USE OF NANOTECHNOLOGY TO IMPROVE PLANT PERFORMANCE IN BOREAL FOREST ECOSYSTEM

by

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Abstract

Nano-priming has been shown to significantly improve the total germination percentage and seedling vigor of different plant seeds including agricultural crops. In these applications, seeds primed with Carbon nanotubes (CNTs) exhibited dramatic improvements in germination rate and seedling vigor (root and stem lengths). Herein, we applied this technique to non-agriculture crop species in an attempt to resolve several different seeds dormancies hindering their propagation and field establishment. Specifically, the seeds of boreal forest plant species with embryo and seed coat dormancy were nano-primed with several carbon-based nanoparticles, as part of a strategy to overcome seed dormancy. Carboxylic acid functionalized multi-walled carbon nanoparticle (-COOH biomolecule coated) was the most effective in breaking physical (seed coat) and morphological dormancy (embryo), as well as increase the germination rate in combination with stratification in green alder (*Alnus viridis L.*), bog birch (*Betula pumila*), and labrador tea (*Rhododendron groenlandicum*). Conversely, a combination of carbon nanoparticles (CNPs), especially the multiwall carbon nanoparticles functionalized with carboxylic acid (MWCNT-COOH), cold stratification, mechanical scarification and hormonal priming (gibberellic acid) was effective in overcoming embryo and hard seed coat dormancy present in buffalo berry seeds (*Shepherdia canadensis L.*). A concomitant increase in the seedling vigor index and the number of normal seedlings was observed in the nano-primed germinated seedlings, indicating its superior ability to be established across a range of environmental sites. The improvement in germination rate and resolution of both embryo and seed coat dormancy appears to be associated with the remodeling of

several membrane lipids as indicated by the segregation of these molecular species in the same quadrant of the biplot as germination rate (GR) and seedling vigor index (SVI), following redundancy analysis. Phosphatidylcholine (PC) (18:1/18:3), phosphatidylglycerol (PG) (16:1/18:3), phosphatidylethanolamine (PE) (18:3/18:2), and digalactosyldiacylglycerol (DGDG) (18:3/18:3) lipids classes were observed to be highly correlated with increased seed germination percentages and the enhanced seedling vigor observed in this study for the evaluated species. Mechanistically, it appears that carbon nano-primed seeds following stratification is effective in mediating seed dormancy by remodeling the seed membrane lipids {Phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA) and digalactosylglycerol (DG)} in both peatland and upland boreal forest species. These findings suggest that nanopriming (20 μgmL^{-1} or 40 μgmL^{-1}) may be a useful approach to resolve seed dormancy issues and improve seed germination in non-resource boreal forest species ideally suited for forest reclamation following resource mining.

Keywords: Carbon Nanoparticles (CNPs), Germination Rate, Membrane Lipid, Seed Dormancy, Seedling Vigor.

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Md. Hosen Ali

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List of Abbreviations and Symbols

ABA	Abscisic acid
ANOVA	Analysis of variance
AOSA	Association of Official Seed Analyst
ANS	Abnormal seedlings
ATS	Acetyl hydrolase
BHT	Butylated hydroxytoluene
C30RP	C30 Reverse Phase Chromatography Column
CL	Cardiolipin
CNP	Carbon nanoparticles
CNTs	Carbon Nanotubes
COOH	Carboxylic acid
DG	Diacylglycerol
DGD	Dialkylglycine decarboxylase
DGDG	Digalactosyldiacylglycerol
DI	Deionized water
EC	Electrical conductivity
EPT	Ethanolamine phosphotransferase
ESI	Electrospray ionization
ER	Endoplasmic Reticulum
GA	Gibberellic acid
GR	Germination rate
HESI	Heated Electrospray Ionization
ISTA	The International Seed Testing Association
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LED	Light Emitting Diode
LPA	Lysophosphatidic Acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethnolamine
LSD	Least significant difference
MGDG	Monogalactosyldiacylglycerol
MS	Mass spectrometry
MWCNT	Multi wall carbon nanotubes
NAIT	The Northern Alberta Institute of Technology
NS	Normal seedlings
PA	Phosphatidic Acid

PAP	Phosphatidic acid phosphatase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidyl glycerol
PGP	Glycerol-3-phosphate Phosphatase
PI	Phosphatidylinositol
PIP	Phosphatidyl inositol 3,4,5-triphosphate
PS	Phosphatidylserine
PSD	Phosphatidylserine decarboxylase
RDA	Redundancy analysis
SNP	Silver nanoparticles
SQDG	Sulfoquinovosyldiacylglycerol
SVI	Seedling vigor index
SWCNT	Single wall carbon nanotubes
UHPLC	Ultra High Performance Liquid Chromatography

Chapter 1: General Introduction

The realization of the potential use of nanotechnology in many sectors, including agriculture, have increased in the last couple of years (Josef and Katarina, 2017). The use of nanoparticles in enhancing plant growth, seed germination, and to modulate how plants interact with their environment at both the cellular and molecular level has been demonstrated in several recent studies (Kottegoda et al., 2011; Zheng et al., 2005). For example, carbon nanoparticles (CNPs) was shown to increase germination rates in tomatoes and onions (Haghighi & Silva, 2014).

Buffalo berry and green alder from upland ecosystem, bog birch and labrador tea from peatland ecosystem, are four common boreal forest species in Canada which are early succession species that would facilitate revegetation of boreal forest. Many scientists suggested that a large portion of the boreal forest sites are disturbed following resource harvesting, and need to be revegetated using native species including these four species (Karin, 2017). For example, disturbances during resource harvesting include linear features (the vast network of seismic lines, pipelines, access roads, utility corridors, and multipurpose trails), especially in large boreal forest mining areas (Shanti et al., 2013). These issues have gained national attention, and restoration of areas disturbed by those activities have become integral to the national forest management strategy in Canada, as well as in other provinces. For instance, in Alberta, the 2010 and 2015 Reclamation standards categorically made forest restoration an obligation for oil and gas companies (Alberta Environment, 1995), though these standards only apply to conventional oil and gas operations. It is mandated that all oil and gas companies must return the forests to their original state or, on a trajectory to get back to their natural state after operations ceased in the mined areas. This provision obligates oil and gas

companies to seek ways to ensure effective restorations, revegetation, or reclamation of the disturbed boreal forest sites using native species following cessation of mining operations (Alberta Environment, 1995). However, a major hurdle to reclamation activities is the inability for traditional propagation techniques to help generate enough seedlings from native non-resource boreal forest species. One of the primary reasons is that many of the native boreal forest species have seed dormancy issues preventing or limiting germination (Bentsink and Koornneef, 2008). As such, there is a need to find effective ways to resolve seed dormancies in these species as part of a long-term strategy to increase their use and success in boreal forest reclamation. Hence, four forest species common in either peatland or upland boreal forest ecosystems were chosen for this study because they exhibit different types of dormancy (seed coat dormancy, embryo dormancy, and physiological dormancy), resulting in poor germination rate, field emergence, and establishment. Measures resulting in successful resolution of the respective seed dormancies of these species would facilitate reclamation efforts and impact broader regeneration in boreal forests (Kobayashi et al., 2010; Bentsink & Koornneef, 2008).

It has been shown that nanotechnologies hold significant promises for improving seed germination in agricultural crops, specifically in increasing germination rate and seedling vigor (Cheng et al., 2016). Nano-priming is a technique whereby seeds are hydrated in CNP solutions to improve seed germination (percent or rate), establish uniform seedlings, as well as resolve or overcome seed dormancy challenges (Farahani et al., 2012; Mahakham et al., 2017). This technique may also improve the vigor of seedlings following germination. For example, Mondal et al. (2011) demonstrated that

carbon nanotubes (CNTs) accelerated seed germination, growth rates as well as seedling vigor in tomato.

Membrane lipids are important in plant germination and growth, and they facilitate different bio-chemical processes during plant germination (Monticelli et al., 2009). Notably, the cell membrane forms the most active part in the germination process as cell membrane is responsible for interaction between the embryo and endosperm (Yan et al., 2014). Hence, successful germination of plant seeds depends on how the seed cell membrane is remodeled, and how membrane lipid metabolism is modulated during the germination process (Yu et al., 2015). However, little is known about how membrane lipid metabolism and membrane remodeling following seed nano-priming can affect germination in plants exhibiting different types of seed dormancy. In this study, the use of nanotechnologies were applied to seeds of non-resource species that commonly occur in the boreal forests of Canada including: buffalo berry (*Shepherdia canadensis)*, green alder (*Alnus viridis)*, bog birch (*Betula pumila*), and labrador tea (*Rhododendron groenlandicum)* with the goal of improving seed germination and seedling vigor of these species.

Furthermore, the extent of nanoparticle uptake in plants and their role in plant development is poorly understood. This research, therefore, seeks to advance our understanding of seed priming with carbon nanoparticles (CNP) as a technique to overcome seed dormancy issues and improve seed germination in non-resource boreal forest species.

1.1. Hypothesis

Seed priming with Carbon Nanoparticles (CNP) can be used to overcome seed dormancy in boreal plant species by modulating membrane lipid metabolism.

1.2. Objectives

- Use select carbon nanoparticles to resolve seed dormancy, improve seed germination and seedling vigor in upland and peatland boreal forest species.
- Determine whether nanopriming induced modulation of seed cell membrane lipid metabolism played a role in resolution of seed dormancy in boreal peatland and upland plant species.

1.3. References

Alberta Environment. (1995). *Reclamation criteria for wellsites and associated facilities - 1995 update*. https://doi.org/ENV-069-OP

Alberta Environment and Sustainable Resource Development. (2013). *2010 Reclamation Criteria for Wellsites and Associated Facilities for Forested Lands (Updated July 2013)*.

Bentsink, L., & Koornneef, M. (2008). Seed Dormancy and Germination. *The Arabidopsis Book*, *6*, e0119. https://doi.org/10.1199/tab.0119

Cheng, H. N., Klasson, K. T., Asakura, T., & Wu, Q. (2016). Nanotechnology in agriculture. *ACS Symposium Series*, *1224*(7), 233–242. https://doi.org/10.1021/bk-2016-1224.ch012

Farahani, H. A., Maroufi, K., & Sani, B. (2012). Seedling Production in Nigella (Nigella sativa L .) under Nanopriming Method, (May).

Haghighi, M., & Silva, J. A. T. (2014). The effect of carbon nanotubes on the seed germination and seedling growth of four vegetable species. *Journal of Crop Science and Biotechnology*, *17*(4), 201–208.

Josef J. and Katarian K. (2017). Application of nanotechnology in agriculture and food industry, its prospects and risks. *Ecol Chem Eng S., 22(3):321-361*.

Karin N. (2017). Vegetation Characterization and Effects Assessment of the Proposed Berens River to Poplar River First Nation All-Season Road. *Interim Report, Manitoba East Side Road Authority, Manitoba, Canada*. https://www.ceaa.gc.ca/050/documents/p80094.pdf

Kobayashi, Y., Nabeta, K., & Matsuura, H. (2010). Chemical Inhibitors of Viviparous Germination in the Fruit of Watermelon. *Plant and Cell Physiology*, *51*(9), 1594–1598. http://dx.doi.org/10.1093/pcp/pcq103

Kottegoda, N., Munaweera, I., Madusanka, N., & Karunaratne, V. (2011). A green slow-release fertilizer composition based on urea-modified hydroxyapatite nanoparticles encapsulated wood. *Current Science, 101*(1), 73–78.

Mahakham, W., Sarmah, A. K., Maensiri, S., & Theerakulpisut, P. (2017). Nanopriming technology for enhancing germination and starch metabolism of aged rice seeds using phytosynthesized silver nanoparticles. *Scientific Reports*, *7*(1), 1–21. https://doi.org/10.1038/s41598-017-08669-5

Mondal, A., Basu, R., Das, S., & Nandy, P. (2011). Beneficial role of carbon nanotubes on mustard plant growth: An agricultural prospect. *Journal of Nanoparticle Research*, *13*(10), 4519–4528. https://doi.org/10.1007/s11051-011-0406-z

Monticelli, L., Salonen, E., Ke, P. C., & Vattulainen, I. (2009). Effects of carbon nanoparticles on lipid membranes : a molecular simulation perspective. (22), 4433–4445. https://doi.org/10.1039/b912310e

Pourkhaloee, A., Haghighi, M., Jamal Saharkhiz, M., Jouzi, H., & Mahdi, M. (2011). Carbon Nanotubes Can Promote Seed Germination via Seed Coat Penetration. *Seed Technology* (33), 47-59.

Sekhon B. (2014). Nanotechnology in agri-food production: an overview. *Nanotechnol Sci Appl.* (Vol. 7), 31-53.

Shanti B., Ann G. and Justin S. (2013). A Community Approach for Landscape

Planning. *Integral Ecology Group Ltd.*, 49-70.

Smirnova, E. A., Gusev, A. A., Zaitseva, O. N., Lazareva, E. M., & Onishchenko, G. E. (2011). Multi-walled Carbon Nanotubes Penetrate into Plant Cells and Affect the Growth of Onobrychis arenaria Seedlings, *3*(8), 99–106.

Srilatha B. (2011). Nanotechnology in Agriculture. *J Nanomedic Nanotechnol*, (2), 123.

Wang, E. C., & Wang, A. Z. (2014). Nanoparticles And Their Applications In Cell And Molecular Biology. *Integrative Biology : Quantitative Biosciences from Nano to Macro*, *6*(1), 9–26. https://doi.org/10.1039/c3ib40165k

Yan, D., Duermeyer, L., Leoveanu, C., & Nambara, E. (2014). The Functions of the Endosperm During Seed Germination. *Plant and Cell Physiology*, *55*(9), 1521–1533. http://dx.doi.org/10.1093/pcp/pcu089

Yanga, J., Cao, W., & Rui, Y. (2017). Interactions between nanoparticles and plants: Phytotoxicity and defense mechanisms. *Journal of Plant Interactions*, *12*(1), 158–169. https://doi.org/10.1080/17429145.2017.1310944

Yu, X., Li, A., & Li, W. (2015). How membranes organize during seed germination: three patterns of dynamic lipid remodelling define chilling resistance and affect plastid biogenesis. *Plant Cell Environ.*, *38*(7), 1391–1403.

Zheng, L., Hong, F., Lu, S., & Liu, C. (2005). Effect of Nano-TiO₂ on Strength of Naturally Aged Seeds and Growth of Spinach. *Biological Trace Element Research*, *104*(1), 083–092.

Chapter 2: Effects of Carbon Nanoparticles on Seed Germination and Vigor in Native Boreal Species

2.1. Introduction

Nanotechnology is a modern approach, where carbon and other nanoparticles are designed to interact with molecules at the cellular level (Wang and Wang, 2014). In agriculture, this approach creates a biocompatibility within a living cell, which then generates a positive biological impact (seedling growth) in plants (Sekhon, 2014). For example, Liu et al., (2009) demonstrated that carbon nanoparticles increase germination rate and seedling growth of soybeans *(Glycine max)*. Hence, carbon nanotubes can penetrate seeds to positively impact the germination process and plant development in general (Pourkhaloee et al., 2011).

The ability of CNP to traverse both the cell wall and seed coat have been proposed as a possible mechanism through which the increase germination rates have been achieved (Haghighi & Silva, 2014; Liu et al., 2009). Seed dormancy is a state whereby seeds are unable to germinate in conditions suitable for germination. Seeds have three main types of dormancy: a) hard seed coats where the seeds are impermeable to water and gases (physical dormancy). As such, water uptake and oxygen exchange are restricted. These types of dormancy can only be overcome by creating fissures in the seed coat via mechanical or chemical means. b) The second is embryo or internal dormancy. Seeds undergoing embryo dormancy have cells which has stop growing. This is usually caused by endogenous hormones such as (cytokinin, indole-3-acetic acid, abscisic acid etc.) (Scott and Neil 2012). Seeds with small and undeveloped embryos do not germinate; c) The 3rd main seed dormancy is caused by germination inhibitors such as

cis-ABA, β-D-glucopyranosyl ester, benzoic acid, salicylic acid, chlorogenic acid, coumarin, etc., which inhibit seed germination and plant growth (Kobayashi et al., 2010).

Seedling establishment and adaptation to diverse environmental conditions depends on seedling vigor (Finch-Savage & Bassel, 2016). Following germination, the seedlings need to be adaptive to its natural environment, and sometimes this environment is not favorable for crop growth. Several factors are known to affect seed germination and plant establishment (FAO, 2011). Many different priming techniques have been applied in agriculture crops to alleviate seed dormancy and improve germination (Bentsink & Koornneef, 2008). Priming is a seed hydration technique, whereby seeds are soaked in either solutions or incubated in the presence of a moist solid matrix to enhance the rate and uniformity of germination. Apart from enhancing germination rates, priming is shown to enhance seedling vigor, allowing the seedlings to have an improved capacity to grow and be established across a range of sites with varying environmental conditions. Several priming techniques have been reported in the scientific literature and include hydrating seeds in the following: salt solution (halopriming), solutions of beneficial microbes (biopriming), osmotic solution (osmopriming), solutions of plant hormones (hormonal priming), presence of a magnetic field (magneto-priming), solutions mixed with a solid carrier (matriconditioning) or in solutions of natural compounds nano priming (hydrating seeds in solutions of nanoparticles) (Bhardwaj et. al., 2012; Mahakham, et al., (2017). Halopriming with inorganic salts such as {sodium chloride (NaCl), calcium sulfate (CaSO4), or calcium chloride (CaCl2,)} have been shown to induce salinity tolerance and enhance seed vigor in wheat (Afzal et. al., 2008; Abbasdokht et. al., 2010) and

pulse crops (Jisha and Puthur 2014). Osmopriming with osmotic solutions {polyethylene glycol (PEG), NaCl, or potassium dihydrogen phosphate (KH2PO4)} was reported to induce drought tolerance in wheat (Abbasdokht et. al., 2010) and legume crops (Jisha and Puthur 2014). Of these techniques, nanopriming is the newest and most promising approach that could be applied not only to agricultural crops but also to boreal forest species with seed dormancy issues.

Nano-priming has also been shown to significantly improve germination and seedling vigor of Nigella seeds (Farahani et al., 2012). Hopbush (*Dodonaea viscosa L*.) seeds primed with MWCNTs resulted in dramatic improvements in the seed germination rate and seedling vigor (root and stem lengths). As such, nano-priming with carbon nanoparticles is recommended as a suitable approach for enhancing seed germination and seedling growth in case of revegetation of Hopbush medicinal plants in harsh conditions (Yousefi et al., 2017). Consistent with the observations of enhancing germination percentages and seedling vigor reported in agriculture species primed with different nanoparticles, the possibility exists that this approach could be suitable to resolve the low germination observed for many non-resource boreal forest species such as green alder, bog birch, labrador tea and buffalo berry.

Bog birch is a deciduous shrub of the genus *Betula,* which is native to the peatland ecosystems in boreal forests across North America. Peatland ecosystems form in wet conditions, where flooding obstructs the flow of oxygen from the atmosphere, slowing the rate of decomposition. The peatland ecosystem is the most efficient carbon sink on the planet, because peatland plants capture 2-3 gigatons CO_2 naturally released from the peat, maintaining an equilibrium (Parish et al. 2008 and Freeman et. al., 2012).

Approximately 25-30% of the forests in boreal region are covered by peatland globally (Wieder et.al., 2006). Bog birch plants grow to a maximum height of 1-4 m and has catkins-type reproductive structure. The seeds of bog birch are 1-3 mm long and have hard seed coats. Besides its hard seed coat, bog birch also has embryo dormancy, resulting in delayed and poor germination rate (Forest Service, 1961).

Similar to bog birch, labrador tea is a peatland plant species common in the boreal forest. The plant is from the genus *Rhododendron* and is popularly known as "Herbal Tea". It is a slow-growing shrub, reaching a height of 0.3 m to 0.8 m, with evergreen leaves. The seeds of labrador tea are very tiny, hard to separate, and have embryo dormancy. Both bog birch and labrador tea are native to peatland or wetland ecosystems in boreal forests, and their germination rates are low due to embryo and seed dormancy (Dampc and Luczkiewicz, 2015).

Buffalo berry on the other hand is a small shrub of the genus *Shepherdia* that produce edible berries. The plant is common in upland boreal forested areas and is described as a deciduous shrub, growing to a maximum of 1–4 m. The seeds of buffalo berry are 4–8 mm long, and seed coat is very hard. Another upland species of importance in the boreal forest landscape is Green alder (*Alnus viridis L.*), due to its nitrogen-fixing abilities in boreal forest soils (Vogel & Gower, 1998). These are large (3–12 m tall) shrubs of the genus *Alnus.* The seeds of green alder are small, 1–2 mm long, light brown, with a narrow encircling wing, and has embryo dormancy. For buffalo berry, the hard seed coat undermines germination (Rosner & Harrington, 2003), while green alder is inhibited by embryo growth – namely, embryo dormancy (Kaur et al., 2015).

Cold stratification, incubation with gibberellic acid (hormone priming) and seed coat scarification increase the germination rate of rosemary seeds having hard seed coat, embryo, and physiological seed dormancy (Angela et al., 2009). This could be a useful approach to apply to seeds such as buffalo berry, which also has multiple seed dormancies. There are many studies in the scientific literature demonstrating the beneficial effects of nanotechnology in agricultural species to address issues with seed dormancy and low germination (Samples, 2008). However, to the best of our knowledge, very few studies have been done to assess the effects of nanopriming, particularly in combination with stratification and hormone priming in resolving seed dormancy in native non resource boreal forest species, such as buffalo berry, green alder, bog birch and labrador tea.

Due to the dormancies common in seeds from these species they have low germination, and it is difficult to produce a large number of seedlings or mass propagate these plants from seeds for use in boreal forest reclamation or for revegetation activities. There is high interest in the propagation of non-resource species for boreal forest restoration, revegetation or reclamation arising from anthropogenic disturbances. For example, oil and gas mining resulted in forest fragmentation due to the construction of cut lines, roads, electrical lines, drill sites, etc. (He et al., 2009). Natural resource extraction companies such as oil and gas companies are mandated by law to return the disturbed sites to a functional upland boreal forest ecosystem, following oil and gas mining, using native plant species (He et al., 2009). In addition, there is limited knowledge surrounding what kind of dormancies are present in some of these native species, and what suitable approach or seed treatments may be applicable to break these dormancies. However, many oil and gas corporations have been unable to produce or

obtain enough seedlings to revegetate these areas, partly due to seed dormancy issues related to low germination in many non-resource native species required to reclaim or revegetate these sites (Ann et.al., 2002).

Germination is an integral part of plant life and important in mass propagation, where its success depends on many factors, including the ability to withstand varying changes in environmental conditions. Germination refers to the emergence of the radicle from a seed (Bewley, 2002). Seed vigor is an important indicator used to assess seedling quality, emergence as well as establishment across a range of field and environmental conditions. The electrical conductivity (EC) test is widely used technique to assess seed vigor (Ramos et al., 2012) and shown to be highly associated or correlated with seedling emergence (Vieira et al., 2004). Electrolytes leaking from cell membrane of poor vigor seedlings increase EC readings. As such, high levels of membrane leakage or EC values are characteristic of poor vigor seedlings and poor field emergence (Ahmed et al., 2004).

Nano-priming was reported to be successful in overcoming embryo and seed coat dormancy and improve germination in agriculture crop species in many studies (Mahakham et al., 2017). We believe seed priming with CNP could be useful in breaking seed dormancy in these boreal forest species needed for reclamation activities. The success of nanopriming to resolve seed dormancy and low germination issues in several agricultural crop species can be applied to boreal forest species distributed in peatland and upland ecosystems that share similar challenges. This is important given the relative lack of research and knowledge on the impact of CNTs on increasing germination in boreal forest species. This study was therefore performed to evaluate the effect of CNTs on two peatland (bog birch and labrador tea) and two upland (green

alder and buffalo berry) boreal forest species with seed coat, physiological and embryo dormancy, to determine the effects of CNTs on seed germination, seedling growth, and vigor under laboratory conditions. The outputs of this research could be applied in forest regeneration efforts by incorporating CNTs into propagation techniques.

2.2. Materials and Methods

This research was conducted at the Boreal Ecosystem Research Facility located on Grenfell Campus, Memorial University - Corner Brook, Newfoundland. The experiments were carried out from September 2016 to June 2018. Seeds from two peatland {bog birch (*Betula pumila* L.) and labrador tea (*Rhododendron groenlandicum* L.)} and two upland {buffalo berry (*Shepherdia canadensis L.*) and green alder (*Alnus viridis L.*)} boreal forest species were provided by The Northern Alberta Institute of Technology (NAIT) Boreal Research Institute. All seeds were stored at –20 ^{0}C before experimentation. The study was designed based on four CNP experimental treatments and each treatment had two concentrations (20 $\mu g ml^{-1}$ or 40 $\mu g ml^{-1}$): I) multiwall CNP (MWCNT), II) multiwall CNP functionalized with the carboxylic group (MWCNT-COOH), iii) graphene, and iv) deionized water (DI) used for control.

Seeds (25) from all species were primed overnight in glass vials containing 8 mL solutions of either 20 $\mu g ml^{-1}$ or 40 $\mu g ml^{-1}$ CNP dissolved in DI water. The carbon nanoparticles were thoroughly dissolved in the DI water following sonication for 15 minutes (Qsonica Q700, Model: CL-334, Boston Laboratory Equipment, USA). The treatments were replicated three times, with one set of seeds primed with and without nanoparticles, cold stratified, and a corresponding set of seeds excluded from cold stratification (incubated at room temperature). Seeds were germinated in condensation free Petri plates (P5481, Sigma-Aldrich) padded with wet germination grade Whatman

filter paper (CA28297-216, VWR). Seeds were incubated for three weeks at room temperature (25^0C) and illuminated with LED lights 24 hrs per day. Water (1mL) was added periodically to the filter paper during germination to keep the seeds hydrated. For the cold stratified seeds, they were primed using the same conditions above, except that seeds were incubated at a temperature of 2–4^0C for 15 days, after which the seeds were then exposed to germination conditions at room temperature (25^0C) under continuous LED lighting. The treatments are summarized as follows:

- **CNP:** Imbibition for 24 hours with select CNP before exposure at room temperature.
- **CNP+ Stratification**: Imbibition for 24 hours with select CNP and incubated in the fridge at 2-4^oC for 15 days prior to exposure at room temperature.

In the case of buffalo berry, two additional experimental treatments were conducted to evaluate the difference between mechanical and chemical scarifications, along with plant hormone treatments on germination, though control was treated with only DI water. During mechanical scarification, CNP was added along with gibberellic acid @ 1000 mg L-1. Mechanical scarification was carried out using sandpaper (Mastercraft) bought from a local hardware store by gently rubbing the surface of the seeds between the sheets of sandpaper to scratch the seed surface. The idea was to create scratches on the seed coat, so water can easily penetrate inside the seed. Each seed was rubbed by hand with sandpaper, and then 25 seeds were placed in each Petri plate and primed with and without nanoparticles and hormone. In the chemical scarification, CNP, gibberellic acid (1000 mg L-1), and 80% sulfuric acid were added. Chemical scarification was made by immersing 25 seeds of buffalo berry from each replication in a beaker with concentrated

sulphuric acid for 5 min, stirring with a glass rod. After draining the acid, the seeds were soaked in water for one hour followed by one minute-washing in running tap water, then surface dried with blotting paper (ISTA, 2006).

After scarification, the seeds were primed overnight in glass vials with each CNP (20 µgmL^{-1} and 40 µgmL^{-1}) and DI water (control). All the Petri plates were placed in a fridge at 2–4 ^{0}C for cold stratification for 120 days. A randomized approach was used to carry out the experiments with three replications per treatment. In summary, the two additional treatments for buffalo berry were as follows:

- **Mechanical scarification + stratification + CNP + gibberellic acid (GA):** First, mechanical scarification was done by sandpaper. Seeds were imbibed for 24 h with selected CNP (MWCNT-COOH, MWCNT, and Graphene) using two concentrations (20 µg/mL and 40 µg/mL) followed by GA (1000 mg L-1). Cold stratification was performed in the fridge (2–4^{O}C) for 120 days, and then seeds were exposed to room temperature for an additional 120 days.
- **Chemical scarification + stratification + CNP + GA:** Chemical scarification was done with both 80% and 90% sulphuric acid. Seeds were imbibed for 24 h with select CNP (MWCNT-COOH, MWCNT, and Graphene) followed by GA (1000 mg L-1). Cold stratification was performed in the fridge (2–4^{O}C) for 120 days, and then seeds were exposed to room temperature.

