

**SEAWEED AMENDMENTS FOR REDUCTION OF DAMPING OFF
CAUSED BY *PYTHIUM ULTIMUM***

by

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I dedicate my M.Sc. program and this thesis to my parents who have been always my nearest reserve for motivation and guidance. Their unconditional love, trust and patience has always motivated me to aim for higher goals. Their never ending belief in me have always made to strive for excellence and to achieve my goals.

Papa – Mummy, I am lucky to be your daughter.

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ABSTRACT

Pythium ultimum is an oomycete which causes serious economic losses in greenhouses by causing damping off disease. *Ascophyllum nodosum* is known to act as a nutrient supplement and its application has shown to reduce diseases caused by microbial pathogens in plants. However, its byproduct, one that is left after the extraction, has not been tested for the same. *In vitro* antimicrobial assay showed that higher concentration of *Ascophyllum nodosum* byproduct (ABP) significantly increased *Pythium ultimum* growth. Its PGPR (Plant Growth Promoting Rhizobacteria - *Bacillus subtilis*, *Pseudomonas putida* and *Penicillium*) digested growth media amendment even at higher concentrations (3% w/w) showed significantly improved growth of cucumber seedlings as compared to the undigested ABP of same concentration, in *in vivo* experiments although not significantly different from control. Digested and undigested ABP did not show significant reduction in damping off caused by *Pythium ultimum*, and hence there was no significant increase in biochemical compounds and defence enzymes in cucumber leaf tissues as compared to the control. Further research is needed to determine the compounds present in the ABP, which can lead to a more targeted approach in utilizing it as an elicitor.

LIST OF ABBREVIATIONS USED

4CL	4-coumaroyl CoA ligase
ABP	<i>Ascophyllum nodosum</i> by-product
AlCl ₃	Aluminium chloride
ANOVA	Analysis of Variance
B.s.ABP	<i>Bacillus subtilis</i> digested <i>Ascophyllum nodosum</i> by-product
C4H	Cinnamate 4-hydroxylase
CHI	Chalcone isomerase;
CHR	Chalcone reductase
CHS	Chalcone synthase
DAHP	3-deoxy-D-arabino-heptulosonate-7-phosphate
DAS	Days after sowing
EDTA	Ethylene di-amine tetra acetic acid
EPSP	5-enolpyruvyl shikimate-3-phosphate
ETI	Effector triggered immunity
FW	Fresh weight
GAE	Gallic acid equivalent
HCL	Hydrochloric Acid
HID	Hydroxy isoflavanone dehydratase
HSD	Honestly Significant Difference
IFS	Isoflavone synthase
ISR	Induced systemic resistance
JA	Jasmonic acid
K ₂ HPO ₄	Di-potassium phosphate

KH ₂ PO ₄	Mono-potassium phosphate
Na ₂ CO ₃	Sodium carbonate
NaOH	Sodium hydroxide
NBS-LRR proteins	nucleotide binding sites with leucine rich repeats
O.D	optical density
P.n.ABP	<i>Penicillium</i> digested <i>Ascophyllum nodosum</i> by-product
P.p.ABP	<i>Pseudomonas putida</i> digested <i>Ascophyllum nodosum</i> by-product
PAL	Phenylalanine ammonia lyase
PAMPs	Pathogens associated molecular patterns
PEP	Phosphoenolpyruvate
PGPB	Plant growth promoting Bacteria
PGPR	Plant growth promoting rhizobacteria
PPO	Polyphenol oxidase
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
PVP	Polyvinylpyrrolidone
RDW	Root dry weight
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SAS	Statistical analysis software
SDW	Shoot dry weight
WAS	Week after sowing

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CHAPTER 1 INTRODUCTION

A considerable portion of vegetables are produced in greenhouses and under tunnels and many vegetable production systems rely on transplanted seedlings raised in nurseries. Greenhouse and nursery growers face serious economic losses due to damping off caused by *Pythium* species. The management of this disease has become a serious challenge due to the emergence of fungicide resistant strains of the pathogen. Losses due to damping off disease occur mostly in the nursery with seedlings most susceptible before they attain the transplant stage (Gravel et al. 2007).

A number of management strategies are adopted to reduce damping off in greenhouses and nurseries. Some of the common methods are: the use of fungicides, managing environmental conditions, and soil solarisation. However, these management practices have limitations. In recent years strains of *Pythium* species that are resistant to common fungicide have been reported (Titone et al. 2009). Soil solarisation is effective in reducing the initial inoculum of the pathogen in the soil, but only effective in locations that have warmer climates. Further, there are no varieties of many vegetable crops including cucumber that are resistant to this pathogen making it one of the most difficult diseases to manage (Punja et al. 2003). Therefore, there is an urgent need to develop methods to reduce the incidence of damping off disease in cucumber.

Composts have been widely used as a soil amendment that may suppress soil borne diseases (Pascual et al. 2002), but they generally show no increase in plant growth, quality or yield (Mugnai et al. 2012). An effective organic amendment should reduce the plant pathogen population, increase activity of beneficial microorganisms in the soil, and improve growth, quality and yield of the crop. Research has shown that extracts from the

brown seaweed *Ascophyllum nodosum* when applied to plants, stimulate shoot and root growth (Temple et al. 1989; Metting et al. 1990), improve nutrient uptake (Yan 1993) and also reduce fungal diseases in greenhouse cucumbers (Jayaraman et al. 2011). *Ascophyllum nodosum* contains phlorotannins and many beneficial polysaccharides, some of which (laminarin and laminarans) also act as elicitors for defence response (Merceier et al. 2000; Khan et al. 2009). Research has shown that this enhanced state of resistance is mediated through the stimulation of biochemical reactions within the phenylpropanoid pathway. Significant increases in the total phenol levels and defence related enzymes such as phenylalanine ammonia lyase (PAL) have been found in cucumber plants treated with products of *Ascophyllum nodosum* (Jayaraman et al. 2011).

Therefore, it is thought that *Ascophyllum nodosum* may be an effective growing media amendment for health and prevention of damping off in cucumber seedlings.

CHAPTER 2 LITERATURE REVIEW

2.1 Damping off

Damping off is a seedling disease that causes serious economic losses in vegetable production every year (Gravel et al. 2007; Hultberg et al. 2000; Moulin et al. 1994). It is an important economic issue throughout the world (Ramamoorthy 2002).

There are two types of damping off; pre and post-emergence damping off. In pre-emergence damping off, the seed and the radicle start to rot prior to seedling emergence, thereby creating gaps in the seedbed. Post emergence damping off occurs at or near the ground surface (Duggar 1909). Symptoms appear as a girdle of brown decaying cortex, which extends upwards and downwards from the ground level. At this point, the stem tissue becomes weak, the seedling collapses and topples over creating dead patches in the nursery seed trays. If the toppled seedlings are left on the damp soil the mycelium can invade the rest of the seedlings and cause complete seedling rot.



Figure 2.1: Post emergence damping off symptoms in cucumber.

2.1.1 Etiology of damping off disease

Damping-off is caused by a number of *Pythium* species, including *P. aphanidermatum* (Edson) Fitzp., *P. ultimum* Trow, and *P. irregular* (Punja et al. 2003). *Pythium ultimum* (genus *Pythium*; family Pythiaceae; order Pythiales; class Oomycetes; phylum Oomycota and kingdom Chromista (Uzuhashi et al. 2010) is a necrotrophic, aseptate and coenocytic plant pathogen which can reproduce sexually through oospores and asexually through sporangia and zoospores (Okubara et al. 2003). Several species of this genus are pathogens with a wide host range. The genus *Pythium* generally causes rotting of fruits, roots and stems, as well as pre- or post-emergence damping-off of seedlings (Uzuhashi et al. 2010).

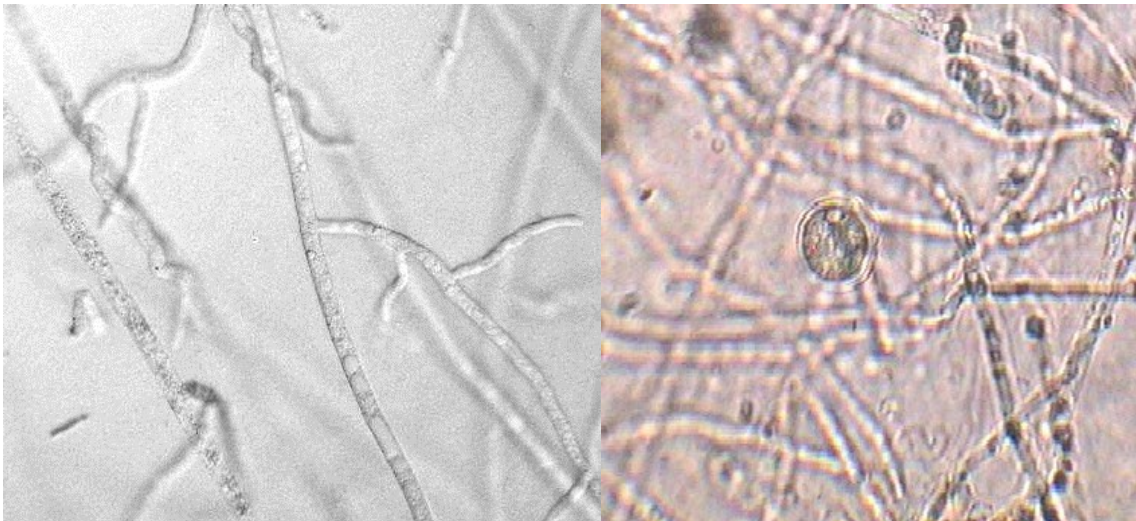


Figure 2.2: Mycelium and sporangium of *Pythium ultimum*

2.1.2 Host Range and Distribution

Pythium ultimum is a ubiquitous soil borne pathogen that is distributed widely throughout the world (Kirk et al. 2008). It is a cosmopolitan root pathogen with a wide range of host plants in horticulture, agriculture and forestry (Larsen et al. 2003). *Pythium ultimum* has been found in Australia, Canada, Brazil, China, Korea, Japan, South Africa and many other countries in the world including most states within the United States of America. It causes *Pythium* blight of turf grass, inflicting very serious damage in golf courses (Allen et al. 2004). It can also infect crops like cabbage, broccoli, cucumber, carrot, melon, cotton and wheat (El-Mohamedy 2012). In general, abundant soil moisture and high soil temperature are the two most important environmental factors that increase the incidence and severity of *P. ultimum* (Chang 2007).

Pythium ultimum is introduced in greenhouses through plug transplants, soil, debris, pond and stream water, and plant growing media. In addition, greenhouse insects like fungus gnats (*Bradysia impatiens*) and shore flies (*Scatella stagnalis*) can also spread this pathogen. Once the plants are infected they can serve as a source of inoculum (spores) that can be splashed onto nearby plants or moved in water films through drainage holes in the containers and spread in irrigation systems (Punja et al. 2003).

Greenhouse and hydroponic culture systems producing uniform, good quality plants and homogeneous yield tend to be prone to infection by *Pythium* species (Kageyama et al. 2002). This problem is especially serious in greenhouses where water is recirculated to prevent the loss of nutrients (Stanghellini et al. 2000).

Long English cucumber (*Cucumis sativus* L.) from family Cucurbitaceae is commercially grown in greenhouses for the fresh market. A significant acreage of greenhouse cucumber are found in the provinces of Ontario, Alberta, Quebec and British Columbia, most of which are under hydroponic production where sawdust or rock wool is utilized as the plant growth medium. Under this high crop density environment, plant pathogens can spread rapidly, leading to significant crop losses (Punja and Yip 2003). Thus cucumber should be handled with utmost care in greenhouses and should be protected from disease.

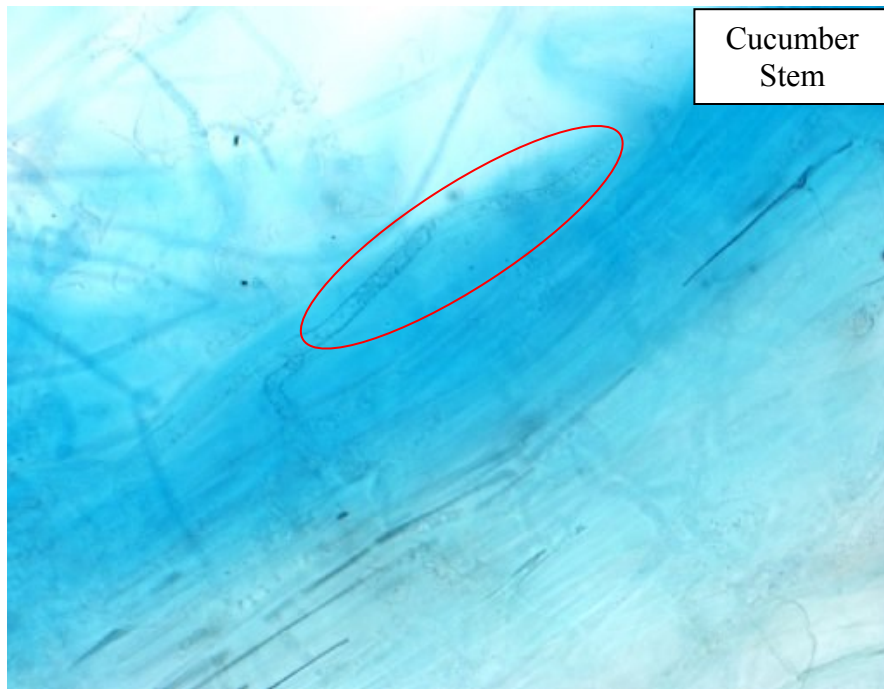


Figure 2.3: *Pythium ultimum* hypha (marked in red circle), mycelium in cucumber stem.

