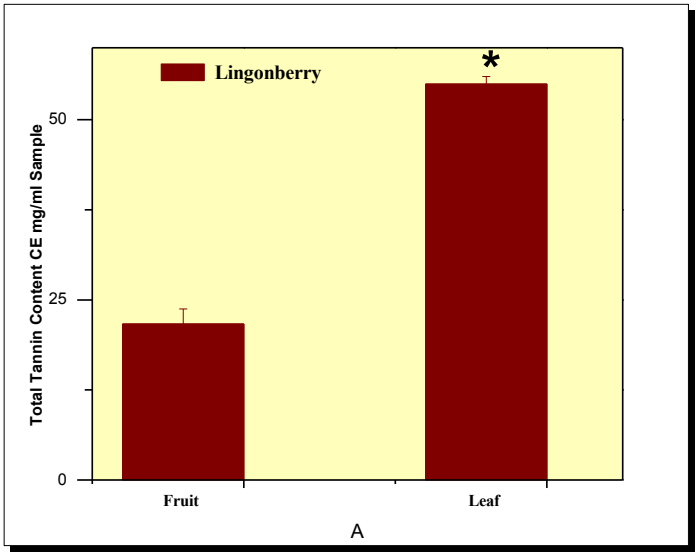

Figure 4.6. The total tannin content in lingonberry fruits and leaves. Means ± SE, n = 3, * – values significantly different at P < 0.05 from the control. CE – Catechin equivalents.

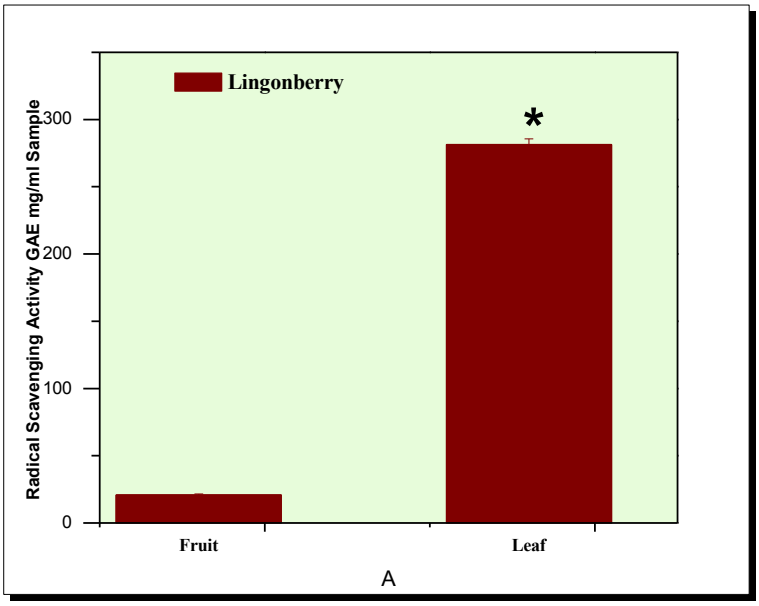

Figure 4.7. The total radical scavenging capacity in lingonberry fruits and leaves. Means ± SE, n = 3, * – values significantly different at P < 0.05 from the control. GAE – gallic acid equivalents.