2.2.1 Tetrazolium test

A tetrazolium test was conducted to evaluate seed viability of the seed lots used in the experiments. A total of 20 seeds of each species were imbibed in DI water under two conditions, with and without each nanoparticle for six hours at 25 ^{0}C (de Paiva *et*

al. 2017). A scalpel was then used to cut open the seeds through their embryo. Half of each seed was then immersed in a tetrazolium solution (2,3,5-triphenyl tetrazolium chloride at a concentration of 0.050% (w/v)) for about six hours (de Paiva *et al.* 2017). The excess stain from the seeds was rinsed in DI water. Using a low power (0.5x) light microscope (Nikon SMZ1500, Valley Microscope, Nikon), the seed viability was assessed. Viable seed stains bright red, while non-viable or dead seeds were either unstained, stained light red, or very dark red. The evaluation led to three classification of seeds: completely stained, unstained, and partially stained (Carvalho et al., 2018).

2.2.2 Electrical conductivity test

An electrical conductivity test was conducted using an electrical conductivity (EC) meter (Field Scout EC Meter, Spectrum Technologies, Inc.) to assess membrane integrity and seed vigor. Seeds (25) for each of the three treatment replicates were soaked in CNPs (MWCNT-COOH, MWCNT, Graphene) or DI water overnight and the conductivity measured and reported as micro-Siemens per centimeter (μScm^{-1}). Higher readings were attributed to lower vigor, while low readings were indicative of superior vigor (Vieira et al., 2004). For each concentration of CNPs, there were three replications and 25 seeds per replication.

2.2.3 Evaluation of abnormal and normal seedlings

The International Seed Testing Association (ISTA) handbook on Seedling Evaluation Procedures (ISTA, 2006) was used for assessing normal and abnormal seedlings following germination. More specifically, seedlings that had stunted, stubby, missing, broken roots, or had split roots from the tip; as well as shoots that were short and thick, or split, constricted, twisted, decayed and the leaves deformed, damaged, or missing, were classified as abnormal.

2.2.4 Germination percentages

The germination rate of seeds and their vigor was measured by observing germination, shoot and root growth over the entire period of the experiment. The number of emerged seeds (visible cotyledons) and the total seeds at each point in time were counted each day to track the germination rate in each treatment. Seed germination rate in each treatment was determined by the following equation:

$$Germination\ (\%) = \frac{number\ of\ germinated\ seeds}{Number\ of\ total\ seeds}\ X\ 100$$

To analyze the emerged seedling lipids, seedlings that were three days old were chosen for observation. The analysis was done by measuring the shoot and root length of each seedling. After measurement, seedlings were stored at –80 ^{0}C for lipid analysis.

2.2.5 Seedling vigor index (SVI)

The seedling vigor index is an important parameter of seed quality and used to assess the enhancement of germination and viability of a seed lot in the field or other conditions. Three days after germination, seedling length (cm) was measured using a ruler to calculate the SVI as follows:

$$\boldsymbol{SVI = \frac{Seedling\ length\ (cm)}{100} \times \%\ germination}$$

2.2.6 Data analysis

XLSTAT Premium Version (Addinsoft, New York, USA) and SigmaPlot 13.0 (Systat Software Inc. CA, USA) were used for data analysis and figure preparation. Statistical analysis was performed by XLSTAT where analysis of variance (ANOVA) was used to determine treatment effects. All the treatments used as independent variables and percentages of normal or abnormal seedlings, electrical conductivity,

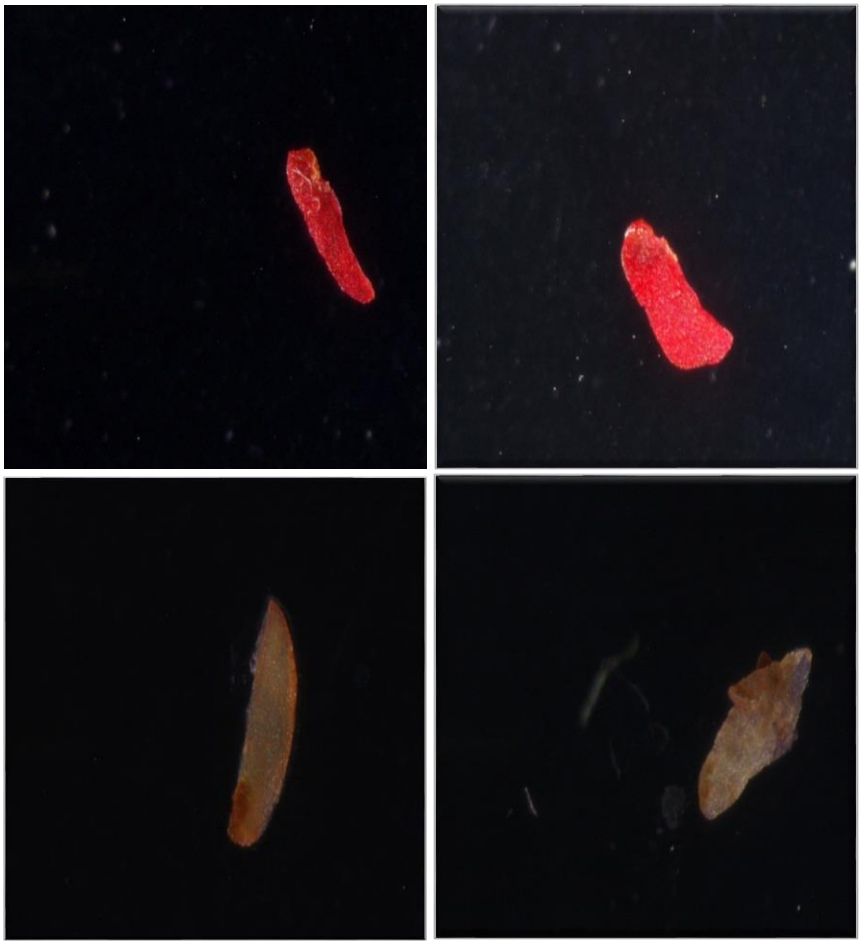
percentages of stained or partial stained seeds, percentages of germination rate, seedling vigor index used as dependent variables. Fisher's and Tukey's LSD test at $\alpha = 0.05$ was used as post hoc test for mean comparison when overall treatment effects were significant though both Fisher's and Tukey's output was same.

2.3. Results

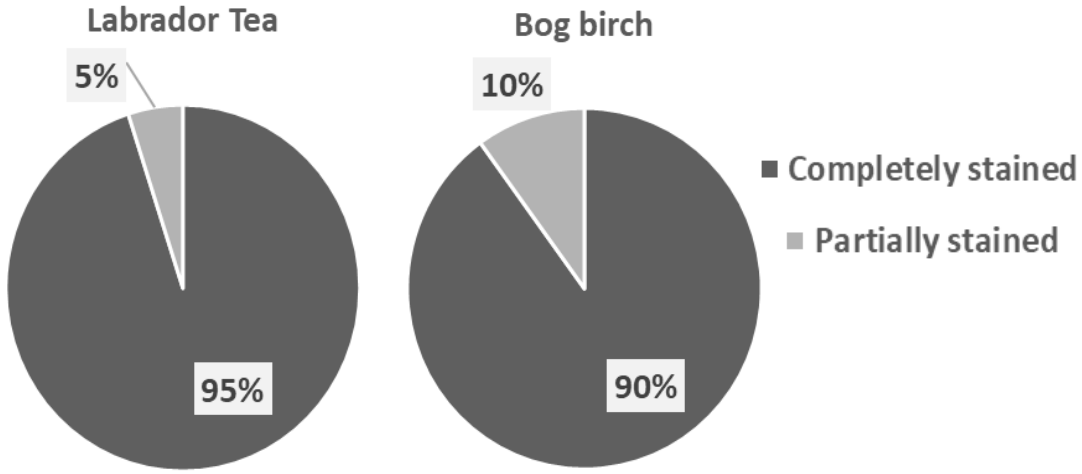
2.3.1 Tetrazolium Test

The tetrazolium test was used to evaluate seed viability. In this test, viable seeds stained bright red color, while the dead tissues stained dark red or pink or not at all {Figure 2.1(a) and 2.1(b)}. For both bog birch and labrador tea species, 90% and 95% seeds were observed as viable seeds, respectively {Figure 2.1(c)}. In contrast, 10% and 5% of seeds were partially stained for bog birch and labrador tea, respectively.

For both green alder and buffalo berry species, 90% and 95% of the seeds were observed to be viable seeds, respectively, as they stained bright red in color {Figure 2.2(a) and 2.2(b)}. In contrast, 10% and 5% of the seeds were partially stained for green alder and buffalo berry respectively {Figure 2.2(c)}.



(a) **(b)**



(c)

Figure 2.1: Tetrazolium test results showing viability of seeds of peatland species following treatments. (a) Stained and partially stained seed of labrador tea, (b) Stained and partially stained seed of bog birch, and (c) Percentages of completely stained and partially stained, n=3.

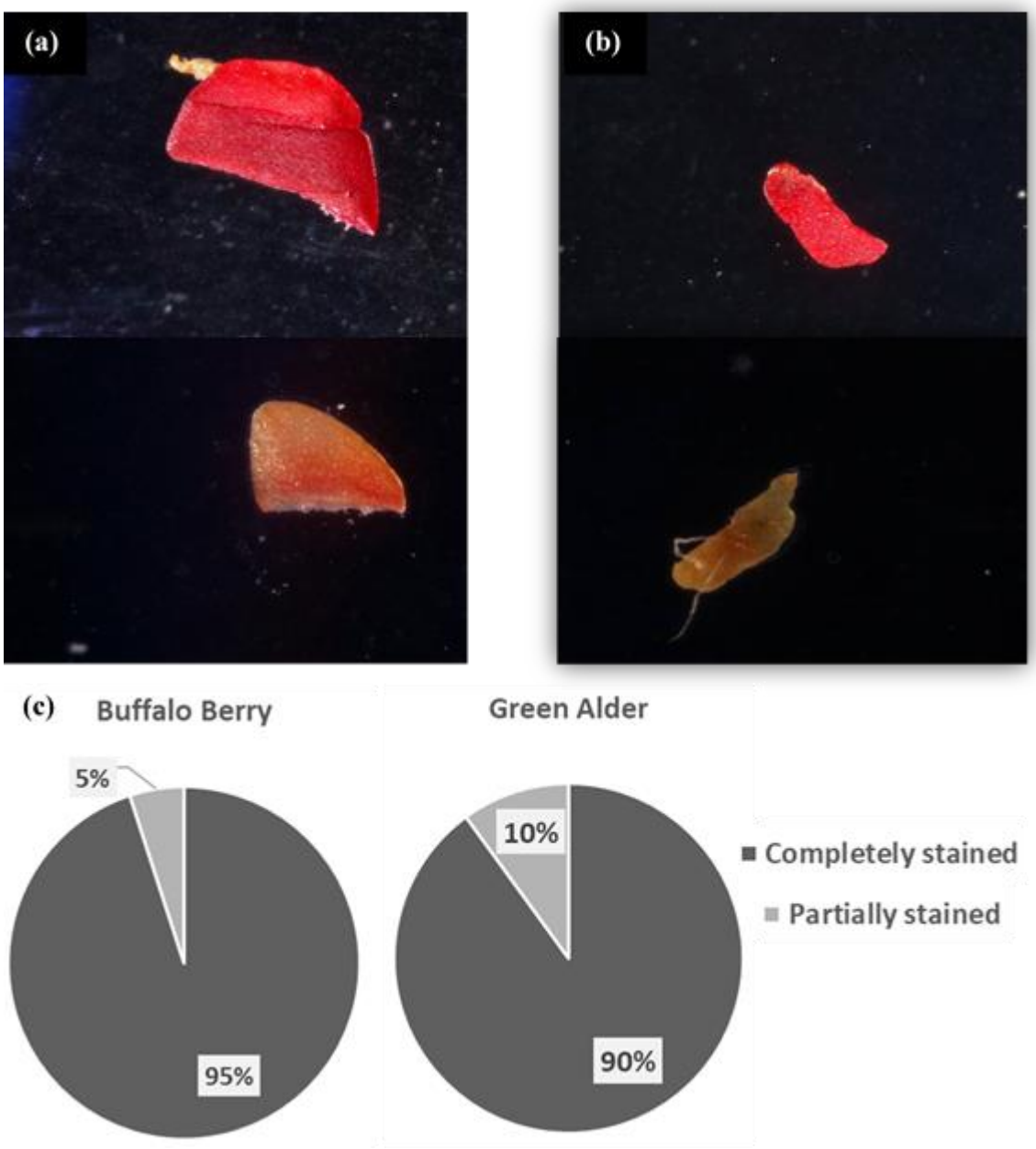


Figure 2.2: Tetrazolium test results showing viability of seeds of upland species following treatments. (a) Stained and unstained seed of buffalo berry, (b) Stained and unstained seed of green alder and (c) Percentages of completely stained and partially stained.

2.3.2 Electrical Conductivity Test

Our ANOVA results indicated that nanopriming treatments had a significant effect on electrical conductivity in all four species ($p<0.05$). Lower electrical conductivity (EC) measures indicate higher seed vigor following nanopriming. For bog birch (peatland) species, the EC was 55 µS cm^{-1} in the control, which was significantly higher than that of all the treatments {Figure 2.3(A)}. In all the treatments, the EC ranged from 20 to 35 µS cm^{-1}. This indicated seeds primed in the presence of nanoparticles had improved vigor than those hydro primed without nanoparticles. In the case of the labrador tea (peatland) species, the EC was 40 µS cm^{-1} in the control, which is significantly higher than all other treatments ($p<0.05$), whereas significantly lower EC values were recorded in all other treatments ranging from 25 to 30 µS cm^{-1} {Figure 2.3(B)}.

An EC of 140 µS cm-1 was recorded for buffalo berry (upland) species in the control, which was higher than all the nanoprimed treatments {Figure 2.4(A)}. In contrast, the EC reading was very low in all the nano primed treatments compared to the control. The electrical conductivity measurements in the primed seeds ranged from 35 to 40 µS cm-1 in all treatments, demonstrating the seeds were viable before seed germination evaluations. Similar trends were also found in the case of green alder (upland) species ($p<0.05$). In the control, the EC was observed to be 90 µS cm-1 compared to the CNP treatments, where it was lower ranging in values, between 25 to 35 µS cm-1 {Figure 2.4(B)}.

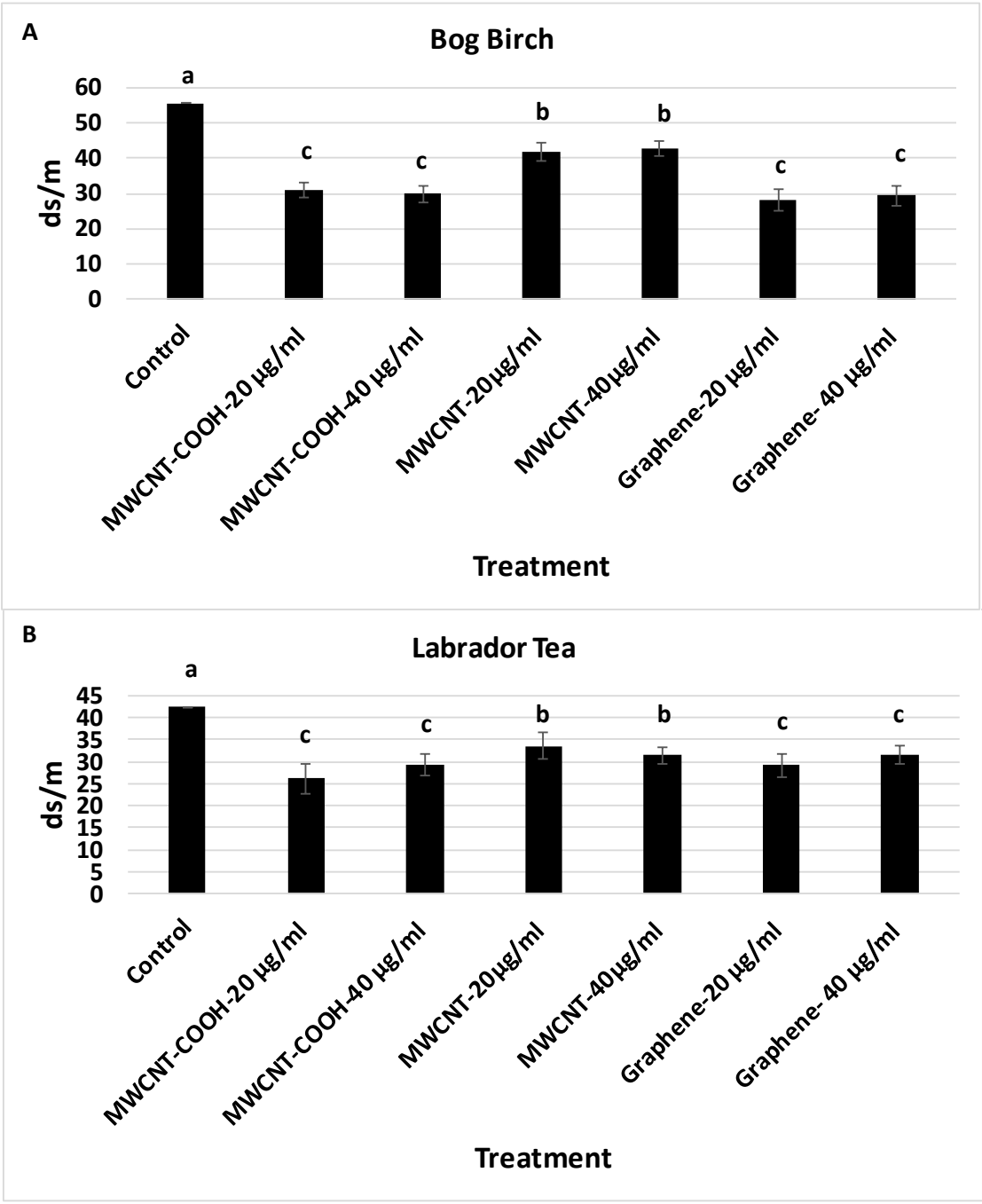


Figure 2.3: Electrical conductivity measures of peatland species A). bog birch and B). labrador tea. The error bar represents ±SE. Different letters indicate significant differences at $\alpha = 0.05$ within the control and treatments, n=3. Control = no CNPs added, MWCNT= multiwall carbon nanotubes, MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid.

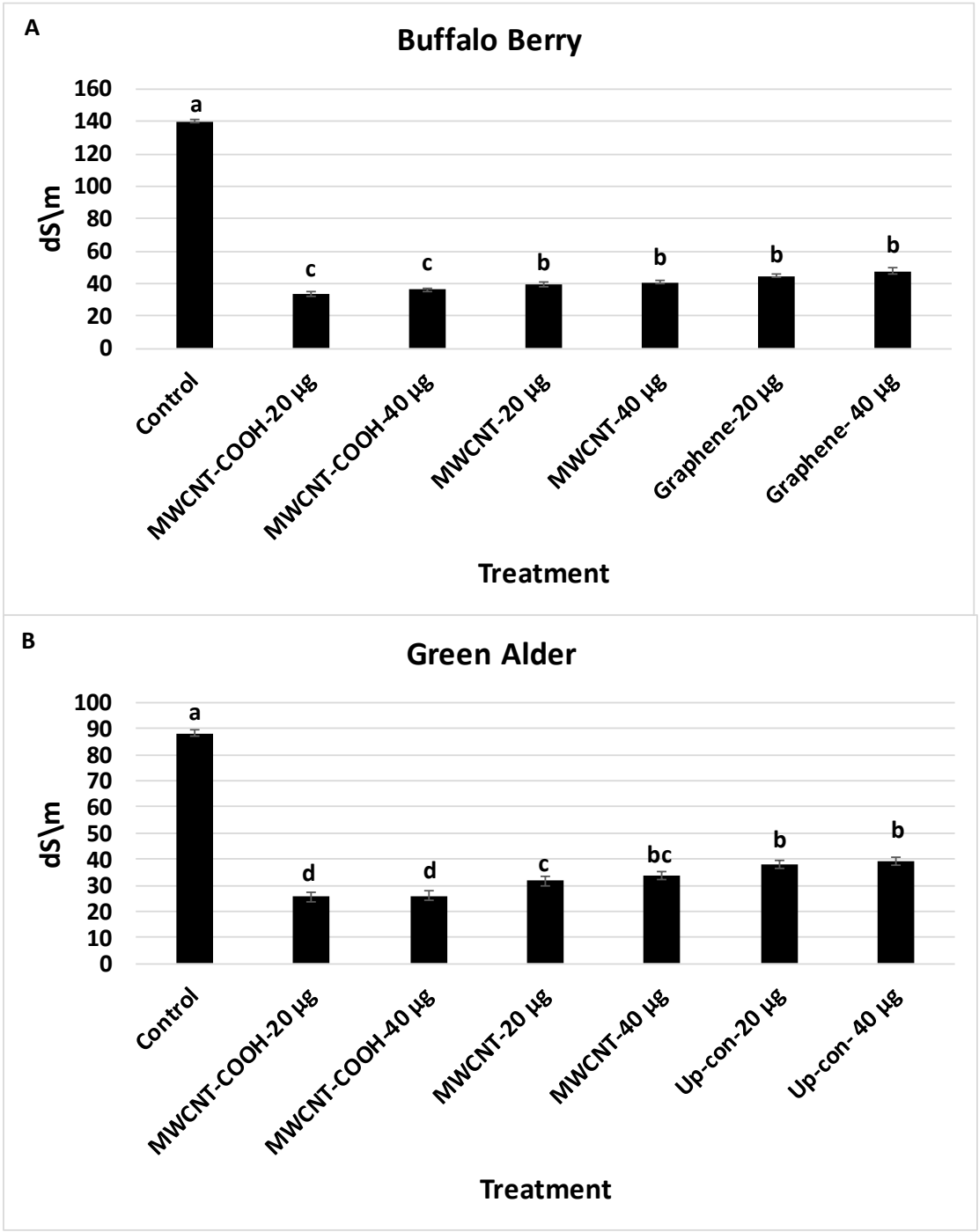


Figure 2.4: Electrical conductivity (µS\m) test of upland species. A). buffalo berry and B). green alder. Values in bar chart represent means ± standard errors and different letters indicated significant difference at $\alpha = 0.05$. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes and Up-con = up-conversion nanophosphors.

2.3.3 Evaluation of normal and abnormal seedlings

In this experiment, CNP with stratification treatment was used to evaluate the normal and abnormal seedlings. The ANOVA results indicated that CNPs treatment has significant effect on normal seedlings production in bog birch and labrador tea (in all four species, $p<0.05$). It is very clear that, due to nanopriming, the number of normal seedlings increased (65% to 75%) in the treatments compared to the control (40%). Following nanopriming with MWCNT-COOH, approximately 75% of bog birch seedlings were observed to be normal after germination compared to the controls {Figure 2.5 (A)}. It was about 70% for MWCNT (20 µg/ml and 40 µg/ml) and 65% for graphene (20 µg/ml and 40 µg/ml), while it was 40% in the control. On the other hand, there were no significant differences observed between treatments and controls for abnormal seedlings. For the labrador tea species, both concentrations of MWCNT–COOH and MWCNT showed 98% germination rate, whereas graphene showed about a 95% germination rate, though it was significantly different ($p<0.05$) between the treatments and the control (about 80%) {Figure 2.5 (B)}. Similar to bog birch, the percentage of normal seedlings in nanoprimed labrador tea increased significantly in all the treatments compared to controls.

Approximately 85% of the seedlings were normal seedlings in green alder primed with MWCNT-COOH (both 20 µg/mL and 40 µg/mL), and this was significantly higher ($p<0.05$) than the control and other nano-primed treatments (Figure 2.6). For MWCNT (20 µg/mL and 40 µg/mL) and up conversion Nano phosphorous (20 µg/mL and 40 µg/mL), the NS were 80% and 70%, respectively, while in the control it was 55%. In the case of buffalo berry, evaluation of normal and abnormal seedlings was done only in M.Sc.+St.+CNP+GA treatments. Both concentrations (20 and 40

µg/mL) of MWCNT-COOH primed seeds had a significantly higher ($p<0.05$) percent (80%) of normal seedlings compared to the control and other treatments (Figure 2.7). The normal seedlings were 65%, 60%, and 50% for MWCNT, graphene, and control, respectively. For MWCNT at 20 µg/mL concentration, it was 70% and for 40 µg/mL it was 65%. Seeds nanoprimed with Graphene had 60% NS seedlings for both concentrations. In the control, the percentages of normal and abnormal seedlings were 50%.