2.1.3 Gaps in current *Pythium* management practices

Pythium sporangium rapidly germinates (1.5 – 2.5 h) in water, and in influence with exudates from seeds or volatiles from roots. This rapid germination of the pathogen makes the management challenging. Fungicides can control *Pythium* infections but they can also leave residues on the crop that impact human health and cause environmental pollution. Other management practices like sanitation (using clean water supplies / sterile water), regulation of temperature and watering are also followed, but they are cumbersome. Soil solarisation is successful in hot climatic zones but ineffective during the winter season and in cool climatic zones (Ramamoorthy 2002).

At present, commercial growers rely on various sanitation measures and fungicide applications to control *Pythium*. The predominant fungicide applied is propamocarb hydrochloride (Previcur) which only has a minor-use registration in Canada (Punja et al. 2003) due to environmental concerns and the presence of residues in the produce. Fungicide use is prohibited in Sweden in plant cultures where continuous harvesting is done (Hultberg et al. 2000). Detergent has shown promising results to reduce the infection by lysing zoospores. However, beyond zoospores, *Pythium* species produce other infective propagules such as oospores, sporangia, and the mycelia that also cause infection in the plant. This indicates that fungicides should be replaced with other safe and eco-friendly solutions such as organic substances.

2.2 Organic Soil Amendments

Pane et al. (2010) stated that the increasing concerns regarding food safety and environmental pollution has forced the progressive restriction of soil fumigant use and fungicides, encouraging the research for an alternative method to control seedling

diseases. To replace or integrate traditional control strategies, the author proposes to exploit the disease suppressive properties of organic amendments.

Peat is the most commonly used organic material for the preparation of potting media because of its homogeneity and favorable agronomic characteristics. However, this organic material is poorly suppressive against soil-borne pathogens (Pane et al. 2010). Compost, an organic material widely used throughout the world, may reduce the effect of the pathogen and suppress soil borne diseases of flower and vegetable crops (Pascual et al. 2002). However, Mugai et al. (2012) observed that in the long term compost had no effect on plant growth, yield and quality of grape.

An effective organic amendment should reduce the population of plant pathogens, increase the activity of beneficial microorganisms, and improve the growth of the crop. The development of formulated amendments is based not only on their effects on soil borne plant pathogens and beneficial microorganisms, but also on nutritional requirements of plants (Huang et al. 1993).

2.3 Plant growth promoting rhizobacteria (PGPRs)

Plant growth promoting rhizobacteria (PGPR) are a group of a wide variety of soil bacteria, which can stimulate healthy growth of host plants (Vessey 2003). Plant growth promoting fungi (PGPF) cause similar beneficial effects as that of PGPRs (Van der Ent et al. 2009). These microorganisms colonize the root surface and closely adhere to soil within the rhizosphere (Compant et al. 2005). Research has shown that PGPRs can promote host plant growth in a number of ways: (i) They can act as bio-fertilizers (supply nutrients to plants such as nitrogen fixing bacteria), (ii) rhizo-remediators (*P. putida* can

use root exudates to degrade naphthalene in soil and protect seeds from being killed and help plants to grow normally), (iii) phyto-stimulators (produces hormones such as auxin, volatiles and cofactor which stimulate plant growth. *B. Subtilis* releases volatiles to promote plant growth), (iv) stress controllers (produces ACC deaminase enzyme which reduces plant ethylene level and hence prevent plant from stress of polyaromatic hydrocarbon, heavy metals, salt, drought and pathogenic bacteria), and (v) as biocontrol agent against many phyto-pathogenic organisms (Lugtenberg and Kamilova 2009). It has been shown that the addition of PGPRs in potting mixture/soil suppresses pathogens by producing specific metabolites (Dukare et al. 2011).

Studies have shown that *Pythium* and other soil borne plant pathogenic microbes can be suppressed by increasing the population of selected organisms that are antagonistic to pathogens and by introducing microbiostasis. This means causing microbial competition for nutrients and ecological niches, antibiosis or parasitism, altered nutrient availability, and thereby inducing host resistance (Dukare et al. 2011; Ntougias et al. 2008).

There are many predictive variables and hence different mechanisms are involved in pathogen suppression depending on physicochemical and/or biological characteristics of compost and the like substances. Disease protection properties may differ dramatically between different composts or substances used instead of compost (Termorshuizen et al. 2006). It will also vary in the same type of compost depending on preparation, maturity characteristics, storage conditions, and timing of application (Ntougias et al. 2008).

2.4 *Ascophyllum nodosum* (L.) Le Jol

Ascophyllum nodosum, a marine macro brown alga from family Fucaceae is the single species in the genus *Ascophyllum*. It is dominant perennial seaweed confined to the intertidal zone of the north Atlantic basin and parts of north-western coast of Europe. *A. nodosum* is commonly known as rockweed (Taylor 1957; Ugarte et al. 2006; Subramanian et al. 2011), Norwegian kelp, knotted kelp, knotted wrack or egg wrack. Shoots of this seaweed arise from a holdfast and develop a complex structure of dichotomous and lateral branches. It forms a single bladder centrally in long, flattened strap-like fronds which hang downwards, draping sheltered intertidal rocks. Many fronds grow from the base and the plant generally regenerates new fronds from the base when the larger fronds are damaged (Ugarte et al. 2001).

2.4.1 Beneficial effects of *Ascophyllum nodosum* on plants

Ascophyllum nodosum is the most commonly used seaweed in commercial extracts and as suspensions used in agriculture and horticulture. There are many beneficial effects of *Ascophyllum* products. The benefits of seaweeds as a source of nutrient, fertilizer and organic matter have led to its use as soil conditioners for centuries (Rayirath et al. 2009; Khan et al. 2009). Annually, 15 million metric tonnes of seaweed products are produced, out of which, a considerable portion is used as biostimulants, nutrient supplements and biofertilizers to increase plant growth and yield (Khan et al. 2009). Temple et al. (1989) reported that extracts of *A. nodosum* when applied to plants, stimulate shoot growth and branching. *A. nodosum* have also shown to increase root growth and lateral root development (Metting et al. 1990), improves nutrient uptake (Yan 1993) and nutrient

imbalance. There is good evidence that *A. nodosum* can be used to provide organic matter and to replace and amend nutrients in the soil.

Besides improving plant growth, development and yield, *A. nodosum* products have also been noted to affect the response of plants to different stresses. It helps in alleviating abiotic stress and imparts tolerance against drought, salinity and frost (Nabati 1991; Nabati et al. 1994; Rayirath et al. 2009).

A few studies also show that application of seaweed extracts to plants resulted in direct or indirect protection against biotic stresses. Featonby-Smith et al. (1983) reported that it enhanced resistance in tomato against nematodes. Foliar sprays of *A. nodosum* extract also showed reduction of downy mildew of grapes, and *Phytophthora capsici* infection in Capsicum (Lizzy et al. 1998). Soil applications of liquid seaweed extracts to cabbage (*Brassica oleracea* var. *capitata*) stimulated microbes that were antagonistic to *Pythium ultimum* (Dixon et al. 2002). This increase in the population of antagonistic microbes inhibits the growth of the pathogen, resulting in reduced incidence of the disease. Recently, Jayaraman et al. (2011) reported that application of commercial extract of *A. nodosum* reduced fungal diseases in greenhouse cucumber.

Ascophyllum nodosum contains many beneficial compounds including phenolic compounds such as phlorotannins and polysaccharides such as laminarin, laminaran, fucoidans, and alginates (Khan et al. 2009). It has been shown that laminarin, and laminarans act as elicitors for plant defence responses. Laminarans induce the formation of antifungal compounds in alfalfa cotyledons, and stimulate phytoalexin accumulation in soybean cotyledons and seedlings (Mercier et al. 2000). The above examples prove *A.*

nodosum product's ability to control many soil borne plant pathogens and maintain soil fertility indicating its potential to have commercial application.

2.4.2 *Ascophyllum nodosum* byproduct

The commercial extracts of *Ascophyllum nodosum* are produced as a result of an Alkali Liquid Extraction Process. During this process, a large amount of leftover solid waste is produced which is not used and is usually discarded. This seaweed solid waste contains significant amounts of beneficial polysaccharides such as alginates, fucoidan and phenolic compounds like phologlucinol, phlorotannins. Phlorotannins have shown important functions such as antimicrobial and antifoulant properties, protection against UV radiation, and most commonly, resistance against herbivores. (Schoenwaelder 2008; Van Alstyne et al. 2001; Amsler et al. 2006). Hence, there is a potential to use this seaweed solid waste as soil amendment to alter the soil physical and chemical conditions that in turn may improve growth of plants and reduce the occurrence of soil borne diseases incited by *Pythium* species that cause major damages in greenhouse seedlings. The composition of this product can potentially be conducive to disease resistance by stimulation of innate physiological pathways within host plants.

2.5. Disease Resistance

Plants are constantly exposed to microbes, some of which are beneficial and some are pathogenic. The ability of plants to identify pathogenic microbes and protect themselves is the key for plants to survive. This ability can be referred as a disease resistance. Plants acquire the resistance from mounting defence responses (Chisholm et al. 2006).

Disease resistance may be an innate defence response or an induced resistance. Innate defence response is a genetically designed ability of a plant to recognize pathogens associated molecular patterns (PAMPs) using cell membrane localized pattern recognition receptors (PRRs), thereby leading to PAMP-triggered immunity (PTI) (Iriti and Faoro 2007). Some pathogens overcome PTI by injecting effectors into plant system to be successful in invading. The plant in response to effectors produces effector triggered immunity (ETI) and protects themselves using intra cellular receptors such as nucleotide binding sites with leucine rich repeats (NBS-LRR proteins) (Kale and Tyler 2011).

Induced resistance is a phenomenon in which a plant once appropriately stimulated, exhibit enhanced resistance upon infection (Van Loon 2000). It is of two types: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is developed when plants activate their defense response against a primary infection by a pathogen, and use the memory of response for latter infections leading to induction of hypersensitive reaction. Similar to SAR, ISR is also effective against different types of pathogens, however in ISR, beneficial microbes (PGPB) induce plants to incur biochemical and physiological changes, and prepare the plant to show defence response upon pathogen attack. Thus in ISR, PGPB does not cause visible symptoms on the host plant unlike SAR. PGPB-mediated ISR has been reported in a number of studies involving free-living rhizobacteria such as *P. putida*.

PGPB-triggered ISR fortifies plant cell wall strength due to callose deposition and alters host physiology and metabolic responses. This leads to enhanced synthesis and accumulation of plant defense chemicals such as phenolic compounds, accumulation of

PAL, peroxidase, polyphenol oxidase and phytoalexins, upon challenge by pathogens at the site of pathogen attack (Compant et al. 2005).

In ISR, plants detect the “non self” molecules of PGPRs called elicitors. These molecules bind to specific receptors and trigger defence responses resulting in protection from pathogens. Cell wall and storage polysaccharides from different seaweeds have been shown to act as elicitors such as fucans, laminarins, alginates, carageenans, and ulvans thereby triggering defence response and hence protecting the plant from pathogenic attacks. Moreover, oligosaccharides produced from seaweed polysaccharides, also have shown to protect plants from fungal and bacterial pathogens mainly through activation of ethylene, salicylic acid (SA) and jasmonic acid (JA) signaling pathway at the systemic level, which further helps in production of defence enzymes such as PAL, and accumulation of phenyl-propanoid compounds (Vera et al. 2011).

Plants resist disease incidences through the production of phytohormones such as JA, SA and stimulation of pathogen associated signaling cascade (PAMP/Effector triggered immunity; induction of physiological processes such as shikimic acid pathway, phenylpropanoid pathway). During these pathways, plants accumulate a large amount of defence/stress response compounds, including many secondary metabolites such as phenolics, flavanoids, terpenoids, glucosinolates and alkaloids to protect themselves from various pathogenic microbial attacks (Pourcel et al. 2013).

