SEAWEED EXTRACT AND HUMIC ACIDS BIOSTIMULANTS TO IMPROVE GROWTH AND POST-HARVEST QUALITY OF SPINACH AND LETTUCE

by

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Table of Contents

List of Tables ..... **v**

List of Figures ..... **vi**

Abstract ..... **x**

List of Abbreviations and Symbols Used ..... **xi**

Acknowledgements ..... **xiv**

Chapter 1 INTRODUCTION **1**

1.1 Plant biostimulants 3

1.2 Seaweeds 3

1.3 *Ascophyllum nodosum* 4

1.4 Humic acids 5

1.5 Lettuce 5

1.6 Spinach 6

1.7 Project hypothesis 6

1.8 Objectives 7

Chapter 2 LITERATURE REVIEW **8**

2.1 Background information on plant biostimulants 8

2.2 Role of plant biostimulants 9

2.3 General composition of humic acids 12

2.4 *Ascophyllum nodosum* extract (ANE) on plant growth and crop yield 14

2.5 Humic acids (HA) on root growth, nutrient uptake, yield and productivity 15

2.6 Importance of bioactive compounds in postharvest shelf life 16

Chapter 3 MATERIALS AND METHODS **20**

3.1 Seed material 20

3.2 Preparation of *Ascophyllum nodosum* and Humic acids extracts 20

3.3 Seed germination assay 21

3.4 Early growth assay 22

3.5 Preparation of *Ascophyllum nodosum* and humic acids extracts to test on the post-harvest shelf life of lettuce and spinach 23

3.6 Plant culture 23

3.7 Sample preparation and storage 24

3.8 Weight loss 24

3.9 Visual quality analysis 25

3.10 Determination of lipid peroxidation 25
3.11 Determination of pigments (chlorophyll a and b, contents) 25
3.12 Determination of anthocyanin content 26
3.13 Determination of total ascorbic acid 26
3.14 Determination of total phenolics content 27
3.15 Determination of total antioxidants 27
3.16 Statistical analysis 28
Chapter 4 RESULTS 29
4.1 Results on seed germination parameters of lettuce seedlings 29
4.2 Results on early growth parameters of lettuce plants 35
4.3 Results on seed germination parameters of spinach seedlings 38
4.4 Results on early growth parameters of spinach plants 44
4.5 Effect of different treatments of ANE, HA and ANE + HA on post-harvest shelf life quality of lettuce during storage period 47
4.5.1 Determination of weight loss of lettuce during storage 47
4.5.2 Visual quality analysis of lettuce at storage period 50
4.5.3 Lipid peroxidation analysis 54
4.5.4 Determination of total ascorbic acid 55
4.5.5 Determination of total phenolics content 56
4.5.6 Determination of total antioxidants 57
4.5.7 Determination of pigments (chlorophyll a, chlorophyll b, and anthocyanins contents) 58
4.6 Effect of different treatments of ANE, HA and ANE +HA on post-harvest shelf life quality of spinach during storage period 60
4.6.1 Determination of weight loss of spinach during storage period 60
4.6.2 Visual quality analysis of spinach during storage period 63
4.6.3 Determination of Lipid peroxidation 68
4.6.4 Determination of total ascorbic acid 69
4.6.5 Determination of total phenolics content 70
4.6.6 Determination of total antioxidants 71
4.6.7 Determination of pigments (chlorophyll a, chlorophyll b, and anthocyanins contents) 72
Chapter 5 DISCUSSION 75
5.1 The effect of ANE, HA, and ANE+HA on seed germination and early growth of lettuce and spinach 76

5.2 Physiological and biochemical studies on lettuce and spinach during storage period to determine post-harvest quality 79
Chapter 6 CONCLUSION **83**
References **85**
APPENDIX – A **108**

List of Tables

Table.1	Composition of *Ascophyllum nodosum* extract	11
Table.2	Elemental compositions of humic acids	13
Table.3	Common growth responses to soil drench or foliar application of seaweed extract or humic acid application as cited in the literature	19
Table.4	Different concentrations/ test solutions of ANE and HA alone, or in combination were evaluated for their efficacy in improving seed germination and early growth parameters of lettuce and spinach	21
Table.5	Different concentrations of ANE, HA and ANE + HA on pigments (chlorophyll a, chlorophyll b, and anthocyanins contents) of lettuce were evaluated on day 10 and day 21 of storage conditions	59
Table.6	Different concentrations of ANE, HA and ANE + HA on pigments (chlorophyll a, chlorophyll b, and anthocyanins contents) of spinach were evaluated on day 14, day 21 and day 28 of storage conditions	73-74

List of Figures

Figure.1 Effect of ANE, HA and ANE+HA on germination percentage of lettuce seedlings after 24 hours 30

Figure.2 Effect of ANE, HA and ANE+HA on mean seed germination of lettuce seedlings after 24 hours 31

Figure.3 Effect of ANE, HA and ANE+HA on radicle length of lettuce seedlings on 7^{th} day of germination 32

Figure.4 Lettuce seedlings at 7 days of germination 33

Figure.5 Effect of ANE, HA and ANE+HA on plumule length of lettuce seedlings on 7^{th} day of germination 34

Figure.6 Effect of ANE, HA and ANE+HA on Seedling vigour index of lettuce seedlings after 24 hours 35

Figure.7 Pre-harvest treatment application of ANE, HA and ANE+HA on lettuce plants to determine (7A) fresh weight, and (7B) dry weight 36-37

Figure.8 Effect of ANE, HA and ANE+HA on germination percentage of spinach after 36 hours of germination 39

Figure.9 Effect of ANE, HA and ANE+HA on mean seed germination of spinach after 36 hours of germination 40

Figure.10 Representing figure of spinach seedlings photographed on 7^{th} day of germination 41

Figure.11 Effect of ANE, HA and ANE+HA on radicle length of spinach seedlings after 36 hours 42

Figure.12 Effect of ANE, HA and ANE+HA on plumule length of spinach seedlings after 36 hours 43

Figure.13 Effect of ANE, HA and ANE+HA on seedling vigour index of spinach seedlings after 36 hours 44

Figure.14 21 day's pre-harvest treatment application of ANE, HA and ANE+HA on spinach plants to determine (14A) fresh weight, and (14B) dry weight 45-46

Figure.15 The postharvest fresh weight loss in lettuce treated with different concentrations of ANE, HA, and ANE+HA held up to 21 days storage period at 0-4°c in the dark with RH ≥ 95% 48

Figure.16 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on dry matter content (DMC)(g/100g FW) of lettuce held up to 21 days storage period at 0-4°c in the dark with RH ≥ 95% 49

Figure.17 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on (17A) colour (17B) Turgor and (17C) Visual quality of lettuce held over 21 days storage period at 0-4°c in the dark with RH ≥ 95% 51-52

Figure.18 Lettuce control leaves, and leaves treated with different concentrations of ANE, HAs, and ANE+HAs held over 21 days storage period at 0-4°c in the dark with RH ≥ 95% 53

Figure.19 The Effect of ANE, HA and ANE+HA on MDA content of lettuce on day 10 and day 21 at storage conditions 54

Figure.20 The Effect of ANE, HA and ANE+HA on total ascorbate content of lettuce on day 10 and day 21 at storage conditions 55

Figure.21 The Effect of ANE, HA and ANE+HA on total phenolics content of lettuce on day 10 and day 21 at storage conditions 56

Figure.22 The Effect of ANE, HA and ANE+HA on total antioxidants content of lettuce on day 10 and day 21 at storage conditions 57

Figure.23 The postharvest fresh weight loss in spinach treated with different concentrations of ANE, HAs, and ANE+HAs held upto 28 days storage period at 0-4°c in the dark with RH ≥ 95% 61

Figure.24 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on dry matter content (DMC) of spinach upto over 28 days storage period at 0-4°c in the dark with RH ≥ 95% 62

Figure.25 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on (A) colour (B) Turgor and (C) Visual quality of lettuce held over 21 days storage period at 0-4°C in the dark with RH ≥ 95% 64-65

Figure.26 Spinach control leaves, and leaves treated with different concentrations of ANE, HA, and ANE+HA held over 28 days' storage period at 0-4°c in the dark with RH ≥ 95% 66-67

Figure.27 Effect of ANE, HA and ANE+HA on lipid peroxidation of spinach on day 14, 21 and day 28 at storage conditions 68

Figure.28 Effect of ANE, HA and ANE+HA on total ascorbates of spinach on day 14, 21 and day 28 in storage conditions 69

Figure.29 Effect of ANE, HA and ANE+HA on total phenolic contents of spinach on day 14, 21 and day 28 in storage conditions 70

Figure.30 Effect of ANE, HA and ANE+HA on total antioxidant capacity of spinach on day 14, 21 and day 28 at storage conditions 71

Abstract

The leafy greens are highly perishable vegetables that are affected by pre-harvest and post-harvest conditions. Biostimulants can be utilized to enhance germination, growth, yield and quality of the crops. In this study, the effect of two plant biostimulants, *Ascophyllum nodosum* extract (ANE) and humic acid (HA) and their combinations (ANE+ HA) were evaluated for their potential to improve growth and minimize post-harvest losses in lettuce and spinach. Thirteen combinations of ANE and humic acids were assessed for seed germination and early growth of lettuce and spinach. Among these, most effective treatments were used to analyze post-harvest quality of lettuce and spinach. In the laboratory, the application of ANE, HA, and ANE+HA significantly ($p \leq 0.05$) improved germination and early growth parameters of lettuce and spinach. The combination treatment, 0.25 % ANE and 0.2 % HA (T_{12}) showed 103.2% and 13.1% increase in the radicle length of lettuce and spinach, respectively. Similarly, plumule length was also higher in the presence of ANE, HA, and ANE+HA. Under the greenhouse conditions the weekly application the biostimulants improved biomass in all treatments but fresh and dry biomass in lettuce treated with 0.25 % ANE and 0.2 % HA (T_{12}) were 103.1% and 113.9% respectively, compared with control. Whereas in spinach T_{12} had increased fresh and dry biomass by 62.9% and 103.3%, respectively. Pre-harvest treatment of lettuce and spinach with the combined ANE and HA significantly reduced fresh biomass loss during storage at 4ºC for up to 21days. Further visual appearance quality (color, turgor and reduced softening of tissue) was also maintained while the nutritional quality of total antioxidants and phenolics were 1.7 and 1.5 folds, respectively, higher than the control. The preliminary results suggest ANE, HA and combination ANE + HA would enhance seed germination, plant growth and retain post-harvest quality of lettuce and spinach.

List of Abbreviations and Symbols Used

ANE	*Ascophyllum nodosum* extract
HA	Humic acids
PHL	Post-harvest losses
ROS	Reactive oxygen species
RH	Relative humidity
Da	Dalton (unit)
C	Carbon
H	Hydrogen
N	Nitrogen
O	Oxygen
S	Sulfur
%	Percentage
CO_2H_2	Methylenedioxy functional group
OH^-	Hydroxide
C=O	Carbonyl group
AsA	Ascorbic acid
α	Alpha
β	Beta
γ	Gamma
BAP	Benzylaminopurine
W/V	Weight/volume
GP	Germination percentage
MSG	Mean seed germination
SVI	Seedling vigour index
FW	Fresh weight
DW	Dry weight
DWL	Dry weight loss

DMC	Dry matter content
MDA	Malondialdehyde
g	Grams
g	Centrifugal force
EtOH	Ethanol
µL	Micro litre
TCA	Tricarboxylic acid
TBA	Thiobarbituric acid
BHT	Butylated hydroxytoluene
Min	Minute (unit of time)
h	Hours (unit of time)
ml	millilitres
MeOH	Methanol
nm	Nano metre
L-ascorbic acid	Vitamin C
m-phosphoric acid	meta-phosphoric acid
O- phosphoric acid	Ortho-phosphoric acid
KH_2PO_4	Monopotassium phosphate
µg	Microgram
EDTA	Ethylenediaminetetraacetic acid
DTT	Dithiothreitol
mM	Milli Molar
$FeCl_3$	Ferric chloride
µmol	Micromole
DPPH	2,2-diphenyl-1-picrylhydrazyl hydrate
TE	Trolox equivalents
IAA	Indole acetic acid
PUFA	Polyunsaturated fatty acids

RCBD	Randomized complete block design
ANOVA	Analysis of Variance
LSD	Least significant difference
SAS	Statistical Analysis system
Fig.	Figure
SE	Standard error

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Chapter 1 INTRODUCTION

Agricultural practices involve using various inputs such as organic, synthetic fertilizers and pesticides to maximize crop growth, yield, and to control pests and diseases. However, particularly prolonged usage of synthetic inputs has resulted in ecological damage, decreased resistance to pests and diseases (Raposo et al.1995; Alfonso et al. 2000) and can affect crop quality (Lurie 1998; Tripathi and Dubey 2004). Consumption of the produce obtained through this method gradually raised health issues in consumers (Harris 1996; Prior 2003). Therefore, modern practices have evolved to reduce excessive synthetic inputs and to minimize the impact of synthetic fertilizers on the environment and on consumers health. Plant biostimulants are new class of inputs, when applied to plants improve seed germination, plant growth and productivity, regulate different physiological processes and improve plants adaptability to abiotic stresses (Bulgari et al. 2015; Van Oosten et al. 2017).

Besides the agricultural practices, better understanding of crop performance is an important strategy to obtain good crop yield and to maintain quality of produce until it reaches consumers. Seed germination and establishment are two important phases that contribute to crop performance resulting in maximum yield and extended post-harvest shelf-life (Finch and William 2015). To achieve this, practices including seed priming, and chemical and physical treatments are deployed to improve seed performance such as seed germination and seedling vigour (Ashraf and Foodlad 2005; Parera and Cantiliffe 1994). Temperature, humidity and soil conditions also affect crop establishment and yield. Thus, ensuring optimal growth conditions is a major factor to obtain vigorous seedlings and crop yield (Nicola and Bassoccu 1994). However, treating seeds with biostimulants provides numerous benefits in stimulating plant growth (Khan et al. 2011).

Besides crop growth and yield, post-harvest losses (PHL) are a major problem in modern agriculture. PHL can be defined as the loss in quality and quantity of produce from the period of harvest to consumption. It was estimated that 5-50 % of crops are lost between field and consumer in developing countries and 2-24 % in developed countries (Lers 2012). Both pre-harvest and post-harvest factors can contribute to these losses. Pre-harvest factors such as temperature, humidity, water availability to the crops, light and soil conditions affect physiological and biochemical changes in produce (Ferguson et al. 1999).

A post-harvest factor contributing to losses is that bioactive compounds such as mineral nutrients, vitamins, antioxidants, flavonoids and phenolic components degrade during storage resulting in loss of nutritional value and reduced shelf life (Kader.1988). However, these bioactive components obtained from fruits and vegetables have an important role in human health, as they promote protective effects against several types of cancer and cardiovascular disease (Kris -Etherton et al. 2002; Williamson 1996). Therefore, it is important to preserve nutrients and phytochemicals in fruits and vegetables despite their short shelf life, to provide health benefits in humans.

Many factors affect post-harvest shelf life and quality of produce. Quality loss in fruits can be noticed by physical properties including softening of tissues, texture, development of off-flavors, weight loss and changes in size and shape. Biological factors like respiration rate, ethylene production and compositional (bioactive compounds, pigments) changes also affect post-harvest quantity of the produce (Siddiqui 2015). Various biotic and abiotic stresses lead to the development of reactive oxygen species (ROS). ROS are molecules produced in plants as byproducts of cellular metabolism and oxidative processes such as photosynthesis and respiration (Apel and Hirt 2004). Excessive ROS react with proteins and lipids causing degeneration of proteins and lipid peroxidation resulting in deterioration

of tissues. The rate of deterioration of produce after harvest is proportional to the respiration rate. Loss of water content in the stored produce reduces weight, loss of appearance (wilting and shriveling) and leads to nutritional losses (Kader 2002). Therefore, to obtain maximum crop yield and minimum post-harvest losses, it is very important to understand biological and environmental factors at the period of crop growth and to follow sustainable agricultural practices. For many years, specific interest has been expressed in the sustainability of using biostimulants in agriculture, horticulture and floriculture systems (Bulgari et al. 2015).

1.1 Plant biostimulants

Plant biostimulants are defined as substances that, when applied to plants, have enhanced positive effects on growth and productivity (Van Oosten et al. 2017). Biostimulants regulate different plant physiological processes and improve plant's adaptability to abiotic stresses (Bulgari et al. 2015). Biostimulants are naturally obtained from diverse sources that are economically and environmentally viable (du Jardin 2015). Currently accepted plant biostimulants include seaweed extracts, humic substances (humic acids and fulvic acids), chitin and chitosan derivatives, amino acids, protein hydrolysates and microbes. Seaweed extracts and humic acids are widely studied for their role in plant growth-promotion (du Jardin 2012; Brown and Saa 2015; Van Oosten et al. 2017). In this study two different biostimulants, seaweeds extract and humic acids, were used to study their effect on seed germination, early growth and post-harvest shelf life, and nutritional quality on leafy vegetables.

1.2 Seaweeds

Seaweeds have been traditionally used as fertilizers and soil conditioning agents since ancient times in coastal Europe (Fleurence 1999). Seaweeds belong to Rhodophyta,

Chlorophyta and Ochrophyta (Guiry 2013). Over the past two decades, seaweeds have been processed and marketed as seaweed extracts in various formulations for use in agriculture and horticulture. Seaweed extracts are rich in micro and macronutrients, polysaccharides, proteins, poly unsaturated fatty acids, polyphenols, phytohormones, and osmolytes (Chojnacka et al. 2012). These compounds elicit multiple beneficial effects in plants, including enhanced seed germination and establishment, overall plant growth and productivity, resistance against biotic and abiotic stresses and increased post-harvest shelf life (Mancuso et al. 2006; Rayorath et al. 2008; Khan et al. 2009). Numerous studies have been reported on beneficial effects of seaweed extracts on crop plants (Blunden et al. 2010) and crop growth, yield and productivity (Craigie 2011).

1.3 *Ascophyllum nodosum*

Ascophyllum nodosum is a macroscopic, marine brown alga belonging to the family Fucaceae, and is found extensively along the Atlantic coast of North America and the coasts of Scotland, Norway and Portugal. It is commonly called rockweed, knotted wrack, knotted kelp and egg wrack. This alga consists of dichotomous branching, vesicles and receptacles and it forms bladders in the central portion of fronds (Sharp 1987). *Ascophyllum nodosum* extract (ANE) is rich in polysaccharides like fucoidan and laminarian, phenolic compounds (Audibert et al. 2010), phytohormones such as cytokinins (Tarakhovskaya et al. 2007), auxins (Khan et al. 2009), traces of gibberellins (Craigie 2011) and essential nutrients like nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), iron (Fe), magnesium (Mg), zinc (Zn), sodium (Na) and sulfur (S) (Rayirath et al. 2009). It contains more polyphenols than other seaweeds (Keyrouz et al. 2011) and contains bioactive compounds like betaines (like γ-aminobutyric acid betaine, δ-aminovaleric acid betaine, laminine (N^6, N^6, N^6-trimethyllysine and glycine betaine) (Blunden et al. 1986).