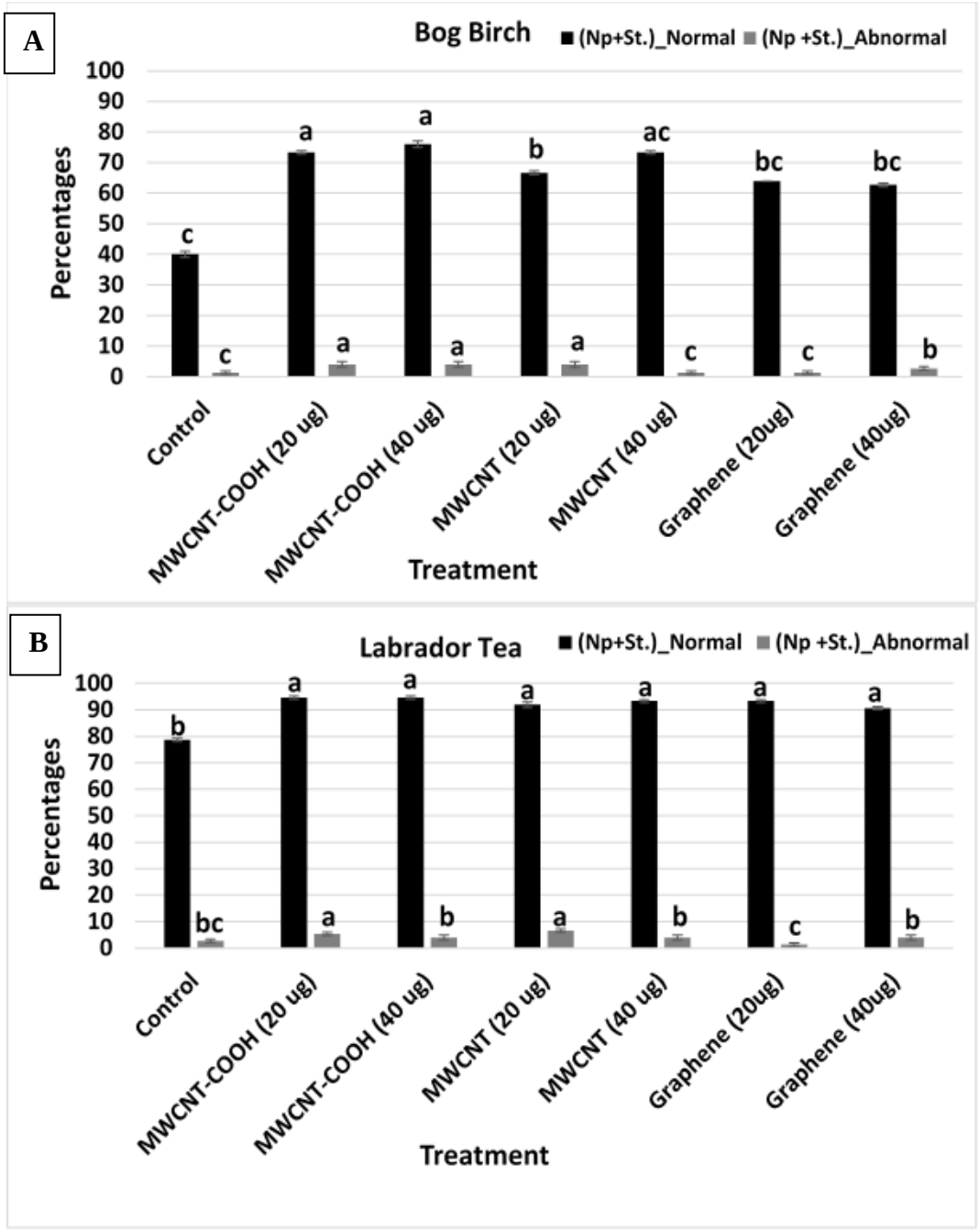


Figure 2.5: Percentages of normal and abnormal seedlings for all the treatments and the control for peatland species A). bog birch and B). labrador tea. The error bar represents ±SE. Different letters indicate significant differences at α = 0.05, Np + st = nanoparticles + stratification. MWCNT-COOH = Multiwall Carbon nanotube funtionalized with carboxylic acid, MWCNT = Multiwall Carbon nanotube

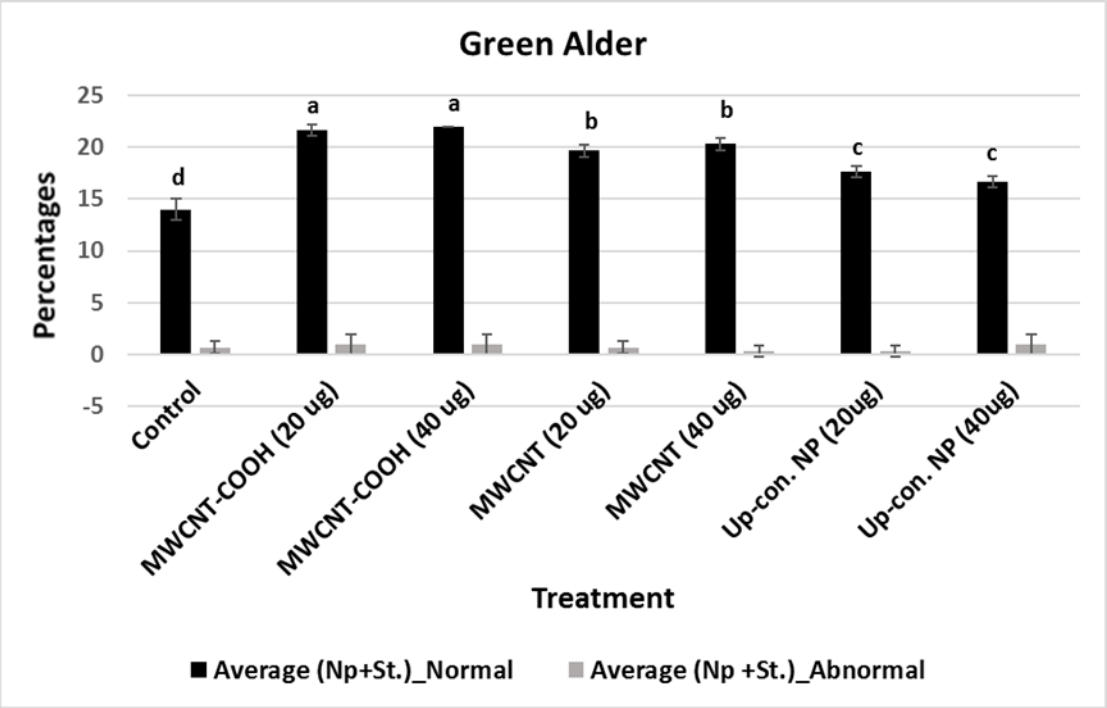


Figure 2.6: Percentages of normal and abnormal seedlings for all the treatments and control for green alder. The error bar represents ±SE. Different letters indicate significant differences at $\alpha = 0.05$, np+st=nanoparticles+stratification. MWCNT-COOH = Multiwall Carbon nanotube funtionalized with carboxylic acid, Up-con. = upconversion nanophosphors, MWCNT = Multiwall Carbon nanotube

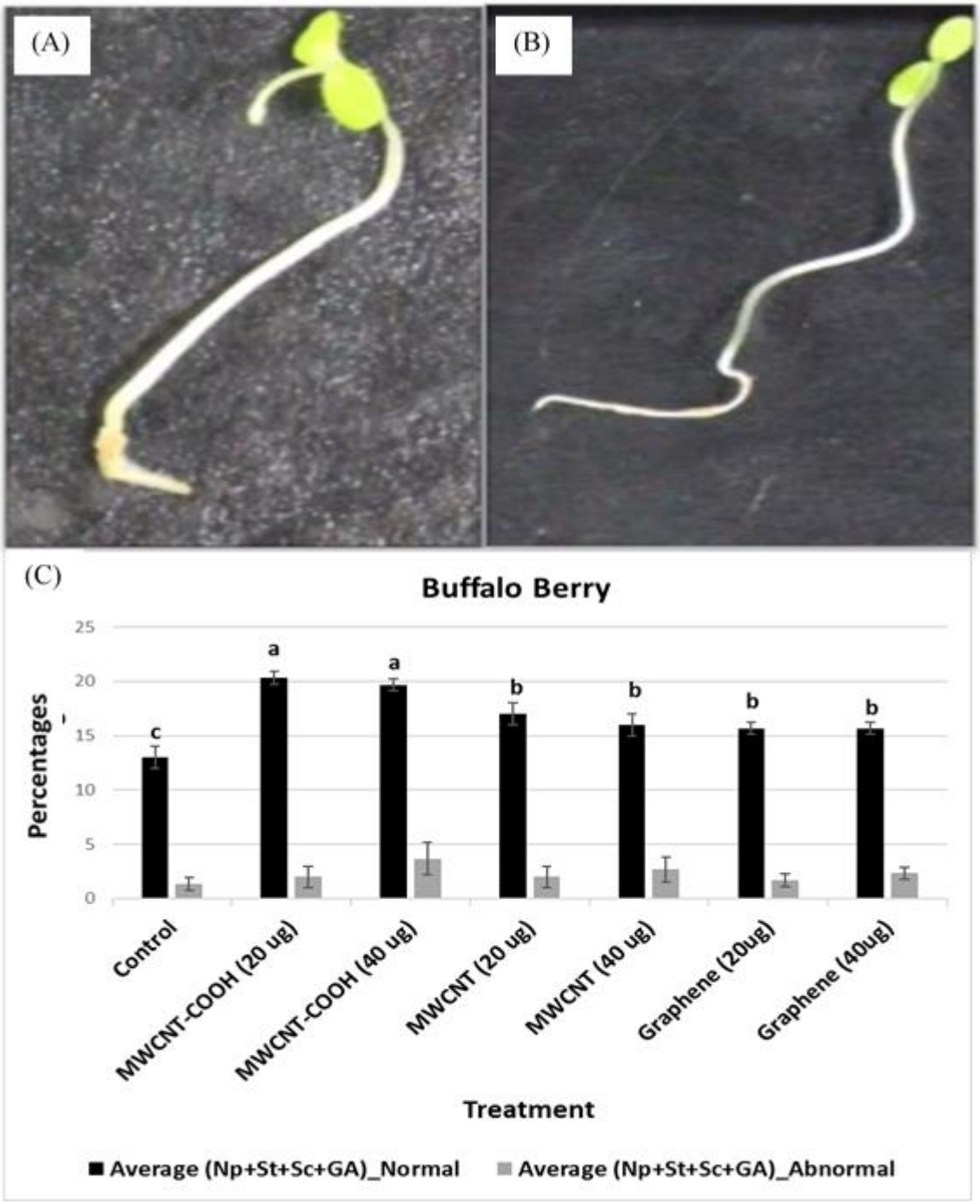


Figure 2.7: Images of normal (B) and abnormal (A) seedlings of buffalo berry. (C) Percentages of normal and abnormal seedlings for all the treatments and the control for green alder. The error bar represents ±SE. Different letters indicate significant differences $\alpha = 0.05$, np + st + sc + GA = nanoparticles + stratification + scarification + gibberellic acid. MWCNT-COOH = Multiwall Carbon nanotube funtionalized with carboxylic acid, MWCNT = Multiwall Carbon nanotube

2.3.4 Effect of carbon nanopriming on seed germination

Seeds nano-primed with MWCNT functionalized with carboxylic acids (MWCNT –COOH) at both 20 and 40 µg/mL had higher germination and germination rate compared to the control treatment, (in all four species $p<0.001$). CNP primed and stratified labrador tea seeds had very high seed germination in all treatments compared to the control. We observed for all the treatments the germination was between 95-100%, while it was about 80% in the control, which is showing significant difference between treatments and control. The highest percent germination (100%) was observed in MWCNT–COOH primed and stratified seeds at 20 µg {Figure 2.8 (A)}. The rate of germination in labrador tea was highest in both concentrations of MWCNT-COOH (20 µg/mL and 40 µg/mL) and MWCNT (20 µg/mL). Germination took place at 2 days after stratification and no additional germinant were detected at 10 days after stratification in all treatments {Figure 2.8 (B)}. Overall, the seeds in MWCNT-COOH (20 and 40 µg/mL) treatment germinated the fastest and most uniformly over the time, and the germination rate was 100%, whereas the control was only 70%. Similarly, up to a 25% increase in germination was observed in bog birch seeds primed with solutions of MWCNT –COOH. For MWCNT (20 µg/mL and 40 µg/mL) and graphene (20 µg/mL and 40 µg/mL), the germination percentages range from 10 to 12%. In the control, the germination rate was less than 10%. All treatments were significantly higher ($p<0.001$) than the control {Figure 2.9 (A)}. Among all the nanoprimed and stratified seed treatments, MWCNT–COOH (20 µg/mL and 40 µg/mL) showed the highest germination at 80%. In the case of MWCNT (20 µg/mL and 40 µg/mL), the percent of seeds germinated was about 70%, while it was about 65% for graphene (20

µg/mL and 40 µg/mL), compared to 40% in the control. For bog birch, emergence began at 3 days following stratification and no additional germinant were detected at 13 days after stratification {Figure 2.9(B)}. Among all the treatments, the rate of germination was highest in the MWCNT-COOH (40 µg/mL) treatment (80%) and the lowest was in the control (40%). Compared to the other treatments, MWCNT-COOH germinated faster and more uniformly in both peatland species.

Similar to peatland species, upland species (buffalo berry and green alder) had faster germination and uniformly in MWCNT-COOH treatment. In case of only nanopriming without stratification, germination was approximately 35% in green alder seeds with MWCNT-COOH at (40 µg/mL) {Figure 2.10(A)}. However, MWCNT-COOH at 20 µg/mL) had a slightly lower percent germination (30%), which was still higher compared to the other treatments (control, MWCNT and up conversion nano phosphorous). For MWCNT and up conversion nano phosphorous at 20 µg/ml and 40 µg/ml, the germination rate was similar between both treatments (20%). In contrast, none of the buffalo berry seeds germinated following nanopriming without seed stratification. Interestingly, when green alder seeds were primed with CNPs and cold stratified, higher germination ($p<0.001$) was observed compared to when the seeds were only nano primed but not stratified. In all cases, the nanoprimed and cold stratified seeds had a higher percent germination compared to the control. Among all the treatments, MWCNT-COOH (both concentrations) had the highest percent germination (about 90%). The germination percent was 80% in MWCNT (both concentrations), 70% in up conversion nano phosphorous (both concentrations), compared to 60% germination in the control. For green alder, first germination occurred 2 days after stratification and attained a 90% rate of germination 12 days after stratification with

both concentrations of MWCNT-COOH treatments {Figure 2.10(B)}. Conversely, buffalo berry seeds primed with CNPs and stratified showed a marginal increase in total germination compared to both the control and nano primed-only seeds. Both concentrations of MWCNT-COOH had a higher percent germination (40 %) compared to all other treatments. The seed germination ranged from 40% for MWCNT (20 µg/ml and 40 µg/ml), 35% for upconversion nanoparticles and 30% for graphene (20 µg/ml and 40 µg/ml) {Figure 2.11(A)}. In the case of Buffalo Berry, first germination occurred 4 days after stratification and attained a 90% rate of germination 12 days after stratification with both concentrations of the MWCNT-COOH treatment {Figure 2.11(B)}. Due to unsatisfactory germination observed in buffalo berry seeds following nano-priming and cold stratification, we changed the germination treatment to include both mechanical and chemical scarification, as well as primed the seeds with plant hormone solutions (GA). We observed none of the seeds germinated in the chemical scarification treatments. In contrast, seeds mechanically scarified, cold stratified, and primed in both CNP and GA solutions had significant increases compare to control in percent germination. Total germination was up to 90% in both MWCNT-COOH (20 µg mL-1 and 40 µg mL-1) treated seeds. Similarly, significantly higher germination ($p<0.001$) was observed in seeds primed with MWCNT and graphene compared to the control, and ranged between 75% and 70 %, respectively. Furthermore, the rate of germination was faster in seeds mechanically scarified, stratified and hormone-treated. Both green alder and buffalo berry seed germination started two days after mechanical stratification. In contrast, germination in both the control and treatments continued to about twelve days after stratification, where no further changes were observed in germination. Though germination started at the same time in both the control and

nanoprimed seeds, but, the number of seeds germinated were significantly higher in the CNPs treated seeds compared to the control {Figure 2.10 (B) and 2.11 (B)}.

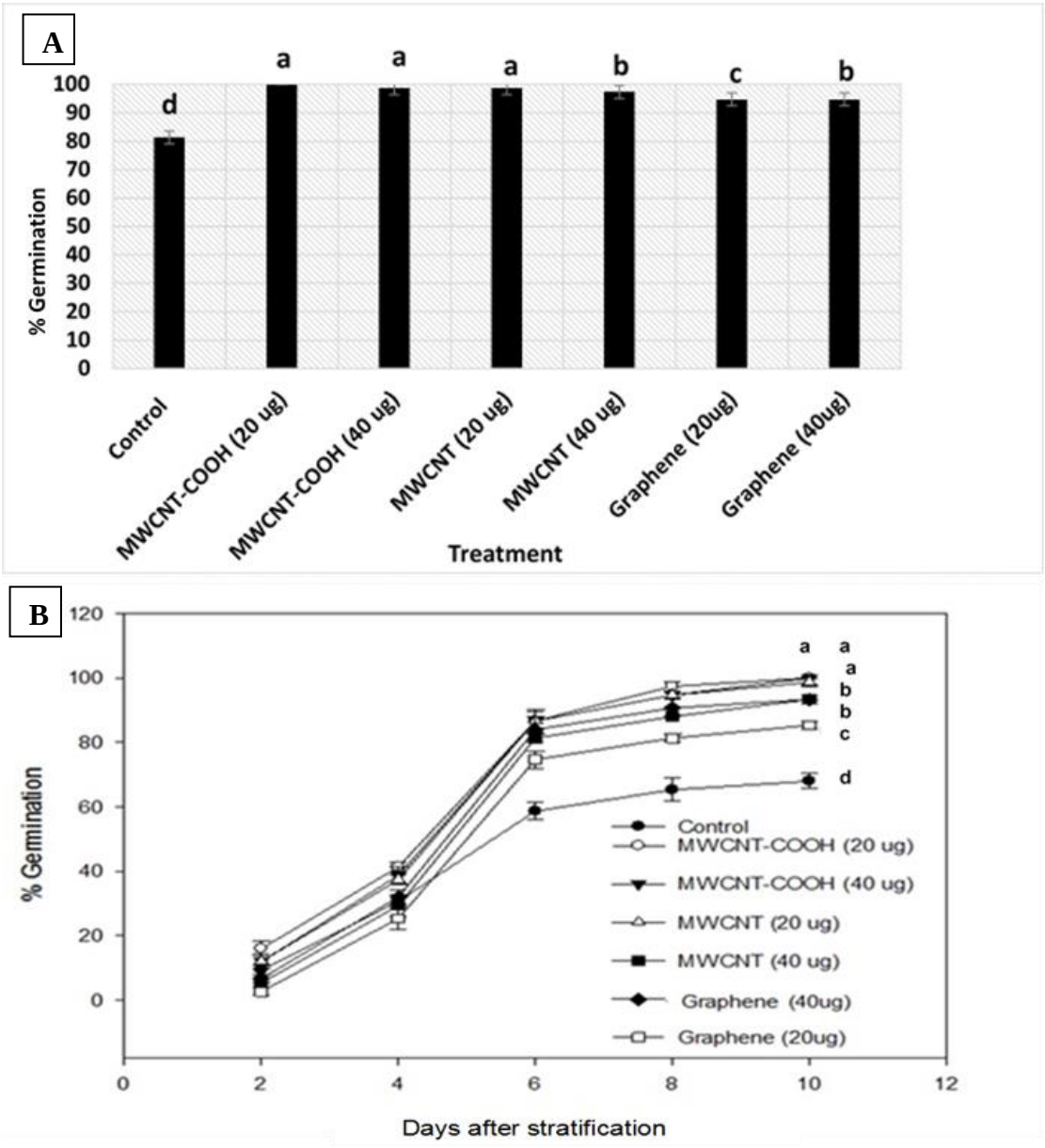


Figure 2.8: Effects of nanopriming on the total seed germination and germination rate of labrador tea. A). Percentages of germination, values in bar chart represent means ± standard errors and all are significantly different at $\alpha = 0.05$. B). Percent of germination over the days after stratification. MWCNT-COOH = Multiwall Carbon nanotube funtionalized with carboxylic acid, MWCNT = Multiwall Carbon nanotube

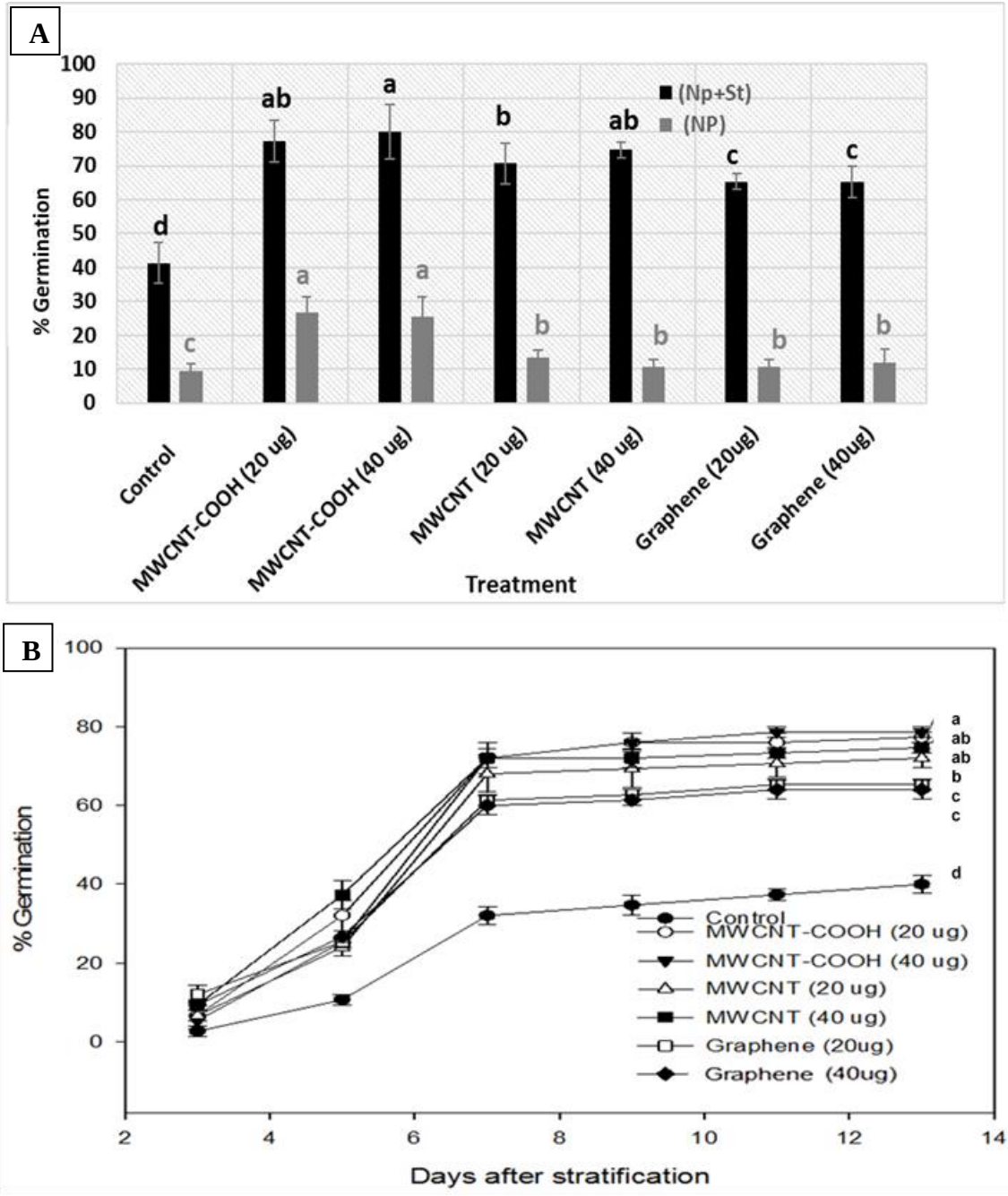


Figure 2.9: Effects of nanopriming on total seed germination and germination rate of bog birch. A). Percentages of germination, values in bar chart represent means ± standard errors and all are significantly different at $\alpha = 0.05$. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes. B). Percentages of germination over the days after stratification period. Np= nanoparticles, Np+st.= nanoparticles + stratification.

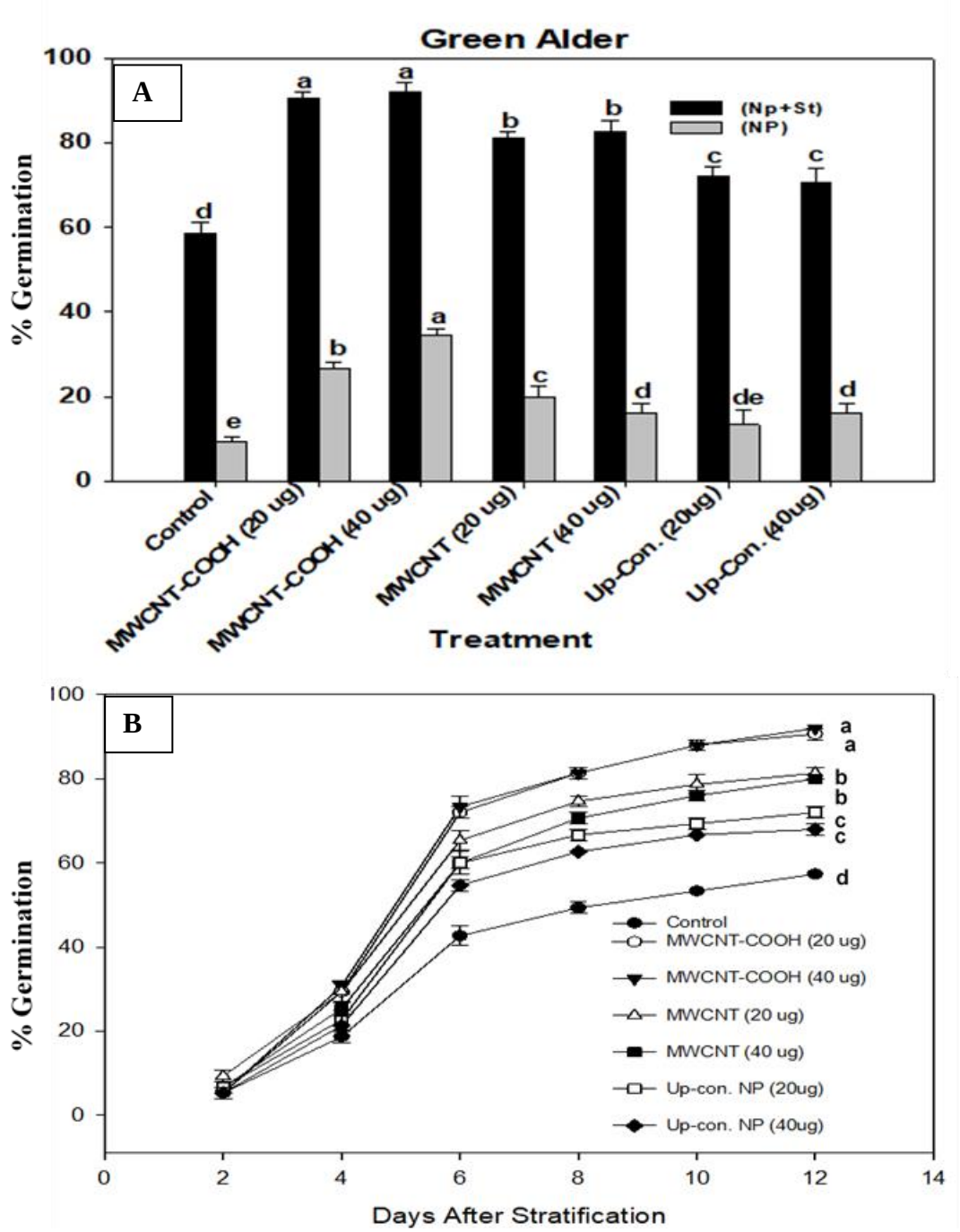


Figure 2.10: A). Effects of nanopriming on the total seed germination and rate of green alder. Values in bar chart represent means ± standard errors and all are significantly different at α = 0.05. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, and up-con = up conversion nanophophors. N = 100 plants per treatment. B). Percentages of germination over the days after stratification period. Np= nanoparticles, Np+st.= nanoparticles + stratification.

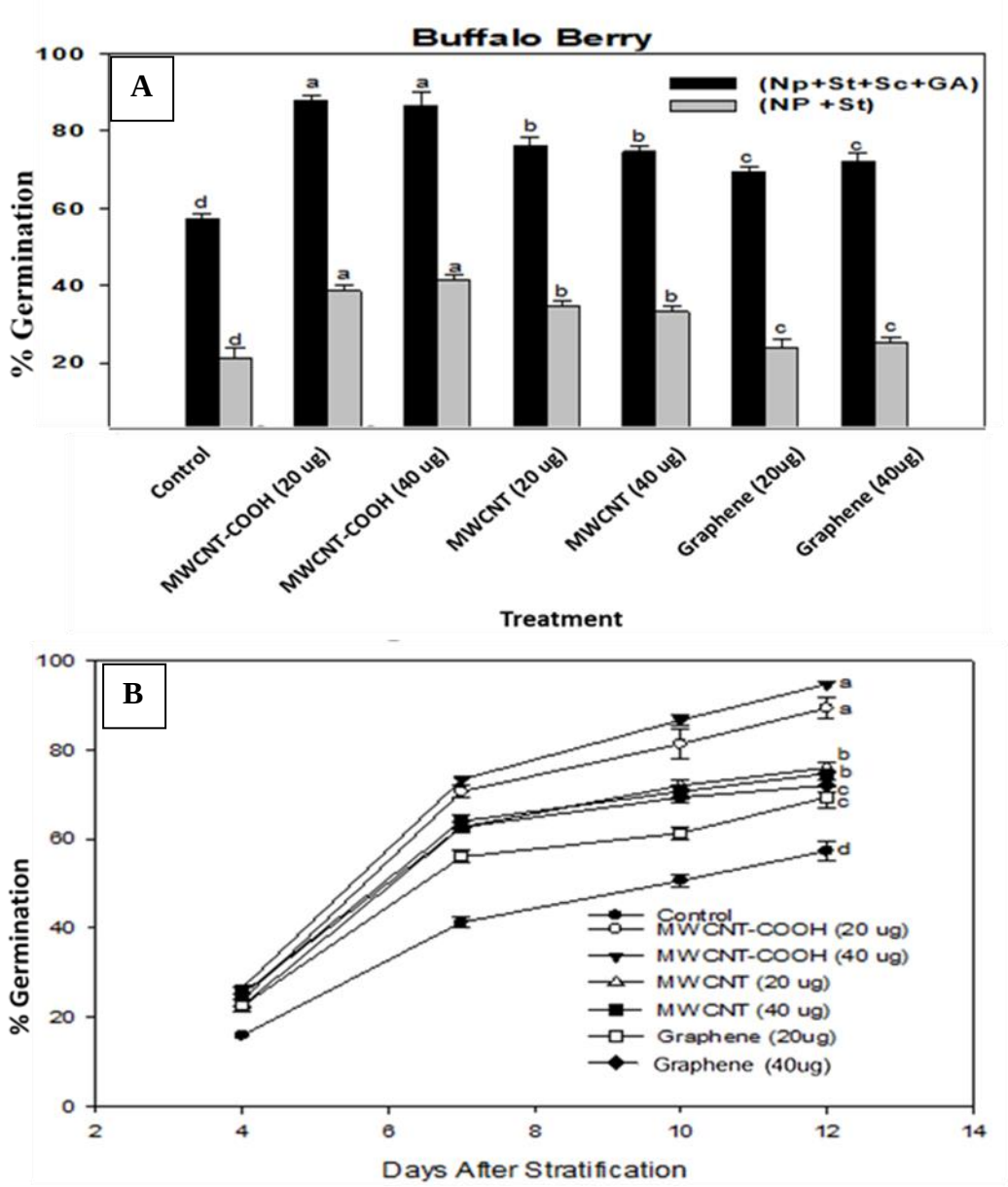


Figure 2.11: A). Effects of nanopriming on the total seed germination and rate of buffalo berry. Values in bar chart represent means ± standard errors and are significantly different at $\alpha = 0.05$. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes. N = 100 plants per treatment. B). Percentages of germination over the days after stratification. Np+St+Sc+GA= nanoparticles+stratification+scarification+gibberellic acid, Np+st.= nanoparticles + stratification.