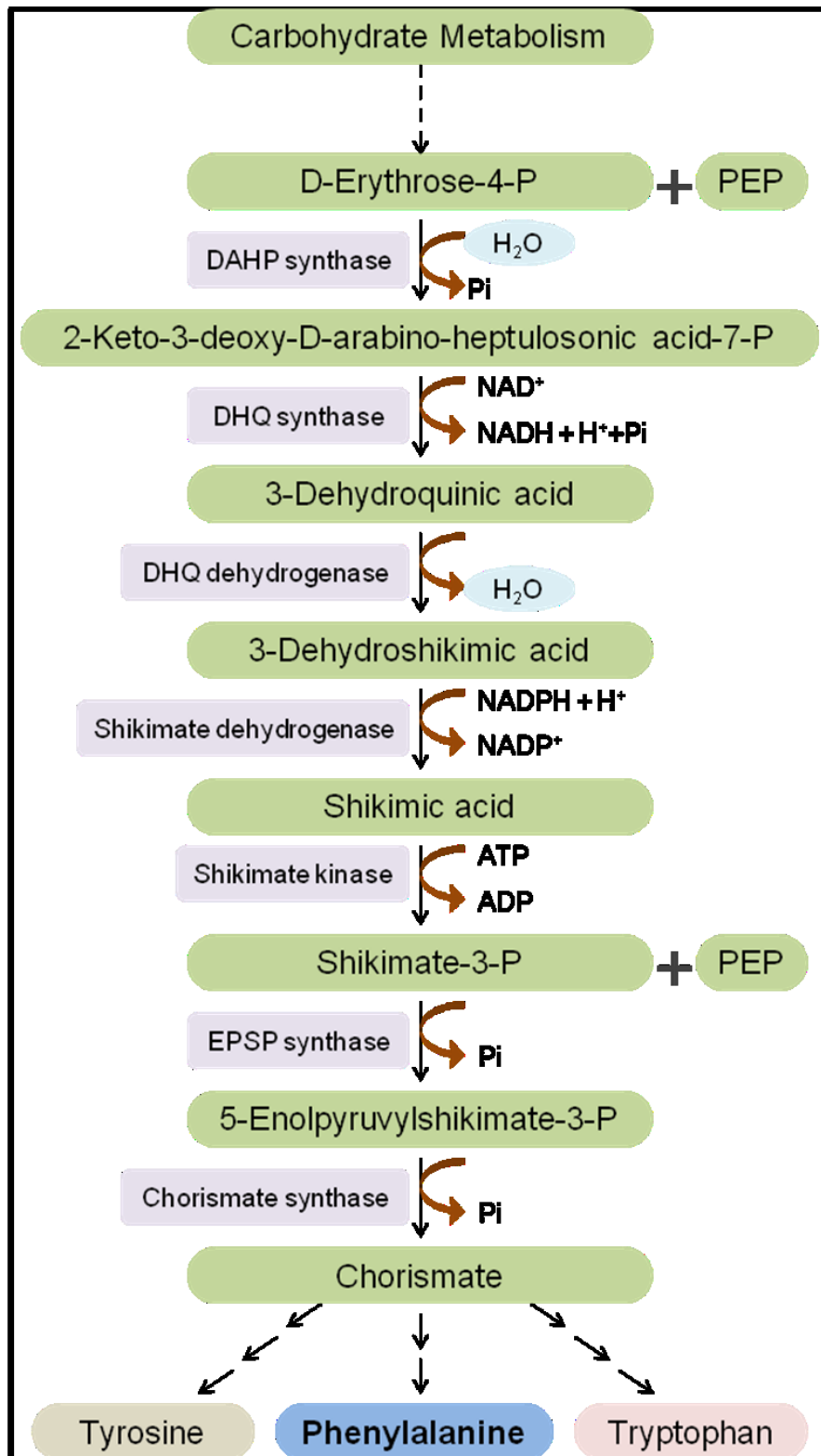


Figure 2.4: Schematic representation of Shikimic Acid Pathway. The broken line represents multiple enzymatic steps. PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; EPSP, 5-enolpyruvylshikimate-3-phosphate (Modified from Rosic and Dove, 2011).

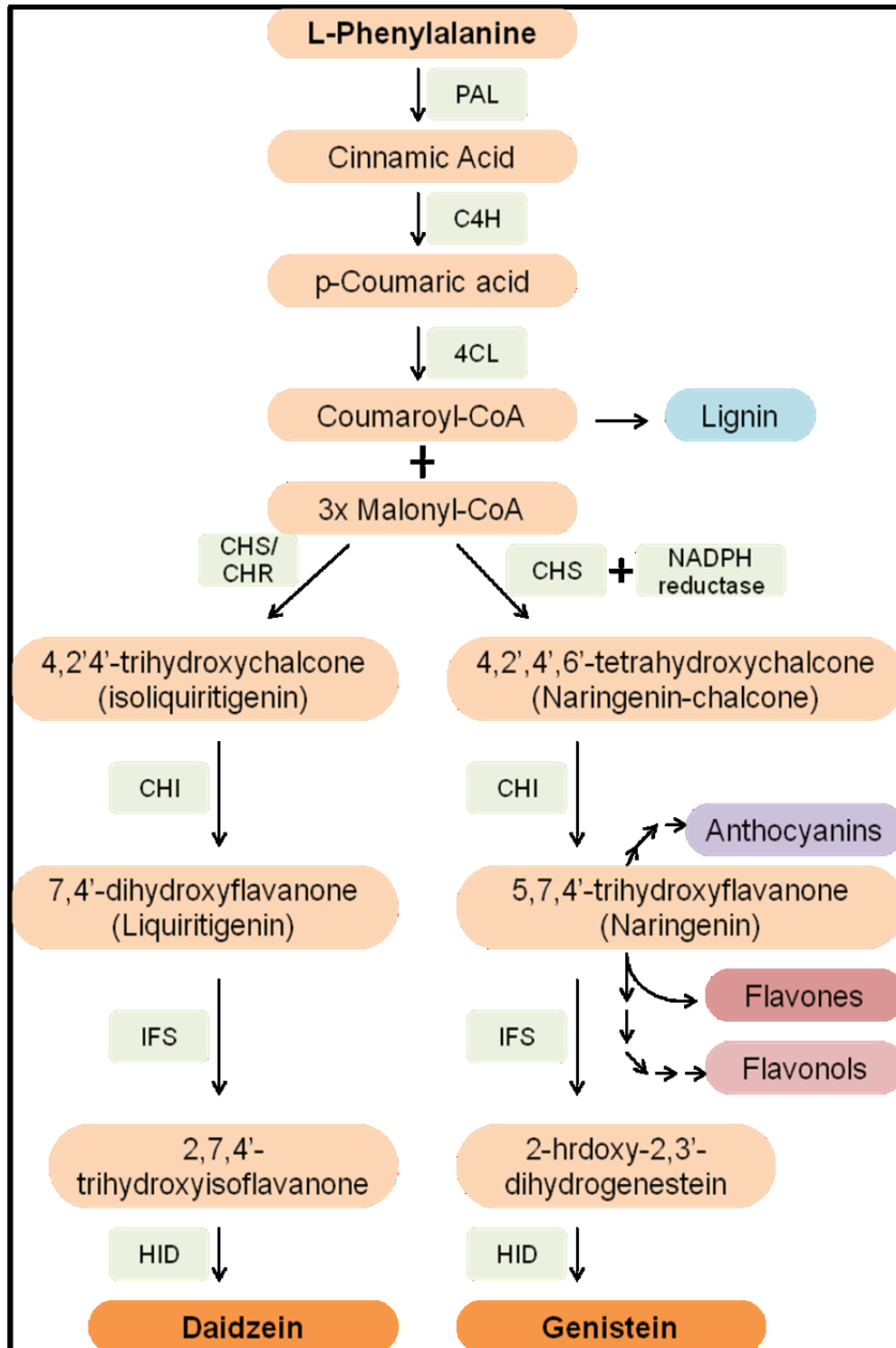


Figure 2.5: Schematic representation of Phenylpropanoid pathway. The broken line represents multiple enzymatic steps. PAL, Phenylalanine ammonia lyase; C4H, Cinnamate 4-hydroxylase; 4CL, 4-coumaroyl CoA ligase; CHS, Chalcone synthase; CHI, Chalcone isomerase; CHR, Chalcone reductase; IFS, Isoflavone synthase; HID, Hydroxyisoflavanone dehydratase (Modified from Misra et. al., 2010).

2.5.1 Defence related compounds

Microbial (PGPR) activity and carbohydrate availability impact suppressive effects against soil-borne plant pathogens (Ntougias et al. 2008). Both the factors combine together to initiate shikimic acid pathway and phenylpropanoid pathway. A detailed description of the pathway is presented in the figure 2.4 and 2.5.

Phenolic compounds are the most common and widespread secondary metabolites in plants. Phenylalanine ammonia lyase is the key enzyme responsible for the production of phenolics in phenylpropanoid pathway. In the pathway, many subclasses of Phenolic compounds are also produced such as flavonoids, tannins, hydroxycinnamate esters, lignin etc. Some of the benefit includes antioxidant activity, ROS quencher and tumor inhibitor (Podsędek 2007; Pourcel et al. 2013).

Flavonoids are a diverse group of phenolic compounds which consists of a C₆–C₃–C₆ carbon structure. These are derived from phenylpropanoid pathway by the help of key enzyme chalcone isomerase. Flavonoids are sub-classified into chalcones, flavones, flavanones, flavandiols, catechins, condensed tannins (proanthocyanidins), and anthocyanins. Flavonoids benefit plants in eco-physiological functions, modulates stress related genes, regulate growth, reproduction, pigmentation and protection from UV radiation. Flavonoids are a good ROS scavenger and its intake reduces risk of cancer, heart disease (Winkel-Shirley 2002; Pourcel et al. 2013).

CHAPTER 3 HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

Amendment of growing media with *Ascophyllum nodosum* byproduct will reduce the incidence of damping off disease caused by *Pythium ultimum*.

Specific Hypothesis

- 1) Direct application of *Ascophyllum nodosum* byproduct will negatively affect the growth of *Pythium ultimum* in vitro.
- 2) Amendment of growing media with *Ascophyllum nodosum* byproduct will increase seedling growth and help to reduce damping off in cucumber caused by *Pythium ultimum*.
- 3) Amendment of soil with *Ascophyllum nodosum* product will increase concentration of defence response compounds (total phenolic content and flavonoid content) and activity of defence response enzymes (Phenylalanine ammonia lyase and Polyphenol oxidase) in cucumber.

3.2 Objectives

- 1) To evaluate the in vitro antimicrobial activity of *Ascophyllum nodosum* byproduct on both vegetative and reproductive growth of *Pythium ultimum*.
- 2) To study the effect of *Ascophyllum nodosum* byproduct on seedling growth and systemic resistance against damping off in cucumber caused by *Pythium ultimum* in vivo.

- 3) To study the changes in defence response enzymes (Phenylalanine ammonia lyase and Polyphenol oxidase) and compounds (Total Phenolic content and Flavonoid content) in cucumber in response to growing media amendment with *Ascophyllum nodosum* byproduct.

CHAPTER 4 MATERIALS AND METHODS

4.1 Materials

Seaweed solid waste left after the seaweed liquid extracts used as biostimulant was obtained from Acadian Seaplants Limited. Cucumber ‘Fanfare’ seeds were obtained from Halifax Seeds Company Limited (Halifax, Nova Scotia, Canada). Culture of the pathogen was obtained from Dr. Paul Hildebrand, AAFC, Kentville, Nova Scotia, Canada.

4.2 *In vitro* anti-microbial assay

4.2.1 Experimental Design

To study the direct effect of the *A. nodosum* product on vegetative and reproductive growth of *Pythium ultimum*, three experiments were conducted; the liquid and the solid culture to study vegetative growth inhibition, and sporangia count to study reproductive growth inhibition.

The experimental designs for all three experiments were completely randomized design with 6 treatments and 5 replications per treatment.

4.2.2 Preparation of culture media

4.2.2.1 Corn meal broth

Forty grams of corn meal (Sigma Aldrich) was weighed and added to 1 liter of distilled water in a clean 2000 ml beaker. It was shaken and heated simultaneously to reach a temperature of 58° C. It was maintained at this temperature for 1 hour. The solution formed was vacuum filtered through four folds of cheese cloth. The filtered corn meal

medium was autoclaved at 121° C for 15 min. This sterile medium was let to cool down before using it in the liquid culture experiment.

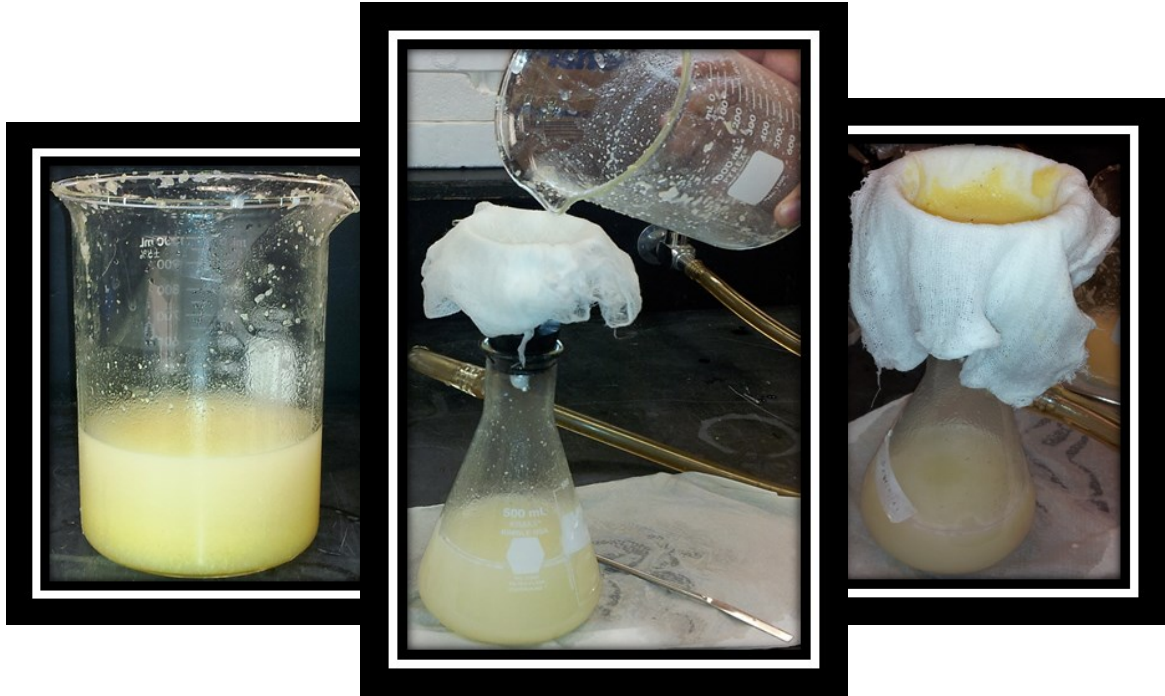


Figure 4.1: Corn meal solution in water; vacuum filtration; prepared corn meal broth without sediments.