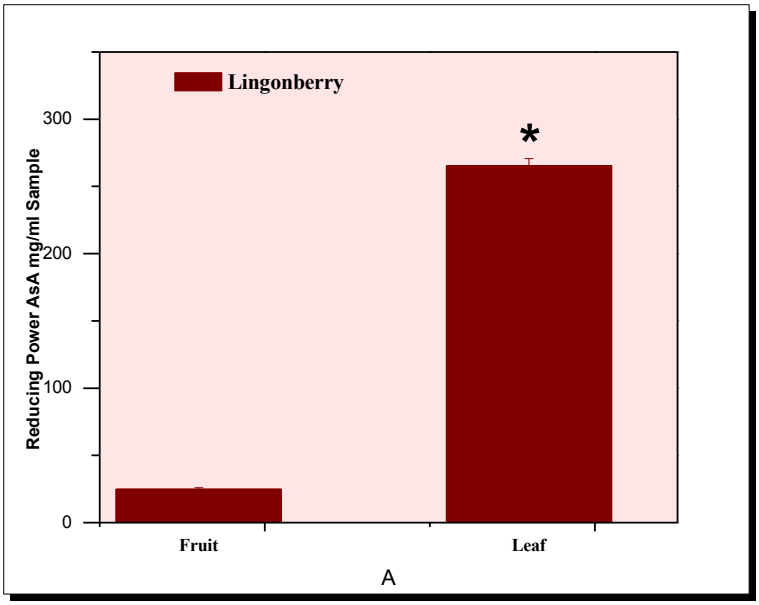

Figure 4.8. The total reducing power in lingonberry fruits and leaves. Means ± SE, n = 3, * – values significantly different at $P < 0.05$ from the control. AsAE – ascorbic acid equivalents.

4.3.2. *Cell culture experiments*

In preliminary studies it was found that treatment of cultures with 10 μM glutamate produced a cell loss of 17.0 ± 5.7 %, which was not statistically significant. However, 100 μM glutamate treatment for 24 hours caused a significant loss of cells of 20.1 ± 6.0 %, therefore a concentration of 100 μM glutamate was used for the remaining experiments. Analysis of cultures using light microscopy indicated that the cells in control (untreated) cultures had intact cell bodies and that cells consistent with neuronal morphology also displayed intact, smooth neurites (Fig. 4.9). After treatment with glutamate for 24 hours, many cells had disrupted cell bodies, and there was an increase in dark punctae, which may be indicative of

condensed nuclei in dead or dying cells (Chen *et al.*, 2000). We quantified the potential neuroprotective effects of the plant extracts by counting the amount of DAPI-stained cells that were present in cultures under various conditions (Figs. 4.10 and 4.11). Treatment with glutamate appeared to increase the amount of brighter, condensed nuclei in cultures, which is often indicative of delayed cell death (Weber *et al*., 2012) (Fig 4.10). Glutamate exposed cell cultures treated with lingonberry fruit extracts showed 79.1 ± 12.4 % of control values and those treated with leaf extracts lingonberry showed 106.9 % ± 8.6 % of control values, which was significantly different versus glutamate treatment alone, indicating a highly protective effect of the leaf extracts (Fig. 4.11). The percentage of cells displaying condensed nuclei were also quantified in the same cultures in which the extent of cell loss was determined. Glutamate caused an increase in the amount of condensed nuclei after 24 hours of exposure, but this increase was not statistically significant. Interestingly, there also appeared to be an increase in condensed nuclei in cultures treated with lingonberry leaf extract, but again these findings were not statistically significant.

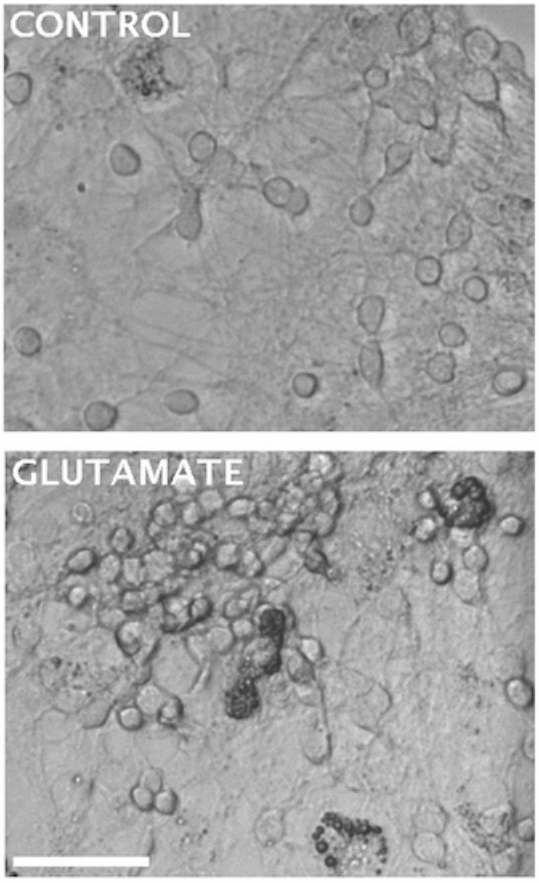

Figure 4.9. Light microscopic images of untreated cells (control; top) and cells treated with 100 μM glutamate for 24 hours (bottom). Note that cells in control cultures had intact cell bodies and that cells consistent with neuronal morphology also displayed intact, smooth neurites. After treatment with glutamate for 24 hours, many cells had disrupted cell bodies, and there was an increase in dark punctae. Scale bar = 50μm and applies to both images. Cell cultures are 12 DIV.