2.3.5 Seedling Vigor Index (SVI)

Our ANOVA results indicated that in all four boreal plant species, nanopriming had a significant effect on SVI ($p<0.05$). The SVI value (2.4) in bog birch seeds primed with MWCNT–COOH (20 µg/mL and 40 µg/mL) treatments were the highest compared to the control and other treatments. For MWCNT (20 µg/mL and 40 µg/mL) primed seeds, the SVI value was approximately 2.0 and for graphene (20 and 40 µg/mL), it was 1.9. The SVI value was significantly lower (1.2) in the control compared to all other treatments {Figure 2.12(A)}. Similar to bog birch, the SVI was significantly higher in labrador tea seeds primed with CNP compared to the control. We observed MWCNT–COOH at 20 µg/mL resulted in the highest SVI (1.7), while the SVI was similar in value (1.4 to 1.5) when primed with the other nanoparticles (MWCNT and graphene) at either 20 or 40 µg/mL. The lowest SVI value (1.1) was recorded in the control treatment {Figure 2.12(B)}.

The results show that SVI values in green alder primed with MWCNT-COOH (20 µg mL-1 and 40 µg mL-1) were significantly higher (SVI = 2.8) than the control and other treatments. In the case of MWCNT (20 µg mL-1 and 40 µg mL-1), it was 2.4, while in up conversion nano phosphorous (20 µg/mL and 40µg/mL) primed seeds, the SVI was 2.2 and 2.1, respectively, compared to 1.6 in the control {Figure 2.13(A)}. For buffalo berry, we measured seedling length, as well as the SVI for only mechanical scarification + stratification + CNPs + gibberellic acid treatments, as this combination of treatment showed the highest percent germination and germination rate {Figure 2.13(B)}. MWCNT-COOH performed better than any other treatments at both concentrations evaluated (40 and 20 µg/mL). The SVI value was 4.00 and 3.75 respectively. Seeds stratified, scarified and primed in solutions of GA and MWCNT

had similar SVI values (3.00) at both 40 and 20 µg/mL. In contrast, graphene treated seeds had higher ($p<0.05$) SVI values at 40 µg/mL (2.90) than seeds primed at 20 µg/mL (2.80). The SVI for the control was lower than the nanoprimed treatments in both species and was 1.75 and 2.25 for green alder and buffalo berry, respectively.

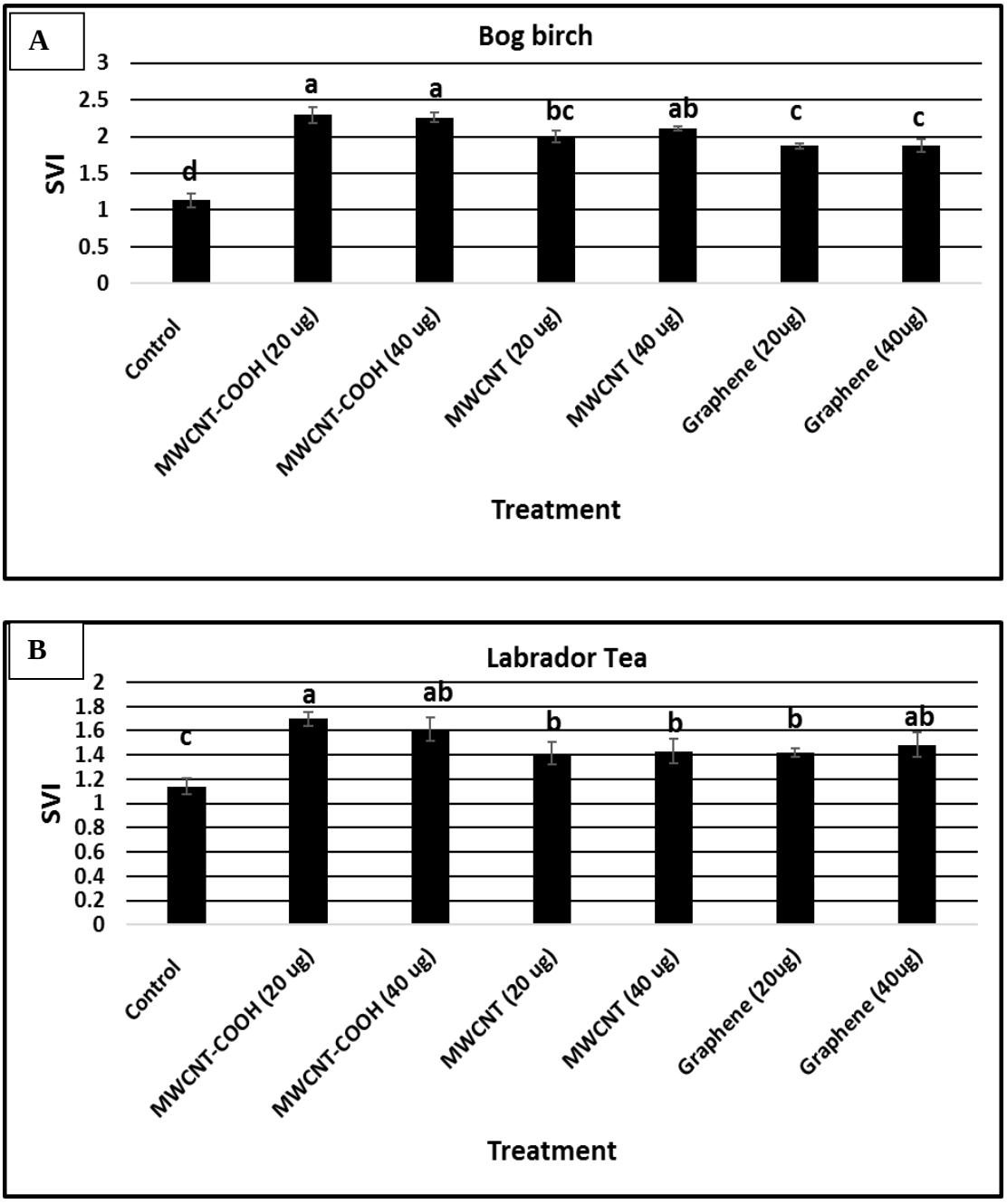


Figure 2.12: Seedling vigor index of A). bog birch and B). labrador tea. Values in bar chart represent means ± standard errors and different letter indicated significant difference at $\alpha = 0.05$. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes.

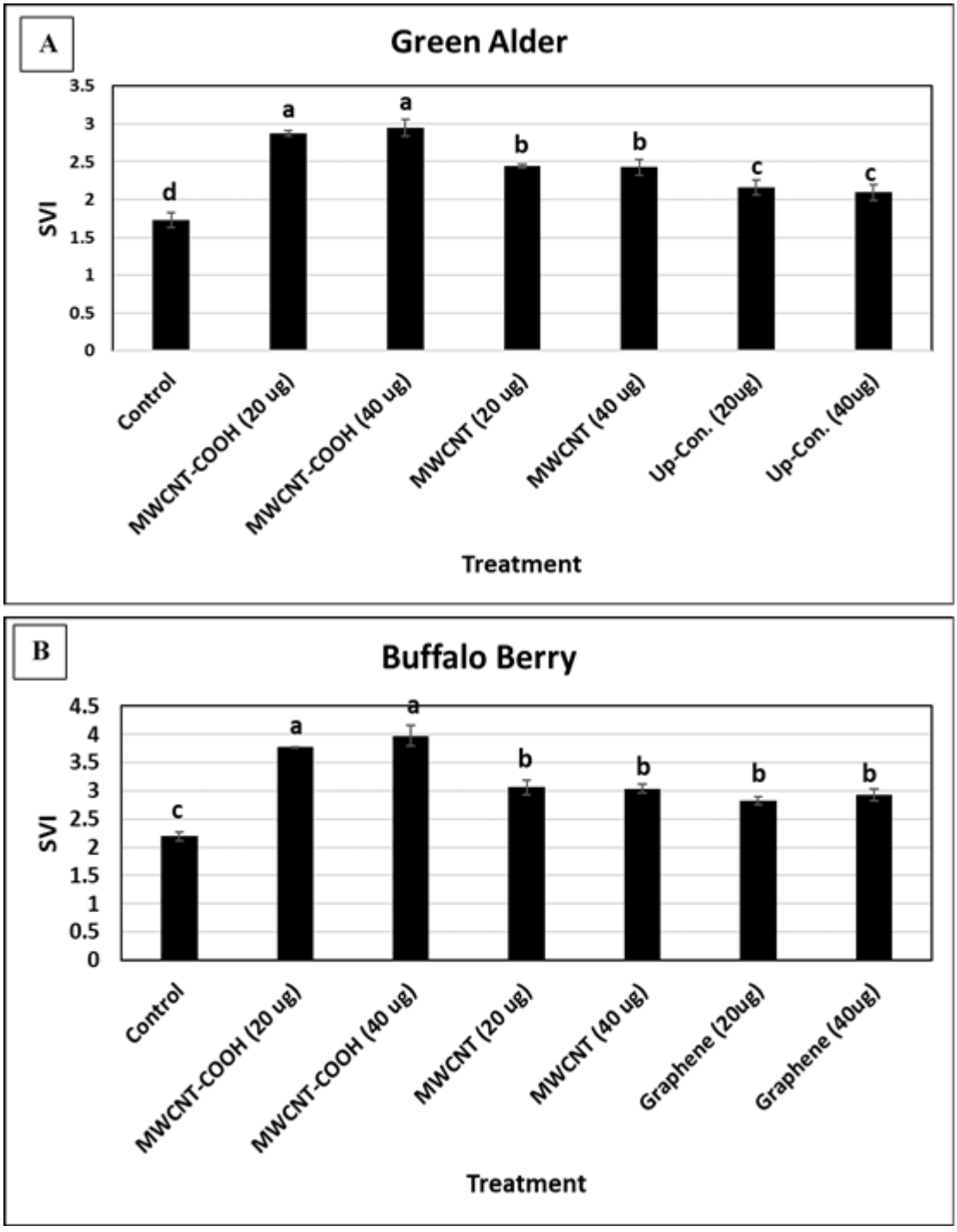


Figure 2.13: Seedling vigor index of A). green alder and B). buffalo berry. Values in bar chart represent means ± standard errors and all are significantly different at $\alpha = 0.05$. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes, up-con = up conversion nanophosphors, N = 100 plants per treatment.

2.4. Discussion

2.4.1 The Effects of Seed Priming with Carbon Nanoparticles on Seedling Quality

In the tetrazolium test, 90% and 100% of the seeds were shown to be viable (completely or partially stained) for peatland species (bog birch and labrador tea), respectively (Figure 2.1). We observed same trend in upland species, approximately 90% of the seeds were viable for both buffalo berry and green alder following nano-priming (Figure 2.2). According to tetrazolium test, all four species have the potential to enhance plant production and thus viable for further study evaluations. Zeng & Wang, (2009) also evaluated the viability of *Nitraria tangutorum* and *Nitraria sibirica* seeds by the tetrazolium test for 12-16 hours at 30 ºC, which is relevant to our experiment. Thus, the seeds we used were viable based on the test output and the accepted use of this test in the literature to assess seed viability. It must be noted that the response of the tetrazolium test depends on many factors. Some of these include the concentration and period of immersion of the seeds in the solution at an optimum temperature. Previous studies show that this test was successfully conducted at an immersion of 0.050% for 6 h at 25 ºC (de Paiva et al., 2017) for an effective result.

In our electrical conductivity (EC) test, EC was higher in the control than all the nanoprimed seed treatments, indicating the amounts of ions leached from the cell membrane of the seeds increased when seeds were hydroprimed (control) but decreased when seeds were nanoprimed with CNP (Figure 2.3 and 2.4). These findings indicated lower membrane potential in nanoprimed seeds compared to the hydroprimed control seeds. High electrolyte leakage from seed membranes increases the EC and is attributed to lower membrane integrity and seed vigor (Sharma et al., 2011). Previous reports have

shown that CNP solutions (fullerenes) had a positive impact on seed membrane integrity and reduced the amount of leached or seepage ions from the cell membrane (Mukherjee et al., 2016). This finding is consistent with our results and supports a role of nanopriming seeds with CNP in improving seed membrane integrity, potential seed vigor and the production or propagation of seedlings without compromising the quality of seeds. Phosphorylation efficiency was reported to be limited by poor membrane integrity, which causes low germination rates in seeds not treated with CNPs (Benamar et al., 2003). Therefore, improved membrane integrity is advantageous for improving seed germination and the quality of the seedlings after germination. It appears that priming of both upland and peatland boreal forest species with CNP could be beneficial in this regard.

We observed a higher percentage of normal seedlings following nano-priming compared to the control primed with only water. Only 5 to 15 % of the seedlings were observed to be abnormal following nano-priming (Figure 2.5, 2.6 and 2.7). This finding implies a positive relationship between seed quality and seed priming with carbon nanoparticles. Seed quality is important in seedling production and achieving high-quality seedlings is paramount to plant survival and establishment (Ferguson et al., 1991). Based on these research findings, MWCNT–COOH produced the best performance regarding seedling production and quality in both upland and peatland forest species evaluated. According to Srinivasan & Saraswathi, (2010), CNPs promoted seedling growth and resolved embryo dormancy in tomato plant seeds. Likewise, Liu et al. (2009), showed that CNTs could penetrate inside tobacco *(Nicotiana tabacum L.)* seeds and increase germination and seedling growth rate without any negative effect on seedling quality. Collectively, our results indicate that

after nanopriming, there is an increased trend of normal seedlings and a decreasing trend of abnormal seedlings.

2.4.2 Effect of Carbon Nanoparticles in Enhancing Germination and Overcoming Seed Dormancy

Bog birch has embryo and seed coat dormancy, and labrador tea has embryo dormancy (Frank and Rowe, 1994; Kaur et al. 2015). These dormancies led to low germination rates in these species. Poff et al., (2016) showed that cold-moist stratification could break embryo dormancy in *Platanthera chapmanii* (terrestrial Orchid) seeds, leading to an 80% increase in germination. Consistent with these findings, we observed that CNP primed seeds had higher germination than the control seeds, where nanopriming combined with cold-moist stratification gave a superior percent and rate of germination compared to when seeds were only stratified. Following nanopriming and cold stratification, we observed approximately 85 to 100% germination in both peatland species. Nanopriming with functionalized CNTs has been reported to have a superior effect on germination compared to non-functionalized CNTs. For example, Cañas et al. (2008) showed that functionalized CNTs is more effective in increasing seed germination rate rather than non-functionalized CNTs in *Onobrychis arenaria*. Similarly, we observed that MWCNT functionalized with carboxylic acid (MWCNT-COOH) was superior in breaking both embryo and seed coat dormancies, thereby increasing seed germination in both bog birch and labrador tea species. The ability of CNPs to penetrate through the seed coat has been proposed as a possible mechanism through which nanoparticles help activate the dormant embryo, resulting in increased seed germination (Haghighi and Silva, 2014). This view is supported by Liu et al. (2009), who noted that CNPs were able to penetrate through

seed coat and influence seed germination. It must be noted that the exact process of how CNPs can influence this is unclear. Several studies have reported that nanopriming is a very effective approach to breaking seed dormancies and improving germination in several agricultural crops species (tomato, rice, etc.) (Mahakham, et al., 2017). However, none of these studies were done using non-resource native boreal forest species.

Mondal et al. (2011) indicated that due to the presence of CNTs, the germination and growth of mustard (*Brassica juncea L.*) increased, when MWCNTs were applied at 2.3 μg mL^{-1}. This is because MWCNTs helped to increase the moisture content and enhance the water absorption machinery of root tissues. Conversely, some studies have reported adverse effects of CNPs on seed germination. For example, CNPs with high doses (2000 mg L^{-1}) led to phytotoxicity and inhibition of seed germination and root growth in tomato. Furthermore, the tendency of nanoparticles to inhibit germination is more common in metal-based nanoparticles (e.g., Zn and ZnO) than with carbon-based nanoparticles (Lin and Xing, 2007). In this study we evaluated whether carbon-based nanoparticles at low concentrations would be effective in breaking dormancies in two boreal forest peatland species (labrador tea and bog birch). In this species, the seeds are very tiny and characterized by also having hard seed coats (seed coat dormancy). Both these dormancies led to reduced seed germination and slow seedling vigor or growth rate (Kaur et al., 2015) in bog birch. Labrador tea seeds are also very tiny and characterized by embryo dormancy; as a result, the germination rate is low in this species (Frank and Rowe, 1994). In our study, the treatments of seeds from both peatland varieties with MWCNT−COOH @ 40 mg L^{-1} led to a significant increase in the total number of seeds germinated, as well as the germination rate, which is

consistent with earlier findings on eleven sedge type wetland plant species (Yin et al. 2012). This work demonstrates that MWCNT-COOH is superior than other CNPs used in this study in terms of breaking embryo and seed coat dormancies in bog birch and labrador tea, and could be a suitable approach to improve the propagation of these species for forest restoration or reclamation activities following resource mining.

Similar to peatland species, upland species also have dormancy issues. Buffalo berry has a hard seed coat (physical dormancy), which impedes germination (Rosner & Harrington, 2003), as the seed coats are impermeable to water and other nutrients, ultimately preventing the germination of viable seeds. On the other hand, green alder is inhibited by embryo growth –Embryo dormancy (Kaur et al., 2015), which is a second category dormancy (morphophysiological dormancy). This dormancy results in low seed germination and stand establishment (Silveira, 2013). The seeds treated with CNP exhibited higher germination compared to the control seeds. The largest increase in germination rate in both upland species was observed when the seeds were primed with MWCNT-COOH, suggesting that the application of functionalized CNTs might have more positive effects on germination (Figure 3.5 and 3.6). Limited research has been conducted on MWCNT functionalized with COOH. In this study, we used MWCNT functionalized with COOH to successfully resolve both embryo and seed coat dormancy, as well as improve seed germination in two upland boreal forest species.

2.4.3 Effect of Carbon Nanoparticles in Enhancing Seedling vigor index

According to the Association of Official Seed Analysts (AOSA), seed vigor is an important measure of seed germination potential, which determines the potential for rapid, uniform emergence and the development of normal seedlings under a wide range

of field conditions, while the seedling vigor index indicates a time-weighted collective germination which quantifies the seedling vigor. Having seeds with high vigor is necessary for effective regeneration. Also, the potential for unsuitable growth temperatures, water excess or deficiency, high heavy metals and poor nutrients in areas mined, mean high vigor seeds are needed for successful regeneration (Bhattacharya, et al., 2012). We observed that seeds primed with CNT had superior vigor, and that the highest vigor was obtained in seeds primed with MWCNT–COOH in both upland and peatland species evaluated. Similar to our findings, silver nanoparticle (SNP)-treated seedlings were reported to attain the greatest growth and about 95% total germination using a concentration of 10-30 µg/ml SNP (Savithramma et al. 2012). This enhanced growth in seedlings may be attributed to increased water and nutrient uptake by seeds treated with SNPs. This view is further supported by the work of Srinivasan and Saraswathi, (2010) which showed that the amount of water intake increased in tomato seeds treated with CNPs, and this contributed to the improved seedling vigor observed.

Cañas et al. (2008) studied the effect of functionalized SWCNTs (functionalized with poly-3-aminobenzenesulfonic acid for higher dispersal ability) and non-functionalized SWCNTs on the root and shoot growth of six crop species: cabbage (*Brassica oleracea L.*), carrot (*Daucus carota L.*), cucumber (*Cucumis sativus L.*), lettuce (*Lactuca sativa L.*), onion (*Allium cepa L.*), and tomato (*Solanum lycopersicum L.*). Their results indicate that the effect of applying CNTs differed, depending on the species. Their results showed that functionalized CNTs are more effective than non-functionalized CNTs for increasing plant shoot and root growth. Enhanced water and

ionic nutrient uptake are stimulated by the use of CNTs, explaining perhaps why growth is then stimulated, as discovered for maize (*Zea mays L.*) (Tiwari et al., 2014).

2.5. Conclusion

In this study we aimed to determine whether select CNP could be used as a seed priming agent to break seed dormancies in two boreal forest peatland and upland species with improved germination and seedling vigor in the test species. We showed for the first time that CNPs could increase germination in boreal forest species common in both upland and peatland ecosystems. The study demonstrates that nanopriming of dormant seeds of four boreal species enhanced seed germination and the seedling vigor index, and that the improved germination and seedling vigor were highly significant in all treatments compared to the control. Of all the treatments in the experiment, stratification combined with MWCNT-COOH nanopriming proved to be the most effective in aiding germination and overcoming seed dormancy. Nano primed seeds with MWCNT-COOH appears to have potential in improving the seed germination and seedling vigor of boreal forest species with morpho and physical seed dormancy issues.

2.6. References

Abbasdokht, H., Mohammad R., and Gholami, A. (2010). The Effect of Hydropriming and Halopriming on Germination and Early Growth Stage of Wheat (*Triticum aestivum L* .). *International Journal of Agricultural and Biosystems Engineering*.,4(8): 984–988.

Afzal, I., Rauf, S. and Murtaza, G. (2008). Halopriming improves vigor, metabolism of reserves and ionic contents in wheat seedlings under salt stress. Plant, Soil and Environment.,9, 382–388.

Ahmet, K., Nusret, O., and Benian, E. (2004). Assessment of Vigor Characteristics of Processing Tomato Cultivars by Using Various Vigor Tests. *Asian Journal of Plant Sciences*, *3*, 181–186.

Amen, R. (1967). The Effects of Gibberellic Acid and Scarification on the Seed Dormancy and Germination in Luzula spicata. *Physiol. Plant.*, *20*, 6–12.

Angela, M., Thomas, H. and Wesley, R. (2009). Stratification, Gibberellic Acid, Scarification, and Seed Lot Influence on Rosemary Seed Germination. *JSTOR*, *31*, 55–65.

Ann, S., Sinton, H., Bietz. J. and Walker, D. (2012). Establishing Native Plant Communities. *Alberta Agriculture, Food and Rural Developement*. ISBN 0-7732-6146-X. 93.

Begum P., Ikhtiari R. and Fugetsu B. (2014). Potential Impact of Multi-Walled Carbon Nanotubes Exposure to the Seedling Stage of Selected Plant Species. *Nanomaterials*, *4*, 203–221.

Benamar, A., Tallon, C., and Macherel, D. (2003). Membrane Integrity and Oxidative

Properties of Mitochondria Isolated From Imbibing Pea Seeds After Priming Or Accelerated Ageing. *Seed Science Research*, *13*(1), 35–45.

Bentsink, L., and Koornneef, M. (2008). Seed Dormancy and Germination. *The Arabidopsis Book*, *6*, e0119. https://doi.org/10.1199/tab.0119

Bewley and Hallett. (2002). Membranes and seed dormancy: beyond the anaesthetic hypothesis. *Seed Science Research*, *12*(2), 69–82. https://doi.org/10.1079/SSR200299

Bhardwaj, J., Anand, A., & Nagarajan, S. (2012). Biochemical and biophysical changes associated with magnetopriming in germinating cucumber seeds. *Plant Physiology and Biochemistry*. 57: 67-73.

Bhattacharya, S., Puri, S., Jamwal, A., & Sharma, S. (2012). Studies on seed germination and seedling growth in Kalmegh (Andrographis paniculata Wall. Ex Nees) under abiotic stress conditions. *International Journal of Science, Environment and Technology* (Vol. 1).

Bottalico, F., Travaglini, D., Fiorentini, S., Lisa, C., & Nocentini, S. (2014). Stand dynamics and natural regeneration in silver fir (*Abies alba* Mill.) plantations after traditional rotation age. *iForest - Biogeosciences and Forestry*, *7*(5), 313–323. https://doi.org/10.3832/ifor0985-007

Canas, J., Long, M., Nations, S., Vadan, R., Dai, L., Luo, M., Ambikapathi, R., Lee, E. H. and Olszyk, D. (2008). Effects of Functionalized and Nonfunctionalized Single-Walled Carbon Nanotubes on Root Elongation of Select Crop Species. *Environ. Toxicol. Chem.*, *27*, 1922–1931.

Carvalho, S. M. C., Torres, S. B., Sousa, E. C., Sousa, D. M. M., Pereira, K. T. O., Paiva, E. P. de, Santos, B. R. V. dos. (2018). Viability of Carica papaya L. Seeds by the Tetrazolium Test. *Journal of Agricultural Science*, *10*(2), 335.

Chaodumrikul, S., Kaewsorn, P., Chulaka, P., & Chanprasert, W. (2016). Breaking seed dormancy in smooth loofah (Luffa cylindrica (L.) M. Roem.) using scarification and dry heat treatment. *Agriculture and Natural Resources*, *50*(2), 85–88. https://doi.org/10.1016/j.anres.2015.09.003

Dampc, A. and M. Luczkiewicz (2015). Labrador tea – the aromatic beverage and spice: a review of origin, processing and safety. *Journal of the Science of Food and Agriculture*. 95 (8): 1577–83. doi:10.1002/jsfa.6889

de Paiva, E. P., Torres, S. B., de Almeida, J. P. N., Sá, F. V. da S., & Oliveira, R. R. T. (2017). Tetrazolium test for the viability of gherkin seeds. *Revista Ciencia Agronomica*, *48*(1), 118–124.

FAO (2011). Optimizing plant population, crop emergence and establishment.

Ferguson, J. M., Keys, R. D., McLaughlin, F. W. and Warren, J. M. (1991). Seed and Seed Quality. *NC State Extension*, 448.

Finch, S. and Bassel, G. (2015). Seed vigour and corp establishment: extending performance beyond adaptation. *J. Exp Bot.*, 67(3):567-91.

Frank A. Blazich and D. Bradley Rowe. (1994). Ericaceae—Heath family *Rhododendron L*., 939–996.

Freeman, C., Fenner, N. and Shirsat, A. (2012). Peatland geoengineering: An alternative approach to terrestial carbon sequestration., 370(1974):4404-4421.

Forest Service. USA. (1961). Brich Seeds will germinate under a water-light treatment without pre-chilling., 124.

Haghighi, M., & Silva, J. A. T. (2014). The effect of carbon nanotubes on the seed germination and seedling growth of four vegetable species. *Journal of Crop Science and Biotechnology*, *17*(4), 201–208.

Harayama, T., & Riezman, H. (2018). Understanding the diversity of membrane lipid composition. *Nature Reviews Molecular Cell Biology*, *19*, 281. Retrieved from http://dx.doi.org/10.1038/nrm.2017.138

Harman, G. E., Khan, A. A., Tao, K. L., & Khan, A. A. (1976). Physiological changes in the early stages of germination of pea seeds induced by aging and by infection by a storage fungus , Aspergillus ruber. *Can. J. Bot.*, (54), 39–44.

He, S., Steven, E., Xuling, G. and Gordon, B. (2009). Narrow-linear and small-area forest disturbance detection and mapping from high spatial resolution imagery. *Journal of Applied Remote Sensing*., 3,1.

ISTA. (2006). *International Rules for Seed Testing. Edition 2006. International Seed Testing Association, Switzerland.*

Jisha K. and Puthur J. (2014). Halopriming of seeds imparts tolerance to NaCl and PEG induced stress in *Vigna radiata (L.)* Wilczek varieties. *Physiology and Molecular Biology of Plants*, 20(3), 303–312.

John O Offem, Emmanuel O Egbe, A. I. O. (1993). Changes in lipid content and composition during germination of groundnuts. *SCI*, *62*(2), 147–155.

Kaur, J., Schoonmaker, A., & Sobze, J. (2015). Length of cold startification period

affects germination in green alder (Alnus viridis (Chaix) DC. subsp. Crispa (Aiton) Turrill) seed collected from Northwestern Alberta. *Native Plants*. 17(2): 95–102.

Kobayashi, Y., Nabeta, K., & Matsuura, H. (2010). Chemical Inhibitors of Viviparous Germination in the Fruit of Watermelon. *Plant and Cell Physiology*, *51*(9), 1594–1598. http://dx.doi.org/10.1093/pcp/pcq103

Kottegoda, N., Munaweera, I., Madusanka, N., & Karunaratne, V. (2011). A green slow-release fertilizer composition based on urea-modified hydroxyapatite nanoparticles encapsulated wood. *Current Science*, *101*(1), 73–78.

Kraszewski, S., Bianco, A., Tarek, M., & Ramseyer, C. (2012). Insertion of short amino-functionalized single-walled carbon nanotubes into phospholipid bilayer occurs by passive diffusion. *PLoS ONE*, *7*(7), 1–11.