4.2.2.2 Corn meal agar media

Seventeen grams of corn meal agar (Sigma Aldrich) was mixed with 1 liter of distilled water in a clean 2000 ml beaker. It was shaken for 15 min. and then transferred into 1000 ml glass bottles supplemented with different concentrations of *A. nodosum* byproduct. Then the media were autoclaved at 121°C for 15 min. Before cooling, 25 ml of *A. nodosum* byproduct supplemented media was poured into 9 cm diameter Petri dishes using a 25 ml pipette. The cooled and settled media plates were stacked overnight and then used in the solid culture and the sporangia counting experiment.

4.2.3 Liquid culture method

Pythium ultimum was cultured on corn meal broth prepared as described in 4.2.2.1. The culture media were supplemented with different concentrations (0g/L, 0.5g/L, 1g/L, 3g/L, 5g/L, and 10 g/L) of the *A. nodosum* byproduct as treatments. A 5mm diameter disc of actively growing culture was inoculated into 125mL conical flasks containing 30mL of medium. The conical flask was incubated in the dark at 25°C for 5 days. At the end of the incubation period the mycelium was washed with sterile distilled water to remove media particles and then placed on a pre-weighed filter paper for drying. The dry weight of the mycelium was recorded after drying in a hot air oven at 70°C for 48h.

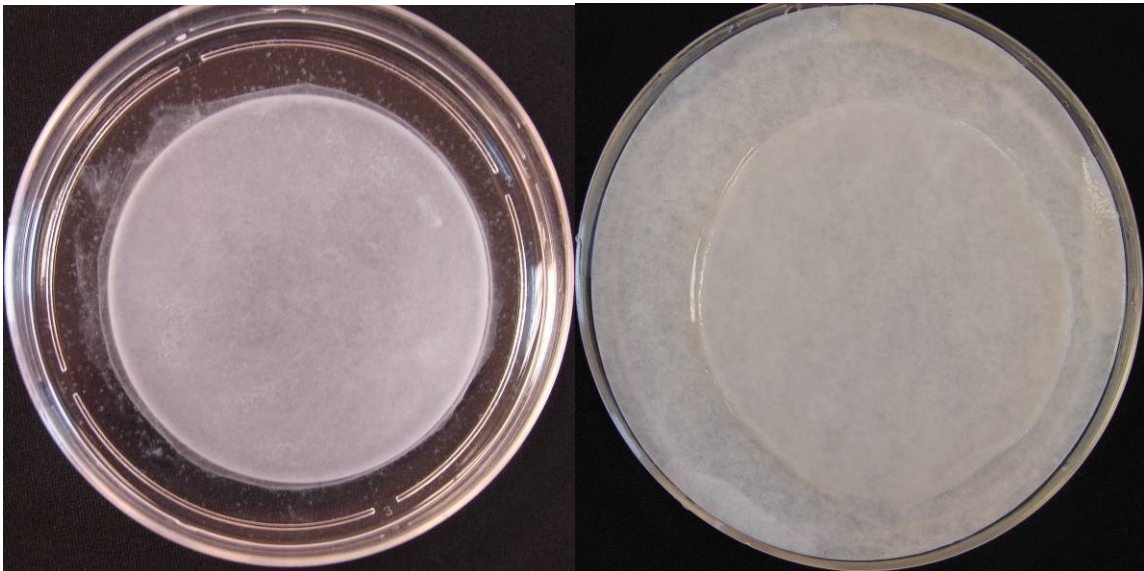


Figure 4.2: Mycelia in water; Mycelia on filter paper.

4.2.4 Solid culture method

Pathogen culture medium and plates was prepared as described in the section 4.2.2.2. The media contain agar as the solidifying agent. The culture media were supplemented with different concentrations (0g/L, 0.5g/L, 1g/L, 3g/L, 5g/L, and 10 g/L) of the *A. nodosum* byproduct as treatments. The media (25mL) were dispensed into 9cm diameter petri dishes. Five mm diameter discs of the actively growing culture were placed in the centre of the petri dish. The plates were incubated in the dark at 25°C for 48 hours. The diameter of pathogen growth was measured using a ruler at every 6 hours after inoculation.

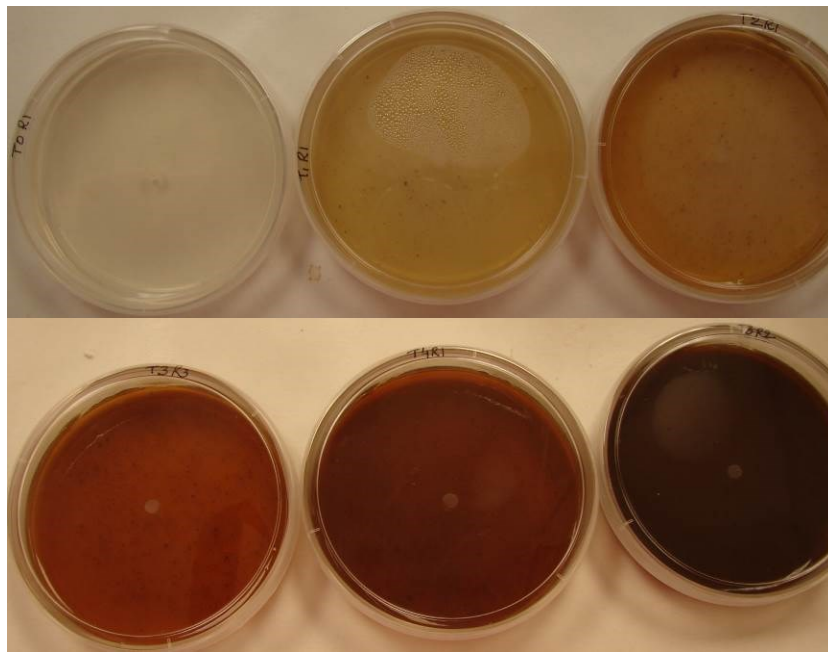


Figure 4.3: Treatments (0g/L, 0.5g/L, 1g/L, 3g/L, 5g/L, and 10 g/L of the *A. nodosum* byproduct supplemented with corn meal agar media in petri dishes inoculated with 5 mm mycelial plug

4.2.5 Effect of *Ascophyllum nodosum* byproduct on reproduction of *Pythium ultimum*.

Pythium ultimum reproduce and cause disease by production of reproductive structures known as sporangia. The effect of the *A. nodosum* byproduct was tested on the ability of *Pythium ultimum* to produce sporangia. In this assay, treatment plates and inoculation was done as described in section 4.2.4. The plates were incubated for 48 hours and then three 5 mm diameter discs of growing culture per plate were used to count sporangia. Each disk was transferred into sterile 1.5 ml micro centrifuge tubes containing 1 ml of sterile water. The tubes were vortexed for 15 sec. and 10 μ L of this sporangial suspension was transferred to each counting chamber of haemocytometer using a sterile micropipette tip. The number of sporangia was counted in haemocytometer using a microscope at 20 x magnification.



Figure 4.4: Haemocytometer and loading of counting chamber of haemocytometer by sporangial suspension.

4.3 *In vivo* Growth room experiments

For these experiments cucumber seedlings were used as a test plant.

4.3.1 Effect of *Ascophyllum nodosum* byproduct on seedling growth and health

4.3.1.1 Preparation of digested *Ascophyllum nodosum* byproduct

Ten grams of *A.nodosum* byproduct was sterilized in magenta jars. Thirty milliliter of sterile water was added to each jar to achieve 60% moisture content. Ten milliliter of 0.5 O.D bacterial cultures (*B. subtilis*, *P. putida*) were inoculated to each jar. A suspension of >10000 *Penicillium* spore per ml was made using five millimeter diameter disk of actively growing culture and sterile distilled water. Ten milliliters of this spore suspension was used to inoculate each magenta jar. The inoculated magenta jars were sealed and incubated at 25° C for three weeks for complete digestion of *A.nodosum* byproduct. These jars were aerated twice every week by turning the contents up and down using spatula.



Figure 4.5: *A. nodosum* byproduct after addition of water and inoculum; aeration of digested *A. nodosum* product.

4.3.1.2 Preparation of potting mixture

Promix was used as the plant growth medium. Three week digested and undigested *A. nodosum* products were mixed in Promix at the rate of 0.5%, 1%, and 3% (w/w). To facilitate the even dispersal of the product into the Promix the mixture was stored in a cool dry place for 10 days before use. A control was kept without mixing anything into the Promix.

4.3.1.3. Experimental Design

A. nodosum byproduct was used in two forms as soil amendments: beneficial microbes digested and undigested. The experimental design for seedling growth and health experiments was randomized block design with 10 blocks or replications. The experimental units were 130 cucumber seedlings with 13 treatment combinations meaning 10 seedlings per treatment.

4.3.1.4 Seedling growth and health experiment

To test the effect of *A. nodosum* byproduct on seed germination and seedling growth, 4” (diameter) plastic pots were filled with the potting mixture (mixture of Promix amended with digested and undigested *A. nodosum* byproduct as described in 4.3.1.1 and 4.3.1.2). Seeds of cucumber cv. Fanfare were planted at a rate of two seeds per pot. Pots were placed on growth room shelves under 21-24°C temperature and 16 h photoperiod. One seedling per pot was retained after germination of seeds. The seedlings were grown for 3 weeks and harvested for measurements.



Figure 4.6: Cucumber grown in different treatment combinations for seedling health experiment. Treatment were undigested *Ascophyllum nodosum* by-product (ABP), *Bacillus subtilis* (B.s.), *Pseudomonas putida* (P.p.) and *Penicillium* (P.n.) digested ABP amendment in growth media.

4.3.1.5 Determination of Seedling Growth

The growth of cucumber plants was measured in terms of physical parameters (biometric measurements). Plant height was measured every week from days after sowing (DAS) using a 15 cm measuring ruler (Fisher scientific). Three weeks after sowing (WAS) number of true leaves per plant were counted, whole shoots were harvested and weighed to determine fresh weight (FW). Shoot and root dry weights (SDW and RDW respectively) were assessed by drying shoot and root samples at 80 °C in an air dry oven for 48 h. True leaf area, root length, and root surface area were measured using WinFOLIA and WinRHIZO software packages from Regent Instruments Inc. respectively.

4.3.2 Effect of *Ascophyllum nodosum* product on suppression of damping off disease

4.3.2.1 Preparation of inoculums

A culture of *Pythium ultimum* was grown in conical flasks containing liquid Corn Meal Medium for 7 days. The mycelial mat was harvested and five grams of the mycelial mat was mixed in 100 ml of sterile distilled water in a blender. It was filtered to maintain an average of 1.5×10^4 sporangia per ml. This microbial inoculum with >10000 sporangia per ml was used to inoculate pots containing cucumber seedlings.

4.3.2.2 Experimental Design

A.nodosum byproduct was prepared as described in 4.3.1.1. The experimental design for all damping off experiments was similar to seedling health experiment which is described in 4.3.1.3. Each pot contained 10 cucumber seedlings. The controls pots were the inoculated pots in which cucumber seedlings were grown in Promix without *A.nodosum* byproduct amendment. All the treatment combinations were inoculated with *Pythium* sporangial suspension.

4.3.2.3 Pre-emergence damping off experiment

Preparation of digested *A. nodosum* byproduct, potting mixture and inoculum was prepared as described in 4.3.1.1, 4.3.1.2 and 4.3.2., respectively. Each 4” pot was planted with 10 cucumber seeds, 5 ml of *Pythium* sporangial suspension was used to inoculate each pot just after seeding before seedling emergence. The pots were covered by transparent polythene bags and placed on a greenhouse bench. Observations were

recorded every 3 days from the date of inoculation on number of seedlings showing damping off symptoms for a total period of 2 weeks.

4.3.2.4 Post-emergence damping off experiment

This experiment was conducted similar to Pre-emergence damping off experiment, except that the inoculation of pots was done just after seedling emergence. Observations were recorded every 3 days from the date of inoculation on number of seedlings showing damping off symptoms for a total period of 2 weeks.

4.4 Biochemical analysis

Chemical components of *Ascophyllum nodosum* are known to act as elicitor of plant disease resistance. To test if the seaweed soil amendment product induces plant defense response in seedlings grown in amended potting mixture, the defense enzyme and chemicals were analyzed. Preparation of digested *A. nodosum* byproduct, potting mixture and inoculum was prepared as described in 4.3.1.1, 4.3.1.2 and 4.3.2.1 respectively. The experiment was conducted similar to 4.3.2.4. Just after seedling emergence, the pots were inoculated.

4.4.1 Experimental Design

The experimental design for all biochemical analysis experiments were randomized block design with 15 blocks. The experimental units were 75 pots, with 5 treatment combinations. Each pot contained 10 cucumber seedlings. All the treatment combinations were inoculated with *Pythium* sporangial suspension. The control pots were inoculated with *Pythium* sporangial suspension, in which cucumber seedlings were grown in Promix without *A.nodosum* byproduct amendment.

4.4.2 Tissue Collection and storage

Approximately 250 mg of cucumber shoot tissue which was grown in the Promix-seaweed amendment mixture were cut with scissors before inoculation. Similarly, shoot tissues were harvested at 24 h and 72 h after inoculation. Tissues from 5 replications per treatment were harvested per time point. The shoot tissue was flash frozen in liquid nitrogen and stored at -80°C until use.