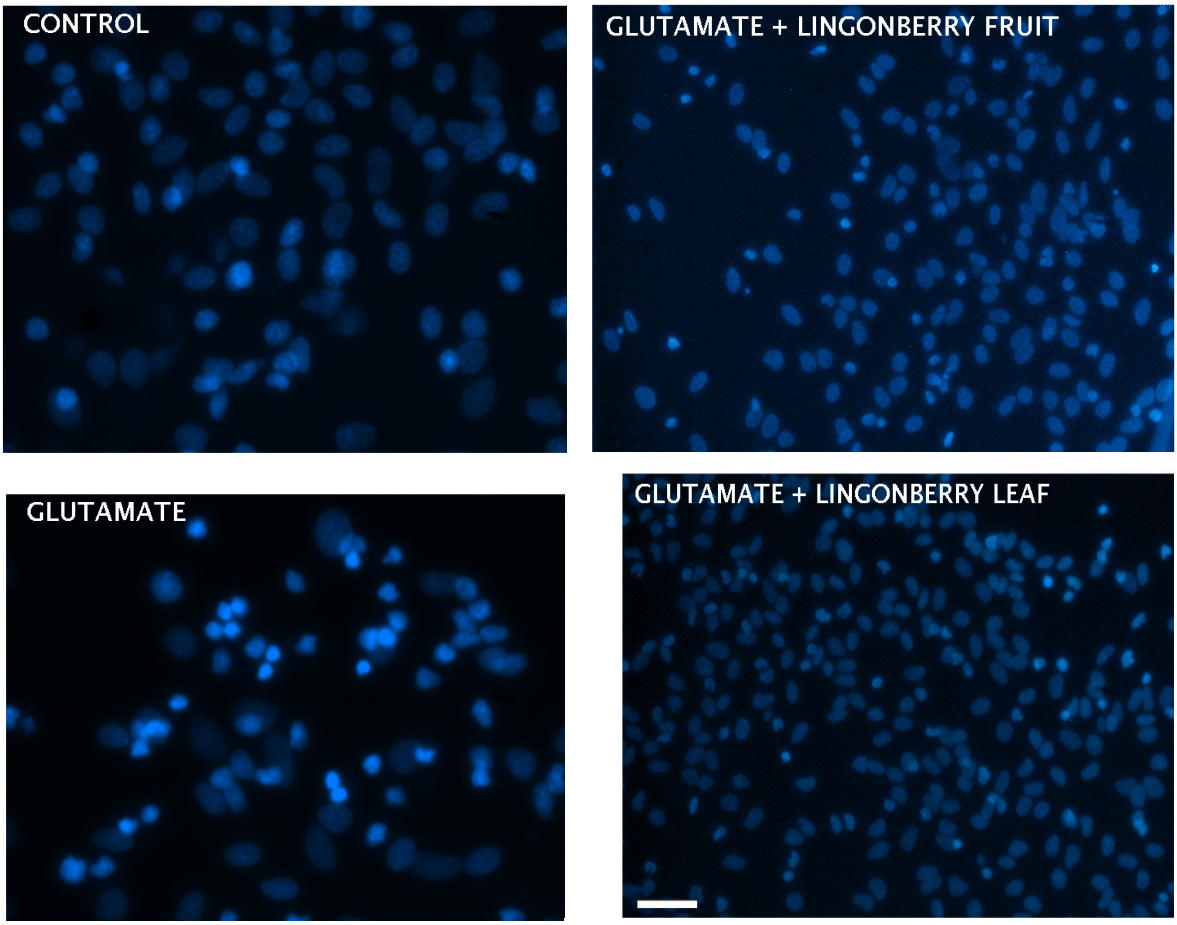

Figure 4.10. Representative images of cortical cells stained with DAPI (blue) in control conditions, after 24 hour treatment with 100µM glutamate, and after 24 hour treatment with 100µM glutamate in the presence of lingonberry fruit and leaf extracts. Note the presence of several condensed nuclei after treatment with glutamate, which may be indicative of delayed cell death. Images on the right are at a magnification of 200x, while images on the left have been enlarged in order to better represent nuclear morphology. Both scale bars = 50µm. The scale bar in the glutamate + blueberry fruit images applies to all three images on the left, while the scale bar in the glutamate + lingonberry leaf image applies to the three images on the right. Cell cultures in images on the left are 16 DIV, while those on the right are 15DIV.

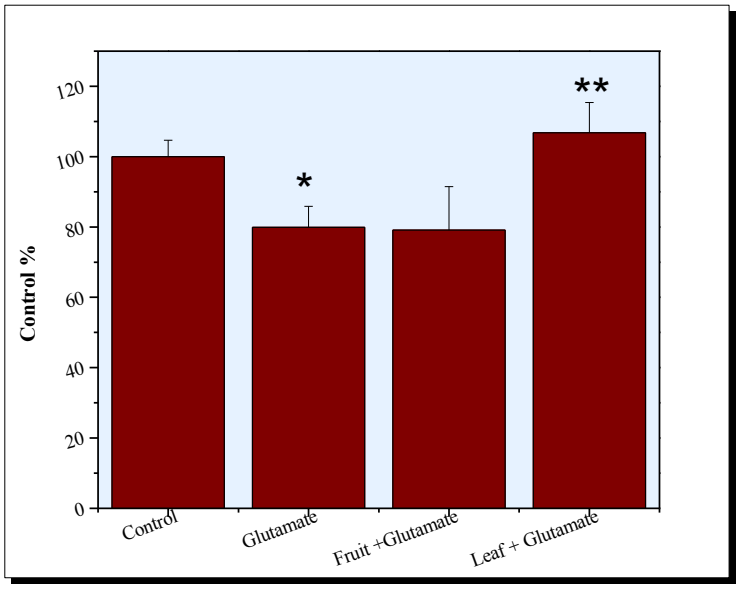

Figure 4.11. Summary of the effects of and lingonberry fruit and leaf extracts on glutamate-mediated cell death. Cells were treated with 100μM glutamate alone or in the presence of extracts. Top graph: the amount of DAPI-positive nuclei were quantified and data is expressed as % of control values. n=6-16. *p<0.05 vs. control; **p<.05 vs. glutamate only (one-way ANOVA with Tukey's post-hoc analysis). Bottom graph: the percentage of nuclei that exhibited a condensed morphology in the same culture wells used to generate the data in the top graphs.

4.4. Discussion