1.4 Humic acids

Humic acids (HA) ($C_{14-20}H_{14-21}O_{6-9}N$) are complex macromolecules, dark yellow to black in colour, and are linked with amino acids, peptides, amino sugars, aliphatic acids, and other organic constituents (Stevenson 1982). Humic acids are defined as soluble alkali and insoluble acid fractions of humic substances (Jones and Bryan 1998). Humic acids are naturally occurring products produced by the decaying of organic materials. Humic acids dissolves in water at higher pH levels (Jones and Bryan 1998) and is commonly present in soil, peat and lignites (Sharif et al. 2002). Humic acids consist of mineral nutrients like sodium (Na), potassium (K), magnesium (Mg), zinc (Zn), calcium (Ca), iron (Fe) and copper (Cu), (Aiken 1985). Humic acids have a high molecular weight and acts as a catalyst in the activities of soil microorganisms that are present in the soil (Chen and Aviad 1990; Sharif et al. 2002). When applied to plants, humic acids stimulate the production of growth-promoting hormones like auxins, gibberellins and cytokinins (Phuong and Tichy 1976).

1.5 Lettuce

Lettuce (*Lactuca sativa L.*) is an annual plant belonging to the Asteraceae family. First grown in the Mediterranean region in 4500 BC, this vegetable crop has now spread world-wide and is marketed as fresh produce (Mou 2008). Lettuce is a cool season crop and mostly grown in temperate and subtropical countries. The ideal temperature for lettuce growth is 16-18 °C. The harvested lettuce is stored at 0-2 °C with 96-98 % relative humidity (RH) (Kader 2002). There are two commercial types of lettuce: 1. Head lettuce and 2. Leaf lettuce. The Head lettuce category is comprised of iceberg, crisp head and butter head lettuce whereas the Leaf lettuce category is comprised of romaine, green leaf and red lettuce. Canada contributes 4110 hectares of area for production and marketed 101,016 metric tonnes of head and leaf lettuce (Statistics Canada 2016). Besides the head and

leaf lettuce, in recent times, baby lettuce (2-3 weeks after germination) has also been freshly consumed (Li and Kubota 2009). Greenhouse lettuce is a major production system in Canada, contributing 19.9 hectares of lettuce production with a farm gate value of $31.7 million (Statistics Canada 2016). Quebec is the leading provincial producer, followed by British Columbia with 14.37 hectares and 2.07 hectares respectively (Statistics Canada 2016). Biotic (diseases and pests) and abiotic (temperature and humidity) factors are major limiting factors of greenhouse lettuce production (www.agr.gc.ca/pmc-cropprofiles).

1.6 Spinach

Spinach (*Spinach oleraceae L.*) is an annual plant belonging to the Chenopodiaceae family. Spinach is a cool season crop and grown throughout the year in temperate areas. The ideal temperature for spinach is 16-20 °C. It is harvested as baby spinach and regular spinach and is generally stored at 2-5 °C (Conte et al.2008). Spinach is rich in bioactive compounds such as vitamins C, E and K, carotenoids and flavonoids (Bergquist et al. 2006). It was first grown in Iran in 400 AD. Canada contributes 732 hectares of total area and marketed 5759 million tons of spinach in 2016 (Statistics Canada 2016). Spinach is categorized into savoy, semi savoy and flat varieties for market production (Spinach vegetable crop production guide for Nova Scotia 2008).

Lettuce and spinach are mostly used for fresh consumption in wraps, mixed salads and as ready to eat vegetables. In this study, romaine lettuce and Sardinia (semi savoy variety) spinach were used.

1.7 Project hypothesis

The main hypothesis of the research project was that the application of ANE, HA and ANE+HA will enhance seed germination, early growth, shelf-life and quality of spinach and lettuce.

1.8 Objectives

The research project had four main objectives:

1. To identify the most effective treatment of ANE, HA and ANE+HA on seed germination and early growth of lettuce and spinach
2. To study the effect of individual treatments of ANE, HA and ANE+HA on early growth of lettuce and spinach
3. To determine the most effective treatments to improve shelf life of lettuce and spinach
4. To determine the most effective treatments for post-harvest nutritional quality of lettuce and spinach.

Chapter 2 LITERATURE REVIEW

2.1 Background information on plant biostimulants

Plant biostimulants were formerly known by many names: plant strengtheners (Torre et al. 2013), agricultural biostimulants (EBIC 2011b), organic biostimulants (Kumar and Shivay 2008), biofertilizers (Parrado et al. 2008), biostimulant plant growth-promoters (Huang 2007) and metabolic enhancers (Doak et al. 2005). Plant biostimulants, as defined by the European Biostimulants Industry Council: "containing substances and/or microorganisms whose function when applied to plants or rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress and crop quality" (EBIC 2012). The EBIC further classified plant biostimulants based on their mode of action (microbial stimulants, humic substances, protein hydrolysates, amino acids and seaweed extracts) (Calvo et al. 2014; du Jardin 2015).

Globally, the biostimulants market has been expanding widely and is expected to reach US $3 billion by 2021 and the annual growth rate is expected to grow 10 to 12 % from 2015 to 2021 (Biostimulant Market 2014). The largest growing national biostimulant market is in Europe with 37 % of the market share, while the Asia-Pacific and North American market ranks second (Anonymous 2016).

2.2 Role of plant biostimulants

Plant biostimulants are collections of heterogeneous organic substances that, when applied to plants at lower concentrations, enhance plant growth and development by stimulating hormone-like activities (Zhang and Schmidt 2000). Biostimulants also augment plant performance and metabolic processes (Posmyk and Szafranska 2016). The composition of biostimulants are mostly undefined because of their complex structures and the presence of a wide range of molecules (Ertani et al. 2012; Guinan et al. 2012). Plant biostimulants act directly on plant metabolism and plant physiology improving crop quality and increasing the yield. Biostimulants enhance nutrient efficiency of plants by increasing nutrient uptake. They also develop good soil conditions by improving soil microbes and soil structure (Calvo et al. 2014).

Seaweed extracts are available commercially in soluble powder and liquid formulations. *Ascophyllum nodosum, Fucus, Laminara* and *Sargassum* are the most commonly used seaweeds. The composition of individual seaweeds varies but generally includes plant hormones, polysaccharides and mineral elements (Shekar et al. 2012). Soil or foliar applications of seaweeds improve plant growth, yield and productivity of many crops (Craige 2011; Khan et al. 2009; Mancuso et al. 2006; Norrie and Keathley 2005). Liquid seaweed extract application led to increased seed germination, root length and crop yield in wheat (Kumar and Sahoo. 2011) and enhanced the nutritional quality of okra (Zodape et al.2008). Seaweed extract obtained from *Ecklonia maxima* improved yield in nutrient-stressed lettuce (Crouch et al. 1990) and in tomato plants (Crouch and Van Staden 1992). Besides plant growth and development, polyphenols and antioxidant properties of seaweeds improves pest and pathogen resistance in many crop plants (Adrian et al. 1996; Hankins et al.1990; Zhang and Ervin 2008). *Ascophyllum nodosum* is one of the brown

seaweeds and an extract from this alga was used in this research. The general composition of the *Ascophyllum nodosum* extract (ANE) is listed in Table 1.

Humic substances are biostimulants categorized into humic acids, fulvic acids and humins that play an important role in both soil and plant functions (Berbera and Garcia 2014). In addition, humic substances are also observed in fresh waterbodies and their interaction with the fresh water biome (Paludan and Jensen 1995; Thomas 1997; Steinberg et al 2008; Jimenez et al. 2017). When applied to plants, humic substances promote morphological and physiological activities of plants by improving soil structure, uptake of nutrients and water holding capacity (Cimrin et al. 2008; Kirn et al. 2010; and Nardi et al. 2007). Humic acids contain auxin-like substances which promote roots and enhanced lateral root development (Canellas et al. 2011 and Trevisan et al. 2010). Studies reported that humic acids improve nutrient uptake, crop yield and productivity when applied as a soil drench or foliar spray in many agricultural and horticultural crops (Canellas et al. 2002; Cavalcante et al. 2013; El-Nemer et al. 2012; Karakut et al. 2009).

Apart from seaweed extracts and humic acids, many studies reported on how protein hydrolysates, amino acids and microbial inoculants play a major role in plant growth stimulation, yield and nutrient uptake (Maini 2006; Morales and Stall. 2003; Parrado et al. 2008; Schiavon et al. 2008; Vranova et al. 2011). However, the mode of action of biostimulants on plant response is still not clearly understood because of their complex molecules, although many articles reporting polysaccharides, macro and micronutrients and phyto-hormone like activities in biostimulants help to explain plant activities and response (Muscolo et al 1999; Schiavon et al. 2010; Strik et al. 2004; Wally et al. 2013).

Table.1 Composition of *Ascophyllum nodosum* extract powder

Composition	Typical analysis
Organic matter content	45-55 %
Alginic acid	12-16 %
Fucose Polymers	13-17 %
Mannitol	4-6 %
Amino Acids	4-6 %
Other inorganic compounds	10-12 %
Ash	45-55 %
Nitrogen	0.8-1.5 %
Phosphorus	0.5-1.0 %
Potassium	14-18 %
Calcium	0.3-0.6 %
Iron	75-250 ppm
Magnesium	0.2-0.5 %
Manganese	8-12 ppm
Sodium	3.0-5.0 %
Sulfur	1.0-2.0 %
Zinc	10-25 ppm

(Adapted from Acadian Seaplants Limited, technical information)

2.3 General composition of humic acids

Humic acids are the major organic constituent formed in soil organic matter by microbial decomposition also known as humic substances (Arancon et al. 2006; Brady and Weil 2002). Based on solubility and range of pH, humic substances are divided into humic acids, fulvic acids and humins (Berbera and Garcia 2014; Senesi and Loffredo 1999). The composition of humic acids varies with the varying amounts of humification, the type of soil and environmental changes (i.e. temperature and moisture) (DiDonato et al. 2016; Kumada 1987; Watanabe and Takada 2006). Humic acids are defined as insoluble acid and soluble alkali (MacCarthy 2001). Literature has reported humic substances to be collections of heterogeneous compounds (Stevenson 1994) containing molecules ranging from 500-1,000,000 Da (Piccolo 2002). Generally, humic acids are rich in proteins, carbohydrates, lignins and contain various functional groups such as aliphatic, aromatic structures, carboxyl, phenolic, alkyl and quinone groups (Maie et al. 2006; Tate et al. 1990; Watanabe et al. 2005; Velasco et al. 2004). The general composition of commercial humic acids were obtained from different substances (soils and organic waste) were listed in Table 2.

Table.2 Elemental compositions of humic acids

HA	C(%)	H(%)	N(%)	O(%)	S(%)	References
Commercial (Sigma-aldrich)	55.6	5.5	4.5	34.4	1.2	Siddiqui et al.2009
Soil IHSS standard	58.1	3.7	4.1	34.1	-	Velasco et al.2004
Peat soil	50.4	4.9	2.8	39.1	0.7	Sato et al.1986
Sediments	43.7-53.8	4.1-5.8	3.5-6.2	31.1-37.1	-	Rensburg 2015
Sewage sludge	52.8	6.8	6.5	33.9	0.1	Klavins and Purmalis 2010
Empty fruit bunch	56.3	5.7	4.4	32.9	1.2	Hassett et al. 1987
River	51.2	4.7	2.6	40.4	1.9	Rice and MacCarthy 1991
Leonardite	63.8	3.7	1.2	31.3	-	Velasco et al.2004

(Adapted from de Melo et al. 2016)

2.4 *Ascophyllum nodosum* extract (ANE) on plant growth and crop yield

Several commercial seaweed extracts are reported to enhance seed germination, plant growth and development, and increase crop yield, biomass and quality (value) (Crouch et al.1990; Fan et al. 2010; Khan et al. 2009; Rayorath et al. 2008). *Ascophyllum nodosum* extract (ANE) is a widely studied seaweed for its beneficial bioactive compounds such as polysaccharides, alginates, vitamins, organic osmolytes and hormone-like substances that aid in plant growth and establishment (Kandasamy et al. 2012). One of the potential mode of action ANE acts on plant growth is by regulating endogenous phyto-hormone biosynthesis (Wally et al. 2013).

Commercial ANE application on barley has shown enhanced seedling emergence and improved root and shoot development (Rayorath et al. 2008). Studies reported on the application of ANE and other seaweeds showed improved nutrient content in various plants (Dobromilska and Gubarewicz 2008; Fan et al. 2011) and enhanced shelf life was demonstrated in spinach (Fan et al. 2011). The combination treatments of ANE with propiconazole (a fungicide) and humic acids (HA) have shown improved thermal stress tolerance of Kentucky bluegrass during storage (Zhang et al. 2003). Similarly, ANE application along with *Sinorhizobium meliloti*, a soil bacterium, enhanced root colonization in alfalfa plants, showing that ANE acts positively on root nodulation and plant growth (Khan et al. 2009). Studies on the application of ANE on few ornamental crops reported enhanced root length, development of shoots, fresh and dry weight of inflorescence (Aziz et al. 2011 and Neily et al. 2010).

2.5 Humic acids (HA) on root growth, nutrient uptake, yield and productivity

HA plays an important role in the uptake of essential macronutrients N, S and P (Chen and Aviad 1990; Varanini and Pinton1995). HA improves soil structure, soil fertility, root architecture and plant metabolism (Mylonas and Mccants 1980; Trevisan et al. 2010). Humic acids contain several oxygenated functional groups (CO_2H_2, OH, phenols, and C=O) that chelates metal ions, by forming water soluble and water insoluble complexes, and promotes plant growth and nutrient activities (Schiavon et al. 2010). Auxin-like substances stimulating root growth and other plant growth activities were demonstrated in many plants (Arancon et al. 2006; Baldotto and Baldotto 2013; Muscolo et al. 1999).

Plant growth regulator-like activity (auxin-like, gibberellin-like and cytokinin-like substances) was observed in humic acids stimulated plant growth (Phuong and Tichy 1976). However, it is very difficult to elucidate the mode of action of humic substances because of their complex structural properties and different origins (Hayes 1997). Numerous studies reported the application of lower quantities of humic acids increased cell permeability, enhanced shoot and root growth and increased lateral root development in many crops (Adani et al. 1998; Akhtar et al. 2015; Canellas et al. 2011; Canellas et al. 2002; Dobbss et al. 2010; Eyheraguibel et al. 2008; Jindo et al. 2012; Mora et al. 2010; Tahir et al. 2011). The addition of humic acids as soil drench or foliar spray has shown improved yield and productivity of vegetable and fruit crops (Arancon et al. 2006; Karakurt et al. 2009; Kirn et al. 2010; Morard et al. 2010; Selim and Mosa 2012; Yildrim 2007).

Humic acids in combination with different microbial based biostimulants have shown increased biomass, N content, chlorophyll content and growth of lettuce plants (Pishchik et al. 2016; Rouphel et al. 2017). The combined application of seaweed extracts and HA have shown root growth, nutrient uptake in *Brassica napus* (Billard et al. 2014) and overall physiological health of creeping bent grass (Zhang and Schmidt 2003).

2.6 Importance of bioactive compounds in postharvest shelf life

Increasing post-harvest losses in developed and developing countries over the past few decades have raised the need to develop products and processes that enhance the quality and shelf life of produce. In addition, finding means to enhance bioactive compounds such as antioxidants, ascorbic acid, phenolic compounds and pigments in produce is also key to add inherit value to the product (Lee and Kader 2000; Naczk and Shahidi 2006; Nuutila et al. 2003). Phytochemicals and antioxidants play an important role in reducing post-harvest losses and in sustaining the health of consumers by reducing the risk of cardiovascular diseases, cancers and other age-related health issues (Hung et al. 2004; Prior 2003).

Fruits and vegetables gradually degrade after harvesting due to physiological changes such as metabolism and respiration, which initiate compositional changes and thus affect quality and quantity of the product (Lers 2012). Change in colour, appearance, flavour and softening of tissues leads to qualitative loss and physical properties like change in size, shape, weight loss and skin thickness leads to quantitative loss (Siddiqqui 2015). Pre-harvest factors such as light, temperature, nutrient availability, growth, and soil conditions and post-harvest factors such as harvesting, handling measures, storage and biotic stress, have adverse effects on quality and quantity leading to the degradation of bioactive compounds and to major economic losses (Ferguson et al. 1999; Kader 2002; Weston and Barth 1997).

Ascorbic acid (AsA), an antioxidant, plays a major role in photosynthesis as an electron donor and acts against oxidative stress by reducing reactive oxygen species (ROS) (Padh 1990; Smirnoff 1996; Smirnoff 2000). Higher ascorbic acid concentrations in baby spinach harvested at early growth stages increased visual quality during storage (Bergquist et al. 2006). Pre-harvest agricultural practices affect ascorbic acid, for example, studies

demonstrated that sustainable and organically grown corn and strawberry crops have higher amount of AsA compared with conventionally grown corn and strawberry crops (Asami et al. 2003).

Phenolic compounds, anthocyanin, chlorophyll pigments and other antioxidants collectively contribute to the nutritional quality of fruits and vegetables and reduce free radicals, reducing oxidative damage (Cano et al. 2003; Ilahy et al. 2011). Viacava and Roura (2015) reported that exogenous application of natural elicitors improved the nutritional value of lettuce sprouts by enhancing phenolics, flavonoids and total antioxidant activity. Turf grass treated with ANE enhanced activity of antioxidant enzyme super oxide dismutase (SOD) by scavenging ROS (Fike et al. 2001). Betaines (glycine betaine, gamma-aminobutyric acid betaines) present in ANE improved chlorophyll pigments in tomato, wheat, barley and maize crops (Blunden et al. 1996). The application of commercial ANE enhanced nutritional quality such as total phenolics, flavonoids and antioxidants in spinach leaves (Fan et al. 2013). Numerous studies proved that cytokinin-like elicitors present in ANE increased endogenous antioxidant activity (β- carotene, ascorbates and *α*-tocopherol) by alleviating ascorbate peroxidase, glutathione reductase and SOD in plants (Allen et al. 2001; Zhang and Schmidt 2000; Zhang and Ervin 2004; Zhang and Ervin 2008). Cytokinin promotes delays in senescence and maintains the integrity of membranes by suppressing lipoxygenase, which promotes senescence (Musgrave 1994; Thimann 1987).

Seaweed extracts and humic acids contain auxin-like and cytokinin-like activities (Muscolo et al.1999; Nardi et al.1988; Piccolo et al.1992; Sanderson et al. 1987; Tay 1985). Auxin and cytokinin-like compounds present in seaweed extracts and humic acids improved turf grass quality and delayed senescence (Goatley and Schmidt 1990; Liu et al.1998; Zhang and Schmidt 2000). Post-harvest weight loss of gerbera flowers has decreased with the

application of humic acids (Nikbakht et al. 2008). The presence of auxin-like and gibberellin-like substances and benzylaminopurine (BAP) in humic acids extracted from leonardite stimulated metabolism and enzymatic activities of plants (Chen and Aviad 1990; Eason et al. 2001; Zhang et al. 2003). Consequently, in the cited literature, the bioactive compounds in the plants enhanced quality and quantity of the produce and the additive effect of biostimulants thus aids in storability and reduces postharvest losses of the crop. Some of the crop growth responses treated with seaweeds and humic acids are listed in the Table 3.