4.4.3 Extraction of Crude Enzyme

The enzyme extraction of cucumber shoot tissue was conducted using the protocol as described by Gadzovska et al. (2007) with few modifications. Fresh samples (whole shoots of seedlings) were ground in liquid nitrogen with the help of pestle and mortar. The extraction from sample was conducted using 1 ml cold enzyme extraction buffer in 2ml micro-centrifuge tube. The buffer was composed of 0.1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.5), containing 3 mM EDTA, and 0.5% (w/v) polyvinylpyrrolidone (PVP). The micro-centrifuge tubes were centrifuged at 12,000g for 10 min at 4 °C. The supernatant was then collected and transferred to another 2 ml micro-centrifuge tube. The previous tube was centrifuged again at 12,000g for 10 min at 4°C. The supernatant collected was mixed to the previous supernatant. The total amount of supernatant was used in enzyme analysis.

4.4.4 Estimation of Total Protein

The collected supernatant was used as crude enzyme extract. Protein concentration was measured using the Coomassie Plus – The Better Bradford™ Assay Kit (Pierce, Rockford, IL, USA). 200 µl of Bradford reagent was added to 35µL of water and 5µl of crude enzyme extract. The absorbance was read at 595 nm using BioTek microplate reader (VT, USA) with Gen5™ software. The total amount of protein present in each sample was calculated using bovine serum albumin as a standard reference (125–1500 µg/mL). The samples and the standards were run in triplicate in the same 96 well plate for each replication.

4.4.5 Phenylalanine Ammonia Lyase (PAL) estimation

PAL is the committed enzyme in the phenyl propanoid pathway that results in the biosynthesis of plant phenolic compounds. Phenolic compounds play a significant role in plant disease resistance. Therefore, the effect of seaweed soil amendment product on PAL activity was investigated. Crude enzyme extract as described in 4.4.3 was used in this assay. Estimation of PAL was based on protocol described by Gadzovska et al. (2007) and Rahman and Punja (2005) with some modifications. A 250 µL reaction mixture containing 200 µL of 15mM L- phenylalanine, 0.1 M Tris HCL buffer at pH 8.8, 35µL of water and 5µl of crude enzyme extract was mixed. The mixture was incubated at 37°C for 1 h. The reaction was stopped by adding 10µl of 5M HCL. The absorbance of reaction mixture was analyzed at 290nm against a blank using a BioTek microplate reader (VT, USA) with the help of Gen5™ software. The amount of cinnamic acid formed was calculated using a standard curve of cinnamic acid (10–500 nmol). The samples and the standards were run in triplicate in the same 96 well plate for each

replication. The activity of PAL was expressed as nMol cinnamic acid/h/mg protein with reference to the standard curve.

4.4.6 Estimation of Polyphenol Oxidase

Crude enzyme extraction was followed as described in section 4.4.3. Estimation of Polyphenol oxidase (PPO) was based on protocol described by Wang et al. (2011), with few modifications. A 240 μ L reaction mixture contained 200 μ L of 0.5M catechol, 0.1 M potassium phosphate buffer (pH 6.5), 35 μ L of water and 5 μ L of crude enzyme extract. The reaction mixture was incubated at 24°C for 2 minutes. The absorbance of reaction mixture was determined at 398 nm against blank using a BioTek Power XS2 microplate reader (VT, USA) with the help of Gen5™ software. The samples and the standards were run in triplicate in the same 96 well plate for each replication. The activity of PPO was expressed as mM Catechol/min/mg protein.

4.4.7 Total phenolic content

The total phenolic content was analyzed using the Folin – Ciocalteu method as described previously by Jayaraman and Punja (2011) with minor modifications. Fresh shoot tissue (250 mg) was homogenized in 500 μ L of 80% methanol. It was shaken at 70°C for 15 min. The supernatant was collected into separate 2 ml micro-centrifuge tube and it was again kept at 70°C for 15 min. The supernatant collected was mixed with the previous supernatant and was used for analysis.

The reaction mixture contained 100 μ L of methanolic extract (supernatant collected), 500 μ L distilled water and 25 μ L of 1 N Folin–Ciocalteu reagent. The reaction mixture was incubated at 25°C for 3 min. After incubation 100 μ L of 20% (w/v) Na₂CO₃ and 100 μ L

distilled water were added to stop the reaction. The solution was further incubated for 1 hour at 25°C. The absorbance was then read at 725 nm against blank using a BioTek microplate reader (VT, USA) with the help of Gen5™ software. A standard calibration curve (gallic acid 20–500 mg/L) was prepared using the same procedure as above. The samples and the standards were run in triplicate in the same 96 well plate for each replication. Total phenolics content was expressed as gallic acid equivalents (GAE, mg gallic acid/g FW).

4.4.8 Flavonoid content

Total flavonoid content in cucumber shoots was quantified following a colorimetric method as previously described by Liu & Zhu (2007) with modifications. Fresh shoot tissue (250 mg) was homogenized in 500 µL of 80% methanol. It was shaken at 70°C for 15 min. The supernatant was collected into separate 2 ml micro-centrifuge tube and it was again kept at 70°C for 15 min. The supernatant collected was mixed with the previous supernatant and was used for analysis.

Fifty µL of tissue homogenates or standard solutions was mixed with 35 µL of 5% (w/w) NaNO₂ and 500 µL of 30% (v/v) ethanol. The reaction mixture was incubated at room temperature for 5 min. After incubation, 35 µL of 10% (w/w) AlCl₃ was added, vortexed and incubated for 6 min. After incubation, 250 µL of 1 mol/l NaOH was added. The volume of the mixture was then brought up to 1.25 mL with the addition of 380 µL of 30% (v/v) ethanol. The reaction mixture was mixed well and incubated for 15 min. Absorbance was measured against a blank (80% methanol) at 510 nm using a BioTek microplate reader (VT, USA) with the help of Gen5™ software. A standard calibration curve (catechin 5–100 mg/L) was prepared using the same procedure as above. The

samples and the standards were run in triplicate in the same 96 well plate for each replication. The total flavonoid content was expressed in ug of catechin equivalents/ g FW.

4.5 Statistical Analysis

4.5.1 *In vitro* anti-microbial assay

Each of the three experiments was repeated three times. Data collected from each experiment was analyzed with one way analysis of variance (ANOVA) using Proc MIXED procedure in Statistical Analysis Software (SAS)(SAS Institute, Cary, NC, USA) at $P= 0.05$. In case of significance ($P = 0.05$) further separation of means of treatments and control was performed using Tukeys Honestly Significant Difference (HSD). Test for all assumptions were performed. Repeated measures were used to analyze results of mycelial diameter measured over time in solid culture method experiment.

4.5.2 *In vivo* greenhouse and biochemical analysis

Design of experiment for all *in vivo* greenhouse and biochemical analysis experiments was randomized block design with 10 blocks. Every experiment was repeated 2 times. Statistical analysis for all experiments was performed by Proc MIXED procedure in SAS by using Multiple Mean Comparisons – Tukey adjustment and test for assumptions (Normality, Constant Variance and Independence). Repeated measures were used to analyze results of seedling height.

CHAPTER 5 RESULTS

5.1 In vitro anti-microbial assay

To study the direct effect of the *A. nodosum* byproduct on the vegetative and reproductive growth of *Pythium ultimum*, three experiments were conducted: the liquid and the solid culture to study vegetative growth inhibition, and sporangia count to study reproductive growth inhibition.

5.1.1 Liquid Culture Method

The response of treatments produced significant differences ($P < 0.0001$) in mycelium growth. Results indicate that the supplementation of *Ascophyllum nodosum* byproduct (ABP) in the liquid corn meal media significantly increased mycelial weight of 7 days old *Pythium* culture as compared to control. It was also found that the mycelial weight progressively increased with the increase in the concentration of ABP in the liquid corn meal media (Figure 5.1). The 10g/l of ABP supplementation in media caused significantly highest mycelial weight as compared to other treatments and control. However, *Pythium* grown in 0.5g/l ABP supplemented media, showed no significant difference in mycelial weight as compared to control.

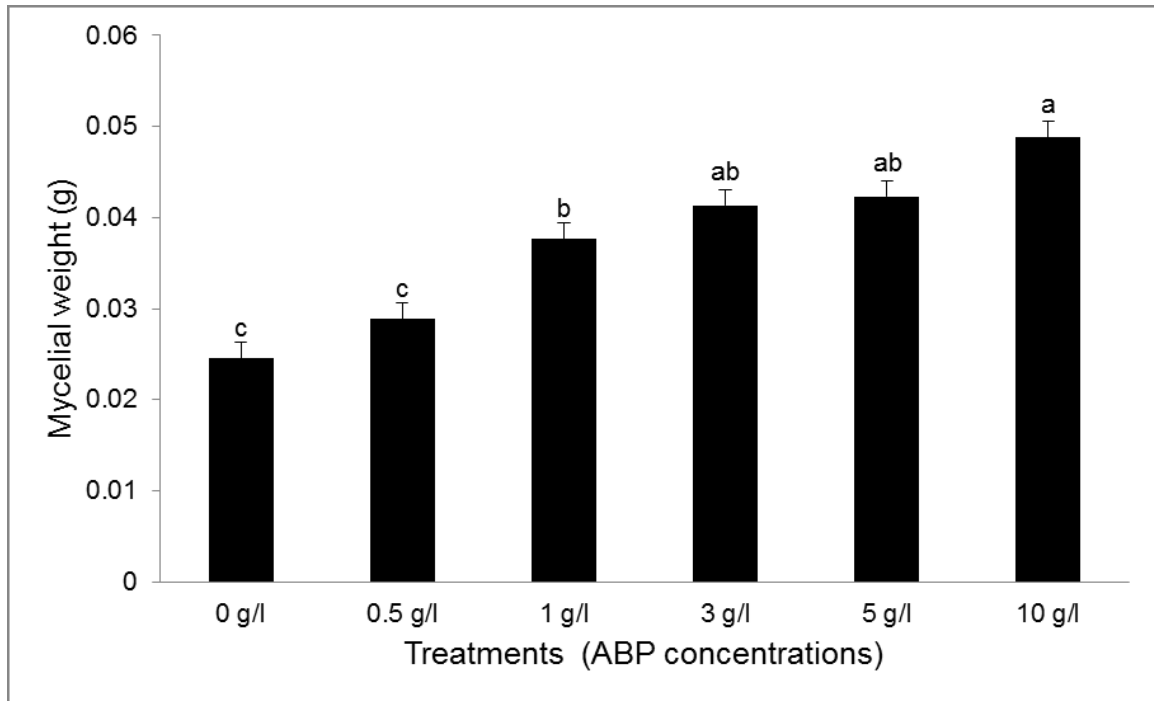


Figure 5.1: The effect of different concentrations of *Ascophyllum* byproduct (ABP) on the weight of *Pythium* mycelia when cultured in liquid medium ($P < 0.0001$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 5 replications per treatment and the experiment was repeated 3 times.

5.1.2 Solid Culture Method

Data were analyzed using hours after inoculation as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and hours after inoculation was significant (P - value < 0.0001). Multiple mean comparisons were conducted using Tukey's test. Results indicate that the supplementation of 0.5g/l and 1g/l of *Ascophyllum* by product (ABP) in the corn meal agar media, showed no significant difference in mycelial diameter increase with the increasing time as compared to control. However, supplementation of 3 g/l, and above concentration of *Ascophyllum* by product (ABP) in the corn meal agar media, significantly decreases mycelial diameter from 18th hour as compared to control (Figure 5.2).

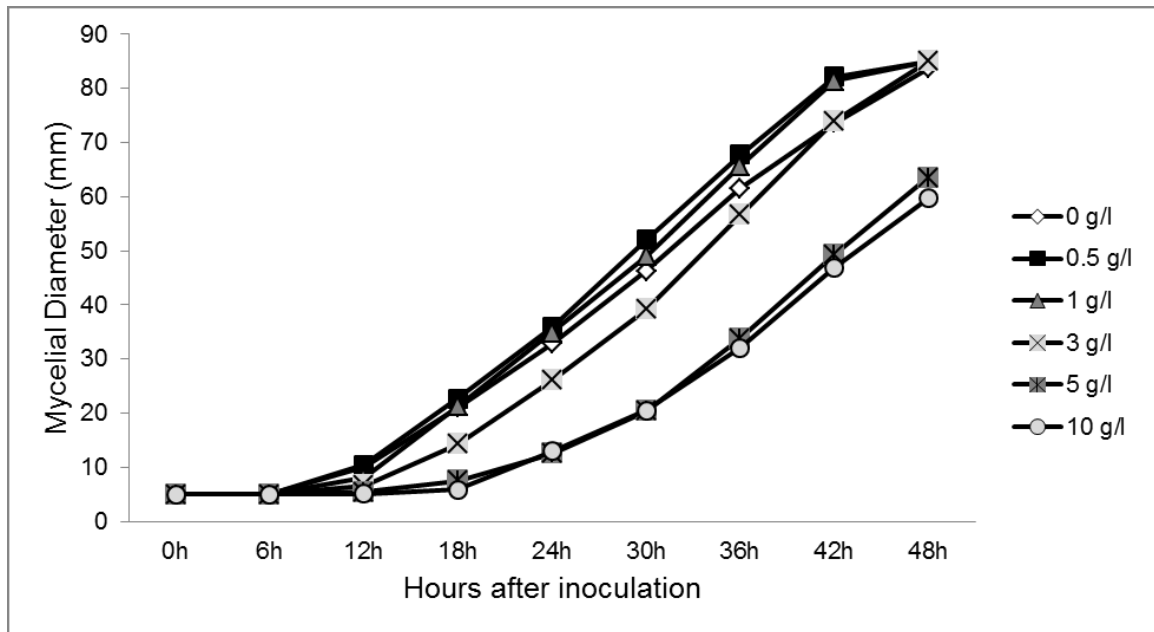


Figure 5.2: The effect of different concentrations of *Ascophyllum* byproduct (ABP) on the diameter of *Pythium* mycelia at every 6 hours over a period of 48 hours, when cultured on a solid corn meal agar medium ($P < 0.0001$). There were 5 replications per treatment and the experiment was repeated 3 times.