In the current study, high levels of phenolic compounds were detected such as anthocyanins, flavonoids and tannins, in lingonberries growing natively in Newfoundland. It was also observed that the level of phenolic compounds were higher in the lingonberry leaves compared to their fruits as indicated by radical scavenging activity and reducing power, which is consistent with the findings in previous lingonberry cultivars (chapter 3). These biochemical data are in line with the biological activities of the extracts on glutamate exposed rat brain cultures. This could be because of the direct correlation of involvement of reactive oxygen species during glutamate excitotoxicity and role of polyphenols in scavenging ROS.

Glutamate plays an important role in normal neurophysiology, such as a variety of cognitive functions (Rahn *et al*., 2012), as well as being involved with synaptic plasticity, which is believed to be the cellular mechanism of learning and memory (Kim & Linden, 2000; Lamont & Weber, 2012). However, increased glutamate levels results in glutamate-mediated excitotoxicity, which can lead to cell damage and death (Goto *et al*., 2009). This phenomenon could be due to excessive glutamate release, or inadequate uptake of glutamate by glial cells through transporters. This pathological process is believed to contribute to brain aging and neurodegenerative over many years, but can occur very rapidly during severe insults such as stroke and traumatic brain injury (Nakamura & Lipton, 2010; Weber, 2004). Excitotoxicity generally causes an excessive elevation of intracellular Ca^{2+} levels (Mehta *et al.*, 2013; Chen *et al.*, 2000), which causes changes in the normal functioning of neurons and can over-activate several Ca^{2+}-dependent enzymes leading to changes in normal cellular processes (Weber,