Table.3 Common growth responses to soil drench or foliar application of seaweed extract or humic acid application as cited in the literature

Crop	Extract	Plant response	Reference
Alfalfa	ANE	Increased growth and root nodulation	Khan et al. 2012
Arabidopsis	ANE	Enhanced drought tolerance	Santaniello et al. 2017
Barley	ANE	Induced amylase activity in seeds	Rayorath et al. 2008
Beans	ANE	Enhanced seed germination	Carvalho et al. 2013
Cotton	HA	Increases plant growth, water use efficiency (WUE) and yield under saline conditions	Rady et al. 2016
Eggplant	ANE	Fruit Yield	Bozorgi 2012
Gerbera	HA	Plant growth uptake, postharvest shelf life	Haghighi et al. 2014
Lettuce	HA	Enhanced P availability	Cimrin and Yilmaz 2005
Lettuce	HA	Root morphology	Busato et al. 2017
Lettuce	HA	Increased yield	Amanda et al. 2009
Lettuce	ANE	Enhanced seedling growth	Moller and Smith 1998
Potato and Spinach	ANE	Yield and increase in N, P, K and Mg uptake	Verlinden et al. 2009
Spinach	ANE	Yield and Nutritional quality	Fan et al. 2013

Chapter 3 MATERIALS AND METHODS

3.1 Seed material

Seeds of lettuce (*Lactuca sativa*., paris island cos variety), and spinach (*Spinach oleracea*., sardinia variety) were procured from Veseys Seeds company (PEI, Canada) and stored in the dark at 4 °C.

3.2 Preparation of *Ascophyllum nodosum* and Humic acids extracts

The commercial Humic acids (HA), Bonadea Gardens Inc. was purchased from Halifax Seed Company (Halifax, NS, Canada) and the extract of *Ascophyllum nodosum* (ANE); Acadian® Marine Plant Extract Powder was procured from Acadian Seaplants Limited (Dartmouth, NS, Canada). Stock solutions of 2 % (w/v) HA and ANE were prepared and stored at 4 ℃. The required volume of stock solutions was mixed with distilled water, to prepare treatments of ANE and HA, which were used alone and in different combinations as listed in Table 4.

Table.4 Different concentrations/ test solutions of ANE and HA alone, or in combination were evaluated for their efficacy in improving seed germination and early growth parameters of lettuce and spinach

Treatment	Concentration of Ascophyllum nodosum (ANE) (%)	Concentration of Humic acids (HA) (%)	Other
C	-	-	Water (control)
T_1	0.1	-	
T_2	0.5	-	
T_3	-	0.2	
T_4	-	0.4	
T_5	0.1	0.2	
T_6	0.1	0.4	
T_7	0.5	0.2	
T_8	0.5	0.4	
T_9	0.05	0.1	
T_{10}	0.05	0.2	
T_{11}	0.25	0.1	
T_{12}	0.25	0.2	
T_{13}	0.25	-	

3.3 Seed germination assay

Lettuce and spinach seeds were sown in germination pouches (CYG germination pouches, Mega International, Newport, US) and treated with different test solutions (Table 4). Each germination pouch contained ten seeds and all experiments were carried out in triplicate. Seeds were incubated in the dark until germination, and later placed under fluorescent lights of approximately (300 $\mu mol.m^{-2}.s^{-1}$) at room temperature with a photoperiod of 16/8 hours (day/night). Seeds were observed for several germination parameters, including germination percentage (GP), mean seed germination (MSG) and

seedling vigour index (SVI), as described by Noorhosseini et al. (2017). These parameters were calculated using the following formulas:

a) Germination percentage (GP) = (Number of seeds germinated)/ (Total number of seeds) ×100
b) Mean seed germination (MSG) = GP / T whereas GP = germination percentage and T = mean number of seeds germinating per day.
c) Seedling vigor index (SVI) = GP× SL whereas GP-germination percentage, SL-seedling length

After germination, seedlings were grown for seven days. On the seventh day, length of roots, and plumules of seedlings were measured for each crop using ImageJ software.

3.4 Early growth assay

Seeds of lettuce and spinach were planted 1.5 cm deep in small pots (size: 8.5 × 9.5 cm) containing PRO-MIX® general purpose, (Premier tech). Each treatment had six replications and were arranged in a randomized complete block design in a green house. After five days of germination, pots were irrigated once a week with 50 ml of HA or ANE for three weeks after germination. Plants were fertilized on the 10^{th} and 20^{th} day post-germination with 50 ml of fertilizer containing 1 g L^{-1} of 20-20-20 NPK. Pots were irrigated with distilled water on every alternate day of treatment. Pots irrigated with water throughout the experiment served as the control treatment. The greenhouse conditions were maintained at 21 °C with a photoperiod of 12/12 hours (day/night) and light was supplemented with fluorescent lights of approximately (300-400 $\mu mol.m^{-2}.s^{-1}$). After three weeks of treatment, growth parameters, fresh weight (FW), and dry weight (DW)) were recorded (DW was recorded after drying plant biomass in an oven at 72 °C for 48 hours). Percentage change in water content was calculated as [(FW-DW)/ FW] ×100 (Shukla et al. 2012).

The best combinations of HA and ANE (those eliciting better seed germination and early growth) were used for further post-harvest analysis and evaluated for their efficacy in improving post-harvest quality of lettuce and spinach.

3.5 Preparation of *Ascophyllum nodosum* and humic acids extracts to test on the post-harvest shelf life of lettuce and spinach

The commercial Humic acids (HA), Bonadea Gardens Inc. was purchased from Halifax Seed Company (Halifax, NS, Canada) and the extract of *Ascophyllum nodosum* (ANE); Acadian® Marine Plant Extract Powder was procured from Acadian Seaplants Limited (Dartmouth, NS, Canada). Stock solutions of ANE and HA were prepared by dissolving the soluble powder in distilled water and were used to prepare different combinations of ANE and HA as listed in Table 4. The most effective six treatments (T_1 - 0.1 % ANE, T_3 – 0.2 % HA, T_4 -0.4 % HA, T_6 -0.1 % ANE+0.4 % HA, T_{12} – 0.25 % ANE+0.2 %HA, T_{13} - 0.25% ANE) were identified and selected for further experiments.

3.6 Plant culture

Seeds of lettuce and spinach were planted 1.5 cm deep in large pots (15.0 × 10.5 cm) containing PRO-MIX® (general purpose) . Each experiment was carried out three times and had three replications. All treatments were arranged in a randomized complete block design in a greenhouse. Plants were treated once a week with different combination of ANE and HA (Table 5) at the rate 100 ml per plant, for 4 weeks after germination. On the 10^{th} and 20^{th} day post-germination, all plants were irrigated with 100 ml of fertilizer containing 1 g L^{-1} of 20-20-20 NPK. To maintain uniform moisture required for optimum growth, plants were irrigated with distilled water on alternate day of treatments. For this study, the controls were grown under similar growth conditions as plants used in treatments and were irrigated with equal volume of distilled water. The greenhouse conditions were maintained at 21 °C with a photoperiod of 12/12 hours (day/night) and

light was supplemented with fluorescent lights (300-400 μmol.m^{-2}.s^{-1}). After four weeks of treatment, plants were harvested to evaluate post-harvest shelf life during storage conditions. However, at the time of experiments, temperature fluctuated between 21 °C - 28 °C at the day time in green house at different seasons (summer, spring, winter).

3.7 Sample preparation and storage

For the studies on changes during post-harvest storage, after 30 days' growth of lettuce and 35 days' growth of spinach, plants were harvested with sharp scissors, fresh weight was recorded immediately, then soaked in ice-cold water, placed in perforated plastic bags, and stored in dark, controlled storage room maintained at 0-5℃ and 95% relative humidity. For lettuce, samples were removed on 10^{th} and 21^{st} days of post-harvest storage. For spinach, leaves were harvested on 14^{th}, 21^{st}, and 28^{th} days of post-harvest storage. At each time point, samples were assessed for their colour, turgor, and other nutritional parameters for post-harvest losses. Plants samples were chopped into pieces, thoroughly mixed and sub-samples were removed immediately for analyzing chlorophyll and MDA (malondialdehyde) content and remaining samples were flash frozen in liquid nitrogen and stored in -80℃ for analysis of other biochemical parameters.

3.8 Weight loss

To determine the effect of different combinations of ANE and HA on weight loss during the storage period, plants were weighed on day 0 i.e. day of harvesting and were subsequently weighed at different time-points as mentioned (Section.3.7). Fresh weight loss was expressed as a percentage of the initial fresh weight as described by Fan et al. (2014). Dry weight loss (DWL) during storage was recorded for each time-point by drying plants in an oven maintained at 65 °C for 48 h and expressed as g/100 g fresh weight (FW).

3.9 Visual quality analysis

The effect of different the combination of ANE and HA on visual quality (colour and turgor) of lettuce and spinach plants during storage was evaluated using 1-15 evaluating scale and expressed as a Visual quality index (appendix-A), as described by the Fan et al. (2010).

3.10 Determination of lipid peroxidation

The malondialdehyde (MDA) content was analyzed using an improved method after Hodges and Forney (2000). Fresh leaf samples (1 g of lettuce and 0.3 g of spinach), were homogenized in 15 ml of 80% ethanol (EtOH), followed by centrifugation at 3,000g at 4 °C for 10 min. The supernatant (100 µL) and 900 µL distilled water were added to a test tube with 1 ml of (i) 20% (w/v) trichloroacetic acid (TCA) and 0.01% (w/v) butylated hydroxytoluene (BHT), (ii) 0.65% (w/v) TBA. The mixture was mixed vigorously, heated at 95 °C in a dry bath for 25 min, cooled and centrifuged at 3,000g for 10 min. Absorbance was measured at 440, 532, and 600 nm. MDA equivalents was calculated using the following formula:

(1) [(Abs 532+TBA) -(Abs 600+TBA) -(Abs 532-TBA-Abs600-59 TBA)] = A,

(2) [(Abs 440+TBA-Abs 600+TBA) 0.0571] = B,

(3) MDA equivalents (nmol/mL) = 106 [(A-B)/157 000].

3.11 Determination of pigments (chlorophyll a and b, contents)

The effect of different combination of ANE and HA on pigments during post-harvest storage was evaluated, as described by Ritchie (2008). Chlorophyll content was analyzed on day 10^{th} and day 21^{st} for lettuce and on day 14^{th}, 21^{st} and 28^{th} for spinach in all treatments. Fresh tissue (1 g of lettuce, 0.3 g of spinach) was immediately grounded using a motor and a pestle with 15 ml of cold methanol (100%) (MeOH). Following extraction,

the grounded mixture was centrifuged at 10,000 g at 4°C for 10 min, and the pellet was re-extracted with 10ml of cold methanol until all the colour was removed. The extracts were combined, and the volume was made up to 25 ml in falcon tubes. Absorbance was measured at 652, 665 and 750 nm using a spectrophotometer. The chlorophyll content was calculated according to (Harmut 1987).

$C_a = 16.72\ A_{665.2} - 9.16\ A_{652}$

$C_b = 34.09\ A_{652.4} - 15.28\ A_{665.2}$

3.12 Determination of anthocyanin content

Anthocyanin content in the stored lettuce and spinach plants treated with different combination of ANE and HA was determined according to the protocol published by Burgos et al. (2013). Leaf samples (1 g of lettuce and 0.3 g of spinach), was macerated instantaneously with 10 ml of methanol acidified with 1% hydrochloric acid. The mixture was centrifuged at 5000g for 10 min at 4℃ and the pellet was re-extracted. Different fractions were combined, and final extraction volume was made up to 25 ml. Absorbance was taken at 545 nm using spectrophotometer. Anthocyanin content was calculated by using the molar extinction coefficient and molecular weight of malvidin-3-*p*-coumaroyl-glucoside (545 nm, 3.02×10^4 L/mol/cm, 718.5 g/mol).

3.13 Determination of total ascorbic acid

Ascorbic acid was analyzed following the protocol mentioned by Hodges and Lester (2006) with minor modifications. L-ascorbic acid was used as a standard. An amount of 1 g of lettuce and 0.3 g of spinach were grounded with 15 ml of ice-cold freshly prepared 5% (w/v) *m*-phosphoric acid. Following the maceration, the mixtures were centrifuged at 8000g for 15 min at 4℃. Then 100 µL of supernatant, 500 µL of 150 mM KH_2PO_4 buffer (pH 7.4) containing 5 mM Ethylene diamine tetra acetic acid (EDTA), and 100 µL 10 mM

Dithiothreitol (DTT) were added and the mixtures were incubated for 50 min at room temperature. 100 µL of 0.5% (w/v) *N*-ethylmaleimide (NEM) was added to remove excess DTT. Reaction mixtures of 400 µL 10% (w/v) trichloroacetic acid (TCA), 400 µL 44% *o*-phosphoric acid, 400 µL 4% (w/v) α-α[1]-dipyridyl, and 200 µL 30 g/L $FeCl_3$ reagent was added in succession to obtain colour. These reaction mixtures were incubated at 40℃ for 60 min in a shaking incubator and absorbance was recorded at 525 nm using spectrophotometer. Total ascorbic acid content was expressed as µmol/g FW.

3.14 Determination of total phenolics content

The amount of total phenolic content was measured according to the method of Hodges and Lester 2006. 1 g lettuce and 0.3 g spinach were macerated with 70% v/v of methanol and centrifuged at 10,000 g for 10 min. 100 µL of extracts was mixed with 2 ml water and 100 µL of 2N Folin-ciocalteu reagent, and incubated for 10 min. Following incubation, 300 ml of 20%(w/v) sodium carbonate was added and the mixture was vortexed for 15 seconds. The mixture was kept at room temperature in the dark for 2 h and the absorbance was measured at 765 nm using spectrophotometer. The standard calibration curve (0-9 µg/ml) was plotted using gallic acid. Total phenolics were expressed as mg/g gallic acid equivalent.

3.15 Determination of total antioxidants

The total antioxidant capacity of lettuce and spinach leaves was determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) assay method developed by Brand Williams et al. (1995). The antioxidants present in the sample react with DPPH and it converts to 1,1-diphenyl-2-picryl hydrazine creating discoloration (from deep violet to light yellow). 1 g lettuce and 0.3 g spinach, were flash frozen in liquid nitrogen (N) and stored at -80℃. Each frozen sample was homogenized in 15 mL pure methanol (MeOH) using a separate

mortar and pestle for each leaf sample. Supernatant was extracted by centrifuging at 10,000 g for 10 min. The pellet was re-extracted with 10 ml MeOH and supernatants were combined, and the total volume was made up to 25 ml. 2850 µL fresh DPPH solution (0.11 mM) was added to 100 µL of each extract and incubated for 6 h at 22 °C. Absorbance was then read at 517 nm against MeOH as a blank. The scavenging activity was calculated using the equation: Inhibition % = [(Ab−As)/Ab] 100, where Ab is the absorption of the blank sample and As is the absorption in the presence of test sample. The results were expressed in µM Trolox equivalents (TE, µg Trolox)/100 g FW through comparison against a Trolox standard calibration curve (0-20ug/ml).

3.16 Statistical analysis

Each experiment was setup in randomized complete block design and results were expressed as a mean ± standard error (SE). The ANOVA (Analysis of variance) with two blocks was carried out using SAS v. 9.4 statistical software (SAS Institute Inc., Cary, NC, USA), with general linear model at a 95 % confidence interval and 5 % level of significance. When P-value was less than 0.05, multiple means comparison was completed using the LSD (least significant difference) method was used to find means that are significantly different from others.The LSD was used due to uncontrollable sources (for example: temperature, humidity in the green house). The significantly different mean values were represented by different letters. Each experimental unit had nine plants and for each response variable, average of the nine values was used for ANOVA.

Chapter 4 RESULTS

4.1 Results on seed germination parameters of lettuce seedlings

Seed germination in lettuce occurred in all treatments and control after 24 hours under favorable laboratory conditions. The effect of *Ascophyllum nodosum* extract (ANE), humic acids (HA) and ANE+HA combinations significantly ($p \leq 0.0001$) improved percent germination, and mean seed germination compared to the seeds treated with water (control) (Fig. 1 and 2).

The combination of ANE + HA had a significant effect on the growth of lettuce seedlings. Seeds treated with the ANE+HA showed substantially higher radicle and plumule length as compared to the control, ANE and HA (Fig. 3 and 4). Seeds treated with ANE alone and HA alone showed less significant differences on radicle and plumule length when compared with other ANE+HA treatments. The combination treatment of 0.25 % ANE + 0.2 % HA (T_{12}) showed 103.27 % increase in the radicle length as compared to the control but was not significantly different compared to the other treatments (Fig. 3). In contrast, seeds treated with 0.2 % HA alone (T_3), showed a 10.1 % decrease in radicle length, while seeds treated with 0.25 % ANE alone (T_{13}), showed an 86 % increase in radicle length (Fig. 3). The plumule length of seeds treated with 0.25 % ANE + 0.2 % HA (T_{12}) showed a significant increase of 113.5 %, while in 0.25 % ANE alone (T_{13}), an increase of 87% in plumule length was observed as compared to control (Fig. 5). In contrast, 0.2 % HA alone (T_3) showed a plumule length reduction of 5.0 % as compared to control (Fig. 5).

The calculated vigour index of one-week-old seedlings showed that the application of ANE, HA and ANE+HA, had improved seedling vigour of lettuce. However, T_6 and T_{13} have shown significantly improved seedling vigour index (SVI) ($p \leq 0.0001$) (Fig. 6). The stimulatory effect of 0.25 % ANE + 0.2 % HA (T_{12}) was more effective, causing a three-

fold increase in seedling vigour index, as compared to the control (Fig. 6). However, the application of HA alone (T_3 and T_4) showed decreased root, plumule length and vigour index compared to the other treatments, but HA provided an additive effect on lettuce when combined with ANE. These results suggest that the application of 0.25 % ANE + 0.2 % HA (T_{12}), in combination, had an additive stimulatory effect on seed germination parameters (root and shoot length) and vigour of lettuce.