Lee, H., & Kim, H. (2012). Self-assembly of lipids and single-walled carbon nanotubes: Effects of lipid structure and PEGylation. *Journal of Physical Chemistry C*, *116*(16), 9327–9333. https://doi.org/10.1021/jp3010663

Li-beisson, Y. N. Y. (2016). *Lipids in Plant and Algae Development* (Vol. 86). Springer. Retrieved from http://link.springer.com/10.1007/978-3-319-25979-6

Li-Shar Huang and Claus Grunwald. (1990). Lipid and Fatty Acid Changes During Germination of Alfalfa Seeds. *Phytochemistry*, *29*(5), 1441–1445.

Lin, D., & Xing, B. (2007). Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth. *Environmental Pollution*, *150*(2), 243–250. https://doi.org/10.1016/j.envpol.2007.01.016

Liu, Q., Chen, B., Wang, Q., Shi, X., Xiao, Z., Lin, J., & Fang, X. (2009). Carbon nanotubes as molecular transporters for walled plant cells. *Nano Letters*, *9*(3), 1007–1010. https://doi.org/10.1021/nl803083u

Liu, X., Zhang, H., Zhao, Y., Feng, Z., Li, Q., Yang, H.-Q., He, Z.-H. (2013). Auxin controls seed dormancy through stimulation of abscisic acid signaling by inducing ARF-mediated ABI3 activation in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(38), 15485–15490. https://doi.org/10.1073/pnas.1304651110

Ma, Z., Bykova, N. V., & Igamberdiev, A. U. (2017). Cell signaling mechanisms and metabolic regulation of germination and dormancy in barley seeds. *Crop Journal*, *5*(6), 459–477. https://doi.org/10.1016/j.cj.2017.08.007

Maatta, S., Scheu, B., Roth, M. R., Tamura, P., Li, M., Williams, T. D., … Welti, R. (2012). Levels of Arabidopsis thaliana Leaf Phosphatidic Acids, Phosphatidylserines, and Most Trienoate-Containing Polar Lipid Molecular Species Increase during the Dark Period of the Diurnal Cycle. *Frontiers in Plant Science*, *3*, 49. https://doi.org/10.3389/fpls.2012.00049

Mahakham, W., Sarmah, A. K., Maensiri, S., & Theerakulpisut, P. (2017). Nanopriming technology for enhancing germination and starch metabolism of aged rice seeds using phytosynthesized silver nanoparticles. *Scientific Reports*, *7*(1), 1–21. https://doi.org/10.1038/s41598-017-08669-5

Martínez-Ballesta, M. C., Zapata, L., Chalbi, N., & Carvajal, M. (2016). Multiwalled carbon nanotubes enter broccoli cells enhancing growth and water uptake of plants exposed to salinity. *Journal of Nanobiotechnology*, *14*(1), 42.

Matthews, S. and Powell, A. . (1981). *Electrical conductivity test. In Vigour Test Handbook. (ed. D. A. Perry). International Seed Testing Association, Zurich.*

Mondal, A., Basu, R., Das, S., & Nandy, P. (2011). Beneficial role of carbon nanotubes on mustard plant growth: An agricultural prospect. *Journal of Nanoparticle Research*, *13*(10), 4519–4528. https://doi.org/10.1007/s11051-011-0406-z

Mukherjee, A., peralta-videa, J., Gardea, J. and White, J. (2016). Effects and Uptake of Nanoparticles in Plants. In: Xing B, Vecitis CD, Senesi N (eds) Engineered nanoparticles and the environment: biophysicochemical processes and toxicity. Wiley, Hoboken. https://doi. org/10.1002/9781119275855.ch20

Parish F, Sirin A, Charman D, Joosten H, Minaeva T, Silvius M (eds) (2008) Assessment on peatlands, biodiversity and climate change. Global Environment Centre, Kuala Lumpur and Wetlands International Wageningen, 179 p.

Poff, K., Sharma, J. and Richards, M. (2016). Cold-Moist Stratification Improves Germination in a Temperate Terrestrial Orchid. Castanea. 81(4):292-301

Pourkhaloee, A., Haghighi, M., Jamal Saharkhiz, M., Jouzi, H., & Mahdi, M. (2011). Carbon Nanotubes Can Promote Seed Germination via Seed Coat Penetration. *Seed Technology*. 155-169. (Vol. 33).

Prasad, R., Bhattacharyya, A., & Nguyen, Q. D. (2017). Nanotechnology in Sustainable Agriculture: Recent Developments, Challenges, and Perspectives. *Frontiers in Microbiology*, *8*, 1014. https://doi.org/10.3389/fmicb.2017.01014

Ramos, K. M. O., Matos, J. M. M., Martins, R. C. C., & Martins, I. S. (2012). Electrical Conductivity Testing as Applied to the Assessment of Freshly Collected

Kielmeyera coriacea Mart. Seeds. *ISRN Agronomy*, *2012*, 1–5.

Rosner L. and Harrington J. (2003). Russet Buffalor Berry Seeds. *Native,* 81-86.

Samples, V. (2008). Accumulation Parameters and Seasonal Trends for PCBs in Temperate and Boreal Forest Plant Species. *Environmental Science & Technology*, 42(16): 5911-5916.

Savithramma, N., Ankanna, S. and Bhumi, G. (2012). Effect of Nanoparticles on Seed Germination and Seedling Growth of Boswellia Ovalifoliolata – an Endemic and Endangered Medicinal Tree Taxon. *Nano Vision*, 61-68.

Scott, C. and Neil, E. (2012). Concurrent profiling of indole-3-acetic acid, abscisic acid and cytokinins and structurally related purines by high-performance-liquid-choromatography tandem electrospray mass spectormetry, *Plant Methods*. *8*, 42.

Sharma, P., Mishra, M., Singh, N. I., & Chauhan, J. S. (2011). Determination of Integrity of Seed Membrane Systems and Mineral Ions Leakage in Horse Gram, *7*(4), 476–479.

Sekhon B. (2014). Nanotechnology in agri-food production: an overview (Vol. 7).

Silveira, F. A. O. (2013). Sowing seeds for the future: the need for establishing protocols for the study of seed dormancy. *Acta Botanica Brasilica*, *27*, 264–269. http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-33062013000200003&nrm=iso

Sivasubramaniam, K & Geetha, R & Kandasamy, Sujatha & K, Raja & Sripunitha, A & Selvarani, R. (2011). Seed Priming: Triumphs and Tribulations. *Madras Agric. J.*, *98*, 197–209.

Smýkal, P., Vernoud, V., Blair, M. W., Soukup, A., & Thompson, R. D. (2014). The role of the testa during development and in establishment of dormancy of the legume seed. *Frontiers in Plant Science*, *5*, 351.

Srinivasan, C., & Saraswathi, R. (2010). Nano-agriculture - carbon nanotubes enhance tomato seed germination and plant growth. *Current Science*, *99*(3), 274–275.

Tan, X. ming, Lin, C., & Fugetsu, B. (2009). Studies on toxicity of multi-walled carbon nanotubes on suspension rice cells. *Carbon*, *47*(15), 3479–3487. https://doi.org/10.1016/j.carbon.2009.08.018

Tanoue, R., Kobayashi, M., Katayama, K., Nagata, N., & Wada, H. (2014). Phosphatidylglycerol biosynthesis is required for the development of embryos and normal membrane structures of chloroplasts and mitochondria in Arabidopsis. *FEBS Letters*, *588*(9), 1680–1685.

Taylorson, R. C.-H. and R. B. (1987). Changes in Lipid Fatty Acids Associated With Dormancy Breaking in *Amaranthus Albus* Seeds. *Journal of Seed Technology*, *11*(1), 15–22.

Tiwari, D. K., Dasgupta-Schubert, N., Villaseñor Cendejas, L. M., Villegas, J., Carreto Montoya, L., & Borjas García, S. E. (2014). Interfacing carbon nanotubes (CNT) with plants: enhancement of growth, water and ionic nutrient uptake in maize (Zea mays) and implications for nanoagriculture. *Applied Nanoscience*, *4*(5), 577–591. https://doi.org/10.1007/s13204-013-0236-7

Vieira, R. D., Scappa Neto, A., Bittencourt, S. R. M. de, & Panobianco, M. (2004). Electrical conductivity of the seed soaking solution and soybean seedling emergence. *Scientia Agricola*, *61*(2), 164–168. https://doi.org/10.1590/S0103-

90162004000200007

Vogel, J. and Gower, S. (1998). Carbon and nitrogen dynamics of boreal jack pine stands with and without a green alder understory. *Ecosystems*, *1*(4), 386–400.

Wang, E. C., & Wang, A. Z. (2014). Nanoparticles And Their Applications In Cell And Molecular Biology. *Integrative Biology : Quantitative Biosciences from Nano to Macro*, *6*(1), 9–26. https://doi.org/10.1039/c3ib40165k

Wieder, B., & Vitt, D. (2006). Boreal peatland ecosystems, ecological studies. *Springer-Verlag*, *1-8*.

Yin, L., Colman, B. P., McGill, B. M., Wright, J. P., & Bernhardt, E. S. (2012). Effects of Silver Nanoparticle Exposure on Germination and Early Growth of Eleven Wetland Plants. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0047674

Yousefi, S., Kartoolinejad, D., & Naghdi, R. (2017). Effects of priming with multi-walled carbon nanotubes on seed physiological characteristics of Hopbush (Dodonaeaviscosa L.) under drought stress. *International Journal of Environmental Studies*, *74*(4), 528–539.

Yu, X., Li, A., & Li, W. (2015). How membranes organize during seed germination: three patterns of dynamic lipid remodelling define chilling resistance and affect plastid biogenesis. *Plant Cell Environ.*, *38*(7), 1391–1403. https://doi.org/10.1111/pce.12494.How

Yuri R., Maryna O., Aravind R., and Jitendra B. (2008). Interaction of Nanoparticles with Lipid Membrane. *Nano Lett.*, *8*(3), 941–944.

Zeng, Y. J., & Wang, Y. R. (2009). Methods of topographical tetrazolium testing for

seed viability of Nitraria tangutorum Bobr. and N. sibirica Pall. *Seed Science and Technology*, *37*(3), 691–698. https://doi.org/10.15258/sst.2009.37.3.16

Chapter 3: Role of Lipid Metabolism in Overcoming Seed Dormancy in Native Boreal Species

3.1. Introduction

The potential use of nanotechnology in many sectors, including agriculture, has increased in the last couple of years (Josef and Katarina, 2017). It broadens the scope for novel applications to resolve challenges in many sectors because carbon nanoparticles (CNP) have unique physiochemical properties, i.e., high reactivity, tunable pore size, and high surface area (Choi and Frangioni, 2010). The use of nanoparticles in enhancing plant growth, seed germination, and its ability to modulate how plants interact with their environment at both the cellular or molecular level has been demonstrated in several recent studies (Kottegoda et al., 2011; Zheng et al., 2005).

The basic structure of the plant cell membrane is formed by different classes of lipids, including phospholipids, glycolipids, sphingolipids, and steroids (Harayama and Riezman, 2018). The capability of carbon nanoparticles to penetrate through the seed cell walls is unique due to their potential to overcome the thickness of the seed cell barrier components (cell wall and membrane) (Srinivasan and Saraswathi, 2010). One potential for the ability of nanoparticles to penetrate the seed and modulate cell biology and biochemistry is that it may resolve some of the issues associated with seed dormancy.

The ability of CNP to traverse both the cell wall and seed coat have been proposed as a possible mechanism through which the increase germination rates have been achieved (Haghighi & Silva, 2014; Liu et al., 2009). For example, multi-walled carbon nanotubes (MWCNTs), which consist of several concentric layers of graphene (forming

a tube shape) can penetrate plant cell walls, accumulate in the cells and tissues, as well as be transported via the vascular system from roots to stems (Smirnova et.al., 2011). Also, MWCNTs have been shown to increase peroxidase activity in plant cells and stimulate the growth of roots and stems (Smirnova, 2011), possibly via modulation of membrane lipids and proteins (Monticelli, 2009). Generally, most nanoparticles can penetrate plant cell walls and membranes, but the penetration into seeds is specifically associated with seed coat thickness during priming with those nanoparticles (Srinivasan & Saraswathi, 2010).

Seed priming with CNP could be useful in breaking seed dormancy in native boreal forest species needed for reclamation activities, possibly via remodulation of the seed membrane lipid metabolism. During seed germination, a seed goes through various physiological (water uptake, respiration, etc.) and biological (lipids, proteins, etc.) alterations (Ma et al., 2017; Harman et al., 1976). Changes in seed membrane lipid metabolism have been reported to be highly correlated with improved seed germination (Ali and A. Elozeiri, 2017). For example, during germination of *Medicago sativa*, it has been reported that membrane lipids (phospholipids, galactolipids, sulfolipids etc.) increase in concentration (Zhang et. al. 2019). Phosphatidylcholine (PC) was observed to increase significantly compared to other phospholipids, particularly linoleic acid enriched molecular species (Li-Shar Huang and Claus Grunwald, 1990). Lipid fatty acid changes have been reported to be directly associated with the breaking of embryo dormancy of seeds by phytochrome (A and B) in *Amaranthus albus L.* and specific fatty acids might play a role in alleviating seed dormancy (Taylorson and Hannel, 1987). Resolution of seed dormancy can be prompted by many factors (temperature, water, monocarboxylic acid, etc.) but the actions of these factors affected through effecting

changes to cell membranes of the plant seeds (Bewley and Hallett, 2002). In many plants during germination, the fats are hydrolyzed into fatty acids and glycerol by lipase enzyme. Fatty acids are further converted into acetyl-CoA by the process of β-oxidation (Matsui et al., 2004). Bewley and Hallett (2002) stated that mechanism of breaking dormancy takes place into the cell membrane of plant seeds.

Similarly, during germination of groundnuts seeds, John et al., (1993) showed changes in lipid contents and compositions; the non-polar lipids were metabolized faster than the polar lipids. Taylorson and Hannel's, (1987) work further supported this point by demonstrating with *Amaranthus albus L.* seed germination that lipid fatty acid changes could help overcome seed dormancy, especially the polar lipids. An increase in linolenic acid content improved seed germination, which was attributed to the presence of phyto-chromes and lipids in the seed. Collectively, these studies demonstrate that altered seed membrane lipid metabolism plays an essential role in seed germination (Ma et al., 2017).

Priming of boreal plant seeds with carbon-based nanoparticles could be helpful in resolving seed dormancy via modulation of the seed membrane lipid metabolism. Given the limited research on CNTs on germination in boreal species with seed dormancy issues, this study aimed at examining how CNTs could assist in breaking seed dormancy and influence germination both in upland and peatland boreal forest species and how membrane lipid metabolism may play a role in this process. As such, we hypothesize that seed priming with Carbon Nanoparticles (CNPs) can be used to overcome seed dormancy issues in boreal plants by modulating the membrane lipid metabolism.

3.2. Material and Methods

Germinated seedlings from each replicate per treatment were collected and then incubated in hot isopropanol for 15 minutes, and homogenized with an OMNI Tissue Homogenizer (Tissue Master 125, OMNI International, GA, United States). The homogenate was used for further lipid extraction and analysis. An adapted version of the Bligh and Dyer method was used for lipid extraction (Bligh & Dyer, 1959). In short, a total of 10 mg of the seedling sample was transferred to glass centrifuge tubes, and the following solvents were added: 1 mL of methanol containing 0.01% butylated hydroxytoluene (BHT), 1 mL chloroform and 0.8 mL water. The mixture of the sample was centrifuged at 5000 rpm for 15 min. After centrifugation, the organic layer at the bottom of the vial containing the lipids was transferred to pre-weighed 4 mL sample vials with a PTFE lined cap (VWR, Mississauga, ON, Canada). The organic layer was dried under a nitrogen stream, and then weighed to determine the amount of lipids recovered (Bligh and Dyer 1959). The recovered lipids in each vial were then transferred and suspended in 1 mL chloroform: methanol (1:1 v/v) and stored at -20 °C for further analysis.

3.2.1 Chemicals

Ultra high-performance liquid chromatography (UHPLC) grade acetonitrile, chloroform, and methanol were purchased from Fisher Scientific (Hampton, New Hampshire, USA). DI water was obtained from the PURELAB Purification System (ELGA Labwater, ON, Canada). HPLC grade acetic acid, formic acid, and ammonium acetate were purchased from Sigma-Aldrich (ON, Canada). Commercial standards of phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

3.2.2 Plant membrane lipids analysis using UHPLC-C30RP-HESI-HRMS/MS

Ultra-high-performance liquid chromatograph (UHPLC), joined to a C30 reverse phase chromatography column and heated electrospray ionization high-resolution tandem mass spectrometry (UHPLC-C30RP-HESI-HRMS/MS), was used to separate the plant membrane lipid species and other molecular classes. The lipid analyses were carried out using a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, MO, USA) linked with an automatic Dionex UltiMate 3000 ultra-high-performance liquid chromatograph system controlled by Chromeleon software program. An Accucore C30 column (150 × 2 mm2 I.D., particle size: 2.6 µm, pore diameter: 150 Å), which was purchased from ThermoFisher Scientific (ON, Canada), was used for lipid separation. The solvent scheme used to resolve all the complex lipids within the C30 column was as follows: "A" solvent contained water (60:40 v/v) with 10 mM ammonium formate and 0.1% formic acid: acetonitrile. "B" solvent contained H_2O (90:10:1 v/v/v) with 10 mM ammonium formate and 0.1% formic acid: isopropanol: acetonitrile.

UHPLC-C30RP chromatography was conducted at 30 ºC (oven temperature) with a flow frequency of 0.2 mL min^{-1}, and 10 µg/mL of the sample extract suspended in methanol (2:1 v/v): chloroform was injected in the machine. The following solvent system gradient was used for separating the different lipid classes and molecular species: 30% B solvent for 3 minutes; increased to 43% over 5 minutes, then to 50% for 1 minute, then to 90% over 9 minutes, then to 99% over 8 minutes, and lastly at 99% for 4 minutes. The column was re-equilibrated to starting conditions (70% solvent A) for 5 minutes before each new injection. Full scan HESI-MS and MS/MS acquisitions were done on the Q-Exactive Orbitrap mass spectrometer in both positive

and negative modes, and the machine was controlled by X-Calibur software 4.0. The following parameters were used for the Orbitrap mass spectrometer, sheath gas: 40, auxiliary gas: 2, ion spray voltage: 3.2 kV, capillary temperature: 300 ^{o}C; S-lens RF: 30 V; mass range: 200-2000 m/z; full scan mode at a resolution of 70,000 m/z; top-20 data-dependent MS/MS at a resolution of 35,000 m/z and collision energy of 35 (arbitrary unit); an injection time of 35 minutes for C30RP chromatography; isolation window: 1 m/z; automatic gain control target: 1e5 with a dynamic exclusion setting of 5.0s. The machine was adjusted to 1 ppm using ESI positive and negative calibration solutions (Thermo Scientific, MO, USA). Tune parameters were optimized using PC 18:1(9Z)/18:1(9Z), Cer d18:1/18:1(9Z), PG 18:1PG 18:1(9Z)/18:1(9Z), SQDG 18:3(9Z,12Z,15Z)/16:0, MGDG 18:3(9Z,12Z,15Z)/16:3(7Z,10Z,13Z) and DGDG 18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z) lipid standards (Avanti Polar Lipids, Alabama, USA) in positive and negative ion modes.

3.2.3 Data processing

All lipidomics data were acquired and processed using X-Calibur 4.0 ThermoScientific, MO, USA) and LipidSearch version 4.1 (Mitsui Knowledge Industry, Tokyo, Japan) software packages. LipidSearch was used for the identification and quantification of the lipid classes and lipid molecular species. Statistical analysis was performed by XLSTAT. Analysis of variance (ANOVA) was used to determine treatment effects at $p = 0.05$. Redundancy analysis (RDA) and Pearson correlation coefficient were also conducted by XLSTAT and used to determine associations between germination or seedling vigor parameters and altered membrane lipids. Treatments and lipid classes were used as independent variables and nmol% of lipid

classes used as dependent variables. All figures were created using Sigma Plot or XLSTAT software packages.

3.3. Results

3.3.1 Roles of lipid metabolism in overcoming seed dormancy in peatland boreal forest species

We attempted to evaluate whether lipid metabolism played a role in nanoprimed alleviation of seed dormancy in both peatland species. The following classes of membrane lipids were present in the seedlings at germination following nano-priming: CL (Cardiolipin), LPC (Lysophosphatidylcholine), LPE (Lysophosphatidylethnolamine), PA (phosphatidic acid), PC (phosphatidylcholine), PE (phosphatidylehanolamine), PG (phosphatidylglycerol), PI (phosphatidylinositol), PS (phosphatidylserine), DGDG (digalactosyldiacylglycerol), MGDG (monogalactosyl diglyceride), and SQDG (sulfoquinovosyl diacylglycerols). Two iterations of RDA analysis were performed to discern the effects of the treatments on seedling membrane lipid composition (Figure 3.1 and Figure 3.2). Seedlings with similar membrane lipids clustered in the same quadrant of the biplot. In the first RDA analysis, most of the physiological parameters (total germination, SVI, or normal seedlings) clustered with the MWCNT-COOH treatment, and this grouping accounted for 80.36% and 85.94% of the total variance for both bog birch and labrador tea, respectively (Figure 3.1 and Figure 3.2). In the second iteration of the RDA analysis, we only included the lipid classes clustered with MWCNT-COOH, since this was the treatment observed with the highest percent germination and seedling vigor {Figure 3.3(A) and 3.4(A)}.

We observed for bog birch, PI (16:0/18:2), PC (18:3/18:3), PC (18:3/18:2), PG (16:1/18:3), LPC (18:2), DGDG (16:0/18:3), PA (18:3/18:2), PC (18:1/18:3) and DGDG (18:3/18:3) were clustered in Q3 with MWCNT-COOH treatment along with germination rate, SVI, and NS (normal seedlings). PE (18:2/18:2), PE (18:3/18:2), PI (16:0/18:3), LPC (18:3), PG (16:1/18:2) and PA (18:3/18:3) were clustered in Q4 with both graphene and MWCNT treatment, along with abnormal seedlings, while EC was clustered with the control. Approximately 92.32% of the variability was accounted for by this segregation {Figure 3.3(A)}.

For labrador tea, PA (18:1/18:2), PC (18:1/18:3), LPC (18:3), PI (16:0/18:30, PG (16:1/18:3), PE (16:0/18:2) and DGDG (18:3/18:3) lipid classes were clustered in Q2 with MWCNT-COOH, along with SVI, germination rate, and normal seedlings, similar to the observation for bog birch. In contrast, DGDG (16:0/18:3), LPC (18:2), MGDG (18:3/18:3), MGDG (18:2/18:3), PA (18:2/18:2), PG (16:1/18:2), PE (16:0/18:3), PI (16:0/18:2) and PC (18:3/18:2) lipid classes were clustered in Q3 with graphene treatments and abnormal seedlings. EC clustered with the control, same as bog birch, and this segregation accounted for 85.29% of the variability in the data {Figure 3.4(A)}.

Following RDA analysis, ANOVA was employed to examine the effects of the lipid molecular species that clustered with germination rate, SVI, and NS, with MWCNT–COOH the best performing treatment, (CNPs was treated as the independent variable). For bog birch, the means for PG (16:1/18:3) and PC (18:1/18:3), were significantly higher ($p<0.05$) than that of MWCNT, graphene, and the control. Also, the means of MWCNT and graphene were significantly different ($p<0.05$) from the control. The means of DGDG (18:3/18:3) were higher in the MWCNT-COOH

treatment and significantly different from the control, but similar to that of MWCNT and graphene. In the case of LPC (18:2), MWCNT-COOH showed significant differences compared to the control and graphene {Figure 3.3(B)}.

For labrador tea, DGDG (18:3/18:3) was significantly higher ($p<0.05$) in the nanoprimed seedlings compared to the control, with the highest level recorded in the seedlings primed with MWCNT-COOH {Figure 3.4(B)}. Similarly, the LPC (18:3) level was elevated in the nanoprimed seedlings compared to the control. Conversely, the PC (18:1/18:3) was significantly lower in the nanoprimed treatments compared with the control. However, PE (16:0/18:2) was observed to be elevated in the nanoprimed seedlings. Though PG (16:1/18:3) was observed to also increase following nanopriming, only in the MWCNT-COOH was the increase significant compared with the control {Figure 3.4(B)}. As such, a Pearsons correlation analysis was conducted among SVI, germination rate (GR) and these molecular species (DGDG (18:3/18:3), PC (18:1/18:3), LPC (18:3) and PG (16:1/18:3) to see if there was any association with improvements in germination and seedling vigor following nanopriming (Figure 3.5). We observed PC (18:1/18:3) and PG (16:1/18:3) were strongly correlated with SVI ($r=0.909$, $p=0.01$; $r=0.906$, $p=0.001$ respectively). On the other hand, we observed only PG (16:1/18:3) was significantly ($P= 0.003$) correlated with GR ($r = 0.912$) (Figure 3.5). Outputs of the correlation analysis for all the lipid molecular species clustered with MWCNT-COOH, but showed no association with either SVI or GR, are presented in Table 3.1 and Table 3.2.

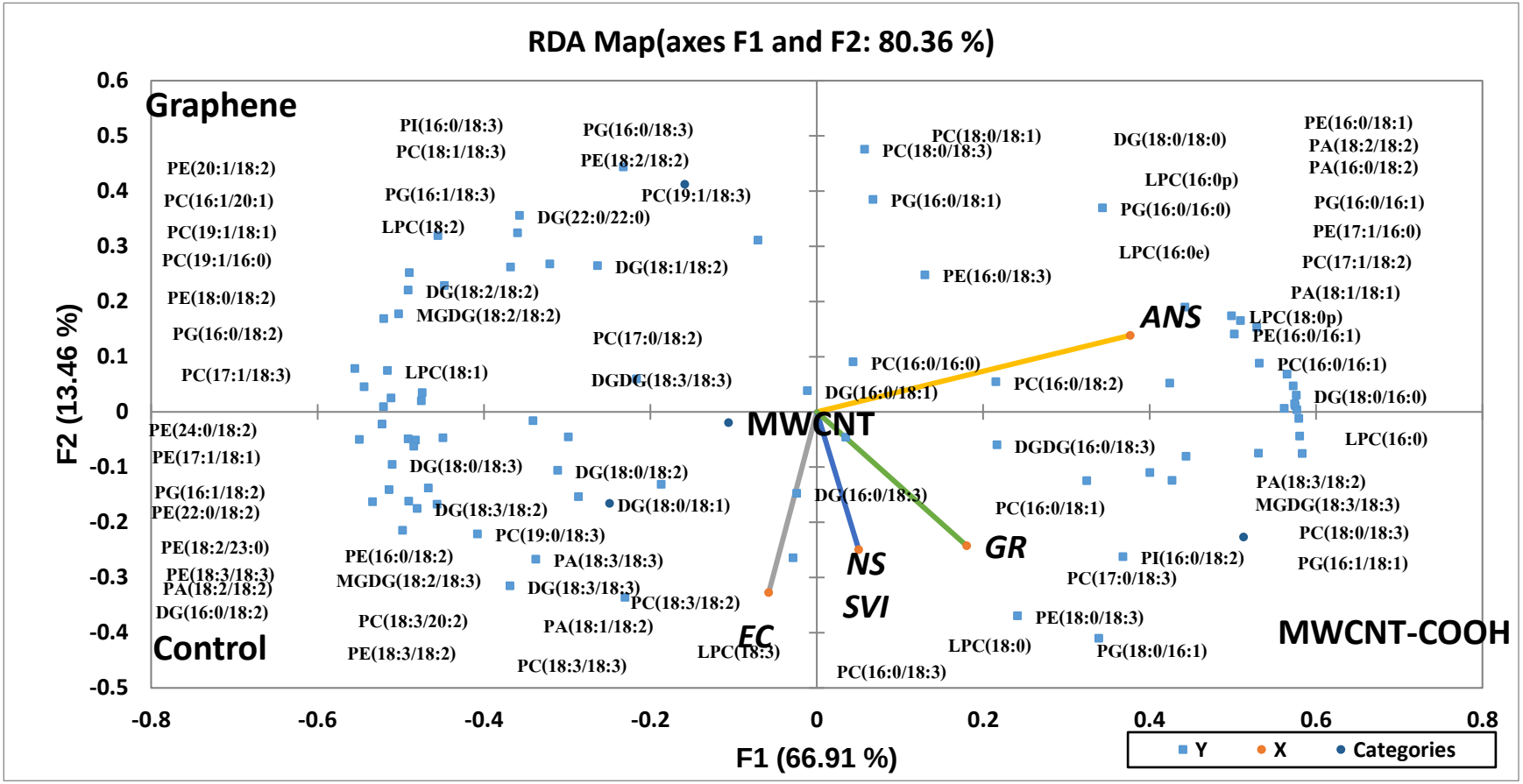


Figure 3.1: First Redundancy analysis (RDA) of bog birch showing the groupings of membrane lipids with seed physiological parameters following seed priming with CNP where n = 100 plants for each treatment. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes, SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings.