2004; Nicholls, 2004; Wojda *et al.*, 2008). As a consequence of activation of calcium-dependent enzymes such as xanthine oxidase (Atlante *et al.*, 2000), phospholipase A_2 (Weber, 2004; Wood, 2003) and nitric oxide synthase, free radicals including ROS and RNS are produced in cells (Mehta *et al*., 2013). Excitotoxicity is also responsible for oxidative dysfunction in mitochondria (Rego & Oliveira, 2003), which could lead to further ROS generation. Therefore, when cell cultures were exposed to glutamate, receptors were over-activated, which likely led to oxidative and nitrosative stress, and ultimately damaged and killed cells. However, elevated levels of Ca^{2+} can also activate other enzymes, such as endonucleases and proteases, which can degrade DNA and proteins respectively (Weber, 2004). Figure 4.12 represents mechanisms of neuronal damage and death caused by elevated calcium mediated cell death. Therefore, cell death may have occurred through mechanisms other than oxidative and nitrosative stress, and the protective effect of our berry extracts may not have been due to antioxidant and and/or anti-nitrosative properties, but rather to other mechanisms. However, Ahn *et al.* (2011) found that 100 μM glutamate causes hippocampal cell death by altered calcium signaling and nitrosative stress. Also, in our culture system, neurons generally only constitute ~12% of the cells, with the remaining cells mostly made up by astrocytes. Approximately ~20% total cell death was observed after glutamate exposure, which suggests that much of the cell death is represented by glial cell death. It is possible that glial cells died due to excessive glutamate uptake and cell swelling, however Chen *et al.* (2000) found that astrocytes exposed to glutamate primarily died due to oxidative stress. This finding suggests that berry extracts would likely protect glia through antioxidant properties. These qualitative finding of increased dark punctae and altered morphology in glutamate-

treated cells is also consistent with astrocytes exposed to glutamate toxicity (Chen *at al*., 2000).