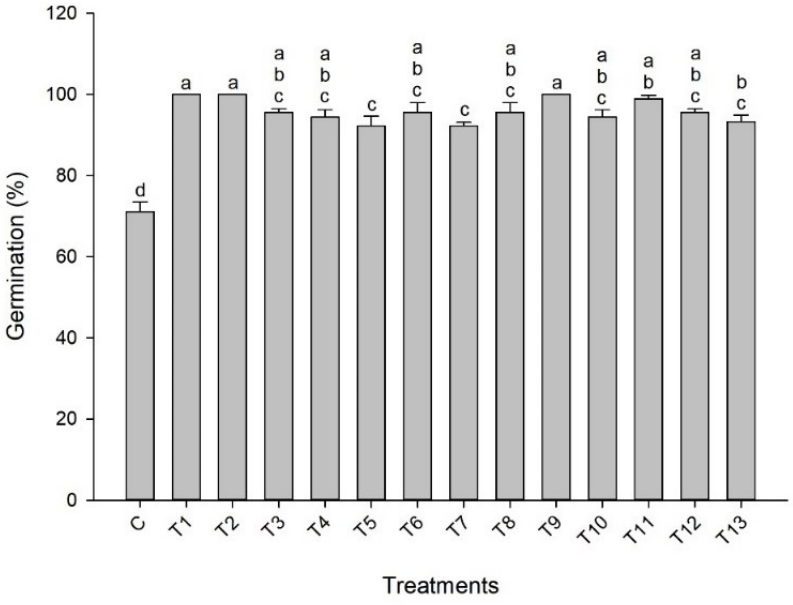


Figure. 1 Effect of ANE, HA and ANE+HA on germination percentage of lettuce seedlings after 24 hours. Treatment solutions were: C – control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5 -ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

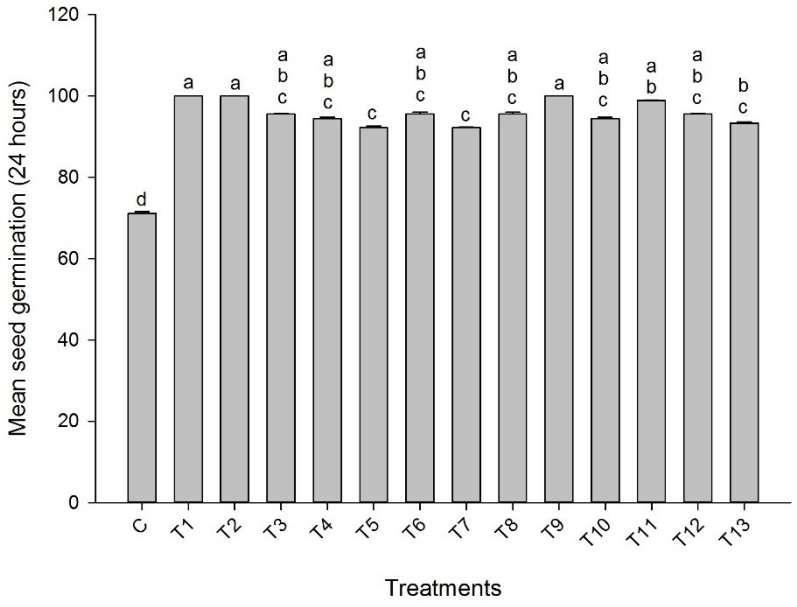


Figure. 2 Effect of ANE, HA and ANE+HA on mean seed germination of lettuce seedlings after 24 hours. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%) +HA (0.2%),T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}-ANE (0.25%)+HA (0.1%),T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

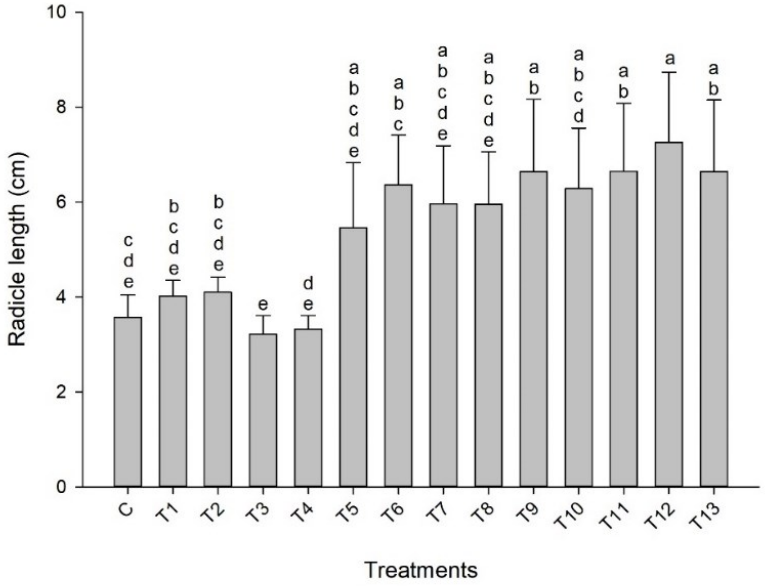


Figure. 3 Effect of ANE, HA and ANE+HA on radicle length of lettuce seedlings on 7th day of germination. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%),T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

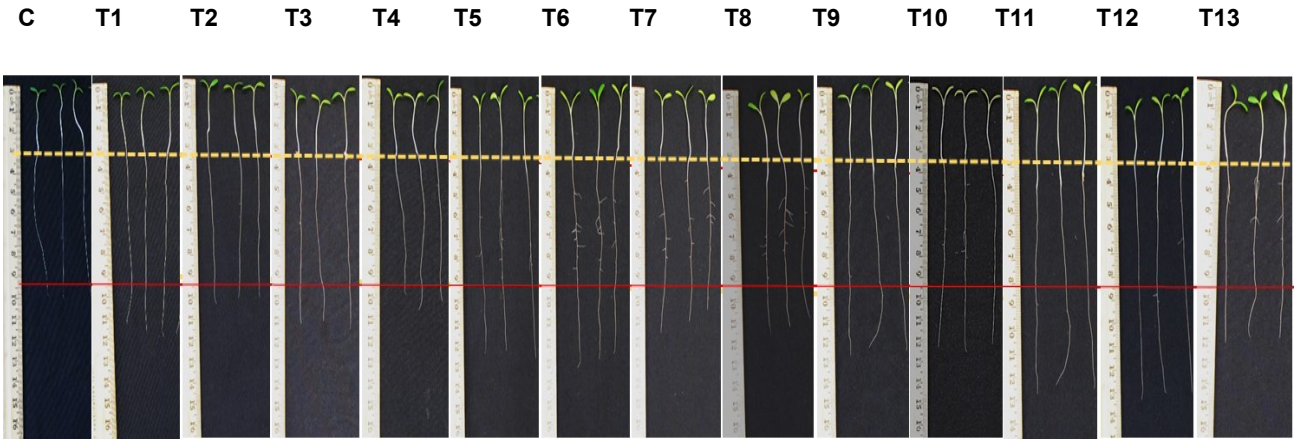


Figure. 4 Lettuce seedlings at 7 days of germination. Treatment solutions were used as: C – control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%),T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA(0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}-ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%).

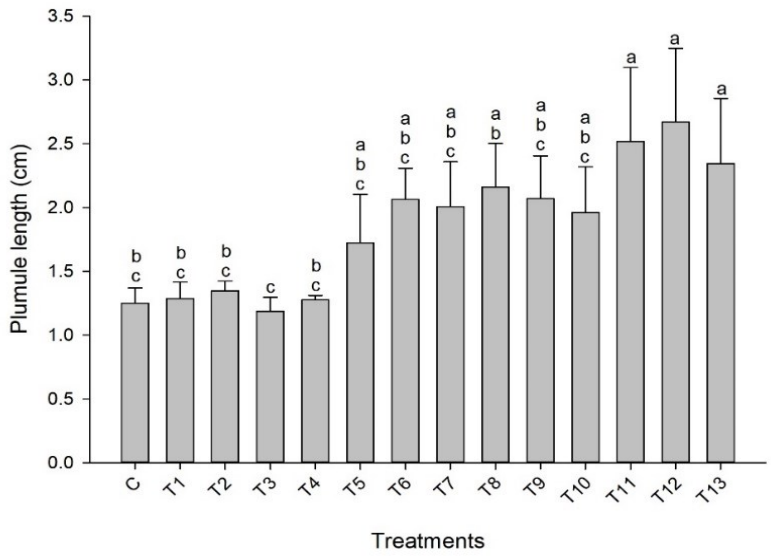


Figure. 5 Effect of ANE, HA and ANE+HA on plumule length of lettuce seedlings on 7th day of germination. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%),T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%),T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

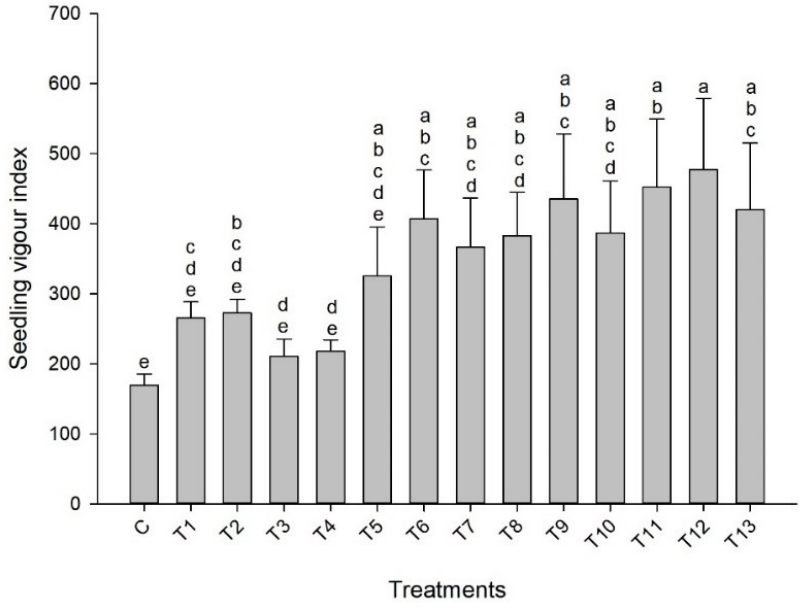


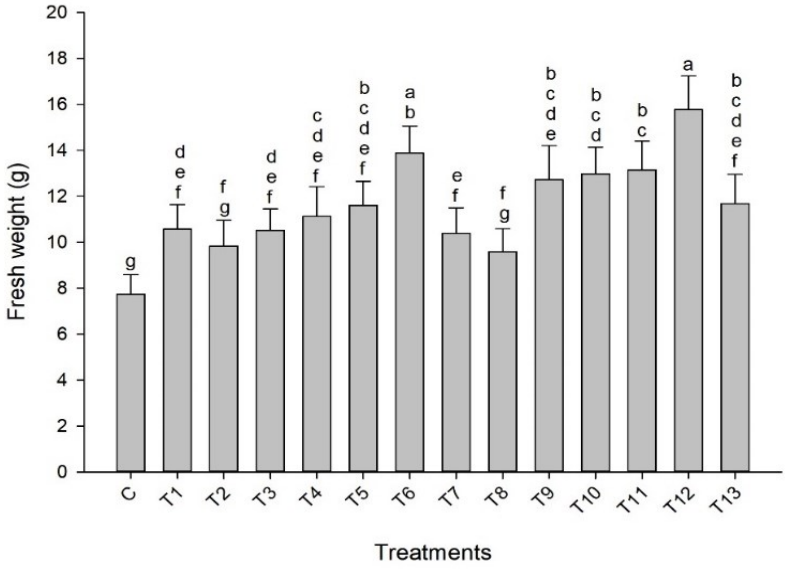
Figure. 6 Effect of ANE, HA and ANE+HA on Seedling vigour index of lettuce seedlings after 24 hours. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%),T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

4.2 Results on early growth parameters of lettuce plants

The addition of treatment solutions containing ANE, HA and ANE+HA combinations to the growth medium (PRO-MIX®) significantly increased lettuce fresh weight ($p \leq 0.0001$) (Fig. 7A). Treatment 0.25 % ANE+ 0.2 % HA (T_{12}), showed significantly higher fresh and dry weight when compared to other treatments and control while, 0.25 % ANE alone (T_{13}), and 0.2 % HA alone (T_3), showed a marginal increase in fresh and dry weight (Fig. 7A and 7B). In contrast, the higher concentrations of ANE+HA treatments (T_7

and T_8) showed reduced fresh and dry weight compared with other treatments (Fig. 7A and 7B), while the application of 0.5 % ANE alone (T_2) and 0.4 % HA alone (T_4) exhibited an increase in fresh weight and dry weight compared to the control. There was no significant difference observed in percentage water content of the plants treated with other treatments, suggesting that ANE and HA significantly ($p \leq 0.0001$) increased plant biomass.

A



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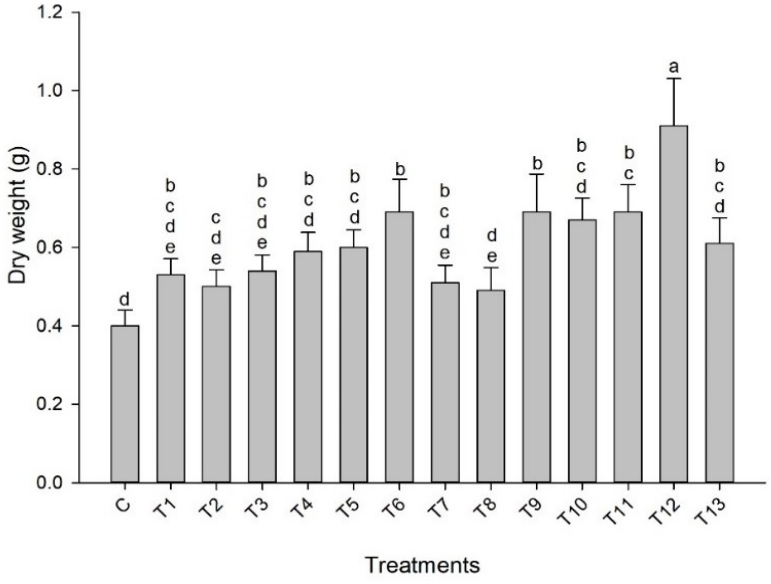


Figure. 7 Pre-harvest treatment application of ANE, HA and ANE+HA on lettuce plants to determine (7A) fresh weight, and (7B) dry weight. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%),T_{11}-ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n=18) and the significantly different mean values are represented by different letters.

4.3 Results on seed germination parameters of spinach seedlings

Seed germination in spinach occurred in all treatments after 36 hours. ANE and ANE+HA showed significantly ($p \leq 0.0001$) enhanced germination of spinach seeds when compared with control (Fig. 8). Maximum increases in the percentage of seed germination were observed in seeds treated with 0.25 % ANE + 0.2 % HA (T_{12}) (13.1 %) as compared to the control, while in 0.25 % ANE (T_{13}), seed germination increased by 10.5 %, as compared to the control (Fig. 9). The individual application of 0.1 % ANE alone (T_1) and 0.2 % HA alone (T_3) reduced seed germination by 14.5 % and 6.6 % respectively, as compared to the control (Fig.8). Interestingly, the combined application of lower concentrations of ANE+HA, i.e. 0.1 % ANE + 0.2 % HA (T_5), showed an increased seed germination of 9.2 % compared to the control.

Interestingly, higher concentrations of ANE+HA treatments, 0.5 % ANE + 0.4 % HA (T_8) also improved germination percentages by 9.2 % as compared to the control (Fig. 8). Treatment 0.5 % ANE (T_2) showed no significant difference whereas, 0.4 % HA (T_4) significantly reduced germination percentage compared with control. A similar trend was observed in the rate of seed germination (ROG), where treatment 0.25 % ANE+ 0.2 % HA (T_{12}), increased by 13.1 % and the mean seed germination (MSG) was higher in 0.25 % ANE + 0.2 % HA (T_{12}) (Fig.9 and 10). Different treatments T_1, T_3, T_4, T_7, T_9, and T_{11} showed reduced seed germination percentage, rate of germination and mean daily germination when compared with control. This test reveals that only few concentrations of ANE, HA and ANE+HA enhances seed germination, rate of germination and mean seed germination of spinach seeds at favorable laboratory conditions.

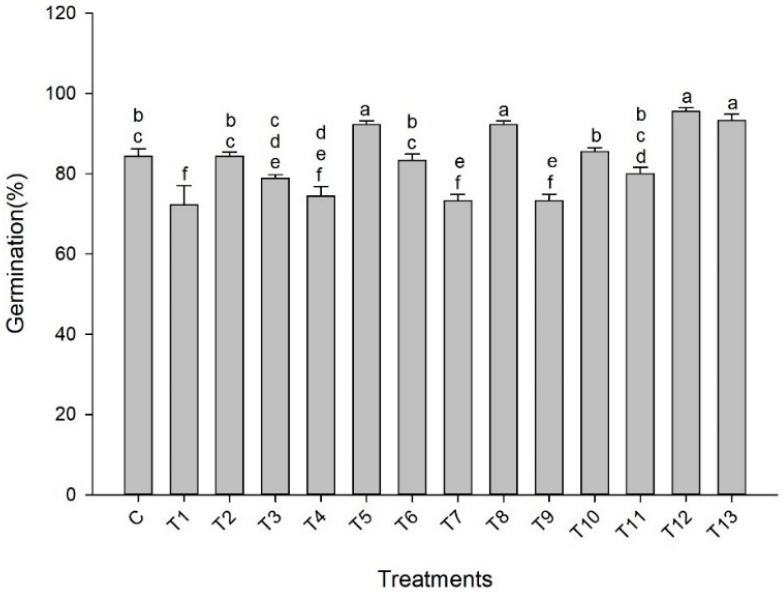


Figure. 8 Effect of ANE, HA and ANE+HA on germination percentage of spinach after 36 hours of germination. Treatment solutions were: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

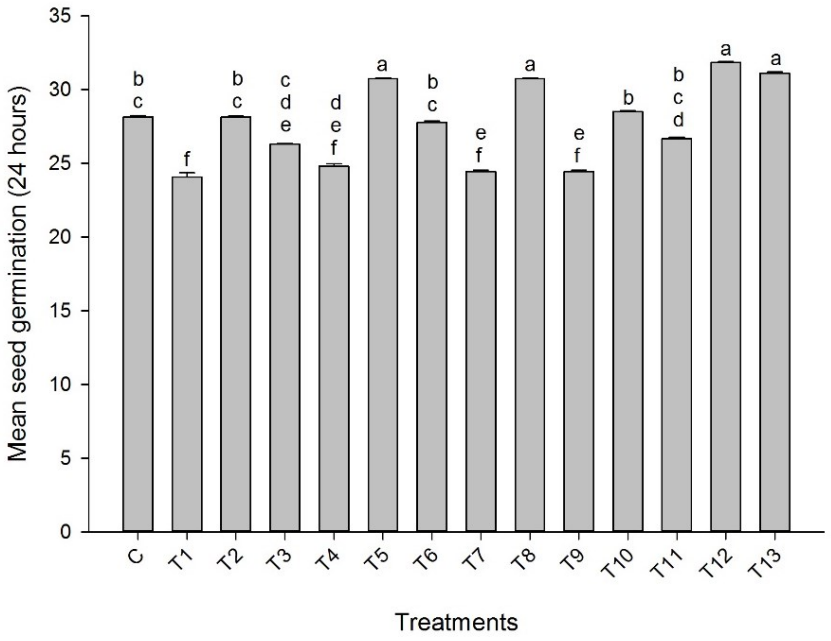


Figure. 9 Effect of ANE, HA and ANE+HA on mean seed germination of spinach after 36 hours of germination. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters

Treatments consisting of ANE, HA, and ANE+HA had growth (radicle and plumule) promoting effects on spinach seedlings (Fig. 10). Treatments 0.1 % ANE + 0.2 % HA (T_5), 0.5 % ANE + 0.4 % HA (T_8), and 0.25 % ANE + 0.1 % HA (T_{11}) showed maximum increase in radicle length respectively 39.67 % ,42.95 % and 46 % as compared to the control (Fig. 11). Similarly, plumule length was also higher in the presence of ANE, HA, and ANE+HA (Fig. 12) as compared to control. The spinach seedlings treated with 0.25 % ANE + 0.1 % HA (T_{11}), 0.1 % ANE + 0.2 % HA (T_5), and 0.5 % ANE + 0.4 % HA (T_8) showed 47.72 %, 48.12 %, and 51.02 % respectively improved seedling vigour index than the control ($p \leq 0.0001$) (Fig. 13). The results suggest that varying concentrations of ANE, HA and ANE+HA showed positive effects on seed germination parameters on spinach under controlled laboratory conditions.