5.1.3 Effect of *Ascophyllum* byproduct on reproduction of *Pythium ultimum*

In this experiment, *Pythium ultimum* was cultured on solid corn meal agar media plates supplemented with different concentrations of *A. nodosum* byproduct, similar to solid culture method. The response of treatments produced significant differences ($P < 0.0001$). Results indicate that the supplementation of 3 g/l and above concentrations of *Ascophyllum* by product (ABP) in the growth media, caused significant increase in *Pythium* sporangial count as compared to control and are also significantly different from each other in an increasing order (Figure 5.3). The 10g/l of ABP supplemented in the growth media showed significantly highest sporangia count increase of approximately 60% as compared to control. However, sporangia count of treatments containing 0.5g/l

and 1g/l of ABP in the growth media was not significantly different than that of control (Figure 5.3).

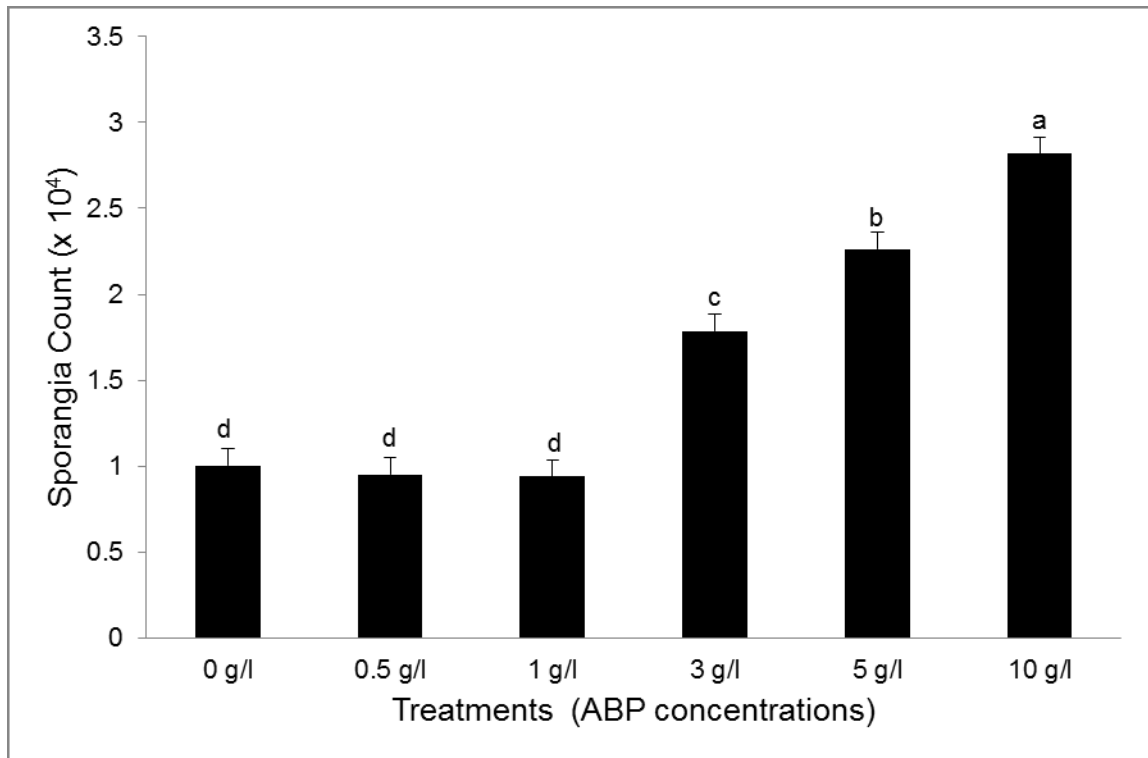


Figure 5.3: The effect of different concentrations of *Ascophyllum* byproduct (ABP) in growth medium on the sporangia count of *Pythium* ($P < 0.0001$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 5 replications per treatment and the experiment was repeated 3 times.

5.2 In vivo Greenhouse Experiments

To study the effect of the *Ascophyllum* byproduct on the seedling health and resistance against damping off disease, three experiments were conducted: seedling health experiment, pre emergence damping off and post emergence damping off experiments.

5.2.1 Effect of Ascophyllum byproduct on seedling growth and health

5.2.1.1 Effect of Ascophyllum byproduct on seedling height

Data were analyzed using weeks as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and weeks was not significantly different ($P = 0.6383$) at 5% level of significance. However, the treatments were significantly different from each other ($P < 0.0001$) at 5% level of significance. So, the data was averaged over weeks for each treatment. Multiple mean comparisons were conducted using Tukey's test. The weeks were also significantly different ($P < 0.0001$). Results indicate that the supplementation of either digested or undigested *Ascophyllum* byproduct (ABP) in the plant growth media, showed no significant difference in seedling height measured every week over a period of 3 weeks (Figure 5.4).

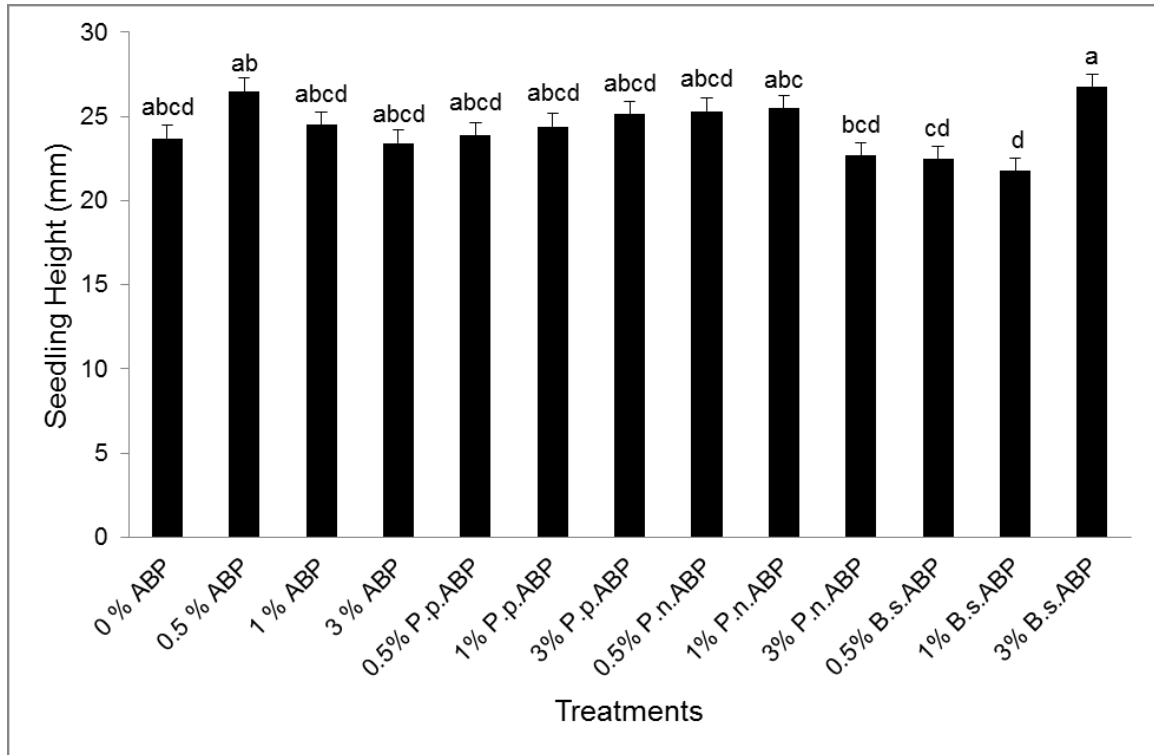


Figure 5.4: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on cucumber seedling height over a period of 3 weeks. P value for treatments * weeks was 0.6383 but P value for treatments was <0.0001. The means are an average of 3 weeks. The error bars represent standard error of experiment. There were 10 replications per treatment and the experiment was repeated 2 times.

5.2.1.2 Effect of *Ascophyllum* byproduct on shoot fresh weight

The responses of treatments were significantly different ($P < 0.0001$). Multiple mean comparisons were conducted using Tukey's test. Blocks were found to be not significantly different ($P = 0.891$). Results indicate that unlike undigested ABP, the supplementation of digested ABP in the plant growth media, showed similar shoot fresh weight of 3 week old seedlings as compared to control, except 1%P.n.ABP (Figure 5.5). With increasing concentration of undigested ABP in cucumber growth media, the 3 week old shoot fresh weight significantly decreases as compared to control and other

treatments. The 3% digested ABP with all three microbes significantly increased shoot fresh weight by approximately 59% as compared to 3% undigested ABP (Figure 5.5).

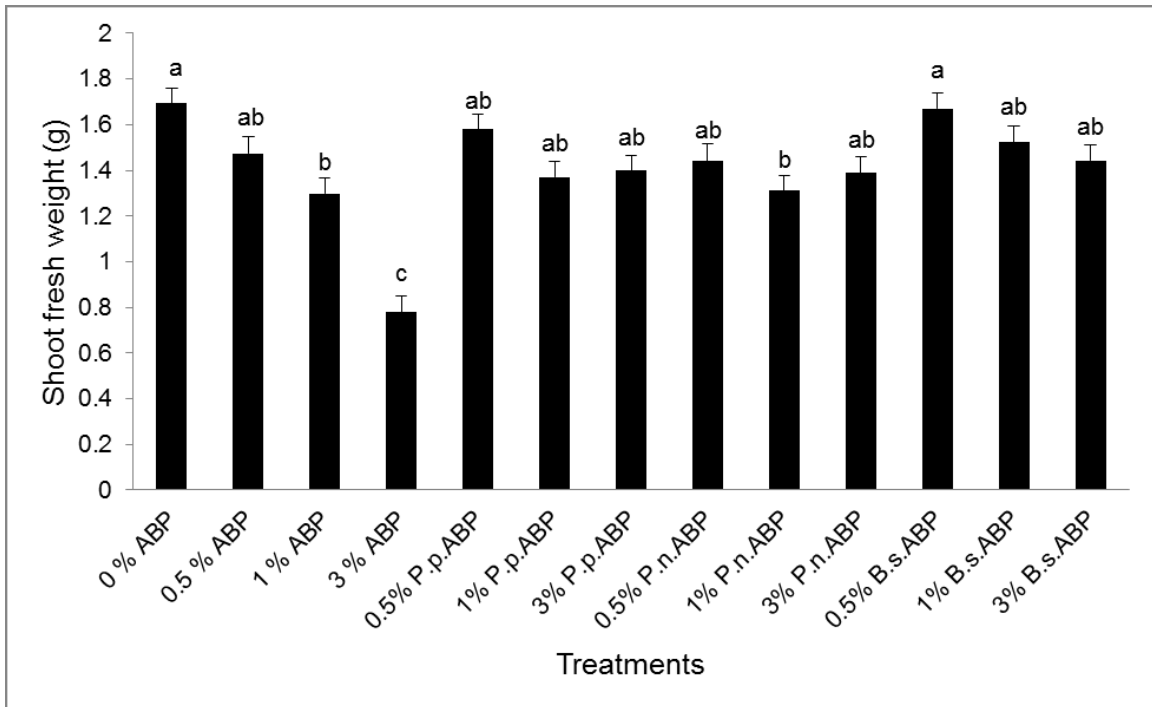


Figure 5.5: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on 3 weeks old cucumber shoot fresh weight ($P < 0.0001$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.1.3 Effect of *Ascophyllum* byproduct on shoot dry weight

The responses of treatments were significantly different ($P < 0.0001$). Multiple mean comparisons were conducted using Tukey's test. Blocks were significantly different ($P = 0.0013$). Results indicate that the supplementation of digested and undigested *Ascophyllum* byproduct (ABP) in the plant growth media, showed no significant difference in shoot dry weight of 3 week old seedlings as compared to control, except 3% ABP and 3% P.n.ABP (Figure 5.6). With the supplementation of 3% undigested ABP in cucumber growth media, the 3 week old shoot dry weight significantly decreased by 50%

as compared to control. All the 3% digested ABP showed significantly higher shoot dry weight by 45-50% as compared to 3% undigested ABP except 3% P.n.ABP (Figure 5.6). 3% P.n.ABP showed no significant difference in shoot dry weight as compared to 3% ABP.