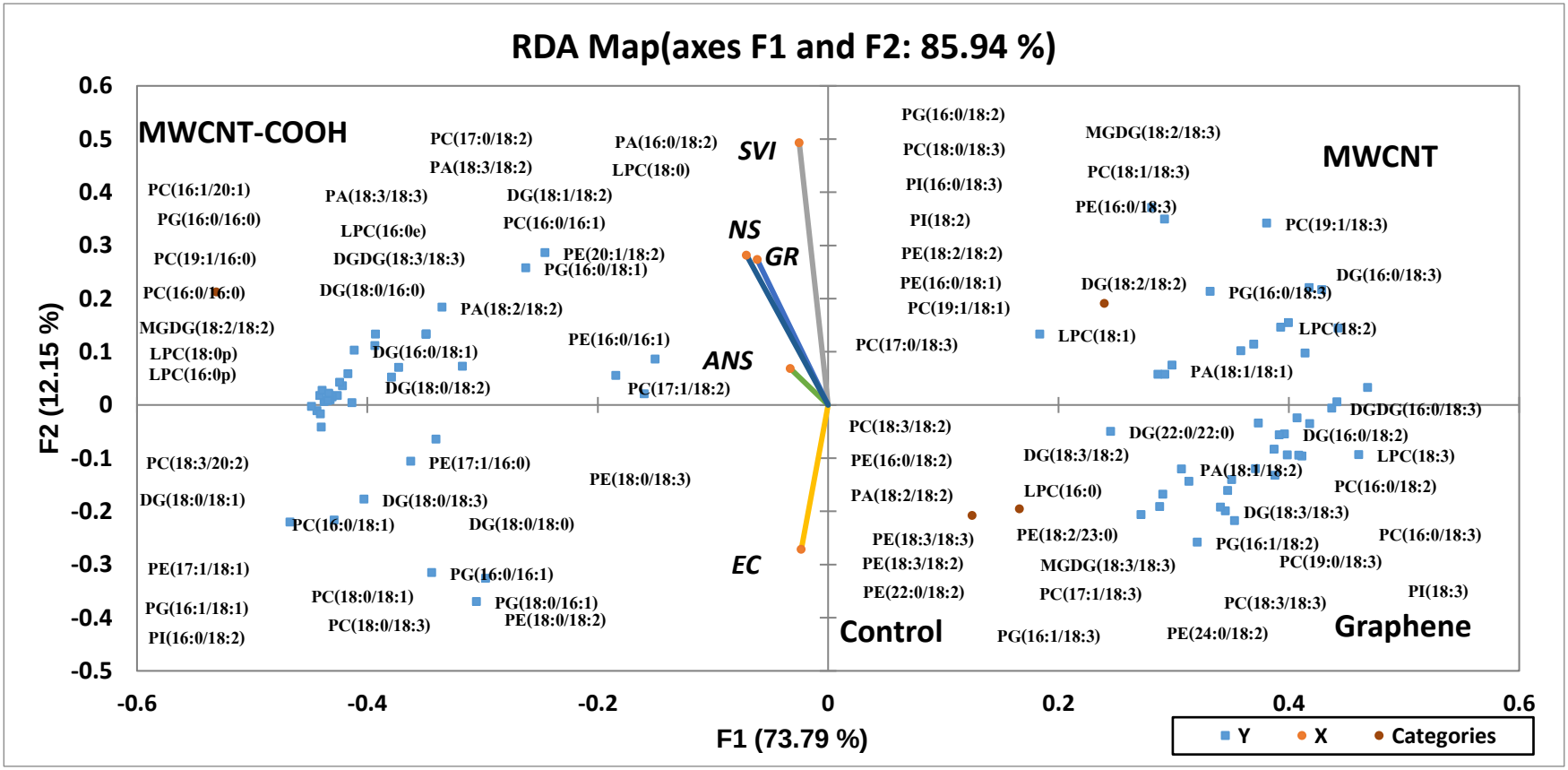


Figure 3.2: First Redundancy analysis (RDA) of labrador tea showing the groupings of membrane lipids with seed physiological parameters following seed priming with CNP, where n = 100 plants for each treatment. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes, SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings.

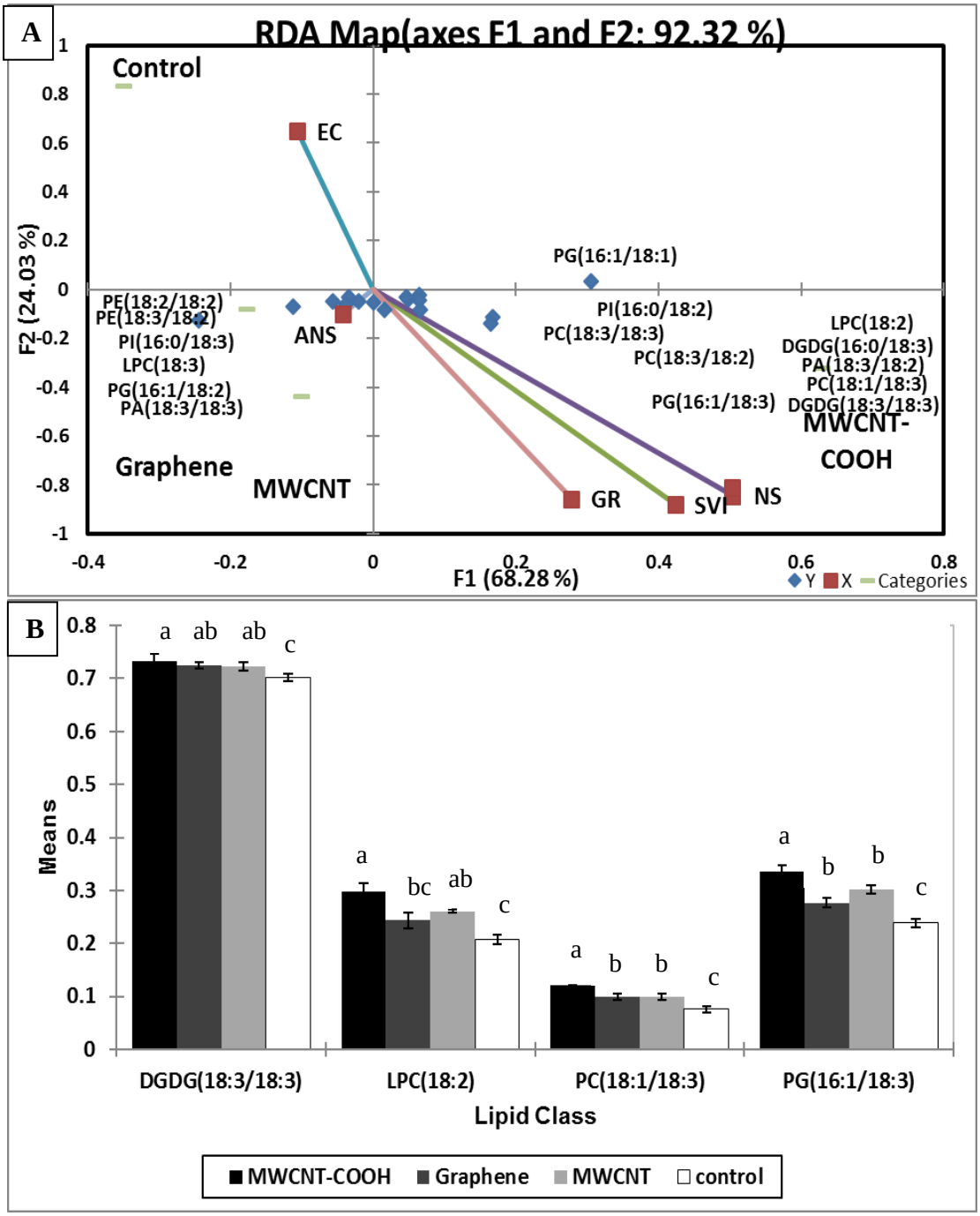


Figure 3.3: A). Second RDA of bog birch for those lipid classes which clustered with MWCNT-COOH treatments during first RDA. B). ANOVA analysis showing the differences in the segregated lipid molecular species following nanopriming. Values represent means ± standard errors and are significantly different at $\alpha = 0.05$, n = 100 plants for each treatment. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes. SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings

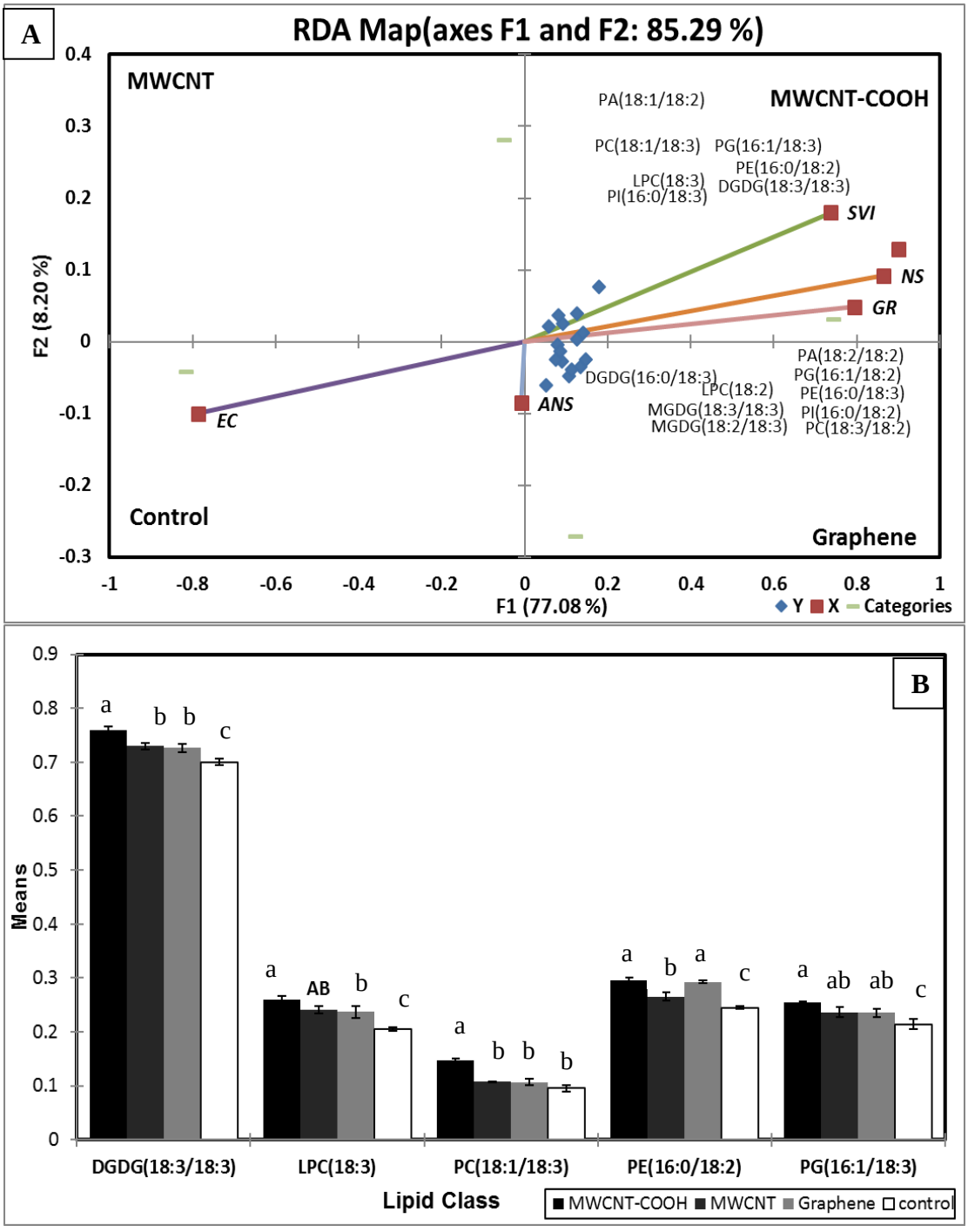


Figure 3.4: A). Second RDA for those lipid classes which clustered with MWCNT-COOH treatments during first RDA in labrador tea. B). Bar chart values represent means ± standard errors and are significantly different at $\alpha = 0.05$, n = 100 plants per treatment. Control = no CNPs added. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes. SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings

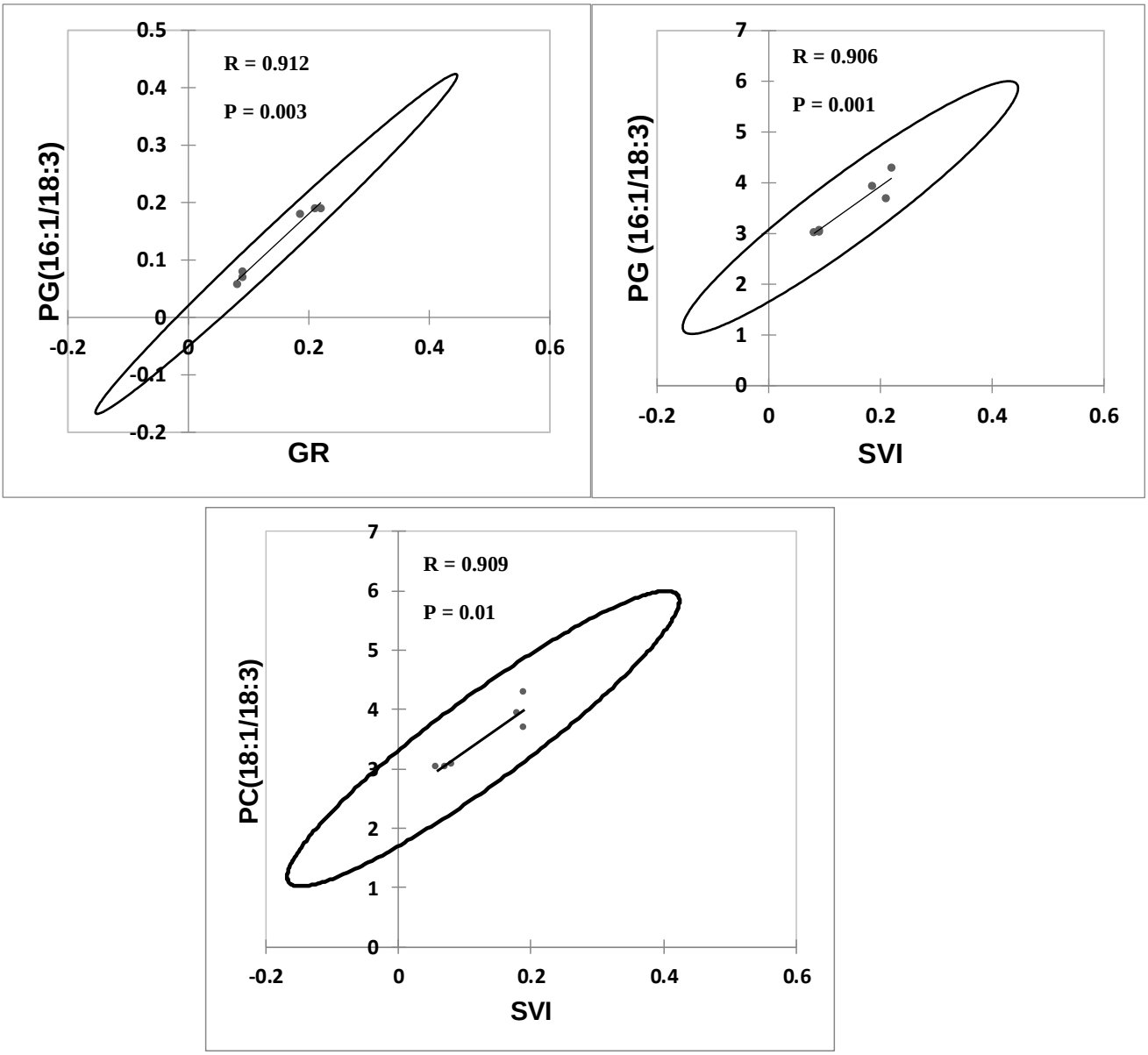


Figure 3.5: Correlations between lipid molecular species and SVI and GR in Peatland boreal forest species. Correlation done for only R1, R2, and R3 of MWCNT-COOH treatments of both bog birch and labrador tea. R = Pearsons correlation coefficient, p = α = at 0.05. n = 100 plants per treatment. SVI = Seedling vigor index, GR = germination rate, PG = phosphatidyl glycerol, PC = Phosphatidylcholine

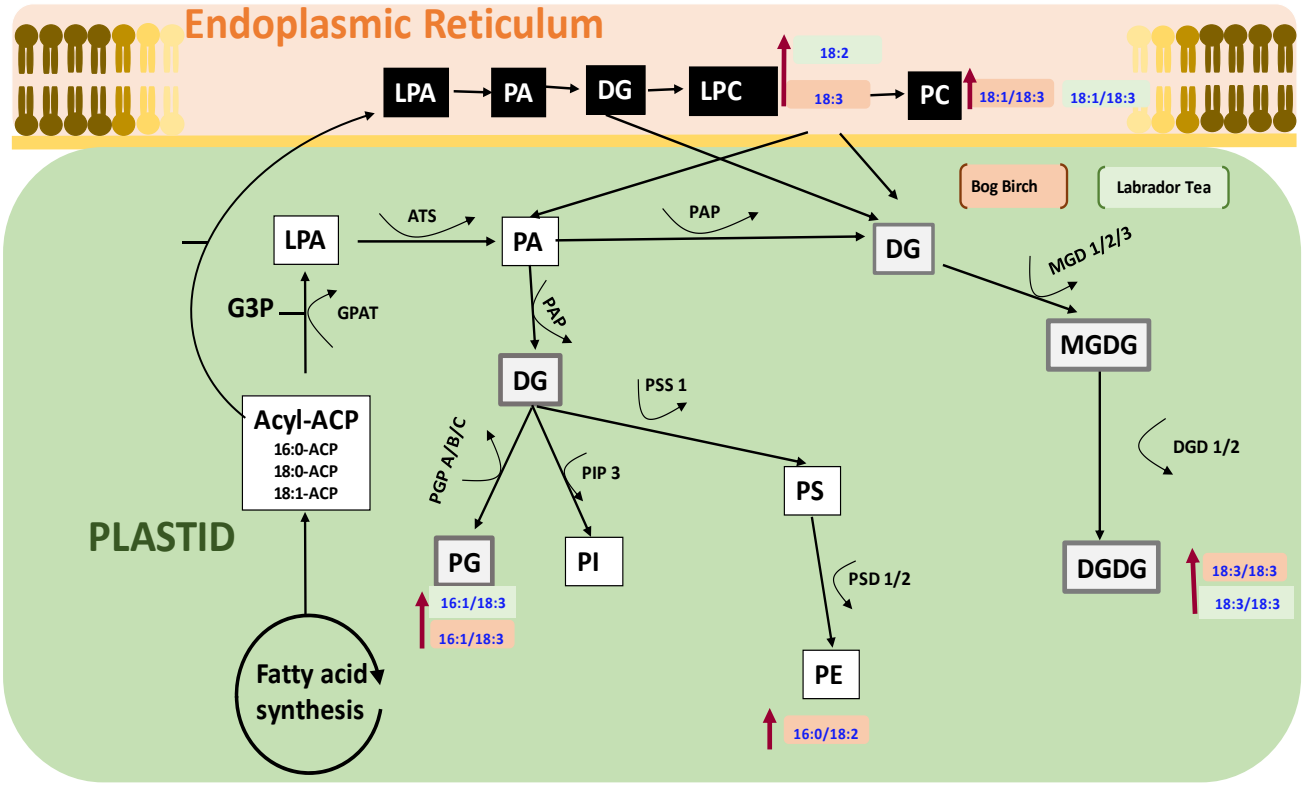


Figure 3.6: KEGG Flow diagram showing the effects of nanopriming with MWCNT-COOH on the membrane lipid metabolism of peatland boreal forest species in response to possible alleviation of seed dormancy. ER = Endoplasmic reticulum, LPA = Lysophosphatidic, PA = Phosphatidic acid, DG = Diacylglycerol, PC = Phosphatidylcholine, PG = Phosphatidyl glycerol, PI = Phosphatidylinositol, PS = Phosphatidylserine, PE = Phosphatidylehanolamine, MGDG = Monogalactosyldiacylglycerol, DGDG = Digalactosyldiacylglycerol, PGP = Glycerol-3-phosphate phosphatase, EPT = Ethanolamine phosphotransferase, DGD = dialkylglycine decarboxylase, PAP = Phosphatidic acid phosphatase, MGDG = Monoalkylglycine decarboxylase, PSD = Phosphatidylserine decarboxylase, PIP = 1-phosphatidylinositol-4-phosphate 5-kinase.

Table 3.1: Lipid molecular species clustered in the same quadrants as SVI and GR following RDA analysis, but are not significantly correlated following Pearson correlation analysis. N = 100 plants per treatment.

Labrador Tea	
Name of lipid Classes	**Level of significance**
PI (16:0/18:2)	NS
PC(18:3/18:3)	NS
PC(18:3/18:2)	NS
PA(18:3/18:2)	NS
DGDG(16:0/18:3)	NS
LPC (18:2)	NS
DGDG(18:3/18:3)	NS

Table 3.2: Lipid molecular species clustered in the same quadrants as SVI and GR following RDA analysis but are not significantly correlated following Pearson correlation analysis. N = 100 plants per treatment.

Bog Birch	
Name of lipid Classes	**Level of significance**
PI (16:0/18:3)	NS
PA(18:1/18:2)	NS
LPC (18:3)	NS
PE (16:0/18:2)	NS
DGDG(18:3/18:3)	NS

3.3.2 Roles of lipid metabolism in overcoming seed dormancy in upland boreal forest species

Approximately 12 membrane lipid classes and associated molecular species were identified in both upland species. These include Cardiolipin (CL), Lysophosphatidylcholine (LPC), LPE (Lysophosphatidylethnolamine), PA (phosphatidic acid), PC (phosphatidylcholine), PE (phosphatidylehanolamine), PG (phosphatidylglycerol), PI (phosphatidylinositol), PS (phosphatidylserine), DGDG (digalactosyldiacylglycerol), MGDG (monogalactosyl diglyceride), and SQDG (sulfoquinovosyl diacylglycerols). First RDA analysis was conducted to discern the effects of the treatments on seedling membrane lipid composition. Seedlings with

similar membrane lipids clustered in the same quadrant of the biplot. For buffalo berry, we analyzed the lipidome for only the treatments of mechanical scarification + stratification + CNPs + gibberellic acid with 20 µg/mL concentration for all treatments, as there was no difference between both 20 µg/mL and 40 µg/mL concentrations in the seedling vigor index, germination rate, or normal and abnormal seedlings in any of the treatments. For green alder, we only evaluated the membrane lipids in CNPs + stratification treatment at 20 µg mL-1 concentrations for the same reason as with buffalo berry.

In first RDA of green alder, PG (16:1/18:1), DG (18:0/18:0), PI (16:0/18:2), PC (17:1/18:2), PA (18:3/18:2), PE (16:0/16:1), DG (18:0/16:0), PA (18:3/18:3), PE (16:0/18:1), PA (18:2/18:2), MGDG (18:2/18:2), PC (18:0/18:1), DGDG (18:3/18:3), PE (16:0/18:3), and PC (17:0/18:3) lipid classes were clustered in Q1 with the MWCNT treatment (Figure 3.7). MGDG (18:3/18:3), PC (18:3/18:3), LPC (18:3), PE (18:3/18:2), DGDG (16:0/18:3), PG (16:1/18:2), LPC (18:2), PE (18:3/18:3), PI (16:0/18:2), PI (16:0/18:3), PA (18:3/18:2), PA (18:2/18:2), DGDG (18:3/18:3), LPC (16:0), PC (18:3/18:2), PG (16:1/18:3), PC (18:1/18:3), PE (16:0/18:2), LPC (18:3), MGDG (18:2/18:3), and PC (19:0/18:3) lipid classes were clustered in Q2 with the MWCNT-COOH treatment , normal seedlings, germination rate, and seedling vigor index. DG (16:0/18:2), PE (24:0/18:2), PA (18:2/18:2), DGDG (16:0/18:3), PE (18:3/18:3), PC (18:3/18:3), MGDG (18:3/18:3), PC (17:1/18:3), LPC (18:0), PC (16:1/20:1), PC (18:0/18:3), PG (16:0/18:3), PE (20:1/18:2), LPC (18:1), PC (19:1/18:3), DG (18:0/18:3), DG (18:2/18:2), PG (16:1/18:2), PG (16:1/18:3), DG (18:3/18:2), PE (22:0/18:2), PE (18:3/18:2), PE (18:2/23:0), and PC (18:3/20:2) lipid classes were clustered in Q3 with the up-con. NP treatment and abnormal seedlings. In

Q4, LPC (16:0e), DG (16:0/18:1), DG (18:0/18:1), DG (18:0/18:2), DG (18:1/18:2), and PC (19:1/18:1) lipid classes were clustered with the control and electrical conductivity. Approximately, 73.2% of the total variability in the data accounted for this segregation (Figure 3.7).

In case of first redundancy of buffalo berry, PC (17:0/18:2), PS (18:2/18:2), PC (16:0/18:3), PS (18:3/18:2), PE (16:0/18:3), PC (16:0/18:2), LPE (18:0), LPC (16:0), and PI (16:0/18:2) clustered in Q1 with the MWCNT treatment. PE (16:0/16:0), PS (17:1/18:2), PE (19:1/ 16:0), PE (17:1/16:0), PE (16:0/16:1), PE (16:0/18:1), LPE (16:0), PA (18:2/18:2), PG (16:0/16:0), PE (16:1/18:1), PC (15:0/18:2), and PA (18:1/18:2) were clustered in Q2 with the Graphene treatments. LPC (18:0), LPC (18:1), PG (16:0/16:1), PE (15:0/18:2), PA (18:0/18:2), PC (16:0/18:1), PC (18:0/18:1), PC (18:0/18:2), PI (16:0/18:1), PC (18:1/18:2), and PI (16:0/18:3) were clustered in Q3 with the control and electrical conductivity. MGDG (18:3/18:3), DGDG (18:0/18:3), LPC (18:2), DGDG (18:3/18:3), PG (16:1/18:3), PI (16:0/18:2), PC (18:1/18:3), LPE (18:2), PC (18:3/18:2), PA (18:3/18:2), PE (18:2/18:2), PE (18:3/18:2), LPC (18:3), PA (18:3/18:3), PS (18:2/18:2), SQDG (16:0/18:1), PI (16:0/18:3), and PG (16:1/18:2) were clustered in Q4 with the MWCNT-COOH treatment, along with germination rate, abnormal seedlings, normal seedlings, and SVI (Figure 3.8).

In the first RDA, quadrant two (Q2) clustered most of the physiological parameters of the seedlings (germination rate, normal and abnormal seedlings, seedlings vigor index) along with the MWCNT-COOH treatment and the associated lipid molecular species for green alder similar to what was observed in the peatland species. A second RDA was also conducted with the lipid classes of Q2{MGDG (18:3/18:3), PC (18:3/18:3), LPC (18:3), PE (18:3/18:2), DGDG (16:0/18:3), PG

(16:1/18:2), LPC (18:2), PE (18:3/18:3), PI (16:0/18:2), PI (16:0/18:3), PA (18:3/18:2), PA (18:2/18:2), DGDG (18:3/18:3), LPC (16:0), PC (18:3/18:2), PG (16:1/18:3), PC (18:1/18:3), PE (16:0/18:2), LPC (18:3), MGDG (18:2/18:3), and PC (19:0/18:3)} clustered with the MWCNT-COOH treatment, as most of the physiological parameters clustered in this quadrant {Figure 3.9(A)}.

Considering the majority of the physiological parameters clustered with MWCNT-COOH, a second RDA was conducted using only the lipid classes/molecular species from Q2 of the first RDA plot to further refine the association between the treatments, altered lipid metabolism, seed germination, and SVI {Figure 3.10(A)}. Following the second RDA of buffalo berry molecular species, most of the lipid classes {DGDG (18:0/18:3), SQDG (16:0/18:1), PE (18:3/18:2), PS (18:2/18:2), LPC (18:2), PC (18:3/18:2), PC (18:1/18:3), PA (18:3/18:2), and PI (16:0/18:3)} were clustered in Q2 with the MWCNT-COOH treatment rather than with the MWCNT, control, or graphene. In case of green alder, most of the lipid classes {PI (16:0/18:2), PG (16:1/18:3), PE (18:3/18:2), DGDG (18:3/18:3), DGDG (16:0/18:3), LPC (18:2), PC (18:1/18:3), PE (18:3/18:3), and PG (16:1/18:2)} were clustered in Q4 and, again, with the MWCNT-COOH treatment {Figure 3.10(A)}. Those clusters were then used to determine the relationship between SVI, germination rate, EC, normal and abnormal seedlings. It was observed from the second RDA map that SVI, germination rate, and normal and abnormal seedlings clustered with the MWCNT-COOH treatment, and the EC were clustered in the control in both upland species. Approximately 72.6% and 90.6% variability in the data accounted for the segregation of buffalo berry and green alder, respectively, in the second RDA analysis.