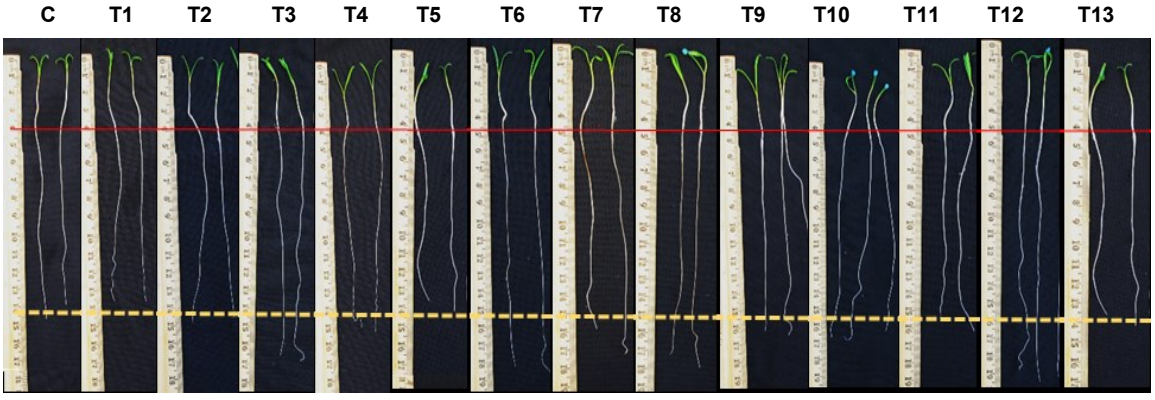


Figure. 10 Representing figure of spinach seedlings photographed on 7th day of germination. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%).

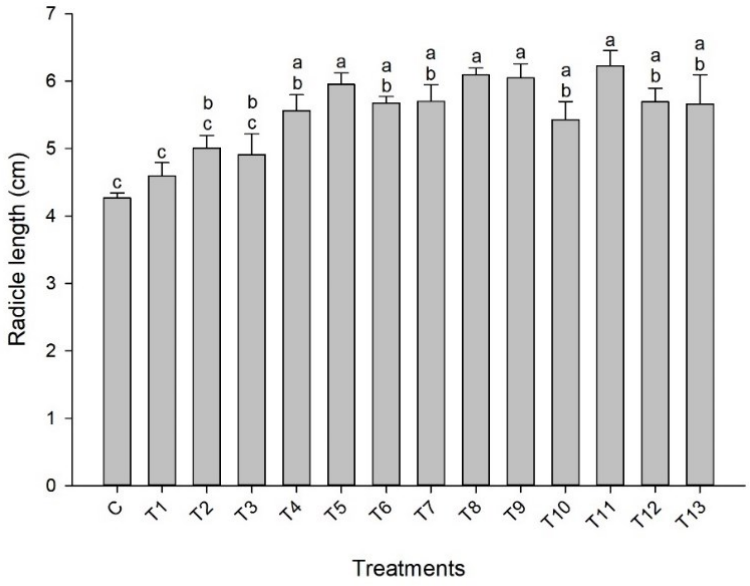


Figure. 11 Effect of ANE, HA and ANE+HA on radicle length of spinach seedlings after 36 hours. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

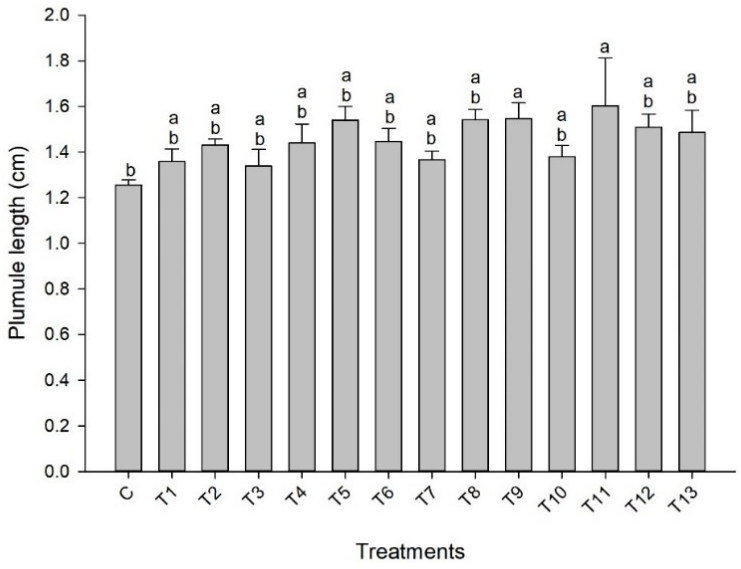


Figure. 12 Effect of ANE, HA and ANE+HA on plumule length of spinach seedlings after 36 hours. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}- ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

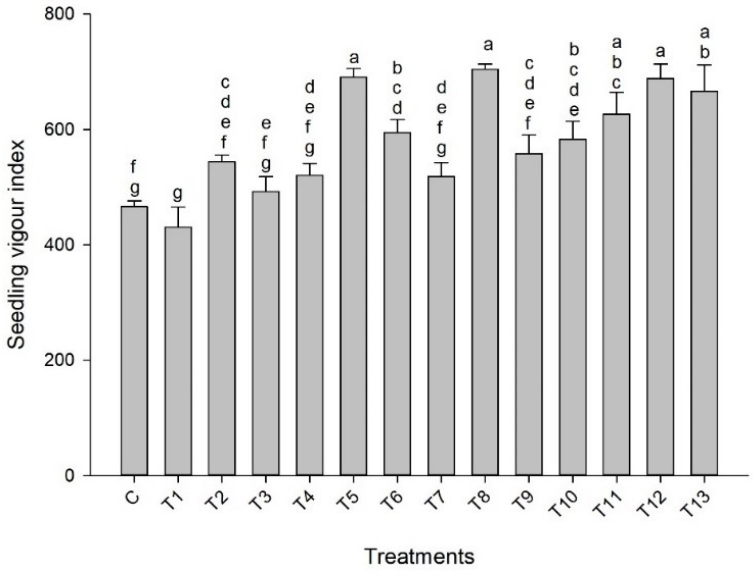


Figure. 13 Effect of ANE, HA and ANE+HA on seedling vigour index of spinach seedlings after 36 hours. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA (0.4%), T_5-ANE (0.1%)+HA (0.2%), T_6-ANE (0.1%)+HA (0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%), T_9-ANE (0.05%)+HA (0.1%), T_{10}-ANE (0.05%)+HA (0.2%), T_{11}-ANE (0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%), T_{13}-ANE (0.25%). Values are the mean ± SE (n≥90) and the significantly different mean values are represented by different letters.

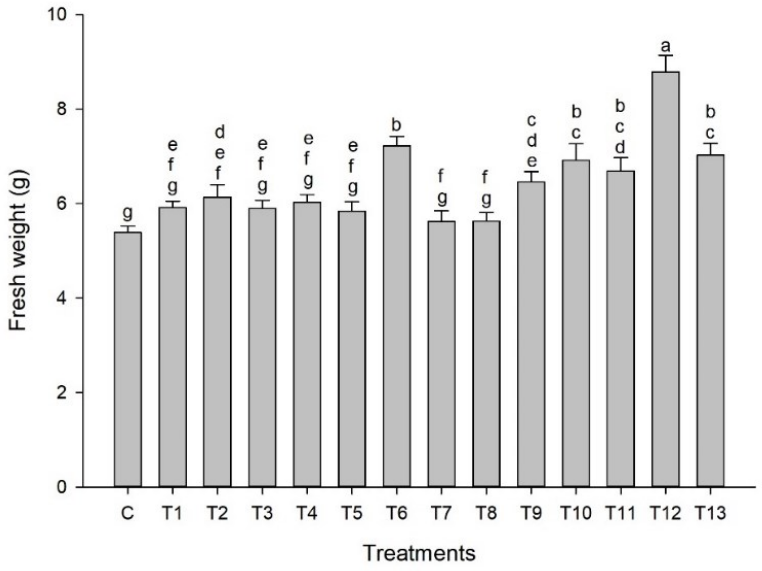
4.4 Results on early growth parameters of spinach plants

Spinach seedlings were root drenched every week with different concentrations of ANE, HA and ANE+HA for 21 days and harvested for determining fresh weight, dry weight and percent water content as discussed in Chapter 3. Seedlings treated with ANE 0.1 % + HA 0.4 % (T_6) and ANE 0.25 % +HA 0.2% (T_{12}) showed increase in fresh weight (34.0 % and 62.9 % respectively) compared to control plants (Fig.14A). Whereas, seedlings treated with T_1 and T_4 showed fresh biomass increases of 9.6 % and 11.7 % respectively,

compared to control. Plants treated with T_3 increased 9.3 % and T_{13} increased 30.2 % of fresh biomass compared with control. In contrast, T_7 and T_8 had showed significant reduction in fresh biomass compared with other treatments i.e. T_6, T_9, T_{10}, T_{11}, T_{12} and T_{13} however, T_7 and T_8, comparatively with the control increased 4.3 % of fresh weight. A similar trend was observed in dry biomass of spinach plants (Fig. 14B). There were no significant increases observed in the percentage water content of plants treated with ANE, HA and ANE+HA treatments.

The overall results obtained from this experiment suggest that varying concentrations of ANE and HA significantly improved plant biomass ($p \leq 0.0001$) during early growth stages of spinach plants.

A



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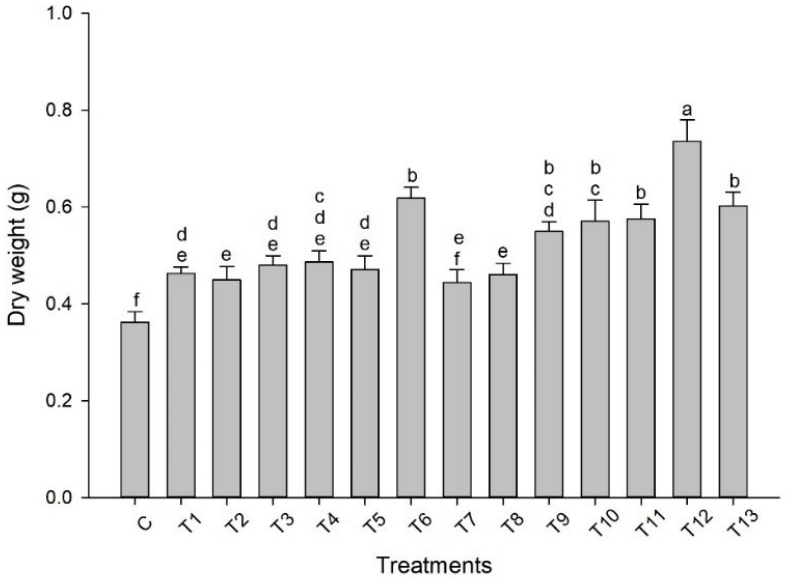


Figure. 14 21 day's pre-harvest treatment application of ANE, HA and ANE+HA on spinach plants to determine (14A) fresh weight, and (14B) dry weight. Treatment solutions were used as: C-control (water only), T_1-ANE (0.1%), T_2-ANE (0.5%), T_3-HA (0.2%), T_4-HA(0.4%), T_5-ANE(0.1%)+HA(0.2%),T_6-ANE (0.1%)+HA(0.4%), T_7-ANE (0.5%)+HA (0.2%), T_8-ANE (0.5%)+HA (0.4%),T_9-ANE (0.05%)+HA (0.1%),T_{10}-ANE (0.05%)+HA (0.2%), T_{11}-ANE(0.25%)+HA (0.1%), T_{12}-ANE (0.25%)+HA (0.2%) , T_{13}-ANE (0.25%). Values are the mean ± SE (n=18) and the significantly different mean values are represented by different letters.

4.5 Effect of different treatments of ANE, HA and ANE + HA on post-harvest shelf life quality of lettuce during storage period

Considering the results obtained from the seed germination experiment and early growth analysis, the most effective six treatments were identified and selected for further experiments. The six treatments and the control used in this experiment are listed in the previous chapter 3 Materials and methods (section 3.5).

4.5.1 Determination of weight loss of lettuce during storage

The effect of ANE, HA and ANE+HA on fresh weight loss of lettuce plants was studied for 21 days at a storage temperature of 0-4 ºC (Fig.15). All treatments and control showed increased post-harvest weight loss with an increase in storage time. A significant reduction in weight loss (59.5 % on day 10 and 50.5 % on day 21) were observed with a corresponding increase in dry matter content (DMC), of 7.4 g on day 10 and 7.3 g on day 21 per 100 g FW) in lettuce plants treated with ANE 0.25% +HA 0.2 % (T_{12}) during the storage period (Fig. 16). DMC for control plants showed 5.4 g/100 g FW and 4.1 g/100 g FW respectively, on day 10 and day 21 of the storage period. Overall, pre-harvest treatment of lettuce leaves with ANE, HA and ANE+HA, significantly reduced fresh weight loss of stored produce when compared with control. The most effective treatment ANE 0.25% +HA 0.2 % (T_{12}) increased lettuce DMC by 76.2 % compared with control on day 21 of storage (Fig. 16).

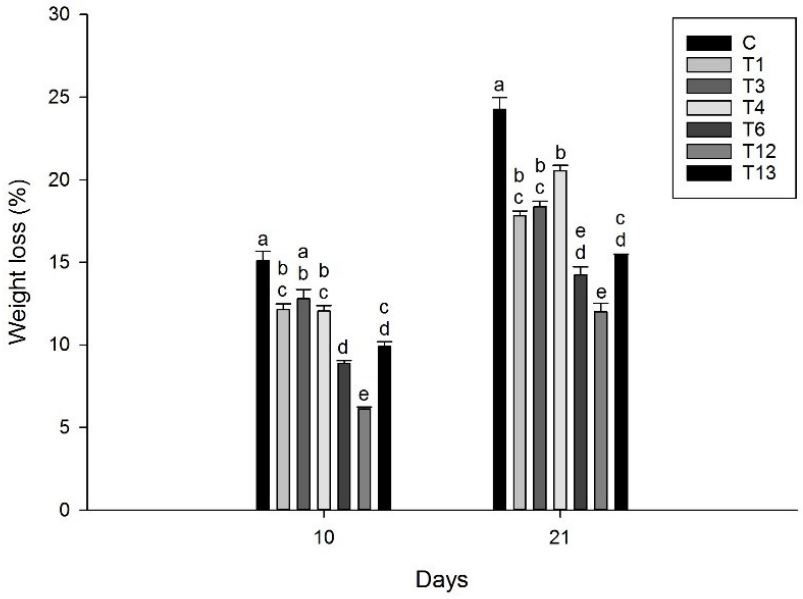


Figure. 15 The postharvest fresh weight loss in lettuce treated with different concentrations of ANE, HA, and ANE+HA held up to 21 days storage period at 0-4°c in the dark with RH ≥ 95%. Bars from left to right for the trearments represent as: C – control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6 ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n=9) and the significantly different mean values are represented by different letters.

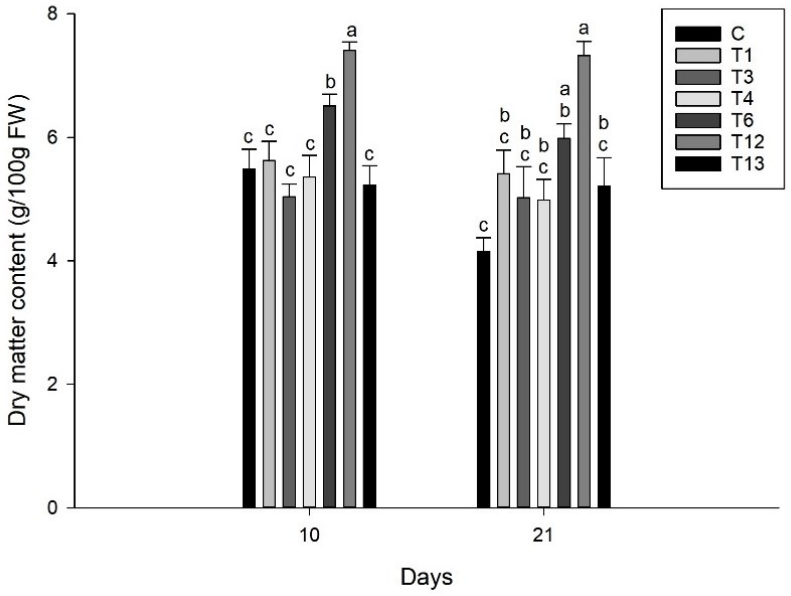
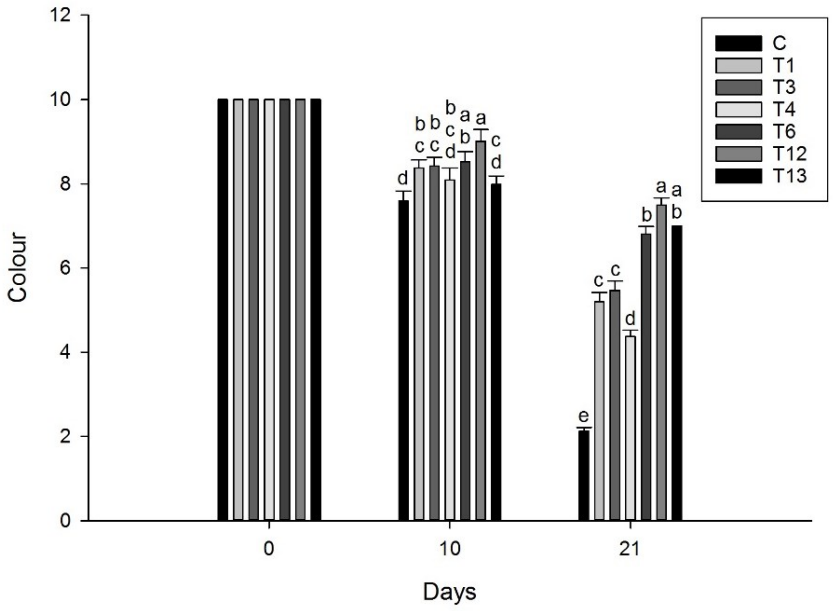


Figure. 16 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on dry matter content (DMC)(g/100g FW) of lettuce held up to 21 days storage period at 0-4°c in the dark with RH ≥ 95%. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%),T_6 ANE (0.1%)+HA(0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n=9) and the significantly different mean values are represented by different letters.