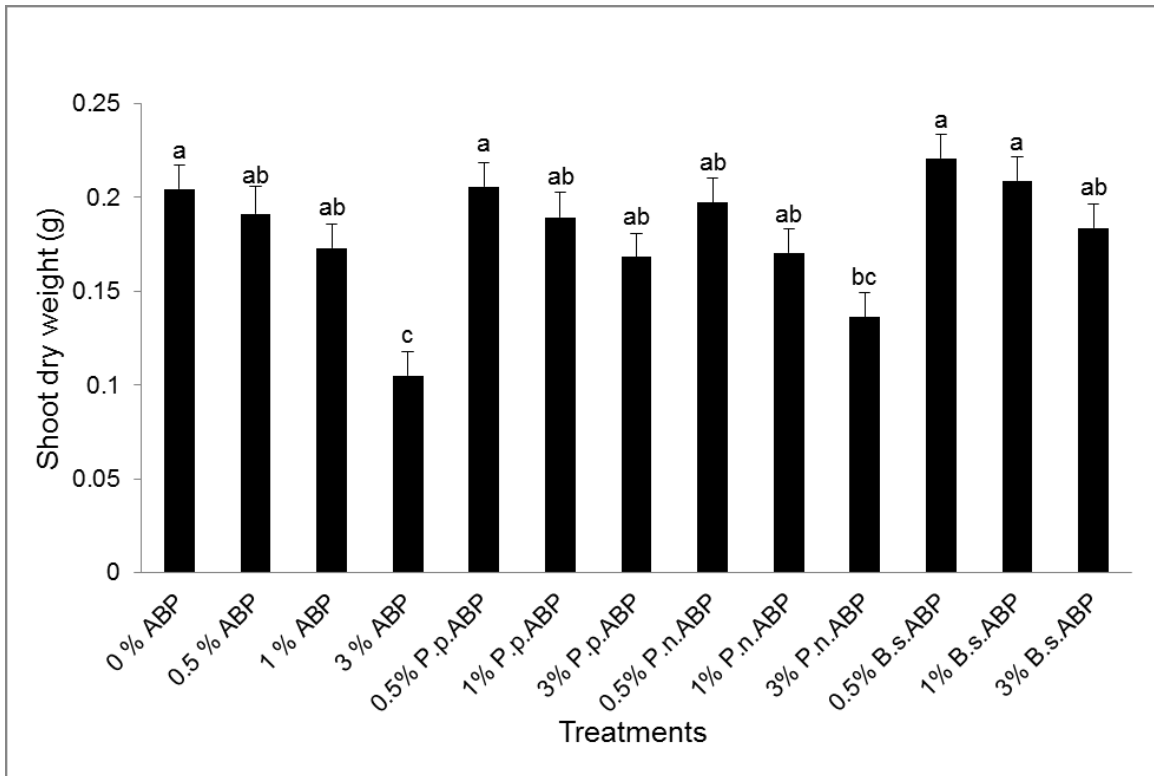


Figure 5.6: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on 3 weeks old cucumber shoot dry weight ($P = 0.0013$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.1.4 Effect of *Ascophyllum* byproduct on root length

The responses of treatments were significantly different ($P < 0.0001$). Multiple mean comparisons were conducted using Tukey's test. Blocks were not significantly different ($P = 0.367$) from each other. Results indicate that the supplementation of digested and undigested *Ascophyllum* by product (ABP) in the plant growth media, showed no significant difference in root length of 3 weeks old seedling as compared to control, except 3% ABP (Figure 5.7). With the supplementation of 3% undigested ABP in cucumber growth media, the 3 week old root length significantly decreased by 53% as compared to control. All the 3% digested ABP showed significantly higher root length by approximately 50% as compared to 3% undigested ABP (Figure 5.7).

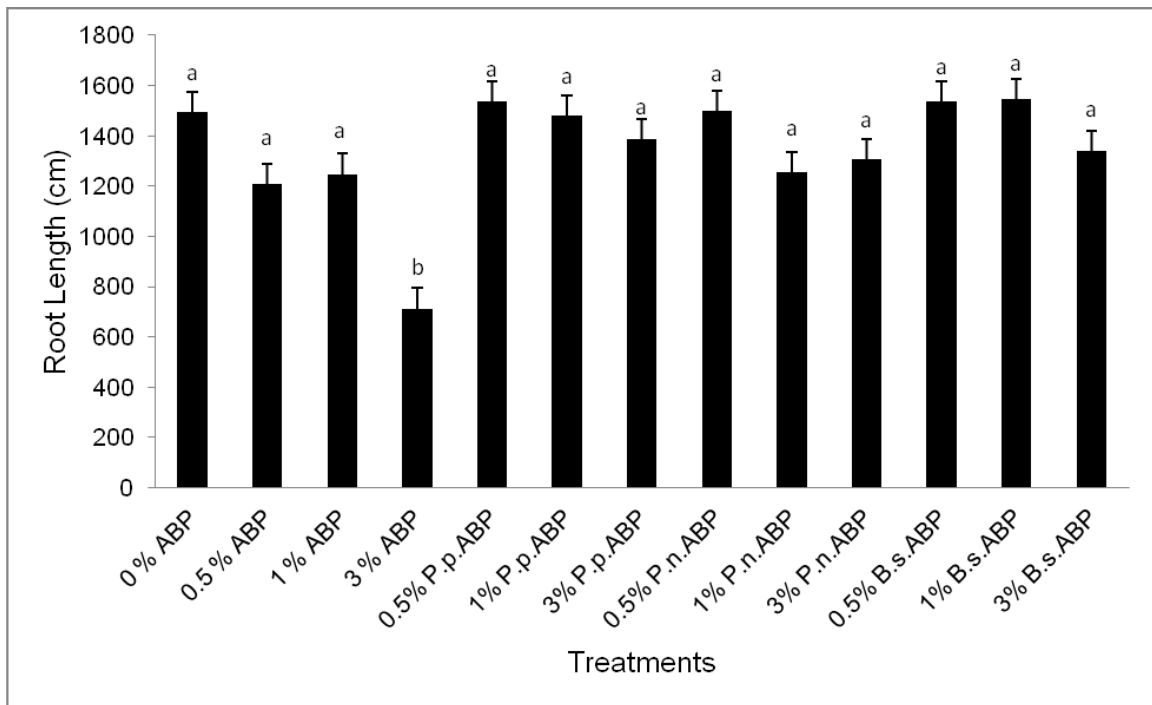


Figure 5.7: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on 3 weeks old cucumber root length ($P < 0.0001$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.1.5 Effect of *Ascophyllum* byproduct on root surface area

The responses of treatments were significantly different ($P < 0.0001$). Multiple mean comparisons were conducted using Tukey's test. Blocks were significantly not different ($P = 0.397$). Results indicate that the supplementation of digested and undigested *Ascophyllum* byproduct (ABP) in the plant growth media, showed no significant difference in root surface area of 3 weeks old seedling as compared to control, except 3% ABP (Figure 5.8). With the supplementation of 3% undigested ABP in cucumber growth media, the 3 week old root surface area significantly decreased by approximately 53% as compared to control. All the 3% digested ABP showed significantly higher root area by approximately 50% than 3% undigested ABP (Figure 5.8).

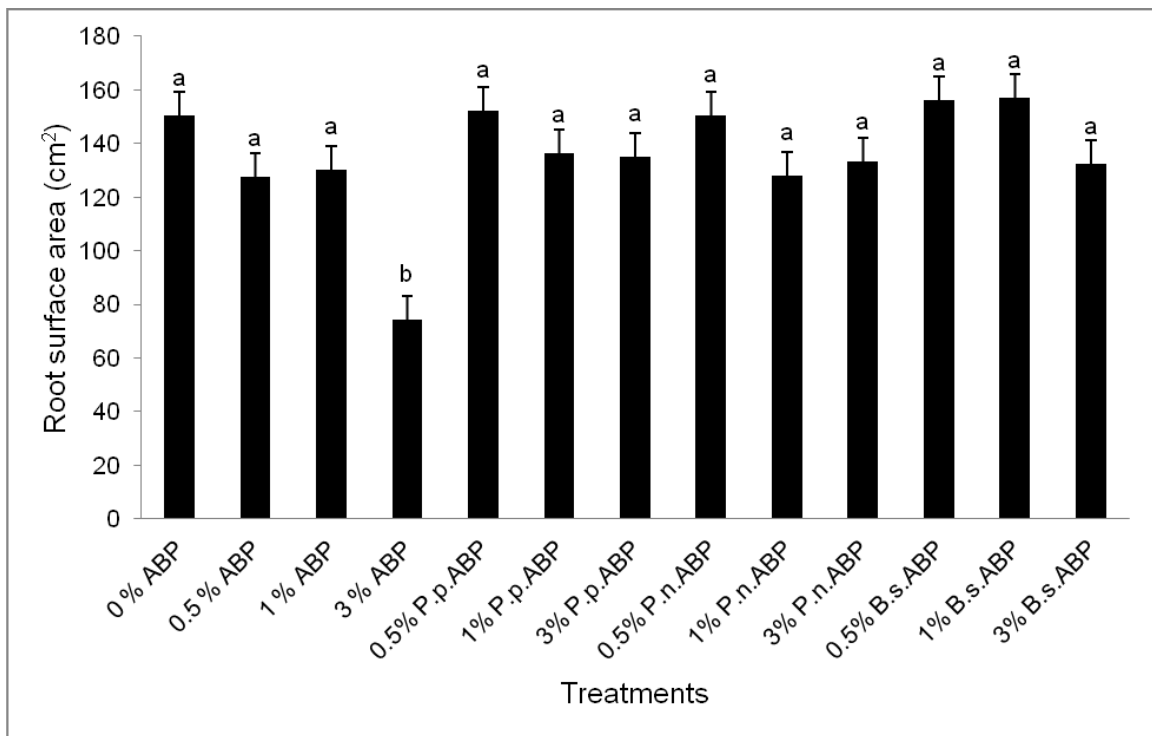


Figure 5.8: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on 3 weeks old cucumber root surface area ($P < 0.0001$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.1.6 Effect of *Ascophyllum* byproduct on root dry weight

The responses of treatments were significantly different ($P = 0.0003$). Multiple mean comparisons were conducted using Tukey's test. Blocks were not significantly different ($P = 0.0520$) from each other. Results indicate that the supplementation of digested and undigested *Ascophyllum* byproduct (ABP) in the plant growth media, showed similar root dry weight of 3 week old seedlings as compared to control (Figure 5.9). However, with the supplementation of 3% undigested ABP in cucumber growth media, the 3 week old root dry weight decreased by approximately 30 - 40% as compared to control and other treatments (Figure 5.9).

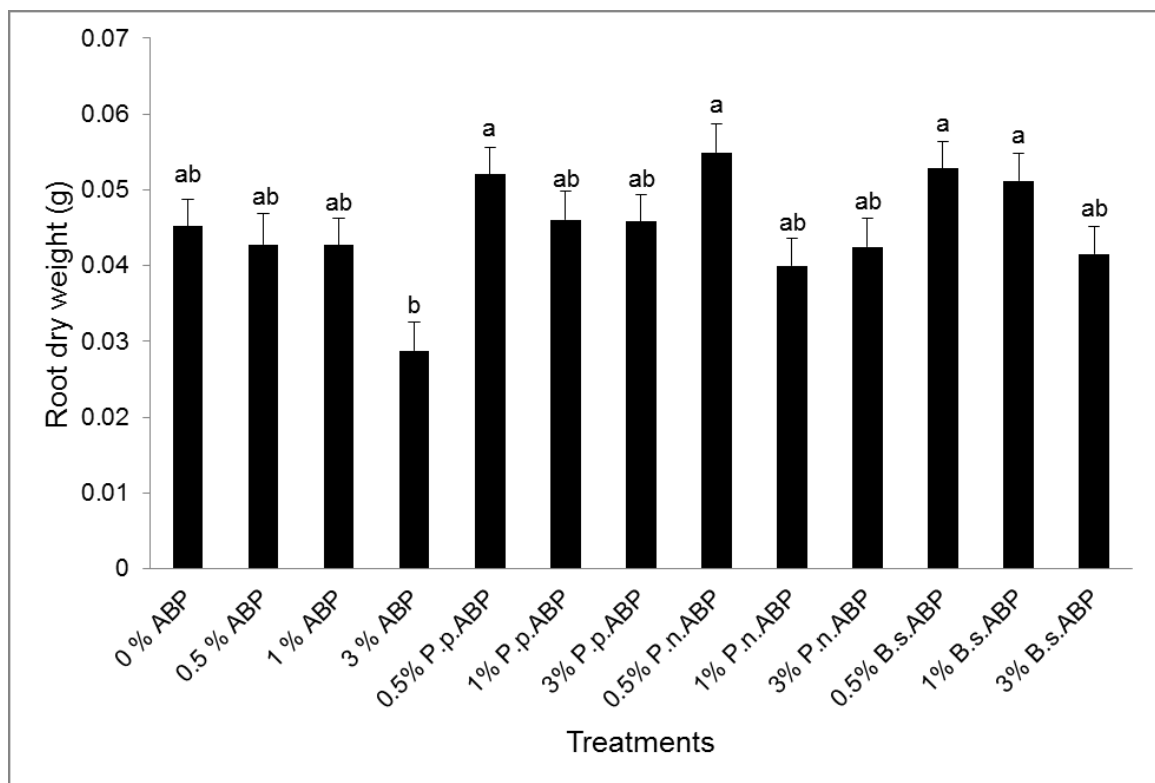


Figure 5.9: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on 3 weeks old cucumber root dry weight ($P = 0.0003$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.1.7 Effect of *Ascophyllum* byproduct on true leaf area

The responses of treatments were significantly different ($P < 0.0001$). Multiple mean comparisons were conducted using Tukey's test. Blocks were not significantly different ($P = 0.34118$) from each other. Results indicate that the supplementation of all concentrations of *Penicillium* and *Bacillus subtilis* digested *Ascophyllum* byproduct (ABP) in the plant growth media, showed significantly similar true leaf area of 3 weeks old cucumber seedling as compared to control (Figure 5.10). The 0.5% undigested and 0.5% all the digested ABP supplementation in the growth media also showed significantly similar leaf area as compared to control. Supplementation of 3% undigested ABP in cucumber growth media, significantly reduced the 3 weeks old cucumber true leaf area by 85% as compared to control. All the 3% digested ABP showed significantly higher true leaf area by approximately 80% as compared to 3% undigested ABP (Figure 5.10).