ANOVA was employed to examine the effects of the lipid molecular species that clustered germination rate, SVI, and NS, with MWCNT-COOH being the best performing treatment. For green alder, PI (16:0/18:2), PG (16:1/18:3), PG (16:1/18:2), PE (18:3/18:2), PC (18:1/18:3), DGDG (18:3/18:3) {Figure 3.9(B)} and for buffalo berry, PI (16:0/18:2), PE (18:3/18:2), PC (18:1/18:3), PA (18:3/18:2) {Figure 3.10(B)} levels were elevated in the MWCNT-COOH treatment compared to the controls ($P<0.05$). Results of the ANOVA showed that for both plant species, priming the seeds with MWCNT-COOH stimulated an increased accumulation of the following lipid molecular species: PI (16:0/18:2), PG (16:1/18:3), PG (16:1/18:2), PE (18:3/18:2), PC (18:1/18:3), DGDG (18:3/18:3), and PA (18:3/18:2). Among all the lipid classes observed to be elevated with MWCNT-COOH after ANOVA, only PC (18:3/18:3) and PE (18:3/18:2) were observed to be common in both upland species, so further correlation was conducted between the seedling vigor index or germination rate and both PC (18:3/18:3) and PE (18:3/18:2) lipid classes using the results from both species (Figure 3.11). We observed PC (18:1/18:3) and PE (18:3/18:2) were positively correlated with the SVI ($r = 0.876$, $p = 0.001$ and $r = 0.921$ and $p = 0.005$ respectively), while PA (18:3/18:2) was strongly correlated with GR ($r = 0.910$ and $p = 0.002$) (Figure 3.11). Lipid molecular species clustered in the same quadrants as SVI and GR following RDA analysis, but are not significantly correlated following Pearson correlation analysis are presented in (Table 3.3 and 3.4).

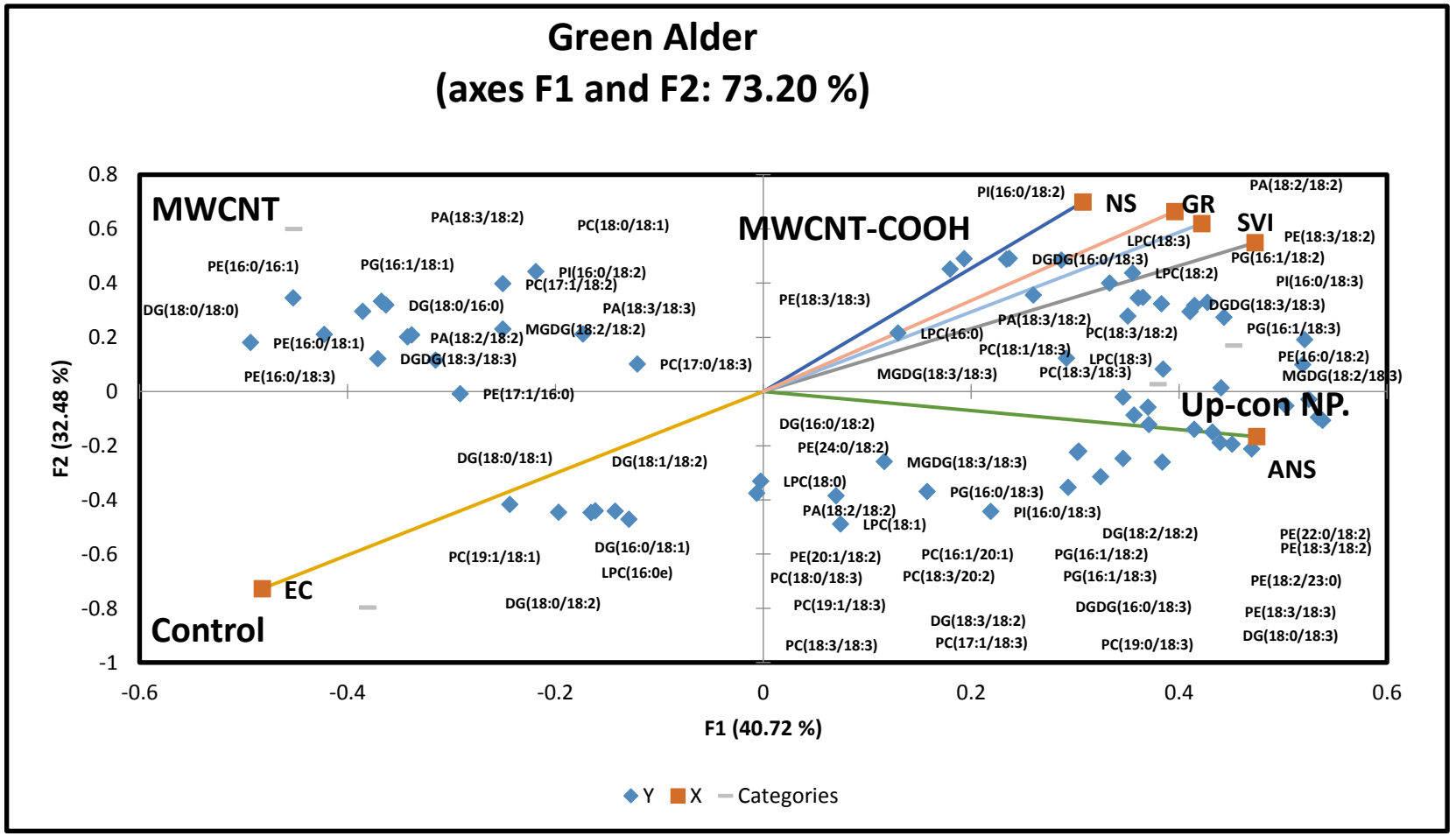


Figure 3.7: First Redundancy analysis (RDA) of Green Alder showing the groupings of membrane lipids with seed physiological parameters following seed priming with CNP where n = 100 plants for each treatment. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes, up-con = up conversion nanophosphors. SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings.

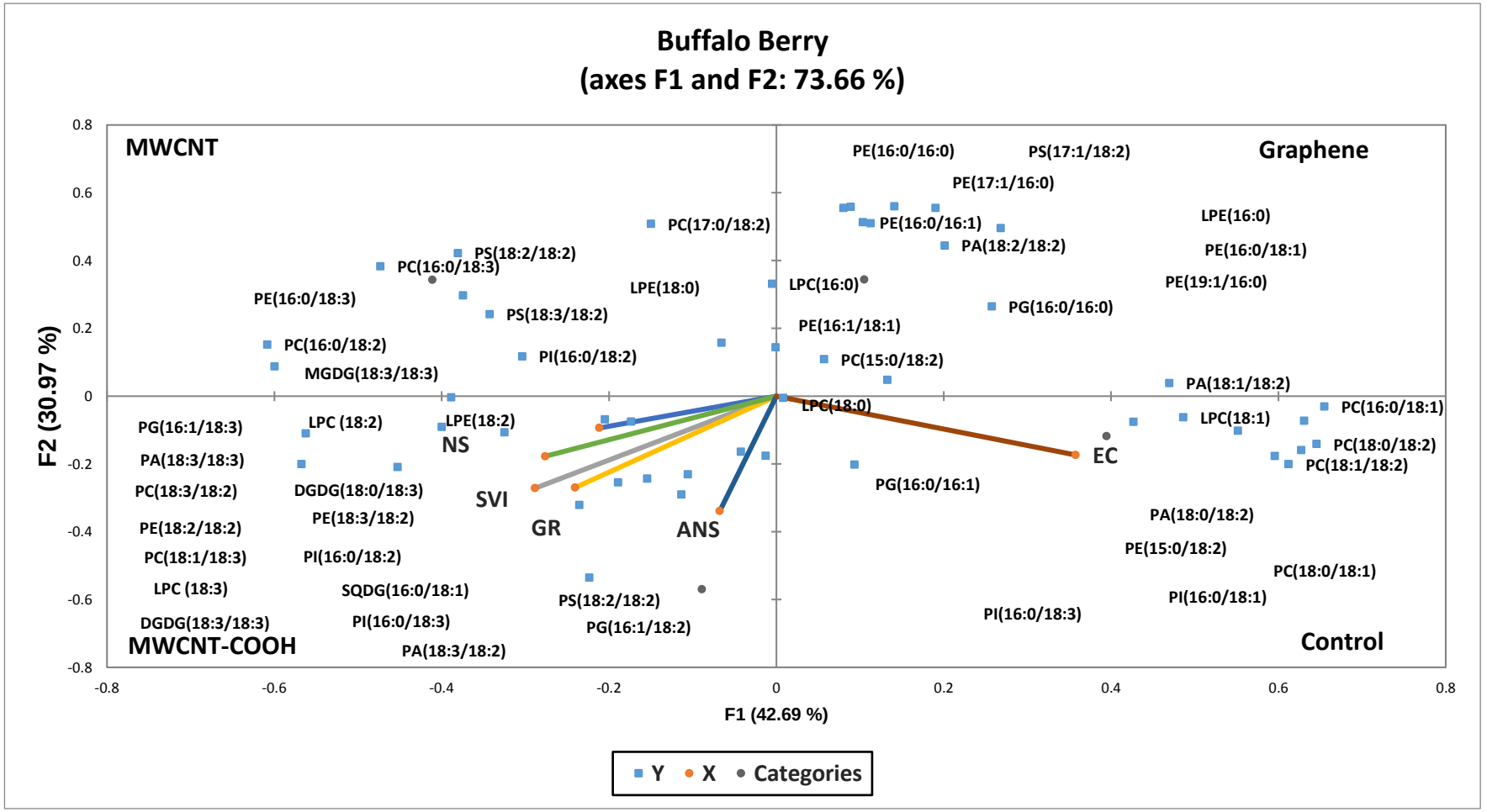


Figure 3.8: First Redundancy analysis (RDA) of Buffalo Berry showing the groupings of membrane lipids with seed physiological parameters following seed priming with CNP where n = 100 plants for each treatment. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, MWCNT= multiwall carbon nanotubes, up-con = up conversion nanophosphors. SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings.

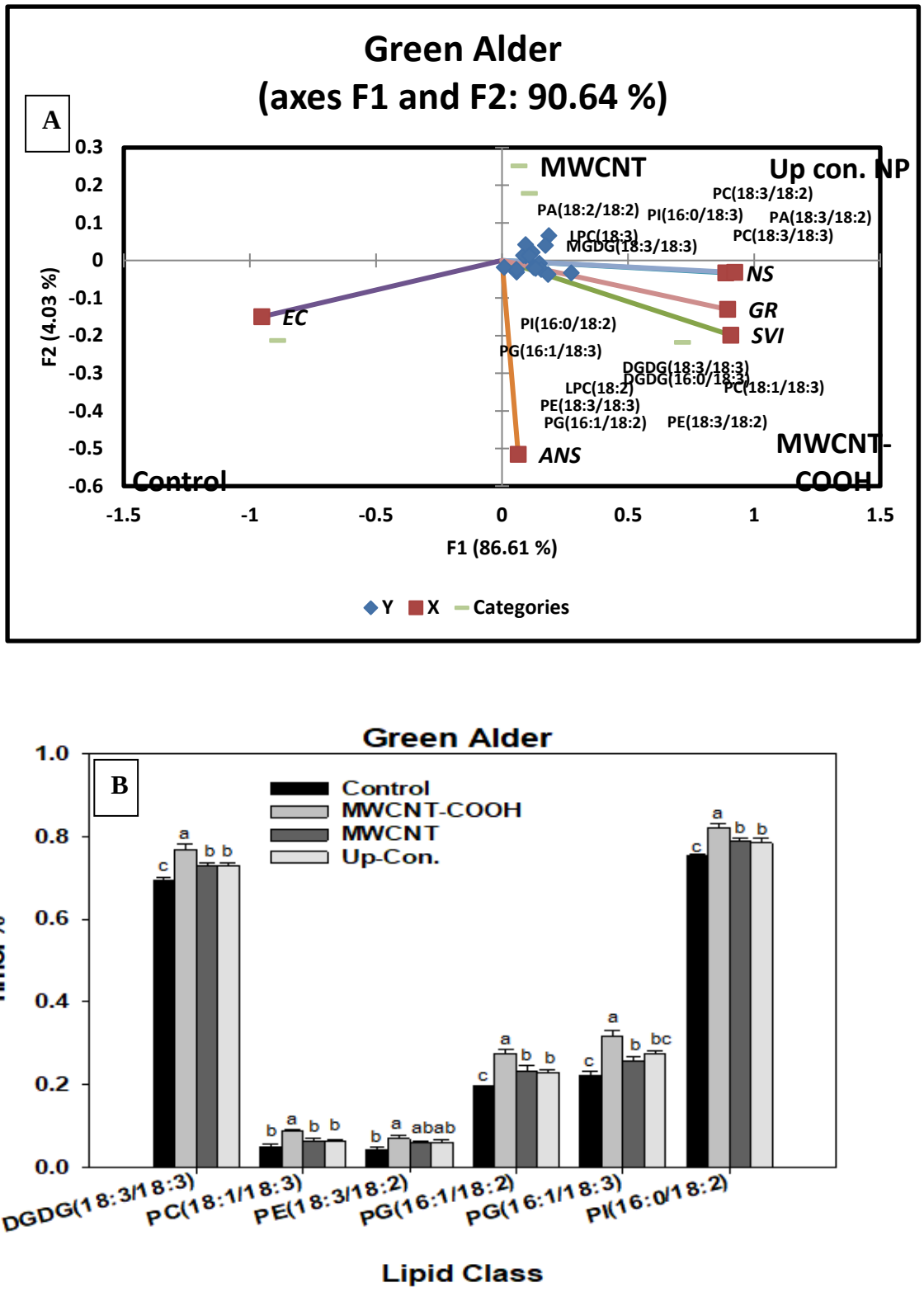


Figure 3.9: A). Second RDA for those lipid classes which clustered with MWCNT-COOH treatments during first RDA in Green Alder. B). Values in bar chart represent means ± standard errors and all are significantly different at $\alpha = 0.05$, n = 100 plants per treatment. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, up con np = up conversion nanophosphors. SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings

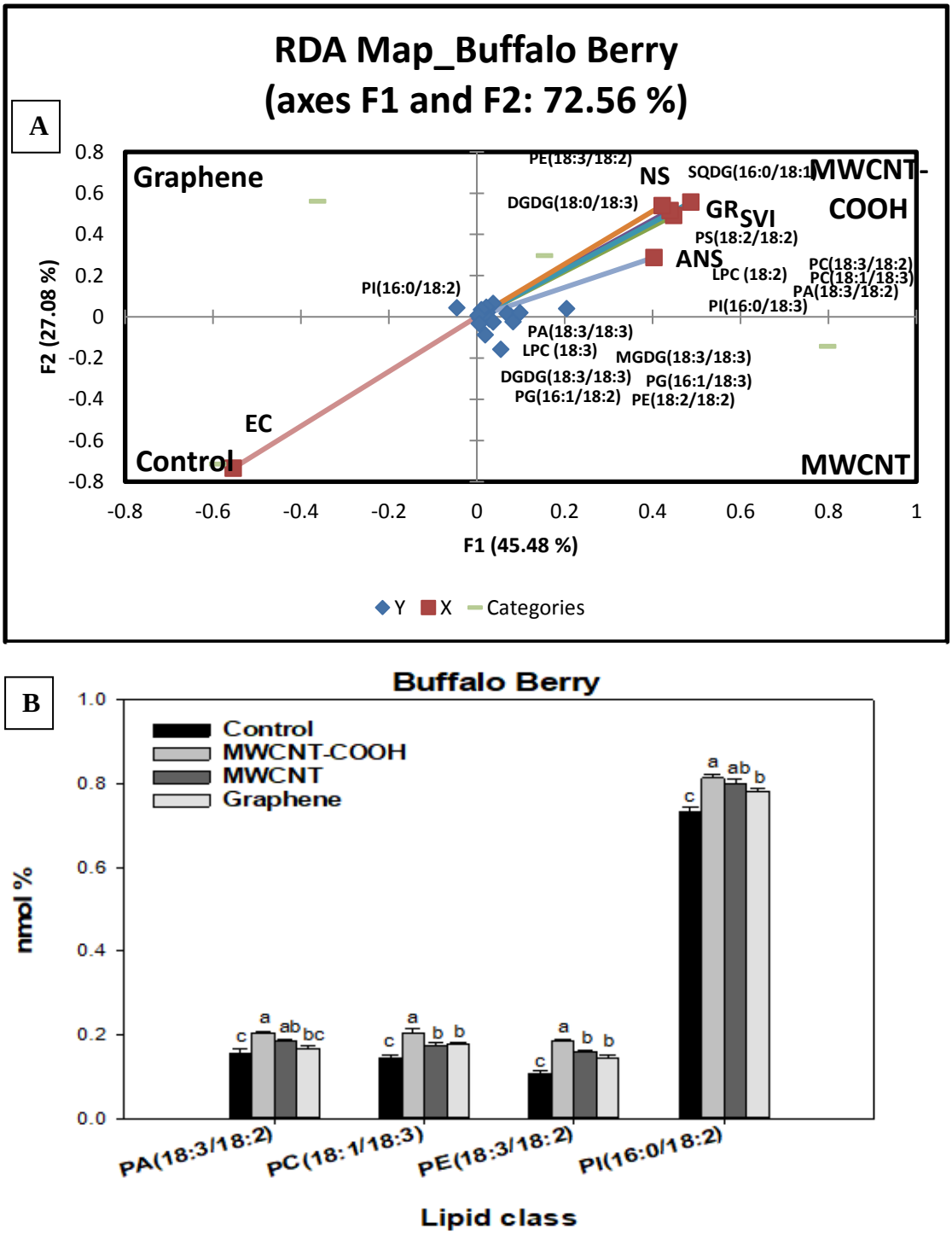


Figure 3.10: A). Second RDA for those lipid classes which clustered with MWCNT-COOH treatments during first RDA in Buffalo Berry. B). Values in bar chart represent means ± standard errors and all are significantly different at α = 0.05, n = 100 plants per treatment. MWCNT-COOH = multiwall carbon nanotubes functionalized with carboxylic acid, SVI = seedling vigor index, EC = Electrical Conductivity, GR = Germination Rate, NS = Normal seedlings, ANS = Abnormal Seedlings

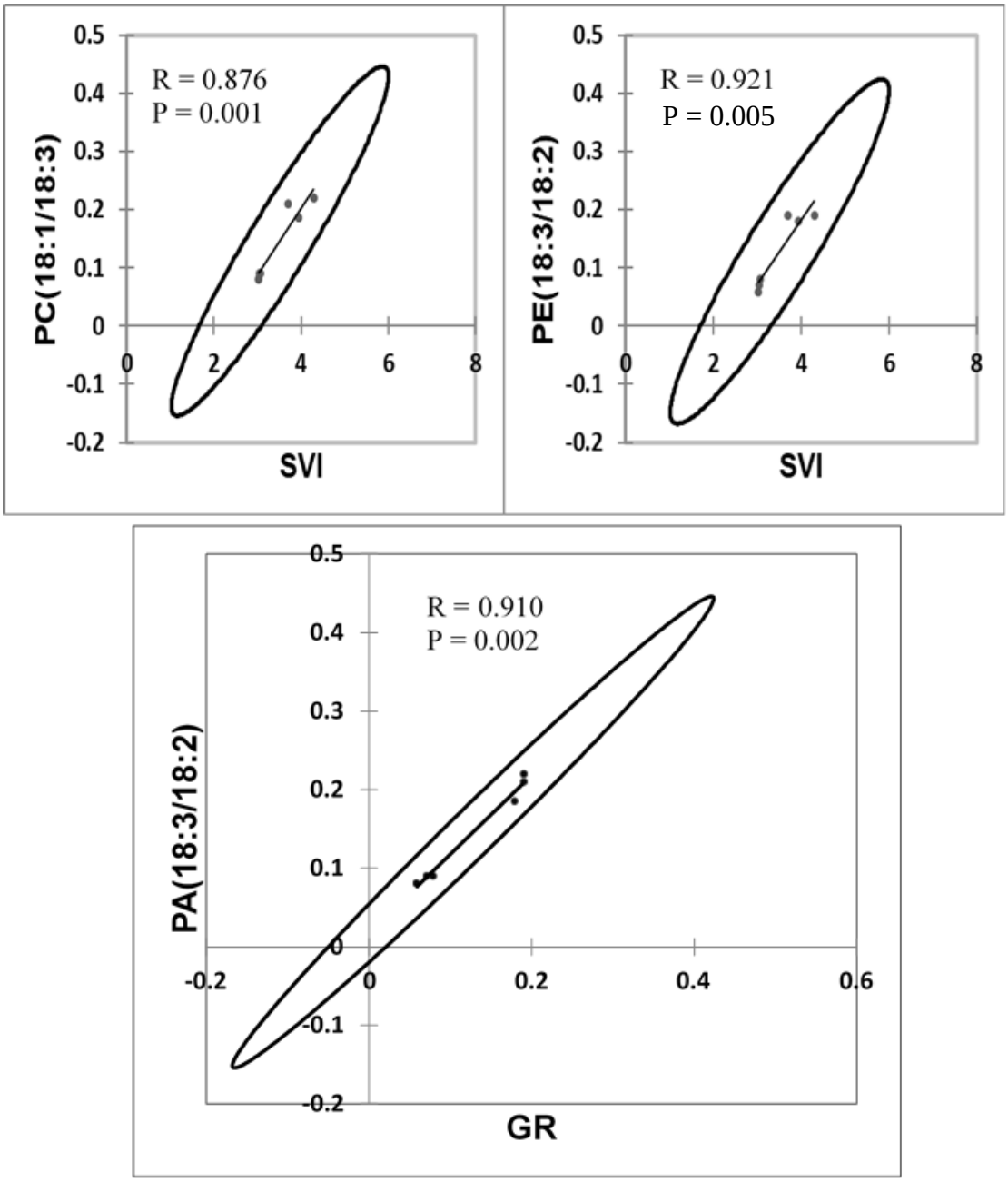


Figure 3.11: Correlation between lipid class vs SVI and GR of upland species. Correlation done for only R1, R2, and R3 of MWCNT-COOH treatments of both buffalo berry and green alder. N = 100 plants per treatment. SVI = Seedling vigor index, GR = germination rate, PC = phosphatidylcholine, PA = Phosphatidic acid, PE = Phosphotidylethanolamine

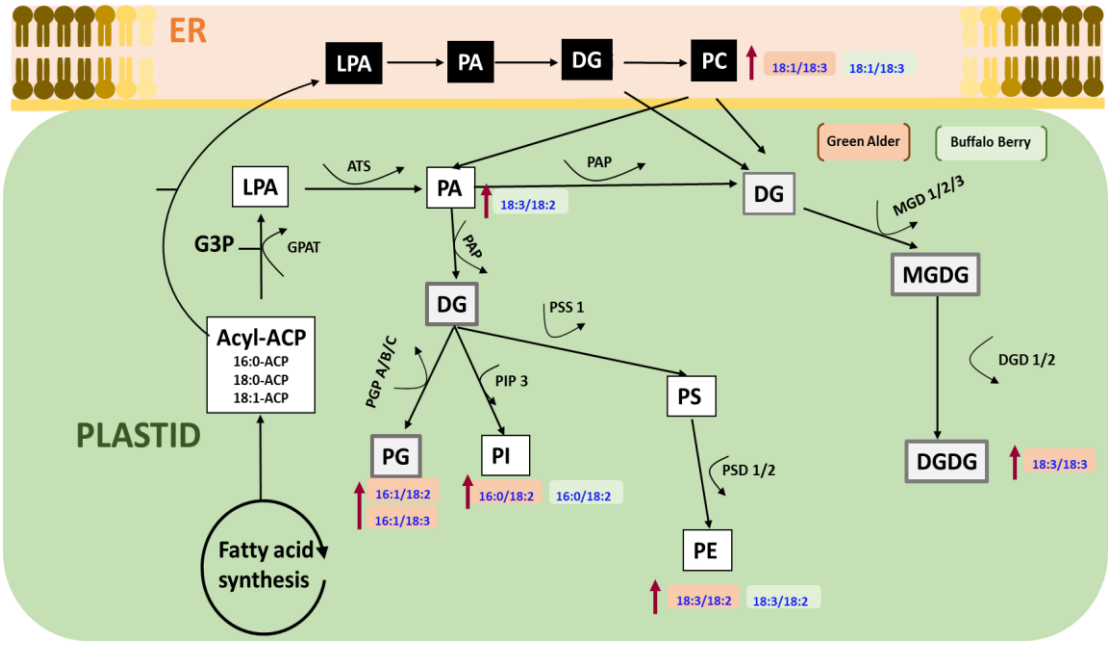


Figure 3.12: KEGG Flow diagram showing the effects of nanopriming with MWCNT-COOH on the membrane lipid metabolism of upland boreal forest species in response to overcoming seed dormancy ER = Endoplasmic reticulum, LPA = Lysophosphatidic, PA = Phosphatidic acid, DG = Diacylglycerol, PC = Phosphatidylcholine, PG = Phosphatidyl glycerol, PI = Phosphatidylinositol, PS = Phosphatidylserine, PE = Phosphatidylehanolamine, MGDG = Monogalactosyldiacylglycerol, DGDG = Digalactosyldiacylglycerol, PGP = Glycerol-3-phosphate phosphatase, EPT = Ethanolamine phosphotransferase, DGD = dialkylglycine decarboxylase, PAP = Phosphatidic acid phosphatase, MGDG = Monoalkylglycine decarboxylase, PSD = Phosphatidylserine decarboxylase, PIP = 1-phosphatidylinositol-4-phosphate 5-kinase.

Table 3.3: Lipid molecular species clustered in the same quadrants as SVI and GR following RDA analysis, but are not significantly correlated following Pearson correlation analysis in Buffalo Berry. N = 100 plants per treatment.

Buffalo Berry	
Name of lipid Classes	**Level of significance**
LPC (18:2)	NS
PC (18:3/18:2)	NS
PI (16:0/18:3)	NS
PS (18:2/18:2)	NS
DGDG (18:0/18:3)	NS
SQDG (16:0/18:1)	NS

Table 3.4: Lipid molecular species clustered in the same quadrants as SVI and GR following RDA analysis, but are not significantly correlated following Pearson correlation analysis in Green Alder. N = 100 plants per treatment.

Green Alder	
Name of lipid classes	**Level of Significance**
DGDG (16:0/18:3)	NS
LPC (18:2)	NS
PE (18:3/18:3)	NS
DGDG (18:3/18:3)	NS
PI (16:0/18:2)	NS
PG (16:1/18:3)	NS
PG (16:1/18:2)	NS

3.4. Discussion

3.4.1 Role of Lipid Metabolism in Overcoming Seed Dormancy in Peatland Boreal Forest Species Following Nano-priming

Nanopriming with CNPs has the potential to modify plant cell structure and physiology (Mahakham et al., 2017). In general, membrane lipids are integral to seed germination processes where they have been observed to undergo significant metabolism and remodeling as seeds germinate (Yu et al., 2015). This involves a series of processes, including a gradual increase of plastidic lipids. For example, the levels of

phospholipids (phosphatidic acid) was shown to be remodeled during germination in soybean seeds, where the level of phosphatidic acid (PA) was observed to initially decrease at the beginning of germination before increasing exponentially during phase I and II , followed by a final decline at the end of germination (Yuri et al., 2008). We observed that the level of different phospholipids changed during the germination of seeds following nanopriming and the resolution of embryo and seed coat dormancy (Figure 2.9 and Figure 2.10).

During the germination of seeds, Elizabeth et al., (1982) found that wheat seeds with high vigor were able to quickly accrue membrane lipids, compared to low vigor seeds which did not; this was attributed to the delay in the start of mitosis by low vigor embryos, in contrast to seeds with high vigor. This implies that the high SVI could be attributed to superior bioaccumulation of membrane lipids, as observed in increasing levels of DGDG (18:3/18:3), PC (18:1/18:3), PG (16:1/18:3), and LPC (18:1/18:3) in the nano primed seedlings, compared to the control.