4.5.2 Visual quality analysis of lettuce at storage period

Lettuce plants treated with different concentrations of ANE, HA, and ANE+HA were evaluated throughout the storage period on day 10 and day 21. Treated lettuce plants had maintained visual quality (colour+ crispness/firmness) compared with control (Fig. 17C). The quality assessment was performed by scoring the individual plant based on colour and turgor (firmness/crispness) of the leaves on a scale of 1-10, Where 10 was given for the best quality and 1 was given for the least/poor quality of lettuce leaves. While, turgor was based on a scale of 1-5, 5 was given for the firm leaves and 1 was given for wet and slimy leaves. Lettuce leaves started mild discoloration from day 10 and notably increased discoloration was found on day 21 (Fig.17A and Fig 18). In storage, the lettuce leaves maintained leaf turgor until 10 days. As the storage time increased, the leaves started to shrivel and yellowing was observed in control plants (Fig. 18). Fig. 17B illustrates that all treated plants at 21- days displayed higher firmness compared with control. The visual quality score was significantly higher in plants treated with ANE 0.25 % +HA 0.2 % (T_{12}) at 21 days in storage (Fig 17C). Overall, the colour and turgor quality of leaves decreased during storage but T_6, T_{12}, and T_{13} showed delayed senescence and maintained crispness for the entirety of 21-day storage period. Significantly higher shriveling and yellowing of leaves was shown by control plants and T_1 on day 21. Overall, treated lettuce plants with ANE, HA and ANE+HA maintained better visual quality parameters compared with control throughout the storage period (Fig 18).

A.


Colour
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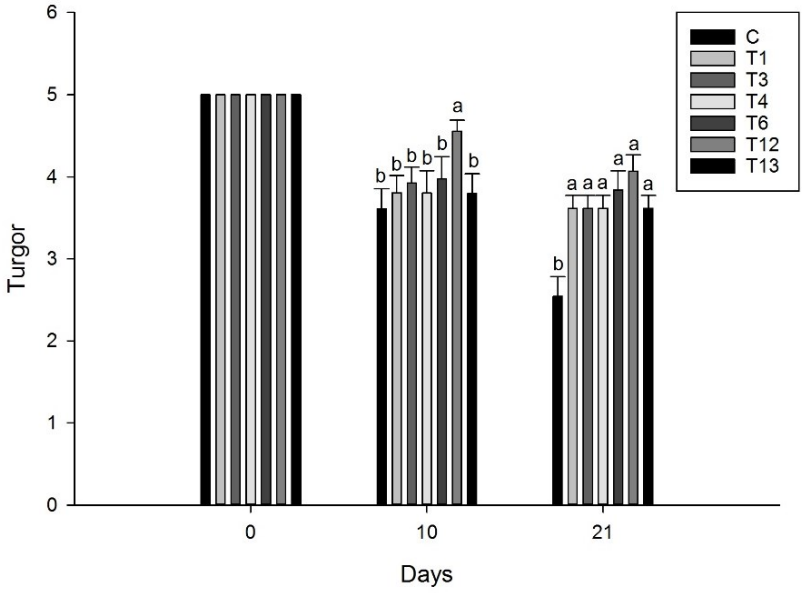
T13

B.


Turgor
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10
21
C
T1
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C.

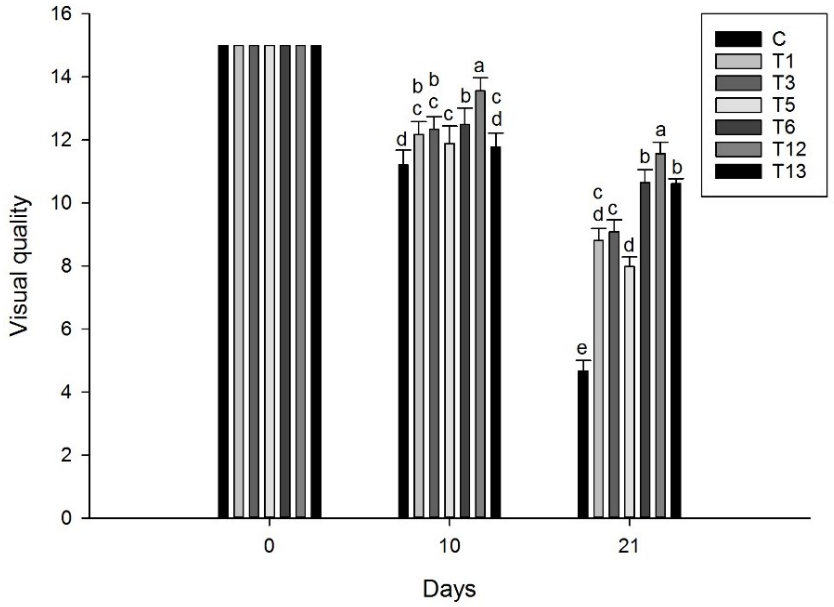


Figure. 17 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on (17A) colour (17B) Turgor and (17C) Visual quality of lettuce held over 21 days storage period at 0-4°c in the dark with RH ≥ 95%. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA(0.4%), T_6 ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n=9) and the significantly different mean values are represented by different letters.

Figure. 18 Lettuce control leaves, and leaves treated with different concentrations of ANE, HA, and ANE+HA held over 21 days storage period at 0-4°c in the dark with RH ≥ 95%. Treatment solutions were: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA(0.4%),T_6 ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%).

4.5.3 Lipid peroxidation analysis

MDA (malondialdehyde) is a biomarker and a secondary product formed during lipid peroxidation and can also be an indicator of oxidative stress. To estimate the amount of lipid peroxidation in lettuce plants during the storage period, Thiobarbituric acid-malondialdehyde (TBA-MDA) complex was examined in lettuce plants on the 10th and 21st days of the storage period. MDA content was reduced significantly in plants treated with T_6 and T_{12} on day 10 compared with control (72.6% and 78.7%, respectively). Increased MDA content in all treatments was shown on day 21 compared to day 10, but a significant 39 % reduction in MDA content was observed in plants treated with T_{12} compared with control (Fig. 19).

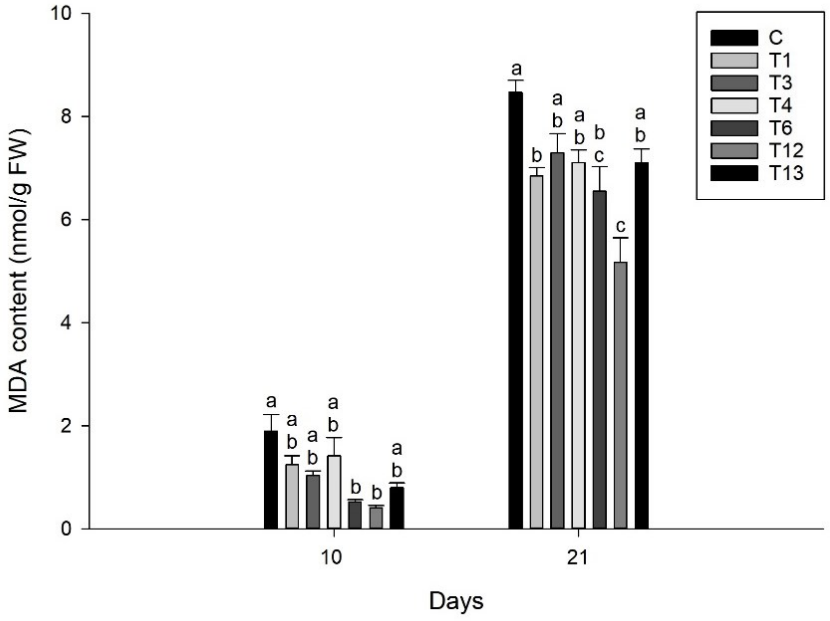


Figure. 19 The Effect of ANE, HA and ANE+HA on MDA content of lettuce on day 10 and day 21 at storage conditions. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE (0.1%)+HA

(0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.5.4 Determination of total ascorbic acid

Total ascorbate content in lettuce plants on day 10 of the storage period was significantly higher in T_6 and T_{12} compared to the control. The rapid decrease in ascorbates were observed on day 21 without any significant differences between treatments. Total ascorbates reduced from 30.7 µmol/g to 9.2 µmol/g FW (Fig. 20). Overall, ANE, HA and ANE+HA treated plants showed significantly higher ascorbic content on day 21 compared with untreated control plants.

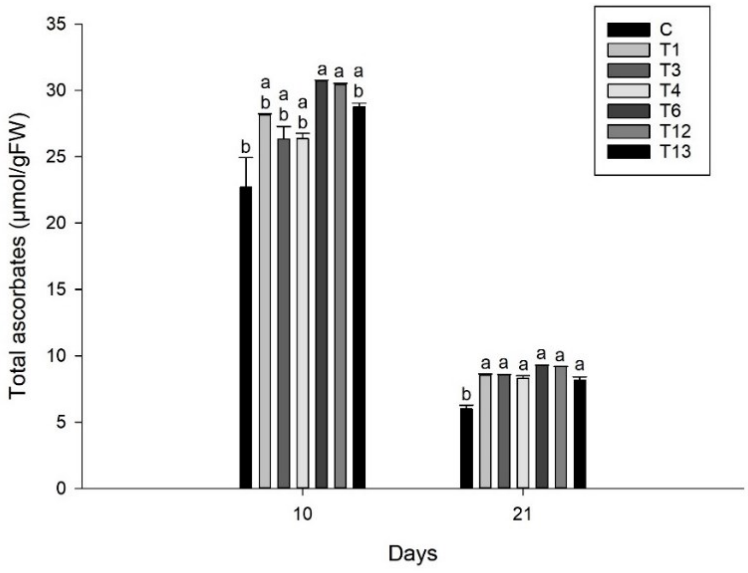


Figure. 20 The Effect of ANE, HA and ANE+HA on total ascorbate content of lettuce on day 10 and day 21 at storage conditions. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are

the mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.5.5 Determination of total phenolics content

The pre-harvest treatment of ANE, HA and ANE+HA on lettuce showed significantly higher ($p \leq 0.01$) phenolics content in T_6, T_{12}, and T_{13} on day 10 of the storage period compared with the control. Phenolics content was less in all treatments on day 21 of the storage period compared to day 10, though plants treated with T_{12} and T_{13} showed 0.7 and 0.5-fold increased phenolics content respectively on day 21 compared with control (Fig. 21). Overall, lettuce plants treated with T_{12} and T_{13} had significantly improved phenolics content after the 21-day storage period compared with control and other treatments.

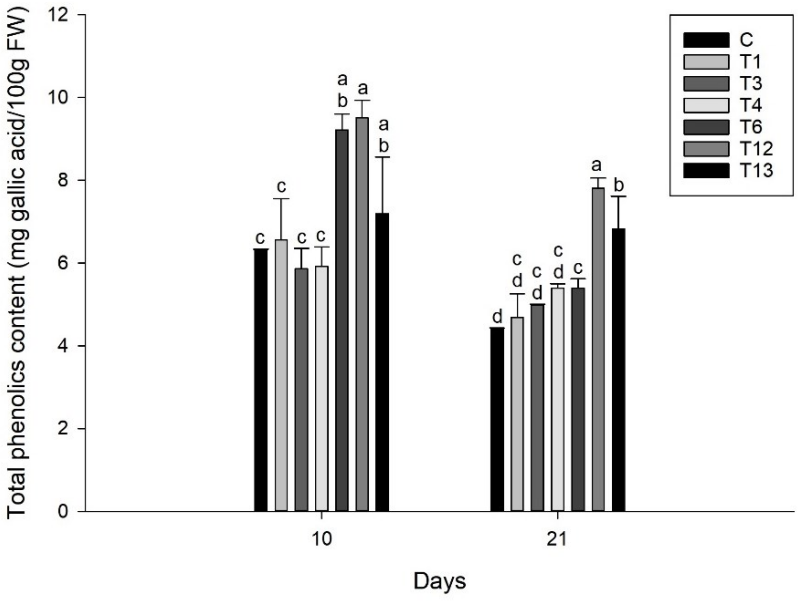


Figure. 21 The Effect of ANE, HA and ANE+HA on total phenolics content of lettuce on day 10 and day 21 at storage conditions. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-

ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.5.6 Determination of total antioxidants

The pre-harvest treatment of ANE, HA and ANE+HA significantly ($p \leq 0.025$) increased the total antioxidants capacity in lettuce during 21-day storage period compared with control (Fig 22). All treated and untreated lettuce plants had reduced total antioxidant capacity as the duration of increased storage period. However, the treated plants showed increased antioxidants on the day 10 and day 21 storage period compared to the control. Overall, the results suggest that the pre-harvest application of ANE, HA, and ANE+HA can improve the total antioxidants capacity of lettuce during 21days storage period.

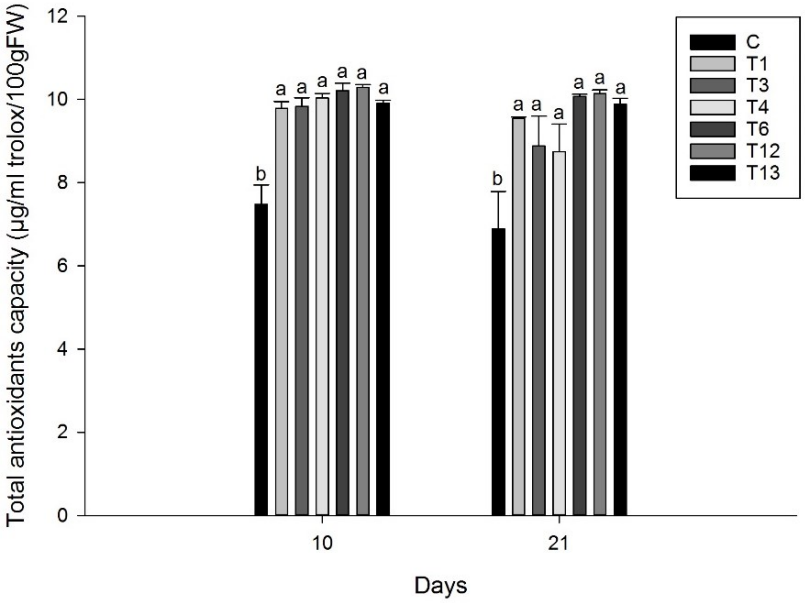


Figure. 22 The Effect of ANE, HA and ANE+HA on total antioxidants content of lettuce on day 10 and day 21 at storage conditions. Bars from left to right for the trearments represent as: C – control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.5.7 Determination of pigments (chlorophyll a, chlorophyll b, and anthocyanins contents)

The treatment effect of ANE, HA and ANE+HA showed no significant increase in chlorophyll a content of lettuce on day 10 of the storage period (Table 6). However, T_{12} (ANE 0.25 % + HA 0.2 %), was significantly higher on day 21 of the storage period. The treatments showed no significant effect on chlorophyll b content of lettuce throughtout the storage period. The anthocyanin content of lettuce plants treated with T_{12} (ANE 0.25 % +HA 0.2 %) was significantly higher when compared with control and other treatments ($p \leq 0.01$). Overall, the chlorophyll content of lettuce was not significantly affected by the application of ANE, HA and ANE+HA for duration of the storage period but improved the anthocyanin content of stored lettuce.

Table. 5 Different concentrations of ANE, HA and ANE + HA on pigments (chlorophyll a, chlorophyll b, and anthocyanins contents) of lettuce were evaluated on day 10 and day 21 of storage conditions. Treatments were: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values were represented by different letters.

Pigments	**Overall mean at day 10**						
	C	**T_1**	**T_3**	**T_4**	**T_6**	**T_{12}**	**T_{13}**
Chlorophyll a (µg/g fw)	91.5±7.4^{ab}	88.7±3.5^{b}	144.2±15.2^{ab}	129.9±23.6^{ab}	162.0±30.4^{a}	142.7±27.0^{ab}	112.8±16.7^{ab}
Chlorophyll B (µg/g fw)	65.3±6.2^{a}	99.1±14.0^{a}	117.3±14.6^{b}	113.0±18.2^{b}	166.5±36.3^{ab}	156.0±39.7^{b}	102.7±23.7^{b}
Anthocyanins (µg/L)	68.2±2.1^{b}	73.1±2.9^{b}	72.6±3.3^{b}	76.0±3.9^{b}	80.0±1.4^{ab}	95.8±6.4^{a}	83.8±7.3^{ab}
Pigments	**Overall mean at day 21**						
	C	**T_1**	**T_3**	**T_4**	**T_6**	**T_{12}**	**T_{13}**
Chlorophyll a (µg/g fw)	47.0±3.6^{d}	52.4±2.2^{cd}	57.6±4.6^{bc}	54.4±3.5^{bcd}	62.8±4.6^{ab}	70.0±4.1^{a}	51.5±3.9^{cd}
Chlorophyll B (µg/g fw)	45.1±1.5^{c}	45.0±3.0^{bc}	28.0±0.3^{ab}	24.4±4.7^{ab}	35.1±1.3^{a}	24.1±3.2^{ab}	25.7±2.5^{ab}
Anthocyanins (µg/L)	51.3±3.1^{b}	54.7±4.6^{b}	55.8±0.6^{b}	61.9±6.5^{b}	74.7±5.8^{ab}	78.6±4.7^{a}	58.8±3.2^{ab}

4.6 Effect of different treatments of ANE, HA and ANE +HA on post-harvest shelf life quality of spinach during storage period

4.6.1 Determination of weight loss of spinach during storage period

The pre-harvest root drench treatment of ANE, HA and ANE+HA showed a significant reduction in fresh weight loss of spinach during the 28-day storage period compared with the control ($p \leq 0.05$) (Fig. 23). Spinach plants showed a gradually increasing percentage fresh weight loss as duration in storage period increased. However, the addition of ANE+HA (T_{12}) showed 49.2%, 45.7 % and 39.3 % reductions in fresh weight loss on days 14, 21, and 28 of the storage period, respectively. Similarly, T_6 reduced the fresh weight loss on the same days by 47.6 %, 39.5 % and 37.4 % compared with control plants.

The higher dry matter content of spinach was observed in all treatments but the DMC decreased as the duration of the storage period increased. However, significantly higher DMC was demonstrated in plants treated with T_{12} (ANE 0.25 %+HA 0.2 %) compared with other treatments (Fig. 24). Overall, different ANE, HA, and ANE+HA treatments showed decreased weight loss and effectively increased DMC during the storage period.

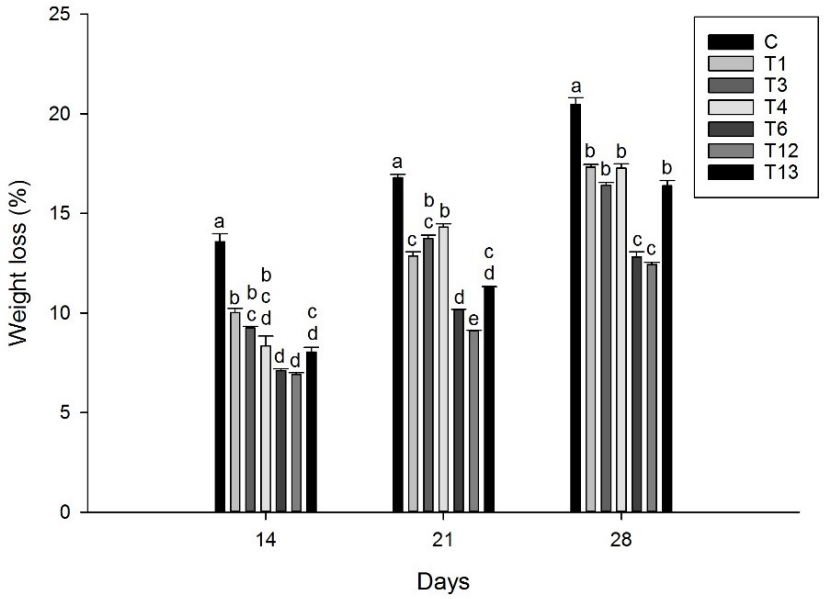


Figure. 23 The postharvest fresh weight loss in spinach treated with different concentrations of ANE, HA, and ANE+HA held upto 28 days storage period at 0-4°c in the dark with RH ≥ 95%. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA(0.4%),T_6 ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n=9) and the significantly different mean values are represented by different letters.