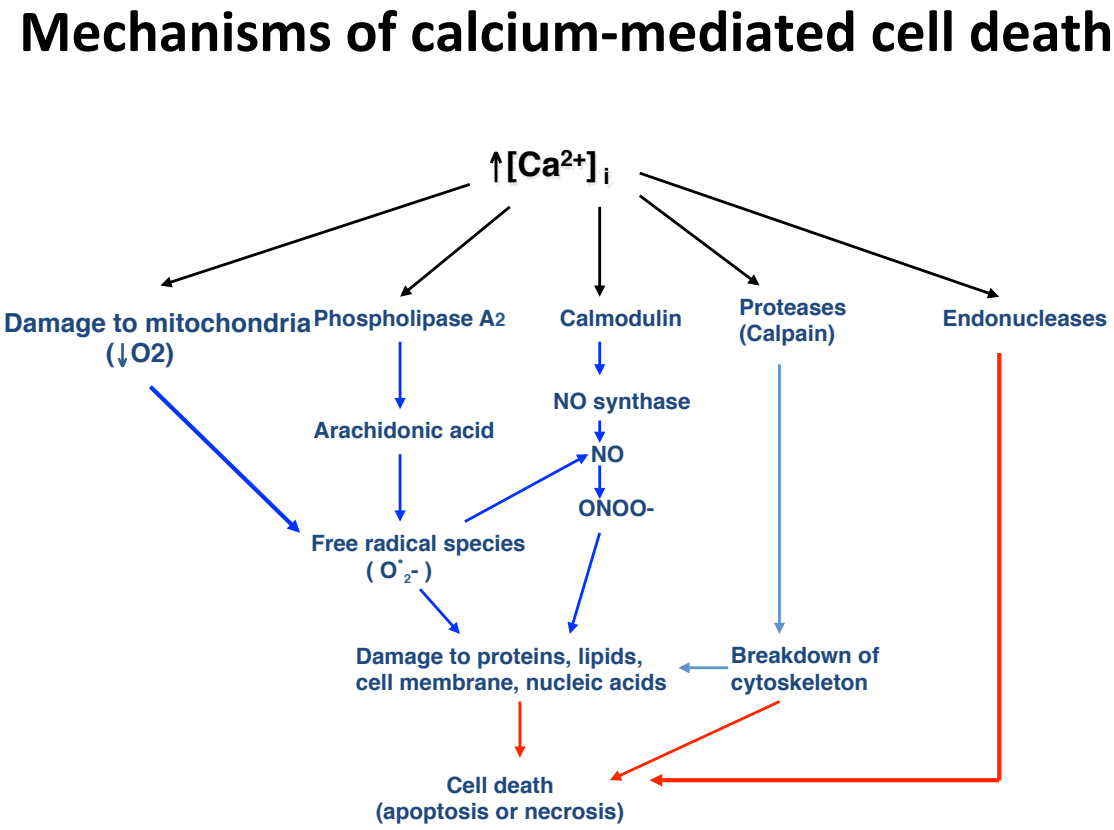

Figure 4.12. A flowchart summarising mechanisms of neuronal damage and death caused by elevated calcium mediated cell death (modified from Weber 2004). NO, nitric oxide; $ONOO^-$, peroxynitrite; O2-•, superoxide.

Many polyphenolic compounds are potent free radical scavengers, and a plethora of literature is available on the antioxidant capacities of phenolic compounds (Naczk & Shaihidi,

2006; Shaidi & Naczk, 2004; Hirano *et al*., 2001; Kahkonen & Heinonen, 2003) and their protective effects (Sun *et al*., 2008; Kong *et al.*, 2003; Rossi *et al*., 2003). Other groups have found similar findings compared to ours using nervous system cell culture models in order to assess potential neuroprotective effects of various polyphenolic compounds. For example, Ahn *et al*. found that a proanthocyanidin extract from grapes could inhibit hippocampal cell death by decreasing nitrosative stress (Ahn *et al.,* 2011). Similar results have been found with other antioxidant compounds, as carotenoids from *Pittosporum tobira* have been shown to protect rat cortical cells from exposure to 100 μM glutamate for 24 hours (Moon & Park, 2010) the same treatment protocol used in the current study. An extract from the fruit of *Alpinia oxyphylla* was shown to protect cortical neurons from exposure to 30 μM glutamate, and had an effect on condensed nuclei, similar to our findings (Yu *et al.*, 2003). However, in a model of glutamate toxicity in hippocampal cultures, a seed extract of *Cassia obtusifolia* provided no protection to both neurons and glia (Drever *et al.*, 2008). It is interesting that only the extract from lingonberry fruit did not show any protection from toxicity in our model. This may be due to the exact chemical profile of the fruit versus the other extracts. Although the overall content of polyphenolics in lingonberry fruit appeared higher than that of blueberry fruit, it is possible that specific compounds in the extracts are responsible for the neuroprotection. For example, Bhuiyan *et al.,* (2011) found that exposure to 50 μM glutamate killed 40% of cortical neurons, but addition of the specific polyphenol, cyanidin-3-glucoside offered no protection.

Previous studies have shown that dietary polyphenols can cross the blood-brain-barrier (Vauzour, 2012), and anthocyanins specifically have been detected in brain tissue after oral

administration to rodents (Andres-Lacueva *et al*., 2005; Talavéra, 2005; El Mohsen *et al.*, 2006). Estimates of specific anthocyanins in brain tissue are generally in the sub-nanomolar range (~0.2-0.25 nmol/g tissue) (Talavéra, 2005; El Mohsen *et al.*, 2006). It is difficult to make direct comparisons to such studies with the current work, the whole extract was added and not specific polyphenols. The final concentration of the extracts we added was 0.833 µg/ml of fruit extract and 0.083 µg/ml of leaf extract. In the previously conducted chemical analysis of commercially available lingonberry extracts (unpublished data), it has been found that these lingonberry extracts contain an estimated 63.7 mg of cyanidin-3-galactoside per 100 mg of fresh extract weight. Assuming that the fresh lingonberry extracts used in the present study contain a similar amount of this compound, this would translate to the cultured cells being exposed to approximately a 10 nM concentration of fruit extract and 1 nM in leaf extract. Talavera *et al.*, detected a level of another cyanidin compound (cyanidin-3-glucoside) of 0.25 nmol equivalent per gram of tissue. Therefore, the amount of extract that was added to the cultures is likely slightly higher than what might be achieved in the brain after oral administration. In addition, the polyphenolic compounds contained in the extracts may not be the predominate forms that would actually enter the brain, as a recent study found that although anthocyanins have a fairly high bioavailability, they also undergo significant metabolism, producing diverse metabolites (Czank *et al.,* 2013). Nonetheless, this system could be used to screen specific polyphenols at various concentrations for potential neuroprotective potential, and also to study the mechanisms of action of protection.