According to RDA, different species of phospholipids clustered with physiological parameters (SVI, GR, NS). This was also MWCNT functionalized with COOH had the highest effect among all physiological parameters (germination rate, SVI, EC, normal and abnormal seedlings, etc.). This finding could be attributed to the ability of functionalized multi walled nanoparticles to penetrate the phospholipid bilayer of the cell to cause a change in the structure while acting as a "nanoneedle" in bilayer (Kraszewski et al., 2012).

To better understand the connections between membrane phospholipid remodeling and SVI or GR, we conducted a Pearson's correlation analysis and discovered LPC (18:2) was highly correlated with GR and PC (18:1/18:3), and PG (16:1/18:3) correlated with SVI. LPC, PC, and PG are vital phospholipids in the cell membrane, and the synthesis or turnover of these phospholipids appears to be associated with improved seed germination and seedling vigor as shown in our proposed KEGG pathway (Figure 2.12). In bog birch, DGDG (18:3/18:3), LPC (18:2), PC (18:1/18:3), and PG (16:1/18:3) molecular species were altered following nanopriming, and the altered lipids were highly correlated with improvement in seed vigor and germination (Figure 2.9). All these molecular species are connected biosynthetically (Maatta et al., 2012), where PG (16:1/18:3) is synthesized from DG in the presence of Glycerol-3-phosphate phosphatase (PGP) A/B/C enzyme, PC (18:1/18:3) synthesized from DG by ethanolamine phosphotransferase (EPT) enzyme, and DGDG (18:3/18:3) synthesized from MGDG by dialkylglycine decarboxylase (DGD) 1/2 enzyme. PG and DGDG synthesis occur in the plastid of the cell, while PC synthesis is in the endoplasmic reticulum (ER) of the cell. C18:3 enriched molecular PG, LPC, PC, and DGDG species appears to be enhanced in bog birch treated with MWCNT–COOH.

In the case of labrador tea, DGDG (18:3/18:3), LPC (18:3), PC (18:1/18:3), PE (16:0/18:2), and PG (16:1/18:3) molecular species were altered in response to nanopriming with CNP, in particular MWCNT–COOH. DGDG (18:3/18:3) is synthesized from MGDG in the presence of DGD 1/2 enzyme, PC (18:1/18:3) is synthesized from DG, and PE (16:0/18:2) is synthesized from PS with the help of PSD

1/2 (Phosphatidylserine decarboxylases) enzyme. PC synthesis occurred in the endoplasmic reticulum of cell, and other types of lipid are synthesized in the plastid. It appears that during the resolution of seed coat and embryo dormancy in Bog birch, MWCNT–COOH modulated the accumulation of C18:3 enriched molecular LPC, PG, PE, PC, and DGDG species (Figure 2.10).

An overall increase in C18:3 enriched molecular species in response to nanopriming in dormant seeds of both peatland species evaluated implies a Δ3 desaturase enzyme played an important role in the modulation of the membrane lipid. In this study, PC (18:1/18:3) and PG (16:1/18:3) were also highly correlated with the seedling vigor index (Figure 2.11). PC, LPC, PG, DGDG, and PE molecular species enriched with C18:3 fatty acids clustered together with MWCNT-COOH primed seeds and may be associated with the improved dormancy SVI and GR observed in this study. This finding indicates that CNP, in particular MWCNT-COOH, appears to play a role in breaking seed coat and embryo dormancies through the modulation of C18:3 enriched molecular species, and suggests that these species are highly associated with improved seedling vigor and overall germination in bog birch and labrador tea (peatland species).

3.4.2 Role of Lipid Metabolism in Overcoming Seed Dormancy in Upland Boreal Forest Species Following Nanopriming

A limited number of studies have shown that alterations in cell membrane lipids may be involved in the alleviation of seed dormancy in agriculture crop species (Yu et al., 2015; Hilhorst, 1998; Taylorson, 1987). Changes in seed fatty acids have been

reported to be directly associated with alleviating seed embryo dormancy, and a remarkable change in linoleic acid levels in *Amaranthus albus* seeds before and after breaking dormancy was observed to be associated with the reported success (Taylorson, 1987). Haghighi et al. (2014) found that the seed germination was higher on media containing CNTs (10 - 40 g mL-1) and alteration of the membrane by CNTs in tomato seeds increased their rates of germination and growth. It was observed among all the treatments, MWCNT functionalized with COOH has a greater effect on all the targeted physiological seed parameters (germination rate, SVI, EC, normal and abnormal seedlings, etc.) than other treatments.

PC (18:1/18:3) and PE (18:3/18:2) were observed to be highly correlated with seedling vigor index (SVI). These lipid molecular species were elevated in both buffalo berry and green alder primed with MWCNT COOH. Conversely, PA (18:3/18:2) was highly correlated with GR (Figure 3.12). PA, PC, and PE are important phospholipids, which exhibited a high rate of synthesis during the germination of soya bean plant seeds (Harwood, 1976). PE is the second most abundant phospholipid in plant cell membranes and plays an important role in cell membrane synthesis and function, and, therefore, in plant growth and development. Also, PC and PE metabolisms are very closely related (Fuqiang et al., 1997). Membrane lipids are known to change during germination (Yu et al., 2015). In our study, CNP may be significantly stimulating these changes considering PA (18:3/18:2), PC (18:1/18:3) and PE (18:3/18:2) were highly positively correlated with SVI and GR.

In the KEGG pathway, the increased trend of 18:3 enriched lipid species means that the Δ3 desaturase enzyme seems to play a vital role in modulating the membrane lipid (Figure 3.13). According to Lee & Kim (2012), self-assemblies of mixtures of carbon nanotube and lipids have affected lipid structure, especially when phospholipids are grafted or engrafted with nanoparticles at different sizes.

As mentioned earlier, in buffalo berry, PI (16:0/18:2), PE (18:3/18:2), PC (18:1/18:3) and PA (18:3/18:2) molecular species were elevated with MWCNT-COOH compared to the control treatment. These molecular species are related biosynthetically, as shown in the lipid metabolism pathway (KEGG) (Figure 3.13). PI (16:0/18:2) is synthesized from DG by the PIP3 {Phosphatidylinositol (3,4,5)-trisphosphate} enzyme, PE (18:3/18:2) is synthesized from PS by the PSD1/2 (Phosphatidylserine decarboxylases) enzyme, PA (18:3/18:2) is synthesized from LPA with the presence of the ATS (Acetyl Hydrolase) enzyme and PC (18:3/18:3) is synthesized from DG with the presence of CDP choline. PI, PE and PA synthesis occur in the plastid, but PC synthesis occurs in the endoplasmic reticulum. C18:3 and C18:2 enriched molecular PI, PE, PC and PA species appear to be enhanced in buffalo berry treated with MWCNT-COOH.

In green alder, the PI (16:0/18:2), PG (16:1/18:3), PG (16:1/18:2), PE (18:3/18:2), PC (18:1/18:3), and DGDG (18:3/18:3) molecular species of lipids are also elevated in the MWCNT-COOH treatment compared to the control and other treatments, and they are also connected biosynthetically (Figure 3.13). PI (16:0/18:2) is synthesized from DG by the PIP3 {Phosphatidylinositol (3,4,5)-trisphosphate} enzyme, PE (18:3/18:2)

is synthesized from PS by the PSD1/2 (Phosphatidylserine decarboxylases) enzyme, and PC (18:3/18:3) is synthesized from DG. But PG (16:1/18:2) and PG (16:1/18:3) is synthesized from DG with the help of the PGP (Phosphoglycolate phosphatase) A/B/C enzyme while DGDG (18:3/18:3) is synthesized from MGDG with the help of the DGD (Dialkylglycine decarboxylase) ½ enzyme.

Recently, nanoparticles have been shown to modulate lipid metabolism in plants. Martínez-Ballesta et. al., (2016) stated that nanopriming with CNPs increased the aquaporins, ion, and water transportation in cell membranes. Also, these authors found that CNPs helped in maintaining electrostatic or homeostasis balance in cell membranes and that the membrane forms new lipid domains, or raft, in broccoli, as a consequence of the CNPs induced lipid metabolism. Our findings demonstrated that that carboxylic acid functionalized MWCNT altered the seed membrane lipid metabolism in dormant seeds having both seed coat and embryo dormancies. Both improved germination and seedling vigor were observed to be highly correlated with the altered lipid species shown in the proposed lipid biosynthetic pathway (Figure 3.13). Specifically, the biosynthetic routes of PC to PA to DG seem to be modulated by MWCNT-COOH during the alleviation of seed coat and embryo dormancies resulting in improved germination and SVI.

3.5. Conclusion

Our study aimed to understand how nanotechnologies could be applied to improve seed germination by overcoming seed dormancy by modulating lipid membrane metabolism. The improved germination and SVI were observed to be

clustered with several membrane lipid molecular species enriched with C18:3 fatty acids. These lipids {PE (18:3/18:2), PC (18:1/18:3), PG (16:1/18:3), LPC (18:1/18:3) and PA (18:3/18:2)} were observed to be significantly elevated compared to the control. Of the elevated molecular species, PE (18:3/18:2), PC (18:1/18:3), PG (16:1/18:3), and LPC (18:1/18:3) were highly correlated with seed germination and seedling vigor index. These results indicate that MWCNT functionalized with carboxylic acids may be useful in resolving seed dormancy in both peatland and upland boreal forest species by modulating cell membrane lipid metabolism. The proposed metabolic pathway of the membrane lipids showed that C18:3 enriched fatty acids play a key role in overcoming seed coat and embryo dormancy. This approach could be useful in improving the propagation of seeds of non-resource species for boreal forest reclamation or restoration, particularly species with seed dormancy issues.

3.6. References

Ahmet Korkmaz, N. O. and B. E. (2004). Assessment of Vigor Characteristics of Processing Tomato Cultivars by Using Various Vigor Tests. *Asian Journal of Plant Sciences*, *3*, 181–186.

Alberta Environment. (1995). *Reclamation criteria for wellsites and associated facilities - 1995 update*. https://doi.org/ENV-069-OP

Alberta Environment and Sustainable Resource Development. (2013). *2010 Reclamation Criteria for Wellsites and Associated Facilities for Forested Lands (Updated July 2013)*.

Ali, A. and Elozeiri, A. (2017). Metabolic Processes During Seed Germination: Advances in Seed Biology. *London: IntechOpen Limited*. https://doi.org/10.5772/intechopen.70653

Amen, R. D. . (1967). The Effects of Gibberellic Acid and Scarification on the Seed Dormancy and Germination in Luzula spicata. *Physiol. Plant.*, *20*, 6–12.

Angela M. Madeiras, T. H. B. and W. R. A. (2009). Stratification, Gibberellic Acid, Scarification, and Seed Lot Influence on Rosemary Seed Germination. *Seed Technology*, *31*, 55–65.

Begum, P., Ikhtiari, R., & Fugetsu, B. (2014). Potential Impact of Multi-Walled Carbon Nanotubes Exposure to the Seedling Stage of Selected Plant Species. *Nanomaterials*, *4*(4), 203–221. https://doi.org/10.3390/nano4020203

Benamar, A., Tallon, C., & Macherel, D. (2003). Membrane Integrity and Oxidative

Properties of Mitochondria Isolated from Imbibing Pea Seeds After Priming or Accelerated Ageing. *Seed Science Research*, *13*(1), 35–45. https://doi.org/DOI: 10.1079/SSR2002122

Bentsink, L., & Koornneef, M. (2008). Seed Dormancy and Germination. *The Arabidopsis Book*, *6*, e0119. https://doi.org/10.1199/tab.0119

Bewley and Hallett. (2002). Membranes and seed dormancy: beyond the anaesthetic hypothesis. *Seed Science Research*, *12*(2), 69–82.

Bhattacharya, S., Puri, S., Jamwal, A., & Sharma, S. (2012). Studies on seed germination and seedling growth in Kalmegh (Andrographis paniculata Wall. Ex Nees) under abiotic stress conditions. *International Journal of Science, Environment and Technology* (Vol. 1), 44-76.

Bligh, G. and Dyer, J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology. 37(8):911-917.*

Bottalico, F., Travaglini, D., Fiorentini, S., Lisa, C., & Nocentini, S. (2014). Stand dynamics and natural regeneration in silver fir (*Abies alba* Mill.) plantations after traditional rotation age. *iForest - Biogeosciences and Forestry*, *7*(5), 313–323. https://doi.org/10.3832/ifor0985-007

Canas, J. E.; Long, M.; Nations, S.; Vadan, R.; Dai, L.; Luo, M.; Ambikapathi, R.; Lee, E. H.; Olszyk, D. (2008). Effects of Functionalized and Nonfunctionalized Single-Walled Carbon Nanotubes on Root Elongation of Select Crop Species. *Environ. Toxicol. Chem.*, *27*, 1922–1931.

Chaodumrikul, S., Kaewsorn, P., Chulaka, P., & Chanprasert, W. (2016). Breaking seed dormancy in smooth loofah (Luffa cylindrica (L.) M. Roem.) using scarification and dry heat treatment. *Agriculture and Natural Resources*, *50*(2), 85–88. https://doi.org/10.1016/j.anres.2015.09.003

Cheng, H. N., Klasson, K. T., Asakura, T., & Wu, Q. (2016). Nanotechnology in agriculture. *ACS Symposium Series*, *1224*(7), 233–242.

Choi, H., & Frangioni, J. (2010). Nanoparticles for biomedical imaging: Fundamentals of clinical translation. *Molecular Imaging*, *9*(6), 291–310.

de Paiva, E. P., Torres, S. B., de Almeida, J. P. N., Sá, F. V. da S., & Oliveira, R. R. T. (2017). Tetrazolium test for the viability of gherkin seeds. *Revista Ciencia Agronomica*, *48*(1), 118–124. https://doi.org/10.5935/1806-6690.20170013

Elizabeth, M., McDonnell, G., & Mirbahar, B. (1982). Membrane lipids and phosphatidyl choline turnover in embryos from germinating low and high vigor wheat. *Journal of Experimental Botany.* 33, pp. 631-642

Farahani, H. A., Maroufi, K., & Sani, B. (2012). Seedling Production in Nigella (*Nigella sativa L* .) under Nanopriming Method. *Journal of Medicinal Plants Research.* 4(16): 1612-1616

Ferguson, J. M., Keys, R. D., McLaughlin, F. W. and Warren, J. M. (1991). Seed and Seed Quality. *NC State Extension*, 448.

Fuqiang, J., and Thomas, S. (1997). Enzymes of the primary phosphatidylethanolamine biosynthetic pathway in postgermination Castor Bean endosperm. *Plant Phisiol.*,

115(4):1589-1597.

Haghighi, M., & Silva, J. A. T. (2014). The effect of carbon nanotubes on the seed germination and seedling growth of four vegetable species. *Journal of Crop Science and Biotechnology*, *17*(4), 201–208.

Harayama, T., & Riezman, H. (2018). Understanding the diversity of membrane lipid composition. *Nature Reviews Molecular Cell Biology*, *19*, 281. Retrieved from http://dx.doi.org/10.1038/nrm.2017.138

Harman, G. E., Khan, A. A., Tao, K. L., & Khan, A. A. (1976). Physiological changes in the early stages of germination of pea seeds induced by aging and by infection by a storage fungus , Aspergillus ruber. *Can. J. Bot.*, (54), 39–44.

Harwood, L. (1976). Synthesis of Molecular Species of Phosphatidylcholine and Phosphatidylethanolamine by Germinating Soya Bean. *Phytochemistry*, (15), 1459–1463.

Hilhorst, H. (1998). The regulation of secondary dormancy. *Seed Science Research*, 8(2), 77-90.

Ibrahim K., and Khalid S. (2017). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*. (10): 21-29.

ISTA. (2006). International Rules for Seed Testing. Edition 2006. *International Seed Testing Association, Switzerland.*

John O. and Emmanuel O. (1993). Changes in lipid content and composition during germination of groundnuts. *SCI*, *62*(2), 147–155.

Josef J. and Katarian K. (2015). Application of nanotechnology in agriculture and food industry, its prospects and risks. *Ecol Chem Eng S., 22(3):321-361*.

Kobayashi, Y., Nabeta, K., & Matsuura, H. (2010). Chemical Inhibitors of Viviparous Germination in the Fruit of Watermelon. *Plant and Cell Physiology*, *51*(9), 1594–1598. http://dx.doi.org/10.1093/pcp/pcq103

Kottegoda, N., Munaweera, I., Madusanka, N., & Karunaratne, V. (2011). A green slow-release fertilizer composition based on urea-modified hydroxyapatite nanoparticles encapsulated wood. *Current Science*, *101*(1), 73–78.

Kraszewski, S., Bianco, A., Tarek, M., & Ramseyer, C. (2012). Insertion of short amino-functionalized single-walled carbon nanotubes into phospholipid bilayer occurs by passive diffusion. *PLoS ONE*, *7*(7), 1–11.

Lee, H., & Kim, H. (2012). Self-assembly of lipids and single-walled carbon nanotubes: Effects of lipid structure and PEGylation. *Journal of Physical Chemistry C*, *116*(16), 9327–9333. https://doi.org/10.1021/jp3010663

Li-beisson, Y. N. Y. (2016). *Lipids in Plant and Algae Development* (Vol. 86). Springer. Retrieved from http://link.springer.com/10.1007/978-3-319-25979-6

Li-Shar Huang and Claus Grunwald. (1990). Lipid and Fatty Acid Changes During Germination of Alfalfa Seeds. *Phytochemistry*, *29*(5), 1441–1445.

Lin, D., & Xing, B. (2007). Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth. *Environmental Pollution*, *150*(2), 243–250. https://doi.org/10.1016/j.envpol.2007.01.016

Liu, Q., Chen, B., Wang, Q., Shi, X., Xiao, Z., Lin, J., & Fang, X. (2009). Carbon nanotubes as molecular transporters for walled plant cells. *Nano Letters*, *9*(3), 1007–1010. https://doi.org/10.1021/nl803083u

Liu, X., Zhang, H., Zhao, Y., Feng, Z., Li, Q., Yang, H.-Q., … He, Z.-H. (2013). Auxin controls seed dormancy through stimulation of abscisic acid signaling by inducing ARF-mediated ABI3 activation in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(38), 15485–15490. https://doi.org/10.1073/pnas.1304651110

Ma, Z., Bykova, N. V., & Igamberdiev, A. U. (2017). Cell signaling mechanisms and metabolic regulation of germination and dormancy in barley seeds. *Crop Journal*, *5*(6), 459–477. https://doi.org/10.1016/j.cj.2017.08.007

Maatta, S., Scheu, B., Roth, M. R., Tamura, P., Li, M., Williams, T. D., … Welti, R. (2012). Levels of Arabidopsis thaliana Leaf Phosphatidic Acids, Phosphatidylserines, and Most Trienoate-Containing Polar Lipid Molecular Species Increase during the Dark Period of the Diurnal Cycle. *Frontiers in Plant Science*, *3*, 49. https://doi.org/10.3389/fpls.2012.00049

Mahakham, W., Sarmah, A. K., Maensiri, S., & Theerakulpisut, P. (2017). Nanopriming technology for enhancing germination and starch metabolism of aged rice seeds using phytosynthesized silver nanoparticles. *Scientific Reports*, *7*(1), 1–21. https://doi.org/10.1038/s41598-017-08669-5

Martínez-Ballesta, M. C., Zapata, L., Chalbi, N., & Carvajal, M. (2016). Multiwalled carbon nanotubes enter broccoli cells enhancing growth and water uptake of plants

exposed to salinity. *Journal of Nanobiotechnology*, *14*(1), 42. https://doi.org/10.1186/s12951-016-0199-4

Matsui, K. and Fukutomi, M. et al. (2004). A tomato lipase homologous to DAD1 (LeLId1) is induced in post-germinative growing stage and encodes a triacylglycerol lipase. *FEBS Letters*. 569(3):195-200.

Matthews, S. and Powell, A. . (1981). *Electrical conductivity test. In Vigour Test Handbook. (ed. D. A. Perry). International Seed Testing Association, Zurich*.

Mondal, A., Basu, R., Das, S., & Nandy, P. (2011). Beneficial role of carbon nanotubes on mustard plant growth: An agricultural prospect. *Journal of Nanoparticle Research*, *13*(10), 4519–4528. https://doi.org/10.1007/s11051-011-0406-z

Monticelli, L., Salonen, E., Ke, C., & Vattulainen, I. (2009). Effects of carbon nanoparticles on lipid membranes : a molecular simulation perspective. *Soft Matter*. (22): 4433–4445. https://doi.org/10.1002/b912310e

Mukherjee, A., peralta-videa, J., Gardea, J. and White, J. (2016). Effects and Uptake of Nanoparticles in Plants. In: Xing B, Vecitis CD, Senesi N (eds) Engineered nanoparticles and the environment: biophysicochemical processes and toxicity. Wiley, Hoboken. https://doi. org/10.1002/9781119275855.ch20

Pourkhaloee, A., Haghighi, M., Jamal Saharkhiz, M., Jouzi, H., & Mahdi, M. (2011). Carbon Nanotubes Can Promote Seed Germination via Seed Coat Penetration. *Seed Technology*. 155-169. (Vol. 33).

Prasad, R., Bhattacharyya, A., & Nguyen, Q. D. (2017). Nanotechnology in Sustainable

Agriculture: Recent Developments, Challenges, and Perspectives. *Frontiers in Microbiology*, *8*, 1014. https://doi.org/10.3389/fmicb.2017.01014

Ramos, K. M. O., Matos, J. M. M., Martins, R. C. C., & Martins, I. S. (2012). Electrical Conductivity Testing as Applied to the Assessment of Freshly Collected *Kielmeyera coriacea* Mart. Seeds. *ISRN Agronomy*, *2012*, 1–5. https://doi.org/10.5402/2012/378139

Sekhon B. (2014). Nanotechnology in agri-food production: an overview (Vol. 7).

Shanti B., Ann G. and Justin S. (2013). A Community Approach for Landscape Planning. *Integral Ecology Group Ltd.,* 49-70.

Sharma, P., Mishra, M., Singh, N. I., & Chauhan, J. S. (2011). Determination of Integrity of Seed Membrane Systems and Mineral Ions Leakage in Horse Gram. *World Journal of Agricultural Sciences. 7*(4), 476–479.

Smirnova, E. A., Gusev, A. A., Zaitseva, O. N., Lazareva, E. M., & Onishchenko, G. E. (2011). Multi-walled Carbon Nanotubes Penetrate into Plant Cells and Affect the Growth of Onobrychis arenaria Seedlings. *Acta Naturae*. *3*(8), 99–106.

Smýkal, P., Vernoud, V., Blair, M. W., Soukup, A., & Thompson, R. D. (2014). The role of the testa during development and in establishment of dormancy of the legume seed. *Frontiers in Plant Science*, *5*, 351.

Srilatha B. (2011). Nanotechnology in Agriculture. *J Nanomedic Nanotechnol*, (2), 123.

Srinivasan, C., & Saraswathi, R. (2010). Nano-agriculture - carbon nanotubes enhance tomato seed germination and plant growth. *Current Science*, *99*(3), 274–275.

Tan, X. ming, Lin, C., & Fugetsu, B. (2009). Studies on toxicity of multi-walled carbon nanotubes on suspension rice cells. *Carbon*, *47*(15), 3479–3487.

Tanoue, R., Kobayashi, M., Katayama, K., Nagata, N., & Wada, H. (2014). Phosphatidylglycerol biosynthesis is required for the development of embryos and normal membrane structures of chloroplasts and mitochondria in Arabidopsis. *FEBS Letters*, *588*(9), 1680–1685. https://doi.org/10.1016/j.febslet.2014.03.010

Taylorson, R. C.-H. and R. B. (1987). Changes In Lipid Fatty Acids Associated With Dormancy Breaking In Amaranthus Albus Seeds. *Journal of Seed Technology*, *11*(1), 15–22.

Tiwari, D. K., Dasgupta-Schubert, N., Villaseñor Cendejas, L. M., Villegas, J., Carreto Montoya, L., & Borjas García, S. E. (2014). Interfacing carbon nanotubes (CNT) with plants: enhancement of growth, water and ionic nutrient uptake in maize (Zea mays) and implications for nanoagriculture. *Applied Nanoscience*, *4*(5), 577–591. https://doi.org/10.1007/s13204-013-0236-7

Vieira, R. D., Scappa Neto, A., Bittencourt, S. R. M. de, & Panobianco, M. (2004). Electrical conductivity of the seed soaking solution and soybean seedling emergence. *Scientia Agricola*, *61*(2), 164–168. https://doi.org/10.1590/S0103-90162004000200007

Wang, E. C., & Wang, A. Z. (2014). Nanoparticles And Their Applications In Cell And Molecular Biology. *Integrative Biology : Quantitative Biosciences from Nano to Macro*, *6*(1), 9–26. https://doi.org/10.1039/c3ib40165k

Yan, D., Duermeyer, L., Leoveanu, C., & Nambara, E. (2014). The Functions of the Endosperm During Seed Germination. *Plant and Cell Physiology*, *55*(9), 1521–1533. http://dx.doi.org/10.1093/pcp/pcu089

Yanga, J., Cao, W., & Rui, Y. (2017). Interactions between nanoparticles and plants: Phytotoxicity and defense mechanisms. *Journal of Plant Interactions*, *12*(1), 158–169. https://doi.org/10.1080/17429145.2017.1310944

Yin, L., Colman, B. P., McGill, B. M., Wright, J. P., & Bernhardt, E. S. (2012). Effects of Silver Nanoparticle Exposure on Germination and Early Growth of Eleven Wetland Plants. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0047674

Yu, X., Li, A., & Li, W. (2015). How membranes organize during seed germination: three patterns of dynamic lipid remodelling define chilling resistance and affect plastid biogenesis. *Plant Cell Environ.*, *38*(7), 1391–1403.

Yuri R., Maryna O., Aravind R., and Jitendra B.(2008). Interaction of Nanoparticles with Lipid Membrane. *Nano Lett.*, *8*(3), 941–944.

Zeng, Y. J., & Wang, Y. R. (2009). Methods of topographical tetrazolium testing for seed viability of Nitraria tangutorum Bobr. and N. sibirica Pall. *Seed Science and Technology*, *37*(3), 691–698.

Zhang, H., Dong, J., & Yu, H. et al. (2019). Research progress in membrane lipid metabolism and molecular mechnism in peanut cold tolerance. *Front Plant Sci.*, *10*, 838.

General Summary

In conclusion, the results of our study revealed that the application of CNPs (nanotechnology) significantly enhanced seed germination and seedling growth potential by overcoming seed dormancy and by changing different plant physiological parameters (germination rate, SVI, normal seedlings, etc.) perhaps by modulating cell membrane lipids. The findings in this study demonstrate that priming seeds with a 20 µg/mL concentration of MWCNT-COOH and GA along with stratification at 2 to 4°C was very effective in resolving seed dormancy in two upland boreal forest species: green alder and buffalo berry and two peatland boreal forest species: bog birch and labrador tea. The results of this study offer a wide range of possibilities of different CNPs in plant research, especially the boreal forest plant species from upland and peatland ecosystems. In this study, we also tried to determine whether remodeling of cell membrane lipidome is associated with the improved germination rate, electrical conductivity, seedling vigor index observed during the resolution of seed dormancy. Among different types of CNPs, seeds primed with MWCNT-COOH appear to mediate seed dormancy by modulating the biosynthesis of membrane lipids. PA, PC, PG, PI, and DG species appear to be remodeled and were associated with the improved germination and seedling vigor observed in the evaluated upland and peatland boreal forest species. Specifically, PC (18:1/18:3) and PG (16:1/18:3) had a positive correlation with SVI and LPC (18:1/18:3) correlated with GR following nanopriming with MWCNT-COOH in both peatland species. Also, PC (18:1/18:3) and PE (18:3/18:2) had a positive correlation with SVI, and PA (18:3/18:2) correlated with GR

in both green alder and buffalo berry seeds primed with MWCNT-COOH characteristic of upland boreal forest ecosystems. In all lipid classes, an overall increase of 18:3 enriched lipid in all four species was observed. This indicates that Δ3 desaturase enzyme played an important role along with the help of nanopriming with MWCNT-COOH in modulating seed membrane lipids, and the alleviation of seed dormancy. The observed increase in SVI and GR, which has great importance in overcoming the challenges associated with mass propagation of these native non resource boreal peatland and upland species for forest reclamation, revegetation or regeneration activities following anthropogenic disturbance such as oil and gas resource mining. Future work to apply the approach to other species with similar and other seed dormancy issues would be useful to further validate the proposed mechanism and effectiveness of the nanopriming approach to enhance germination in other plant species with seed dormancy issues.