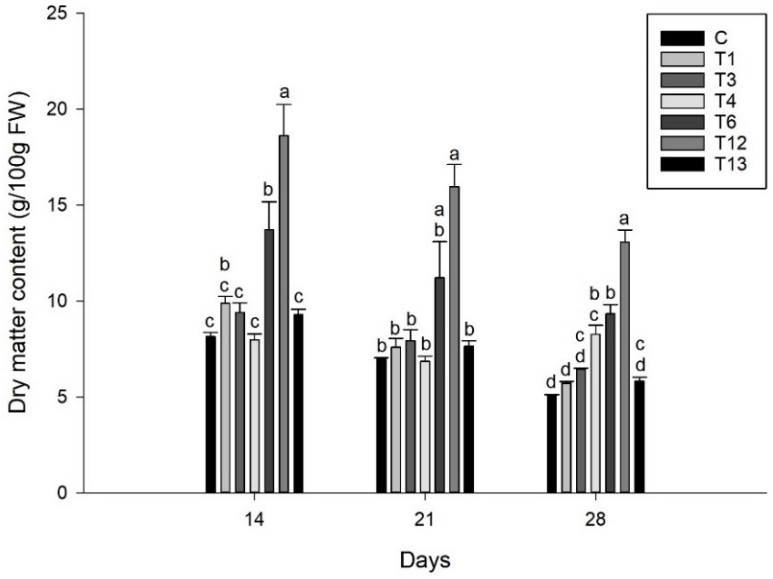
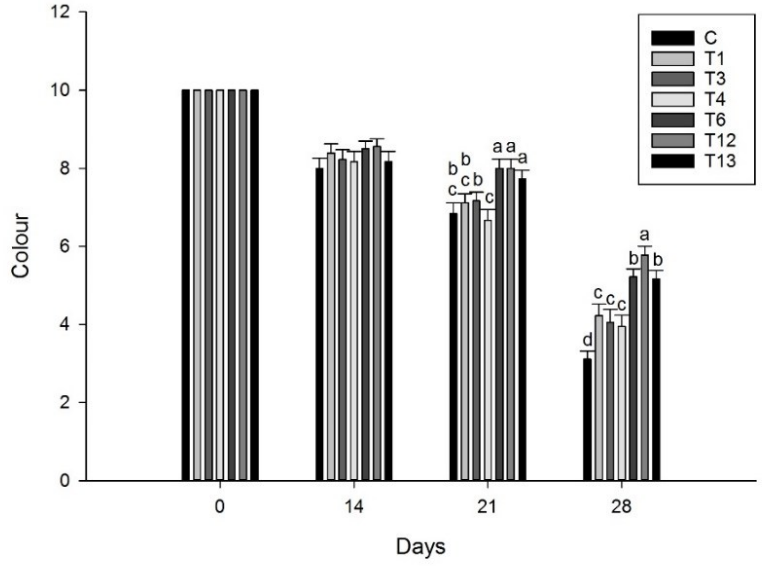


Figure. 24 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on dry matter content (DMC) of spinach upto over 28 days storage period at 0-4°c in the dark with RH ≥ 95%. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA(0.4%),T_6 ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n=9) and the significantly different mean values are represented by different letters.

4.6.2 Visual quality analysis of spinach during storage period

Spinach plants treated with different concentrations of ANE, HA, and ANE+HA were evaluated for their colour and turgor throughout the storage period on days 14, 21, and 28. Treated spinach plants maintained visual quality (colour+ crispness/firmness) on day 14, and gradually reduced throughout the duration of the storage period (Fig. 26). Shriveling and curling of spinach leaves was visibly observed on day 21. The quality assessment for spinach leaves was performed by scoring the individual plant based on colour and turgor of the leaves on two scales: colour was based on a scale of 1-10, while turgor was based on a scale of 1-5. For colour, 10 was given for the best quality and 1 was given for the poor colour quality of spinach leaves, for turgor, 5 was given for the firm leaves and 1 was given for wet and slimy leaves. The colour and turgor quality of treated and untreated spinach leaves significantly decreased over the 28 day storage period (Fig 25A and 25B). However, the visual quality score was significantly higher in treated plants on day 21 except $T_{5,}$ and all the treatments on day 28 (Fig. 25c). The colour of leaves showed no significant effect on day 14 but T_6 – (ANE 0.1 %+HA 0.4 %),T_{12} – (ANE 0.25 % +HA 0.2 %), and T_{13} – (ANE 0.25 %) showed delayed senescence and maintained colour on day 21 and day 28. Shriveling, yellowing and lack of firmness of leaves was observed on control plants, ANE treated, and HA treated plants on day 21 and day 28. Overall, plants treated with T_6, T_{12}, and T_{13} maintained better visual quality compared to control and other treatments (Fig. 25c).

A.


Colour
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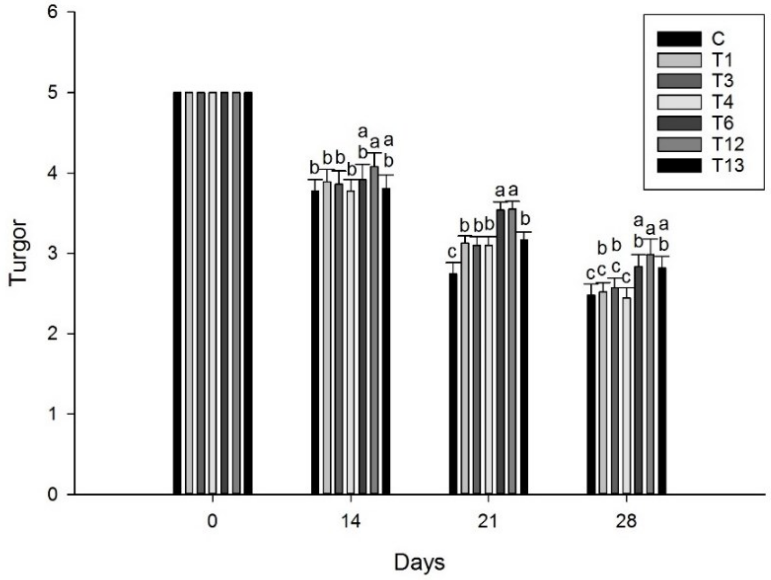
B.


Turgor
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C.

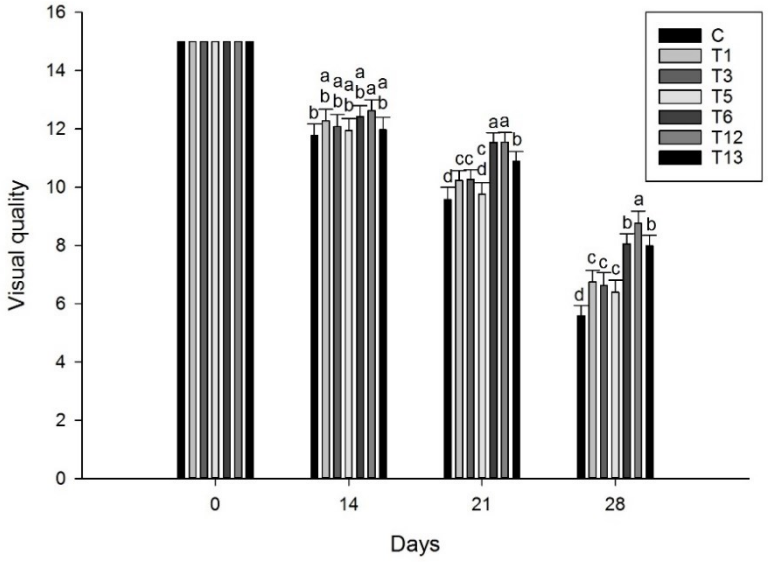

Figure. 25 The pre-harvest treatment effect of different concentrations of ANE, HA, and ANE+HA on (A) colour (B) Turgor and (C) Visual quality of lettuce held over 21 days storage period at 0-4°C in the dark with RH ≥ 95%. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA(0.4%),T_6 ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n=9) and the significantly different mean values are represented by different letters.

Treatments	C	T_1	T_3	T_4	T_6	T_{12}	T_{13}
Day 14							
Day 21							

Figure. 26 Spinach control leaves, and leaves treated with different concentrations of ANE, HA, and ANE+HA held over 28 days storage period at 0-4°c in the dark with RH ≥ 95%. Treatment solutions were: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%),T_6 ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%).

4.6.3 Determination of Lipid peroxidation

Thiobarbituric acid-malondialdehyde (TBA-MDA) complex was examined on days 14, 21, and 28 of the storage period to estimate the amount of lipid peroxidation in the spinach plants. The effect of ANE, HA and ANE+HA on spinach plants moderately reduced peroxidation on days 14, 21, and 28 compared with the control. The gradual increase in lipid peroxidation was observed in all the treated and untreated plants but significantly reduced MDA content was showed in T_6 and T_{12} on day 28 ($p \leq 0.0405$) as shown in Fig. 27.

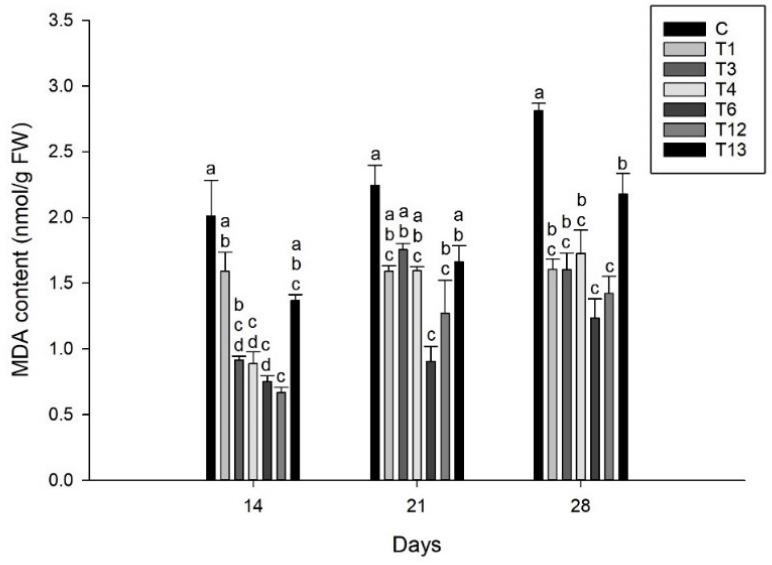

Figure. 27 Effect of ANE, HA and ANE+HA on lipid peroxidation of spinach on day 14, 21 and day 28 at storage conditions. Bars from left to right for the trearments represent as: C- control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the

mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.6.4 Determination of total ascorbic acid

A 1.4 and 2.5-fold increase in ascorbates content was assessed in spinach plants treated with treatments T_6 and T_{12} respectively, compared with the control on day 14 of the storage period. The gradual decrease in total ascorbates was observed throughout the duration of storage period, however, plants treated with T_6 and T_{12} showed significantly higher amounts of ascorbates compared with control (Fig. 28).

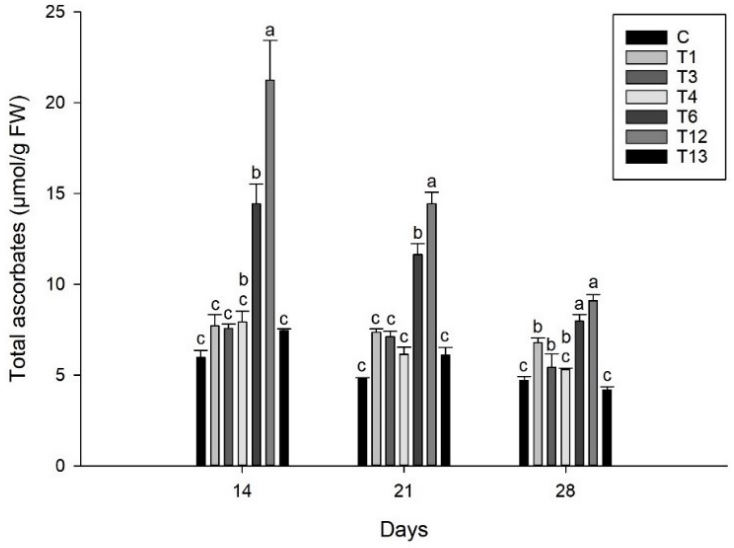

Figure. 28 Effect of ANE, HA and ANE+HA on total ascorbates of spinach on day 14, 21 and day 28 in storage conditions. Bars from left to right for the trearments represent as: C – control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the

mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.6.5 Determination of total phenolics content

The total phenolics content of spinach plants was estimated on Days 14, 21, and day 28 during the storage period. On day 14, a significantly higher phenolics content was shown in plants treated with T_6 (74 %) and T_{12} (112.4 %) compared with control ($p \leq 0.001$). Whereas a rapid decline in phenolics content was shown on day 21 and day 28 with no significant difference between treatments (Fig. 29).

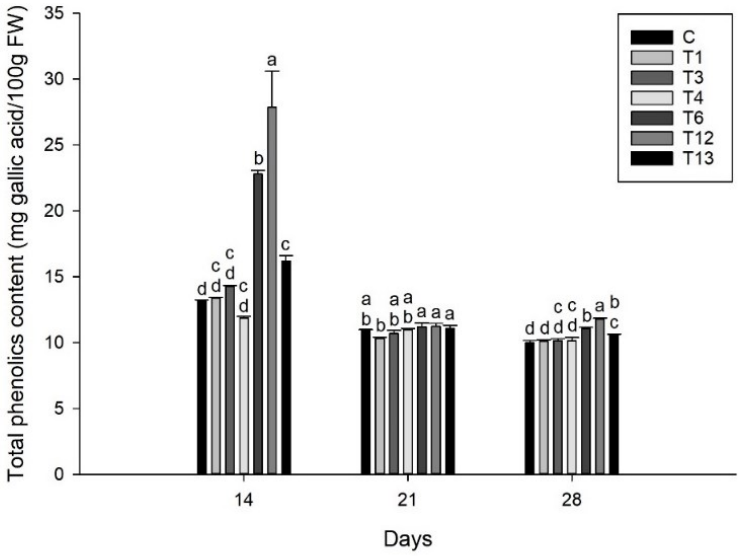

Figure. 29 Effect of ANE, HA and ANE+HA on total phenolics contents of spinach on day 14, 21 and day 28 in storage conditions. Bars from left to right for the trearments represent as: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-

ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA (0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.6.6 Determination of total antioxidants

The pre-harvest treatment with ANE+HA significantly increased the total antioxidant capacity in spinach throughout the duration of the 28-day storage period ($p \leq 0.0013$). All treated and untreated spinach plants reduced the total antioxidants as duration of the storage period increased (Fig. 30). However, treated plants with ANE 0.1 % + HA 0.4% (T_6), ANE 0.25 % +HA 0.2 % (T_{12}), have showed significantly improved antioxidants on day 14, 21, and 28 compared to the other treatments and control. The results suggest that the pre-harvest application of ANE, HA, and ANE+HA can improve the total antioxidants capacity of spinach throughout the storage period.

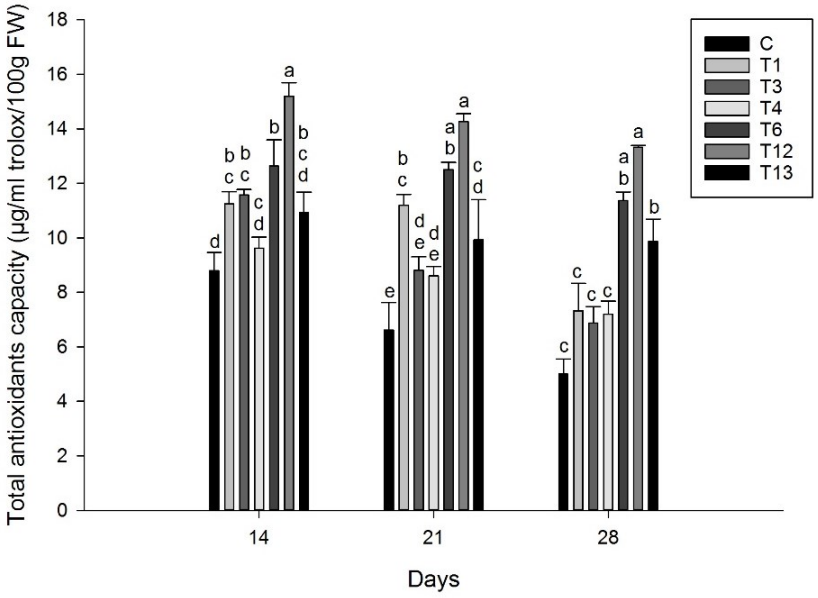

Figure. 30 Effect of ANE, HA and ANE+HA on total antioxidant capacity of spinach on day 14, 21 and day 28 at storage conditions. Bars from left to right for the trearments represent as: C – control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE (0.1%)+HA (0.4%), T_{12}-ANE (0.25%)+HA(0.2%), and T_{13}-ANE (0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values are represented by different letters.

4.6.7 Determination of pigments (chlorophyll a, chlorophyll b, and anthocyanins contents)

The pre-harvest root drench treatment of ANE, HA and ANE+HA showed increased chlorophyll a content of spinach on day 14 of the storage period (table 7). However, no significant improvement was found on day 21 and day 28. Chlorophyll b was higher in all treatments during the same 28-day storage period. Significantly higher anthocyanin content ($p \leq 0.05$) was observed in the plants treated with ANE 0.25 % +HA 0.2 % (T_{12}), and ANE 0.1 %+ HA 0.4 % (T_6) compared with other treatments and control. Overall, there was no significant improvement of chlorophyll a or chorophyll b in spinach on day 21 or 28. The pre-harvest root drench application of ANE+HA to the spinach plants significantly improved the anthocyanin content during the 28 days storage period.