4.5. Conclusion

Overall, the findings suggest that consumption of lingonberries could have a positive effect on human health. For example, the high polyphenolic content and antioxidant capacity of lingonberry leaves would be potentially beneficial for the neuro-protection and brain ageing. It is possible that the consumption of lingonberries, or a tea made from the leaves of this plant, as well as supplements produced from the extracts of leaves, could slow brain ageing or inhibit the development of neurodegenerative disorders. Therefore, ingestion of lingonberry leaves or supplements produced from them could possibly increase the antioxidant and anti-nitrosative capacity of the brain. Therefore, future studies are aimed at analyzing the threshold of dietary consumption of lingonberry products and potential neuroprotection.

4.6. References

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Chapter 5

Conclusion

The results from the present study indicate that lingonberry (*Vaccinium vitis-idaea L.*) plants contain compounds like metabolites and enzymes of the ascorbate-glutathione cycle in leaves and phenolics in fruits and leaves that contribute to the wide array of antioxidant activities. The levels of these enzymes and metabolites are markedly affected by the propagation method. *In vitro* propagated plants, especially those obtained from leaf cultures are a good choice to enhance antioxidant enzyme levels in leaves and phenolic compounds in fruits along with more advanced plant morphology which includes higher plant height, increased number of rhizomes, branches and leaves. On the other hand, the *ex vitro* obtained plants are better option in obtaining bigger in size and higher number of berries per plant. In general, *in vitro* propagation methods are of great benefit in enhancing the levels of antioxidant compounds that could be used by nutraceutical companies.

Extracts of lingonberries were effective in inhibiting the levels of lipid oxidation in pork model systems. The protective effect of berry extracts from *in vitro* derived plants was more profound as compared to those obtained by *ex vitro* propagation.

The levels of antioxidant compounds differed in plants from different origin (with different genotypes). The two subspecies of lingonberry *V. vitis-idaea* L. ssp. *vitis-idaea* (European origin) and *V. vitis-idaea* L. ssp. *minus* (North American origin) differed greatly

in levels of antioxidants and phenolic content. These levels were observed to be much higher in the North American lingonberry *V. vitis-idaea* L. ssp. *minus* as compared to the European lingonberry*V. vitis-idaea* L. ssp. *vitis-idaea.* Harsher climatic conditions at high latitudes corresponded to higher antioxidant levels. Newfoundland is a subject to high climatic fluctuations caused as a result of its proximity to the ocean. This can explain higher levels of antioxidants in lingonberry plants grown there. The levels of antioxidant compounds correlated positively with latitude, altitude, reduced temperature and increased precipitation. Although all the studied clones were maintained in the greenhouse environment under similar conditions for about 10 years, some variations in their phenolic compounds were observed. The variations in the levels of the phenolic compounds and antioxidant activities of plants from different regions could be the result of the adaptation to the climatic conditions where they initially grew and developed and hence influenced their genotypes.

Leaf extracts of wild lingonberry clones from Newfoundland showed a significant neuroprotective effect against oxidative stress in brain cell cultures of one day old rat pups while fruit extracts showed no protective effect. The levels of antioxidant activity and phenolic content in leaves were much higher than in fruits and this correlates with neuroprotective activity.

In summary, this study has three major aspects: the effect of propagation of lingonberry plants on the level of antioxidants; the effect of geographical location on antioxidant accumulation; neuroprotective effects of lingonberry leaf and fruit extracts.

The study reveals major differences in antioxidant metabolism of lingonberry plants obtained by different methods of propagation and having different geographical locations and suggests that lingonberry leaf extracts possess significant neuroprotective activity.