Table. 6 Different concentrations of ANE, HA and ANE+HA on pigments (chlorophyll a, chlorophyll b, and anthocyanins contents) of spinach were evaluated on day 14, and day 21 of storage conditions. Treatments were: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE(0.1%)+HA(0.4%), T_{12}-ANE(0.25%)+HA(0.2%), and T_{13}-ANE(0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values were represented by different letter

Pigments	**Overall mean at day 14**						
	C	**T_1**	**T_3**	**T_4**	**T_6**	**T_{12}**	**T_{13}**
Chlorophyll a (µg/g fw)	144.6±9.2^{e}	190.0±5.1^{d}	242.7±2.4^{bc}	196.8±4.9^{d}	292.6±11.7^{a}	265.4±1.02^{ab}	227.2±12.1^{c}
Chlorophyll b (µg/g fw)	116.6±6.4^{b}	118.2±5.05^{b}	119.1±1.5^{b}	117.0±7.0^{b}	143.6±11.2^{a}	142.4±7.1^{a}	129.0±9.8^{ab}
Anthocyanins (µg/L)	204.4±6.5^{b}	211.2±9.4^{b}	231.5±19.9^{b}	227.3±12.7^{b}	330.9±45.5^{a}	351.7±17.2^{a}	192.6±9.5^{b}
Pigments	**Overall mean at day 21**						
	C	**T_1**	**T_3**	**T_4**	**T_6**	**T_{12}**	**T_{13}**
Chlorophyll a (µg/g fw)	136.0±2.04^{a}	166.9±10.3^{a}	168.9±13.6^{a}	155.3±7.8^{a}	174.0±4.0^{a}	189.7±11.7^{a}	164.9±3.2^{a}
Chlorophyll b (µg/g fw)	81.7±5.2^{c}	102.2±4.2^{bc}	116.1±1.8^{ab}	125.1±6.6^{ab}	132.9±2.6^{a}	123.4±5.4^{ab}	120.4±1.5^{ab}
Anthocyanins (µg/L)	151.3±3.1^{a}	162.9±5.5^{b}	180.4±3.2^{b}	166.4±6.6^{b}	225.4±10.8^{a}	229.3±12.2^{a}	182.2±0.9^{b}

Table. 6 Different concentrations of ANE, HA and ANE+HA on pigments (chlorophyll a, chlorophyll b, and anthocyanins contents) of spinach were evaluated on day 28 of storage conditions. Treatments were: C-control (water only), T_1-ANE (0.1%), T_3-HA (0.2%), T_4-HA (0.4%), T_6-ANE(0.1%)+HA(0.4%), T_{12}-ANE(0.25%)+HA(0.2%), and T_{13}-ANE(0.25%). Values are the mean ± SE (n≥9) and the significantly different mean values were represented by different letter

Pigments	**Overall mean at day 28**						
	C	**T_1**	**T_3**	**T_4**	**T_6**	**T_{12}**	**T_{13}**
Chlorophyll a (µg/g fw)	134.8±6.8^{a}	167.7±3.7^{a}	154.0±9.2^{a}	142.0±6.3^{a}	166.0±2.3^{a}	167.7±6.3^{a}	138.6±5.9^{a}
Chlorophyll b (µg/g fw)	75.0±0.5^{b}	87.3±6.2^{ab}	88.2±4.8^{ab}	108.6±4.7^{a}	98.3±10.3^{ab}	86.9±6.5^{ab}	82.4±3.4^{ab}
Anthocyanins (µg/L)	158.3±1.9^{bcd}	150.9±3.8^{cd}	171.9±4.3^{b}	146.3±1.8^{d}	188.3±1.2^{a}	198.9±2.5^{a}	163.6±1.7^{bc}

Chapter 5 DISCUSSION

Changes in the global environment can lead to enormous post-harvest losses in crop productivity. Researchers are focusing on natural, environmentally friendly practices to increase agricultural productivity. Biostimulants derived from natural resources are widely used in modern agricultural practices (Bulgari et al. 2015). Biostimulants are complex mixtures of compounds and possess distinct modes of action involved in the growth promotion of plants (Van Oosten et al. 2017).

Ascophyllum nodosum extract (ANE) is a biostimulant derived from *Ascophyllum nodosum* and is widely used for its plant growth-promoting activity and its role in mitigating biotic and abiotic stresses (Shukla et al. 2017; Santaniello et al. 2017, Jithesh et al. 2012, Khan et al. 2009). Humic acids (HA) are naturally occurring heterogenous substances produced through the decaying of organic materials. HAs stimulate plant growth through improving nutrient uptake and metal chelation (Baldotto and Baldotto 2013; Calvo et al. 2014; Van Oosten et al. 2017) but also impart stress tolerance in plants through various mechanisms (i.e. increasing membrane stability, ion homeostasis, osmotic adjustments and ROS scavenging) (Chen and Aviad 1990; Van Oosten et al. 2017).

Lettuce and spinach are leafy vegetables widely consumed throughout the world, contributing significantly to the agricultural economy. In the present study, we evaluate the effects of *Ascophyllum nodosum* extract (ANE), humic acids (HA) and ANE+HA for their potential to improve seed germination, early growth characteristics, and post-harvest quality of lettuce and spinach.

5.1 The effect of ANE, HA, and ANE+HA on seed germination and early growth of lettuce and spinach

This study demonstrated that the root drench application of ANE, HA, and ANE+HA stimulates seedling establishment and augments the early growth of lettuce and spinach. The addition of HA improved seed germination of both lettuce and spinach. Similarly, Yildrim et al. (2000) reported that the application of HA improved the seed germination of different leafy vegetables such as parsley, celery and leek. HA also mitigated the negative effect of salinity and drought on the germination of soybean seeds (Gawlik et al. 2016). In this experiment, the higher concentrations of ANE and HA, 0.5 % ANE + 0.4 % HA (T_9) illustrated no phytotoxic effect on seed germination parameters of lettuce and spinach. The effect of ANE+HA on seed germination of both lettuce and spinach was more pronounced as compared to individual treatments. In previous studies, it was found that ANE is rich in hormone-like substances, major and micro plant nutrients and polyphenols (Cardozo et al. 2007; Wally et al. 2013). While, humic acid is rich in mineral nutrients, polyphenolic compounds, hormone-like substances and has various plant growth-promoting activities like nutrient uptake by chelating metal ions, protein synthesis, oxidative phosphorylation and photosynthesis (Atiyeh et al. 2002). In our study, the combined activities of both ANE and HA elicited higher root growth and plumule growth in both lettuce and spinach seedlings.

Seed dormancy and germination are complex physiological processes regulated by various developmental and climatic factors (Koornneef et al. 2002). Germination of the seed is initiated with the imbibition of water and terminates with the elongation of the embryonic axis (Bewley 1997). Lettuce and spinach seeds treated with lower concentrations of ANE, HA, and ANE+HA showed better germination rates associated with higher mean daily germination and seedling vigour indexes. This improvement may be due to the increased biosynthesis of gibberellins, which would have induced the activity

of α-amylase that promoted early germination by enhancing the availability of starch assimilation. Previously, Rayorath et al. (2008) showed that the extract of brown seaweed *Ascophyllum nodosum* induces GA independent α-amylase activity in barley seeds. Sanfilippo et al. (1990) showed that HA contained a significant amount of both free and conjugated GA. Thus, a plausible reason behind the combinatorial effect of ANE and HA on seed germination of lettuce and spinach might be that ANE induced GA independent amylase activity and GA-like properties of HA. GA-like compounds present in HA (Sanfilippo et al. 1990) might act as a signal in germinating the seed by activating the α-amylase genes in the aleurone cells which in turn secretes α -amylase (Sun and Gubler 2004).

Previously published reports show that the application of ANE improved root growth and lateral root development (Rayorath et al. 2008; Khan et al. 2009). Zhang et al. (2003) showed that application of ANE and HA, along with propiconazole improves post-transplant rooting in tall fescue sod. Foliar application of seaweed extract prepared from *Ecklonia maxima* improved yield of nutrient-stressed lettuce (Crouch et al. 1990) and treatment with HA showed a greater effect on increased root growth and root surface area in hydroponically grown wheat (Vaughan and Malcolm 1985). Auxins are major phytohormones that control root growth by regulating cell proliferation and enlargement (Fu and Harberd 2003). A commercial extract of ANE contains approximately 50 mg g^{-1} of indole acetic acid (IAA) by dry weight (Kingsman and Moore 1982). Rayorath et al. (2008) showed that the organic fraction of ANE activates the expression of GUS driven by the synthetic auxin responsive promoter DR5 in transgenic *Arabidopsis thaliana*. Similarly, HA also contain auxin-like substances and polyamines that act directly as plant growth regulators by stimulating plant growth (Dell Agnolla and Nardi 1987). Young and Chen (1997) showed that the application of humic acids in lettuce seedlings increased root

growth associated with stimulation in polyamine content. HA isolated from earthworm compost enhanced the root growth and lateral root emergence of *Zea mays* seedlings (Canellas et al. 2002). The structural analysis of HA derived from earthworm compost revealed the presence of exchangeable auxin groups that are involved in the induction of lateral root development (Canellas et al. 2002). Trevisan et al. (2010) also reported the auxin-like activity of HA and their role in the initiation of lateral roots in *Arabidopsis thaliana.* The application of HA activated the expression of DR5: GUS and showed enhanced expression of an early auxin responsive gene *IAA19* (Trevisan et al. 2009). The application of lower concentrations of ANE+HA had a more pronounced effect on the root growth of lettuce and spinach seedlings as compared to the higher concentrations of the individual applications (HA and ANE) by an additive effect of auxin-like activity possessed by both biostimulants.

In this study, the application of ANE+HA showed better early growth in terms of shoot and root length of lettuce and spinach. Similarly, Billard et al. (2014) showed that the application of the *Ascophyllum nodosum* extract (AZAL5) and humic acids (HA7) to *Brassica napus L.* improved plant biomass and nutrient uptake. Overall, the findings of this study suggest that the application of ANE, HA, and ANE+HA improves seed germination and early growth of spinach and lettuce. However, there is less understanding of the working mechanisms of ANE and HA, due to their vast composition and properties, but based on prior investigations we speculate that plant hormone-like substances, osmolytes and nutrients present in the biostimulants promote these physiological responses.

5.2 Physiological and biochemical studies on lettuce and spinach during storage period to determine post-harvest quality

Post-harvest quality evaluations play an essential role in agricultural and horticultural crops. It is important to maintain quality and minimize losses of a crop. The management of post-harvest losses provides food for a growing human population, conserves environmental resources, and creates profit for growers, retailers and consumers.

In this study, different combinations of biostimulants were used to determine their efficacy on improving quality and reducing post-harvest losses of leafy vegetables. The pre-harvest root drench treatment of ANE, HA and ANE+HA can maintain post-harvest quality of spinach and lettuce at different storage times. In this experiment, the pre-harvest root drench treatments were applied to plants starting at the early growth stage up until one week before harvest. Spinach and lettuce are important and commonly grown leafy vegetables, they are most often consumed raw in salads and sandwiches. This leafy vegetable contains nutrients which have positive impacts on human health. Spinach and lettuce are cool season crops, which mature within a short growing season and also perish quickly after harvest. The storage life period of these leafy vegetables varies depending upon their genotype, and, most importantly, upon pre-harvest growth conditions. The marketable shelf life of spinach and lettuce are ±14 days (Kader 2002). In this study, treated lettuce and spinach plants exhibited extended shelf life up to 21 days over control plants.

Fresh weight loss, colour and turgor quality are important parameters of produce during storage conditions as these attributes decide the market value of the commodity (Ansah et al. 2018). Factors such as temperature and relative humidity play an important role in the post-harvest quality of fruits and vegetables during storage (Hodges et al. 2004). In

this experiment, regardless of the plant species, the pre-harvest root drench treatment, T_{12} (0.25 % ANE+0.2 % HA) showed significantly reduced fresh weight loss and increased dry matter content (DMC); for lettuce, this was observed over a 21-day storage period while in spinach, T_{12} (0.25 % ANE+0.2 % HA) and T_6 (0.1 % ANE+0.4 % HA) treatments showed reduced fresh weight loss over a 28-day storage period. This indicates that ANE+HA can reduce fresh weight loss and increase dry biomass during the storage period. This might be due to the presence of micro- and macronutrients present in *Ascophyllum nodosum* extract (Mancuso et al. 2006); as well as the possible enhancement of the plants nutrient uptake through the chelation effects of the addition of humic acids with ANE (Khaled and Fawy. 2011).

Changes in colour and water loss are other major challenges that occur during the storage period of leafy vegetables which lead to faster deterioration of the commodity. Post-harvest senescence is an accelerated process developed by plants during storage conditions associated with different stresses such as non-availability of nutrients, water, and inhibition of photosynthesis which leads to the development of reactive oxygen species (ROS) and causes cell death (Canetti et al. 2002). In this study, spinach and lettuce plants exhibited significantly improved visual quality parameters (colour, firmness and delayed senescence) on the combined application of ANE+HA. Retained colour and turgor of the spinach and lettuce after 28-day and 21-day storage period respectively, were observed significantly in plants treated with ANE+HA compared to the control. Control plants turned yellow during the storage period, indicating senescence. Cytokinins and gibberellins are plant growth regulators that aid plants in delayed leaf senescence; (Nooden 1989; Van Staden and Harty 1988). Cytokinins show antisenescence properties by suppressing lipoxygenase and maintaining cell membrane integrity (Srivastava and Strivastava 2002; Thimann 1987). ANE and HA contains plant hormone-like substances

such as cytokinins and gibberellins (Khan et al. 2011; Pizzeghello et al. 2001; Zhang and Ervin 2004). The pre-harvest root drench application to the leafy vegetables might stimulate the biosynthesis of gibberellins and cytokinin-like substances which could be the plausible reason for retarding plant senescence during the storage period.

The increased ROS during the storage period leads to lipid peroxidation caused by oxidative damage of cells and tissues (Ayala et al. 2014). Malondialdehyde (MDA) is used as an indicator of lipid peroxidation and is a stable end-product formed during decomposition of polyunsaturated fatty acids (PUFAs) (Hodges et al.1999). The presence of antioxidants and phytochemicals such as anthocyanins and phenolics scavenge the free radicals and protects plants from oxidative damage (Bergquist et al. 2006). Our results demonstrated the increased lipid peroxidation in all the treated and untreated plants during the increased storage duration. Significant reduction of MDA content was observed in stored spinach and lettuce at different storage periods in the plants treated with 0.25 % ANE+0.2 % HA (T_{12}). The antioxidants present in the ANE and HA reduced the free radicals preventing plants from oxidative stress.

Lettuce plants treated with ANE, HA and ANE+HA exhibited significantly higher total antioxidants compared with control; this might be one of the reasons the lettuce plants maintained visual quality and shelf life at storage. Spinach plants treated with 0.25 % ANE+0.2 % HA (T_{12}) exhibited significantly higher antioxidants compared with other treatments. Similarly, 1.0 g/L ANE treated plants improved antioxidants with enhanced activities of glutathione reductases (Fan et al. 2010). In this study, total ascorbic acid in lettuce and spinach was significantly higher in plants treated with ANE+HA, but surprisingly, on day 21, ascorbic contents were noticeably reduced in all treatments and control, however, all treated plants showed higher ascorbic content. Albrecht (1993) reported that lettuce plants are the least source of vitamin C content, and also stated that

vitamin C was lost in whole lettuce at storage. Similarly, the results showed loss of ascorbic content in lettuce plants at 21 days storage. Though the amount of vitamin C in spinach plants gradually reduced as storage time increased, 0.1 % ANE+0.4 % HA (T_6) and 0.25 % ANE+0.2 % HA (T_{12}) treated plants exhibited decreased loss of ascorbic acids compared to control and other treatments. ANE and HA may have imposed secondary metabolism by acting through non-enzymatic antioxidant systems such as ascorbates, phenols and glutathione (Allen et al. 2001; Schiavon et al. 2010). Furthermore, in this study, phenolics content and anthocyanin content were enhanced during storage in lettuce and spinach plants treated with ANE+HA. However, in spinach, phenolics content was reduced on 21 Day and 28 Day storage periods. ANE, HA and ANE+HA had no significant effect on chlorophyll A and chlorophyll B of spinach and lettuce during storage. ANE and HA are heterogeneous compounds and their mode of action is unknown during storage. In this study, ANE+HA treated plants maintained better visual quality, increased total antioxidants, and reduced lipid peroxidation.

Chapter 6 CONCLUSION

Plant biostimulants came into existence to reduce excessive usage of synthetic inputs and to promote plant growth and yield. Seaweed extracts and humic acids are widely used biostimulants to enhance agricultural and horticultural crop performance.

The objective of the present research was to investigate the effective treatments on *Ascophyllum nodosum* extract (ANE), humic acids (HA), and ANE+HA on seed germination and early growth of lettuce and spinach and more specifically, to determine the most effective treatments on post-harvest shelf life of lettuce and spinach.

The results of the present research showed that the application of ANE, ANE+HA solutions to the seeds of lettuce and spinach for 7 days under green house conditions had generally beneficial effects enhancing germination percentage, radicle and plumule length. Whereas, HA alone reduced radicle and plumule length. The seeds treated with ANE + HA had an additive stimulatory effect on radicle length, plumule length and seedling vigour index. The root drench application of 13 treatments on one-week old lettuce and spinach plants grown on growth medium (PRO-MIX®) for 21 days had showed significantly increased fresh and dry biomass. The higher concentrations of ANE + HA (T_7 and T_8) showed slightly reduced biomass compared to other combinational treatments, however, T_7 and T_8 treatments were showed effective biomass compared to control.

The pre-harvest root drenching of ANE+HA over 30 and 35 days of lettuce and spinach respectively, showed significantly improved visual quality after storage compared to control. Lettuce stored over a 21-day period showed retained colour, turgor (firmness/crispness) and delayed leaf senescence. All treatments reduced the fresh weight loss up to 21 days. Moreover, plants treated with T_{12} showed higher phenolics, antioxidants and anthocyanin contents. Likewise, spinach stored over 28 days retained

colour and turgor until 21 days, but quality decreased as the storage period increased beyond 21 days. However, the spinach plants treated with T_6 and T_{12} maintained visual quality up to 28 days and demonstrated increased dry matter content and reduced fresh weight loss. Total ascorbic content, phenolics and total antioxidants were improved in treated plants compared with the control.

Our results demonstrated that ANE, HA and ANE+HA can improve plant growth and establishment. Plants treated with T_{12} had the highest post-harvest shelf life of lettuce and spinach. Plants treated with T_1, T_3, T_4, T_6 and T_{13} also improved quality of spinach and lettuce when compared to control. More importantly, the combinations of ANE + HA showed additive effects on leafy vegetables.

The combination of plant biostimulants, ANE+HA can be used by agriculture companies and growers to enhance the plant growth and to cultivate crops more efficiently. Further, laboratory studies on molecular characteristics of biostimulants could help to understand how and why the use of biostimulants results on specific plant responses. Further studies on the crop growth responses comparing with the synthetic inputs and biostimulants on different species of leafy vegetables or any agricultural crops will be helpful to address the food losses and environmental losses.

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APPENDIX – A

Quality assessment scores for senescence in lettuce and spinach (adapted from Di fan 2010 with minor modifications)

Color	Firmness/Turgor
10 vibrant green (best quality)	**5** firm
9 dark dull green-vibrant green	**4** lack of turgor
8 dark dull green-yellow or dry tip	**3** shriveled/folding/rolling
7 green-yellowing of ½ of leaf	**2** partly wet and slimy
6 mostly yellow with light green	**1** no form-wet and slimy
5 all yellow or slight green	
4 yellow-dull green (10% black tip)	
3 yellow- dull green (50% black tip)	
2 yellow-dull green (75% black tip)	
1 100% black (poor quality)	

Visual quality assessment = colour score + Firmness score