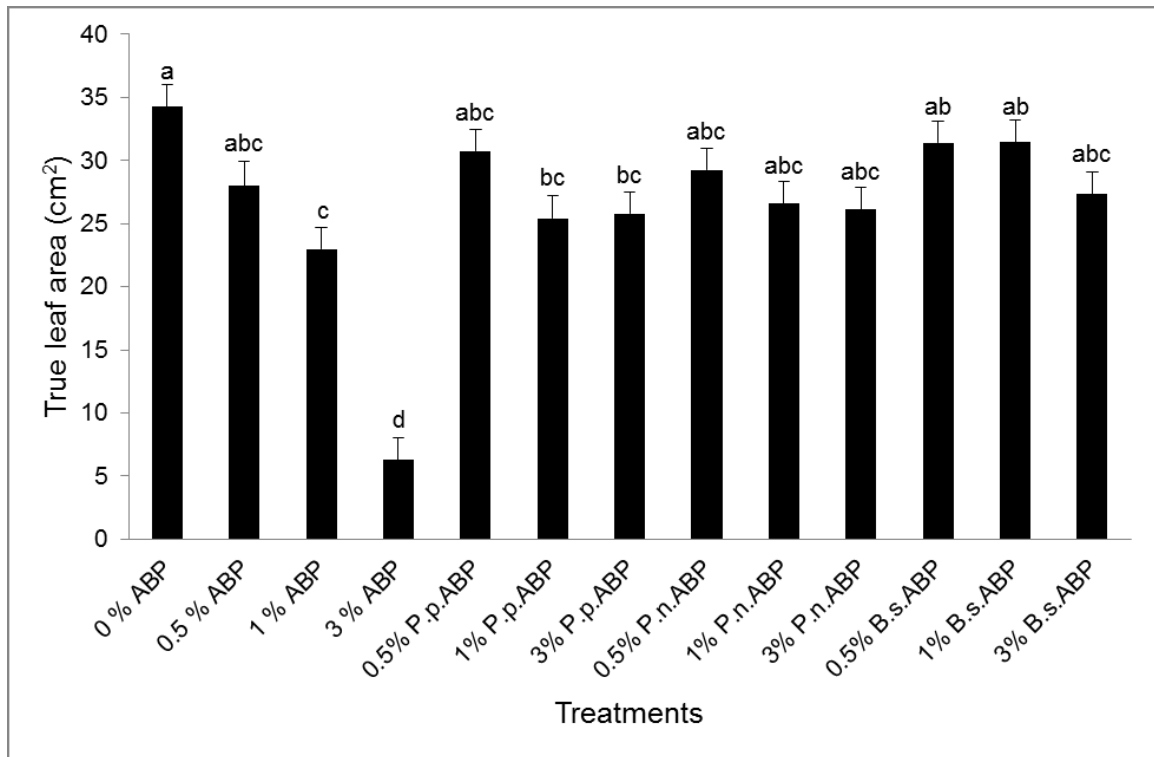


Figure 5.10: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on 3 weeks old cucumber true leaf area ($P < 0.0001$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.1.8 Effect of *Ascophyllum* byproduct on number of true leaves

The responses of treatments were significantly different ($P = 0.0073$). Multiple mean comparisons were conducted using Tukey's test. Blocks were not significantly different ($P = 0.2664$) from each other. Results indicate that the supplementation of digested and undigested *Ascophyllum* byproduct (ABP) in the plant growth media, showed similar number of true leaves as compared to control in 3 weeks old cucumber seedlings (Figure 5.11).

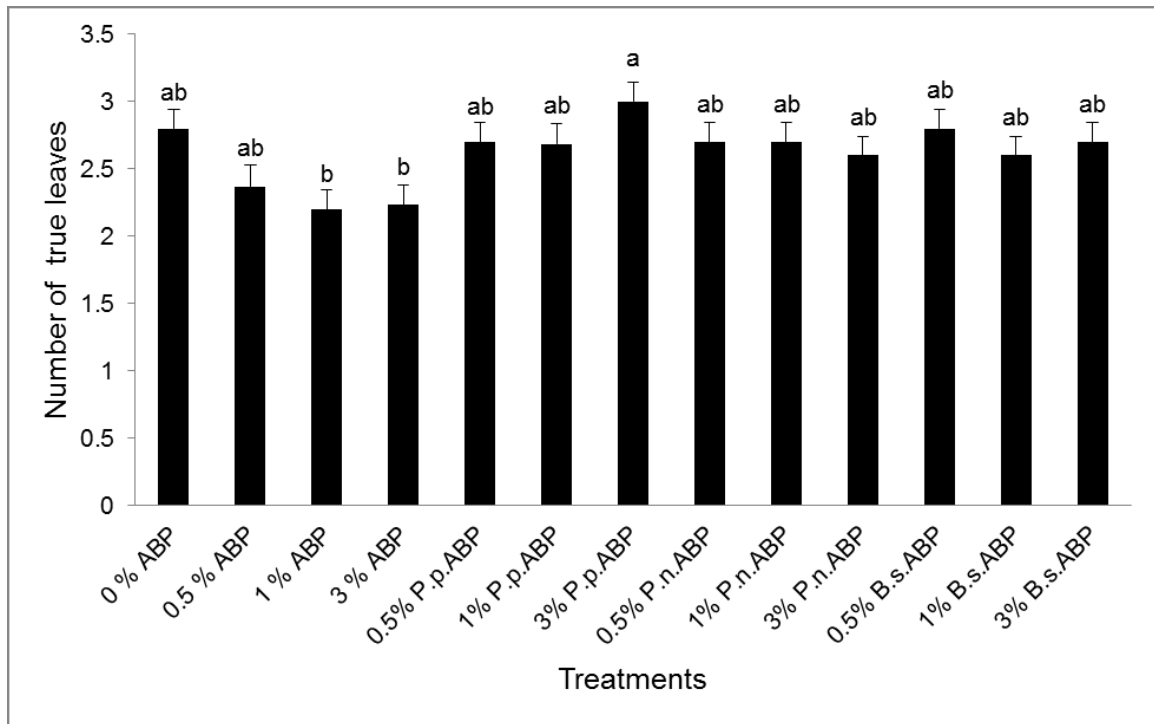


Figure 5.11: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on 3 weeks old cucumber true leaf numbers ($P=0.0073$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.2 Effect of *Ascophyllum* byproduct on damping off disease

5.2.2.1 Effect of *Ascophyllum* by-product on pre emergence damping off disease

Data were analyzed using days as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and days was not significantly different ($P = 1.000$) at 5% level of significance. However, the treatments were found to be significantly different from each other ($P < 0.0001$) at 5% level of significance. So, the data were averaged over days to obtain mean for each treatment. Multiple mean comparisons were conducted using Tukey's test. The days were also significantly

different ($P < 0.0009$). Results indicate that the supplementation of either digested or undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, had no significant difference in number of cucumber seedling showing pre emergence damping off symptom measured every 3rd day over a period of 2 weeks. The treatments were similar to control. However, among the treatments, supplementation of 1% P.n.ABP in growing media showed highest damping off symptoms and was significantly higher than 1%, 3% undigested ABP by 67-70% (Figure 5.12).

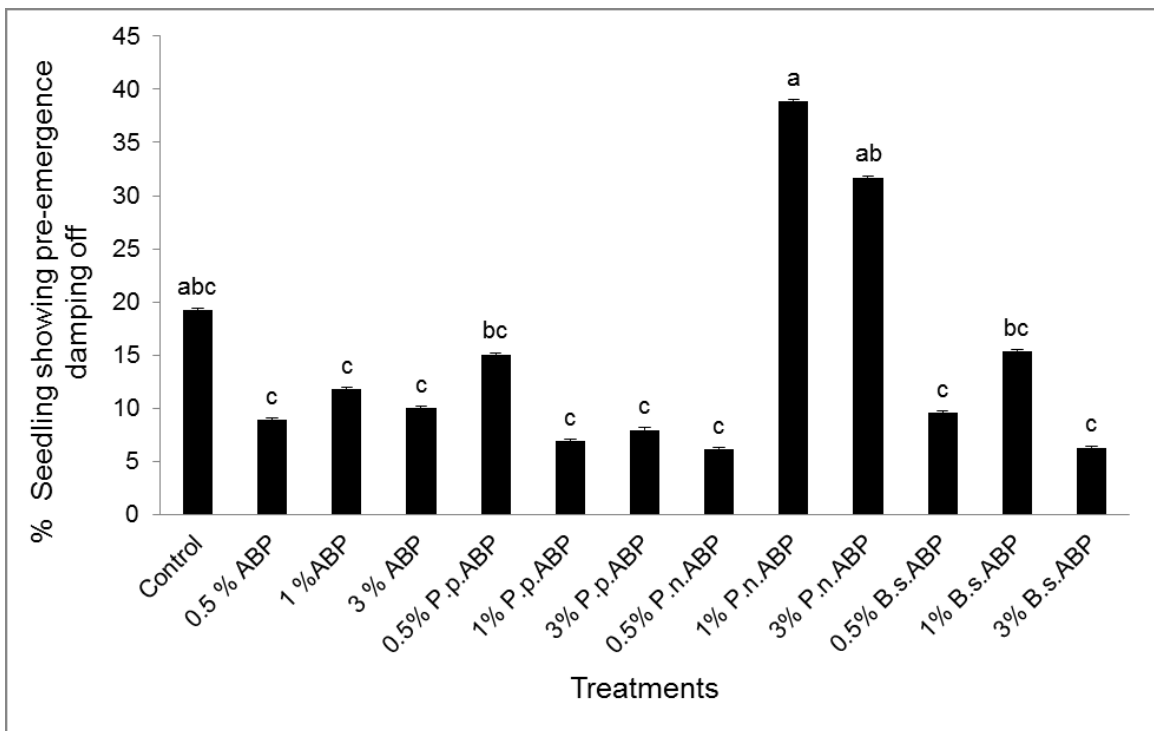


Figure 5.12: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum nodosum* byproduct (ABP) on number of cucumber seedlings showing pre emergence damping off symptom at every 3 days over a period of 2 weeks. Means are an average of 2 weeks data. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.2.2 Effect of *Ascophyllum* byproduct on shoot fresh weight during pre-emergence damping off experiment

The responses of treatments were significantly different ($P=0.0017$). Multiple mean comparisons were conducted using Tukey's test. Blocks were not significantly different ($P=0.0645$). Results indicate that the supplementation of digested and undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, showed similar shoot fresh weight as compared to control in 3 weeks old cucumber seedlings (Figure 5.13).

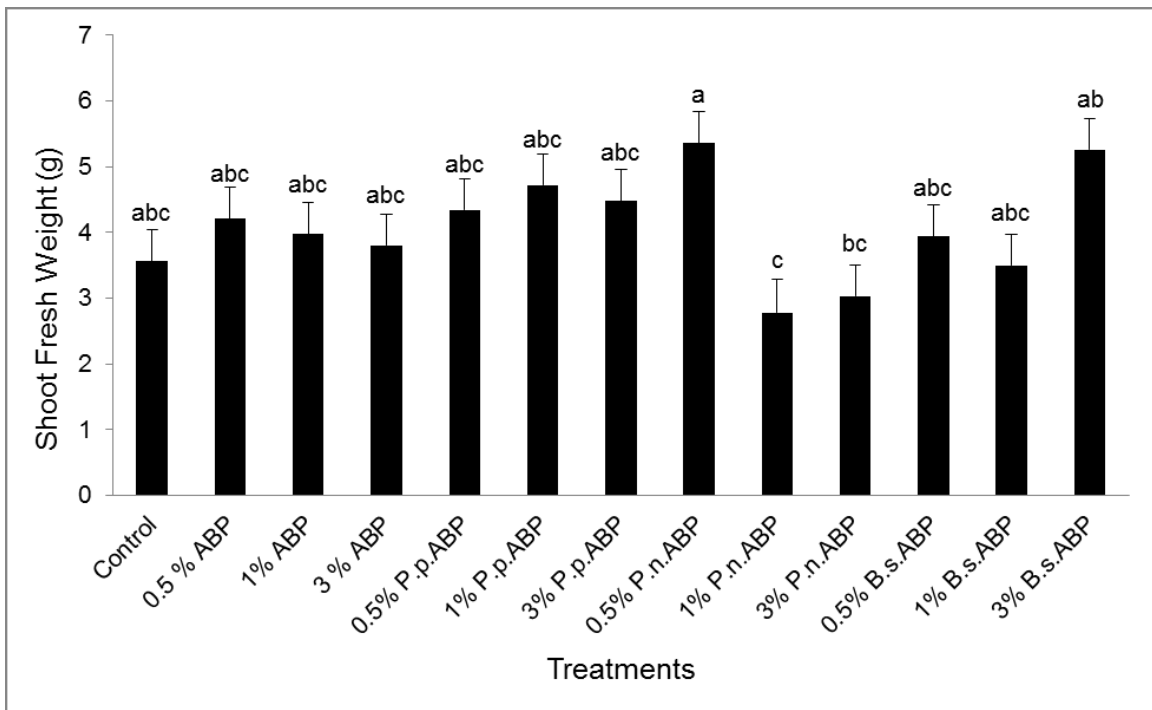


Figure 5.13: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum nodosum* by-product (ABP) on 3 weeks old healthy cucumber shoot fresh weight ($P=0.0017$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.2.3 Effect of *Ascophyllum* byproduct on shoot dry weight during pre emergence damping off experiment

The responses of treatments were significantly different ($P = 0.0002$). Multiple mean comparisons were conducted using Tukey's test. Blocks were significantly different ($P = 0.0006$). Results indicate that the supplementation of digested and undigested *Ascophyllum* by-product (ABP) in the plant growth media, showed similar shoot dry weight as compared to control in 3 weeks old cucumber seedlings (Figure 5.14). However, among the treatments, 1% P.n.ABP supplementation in growth media showed reduced shoot dry weight by approximately 45% as compared to control and 1% undigested ABP (Figure 5.14).

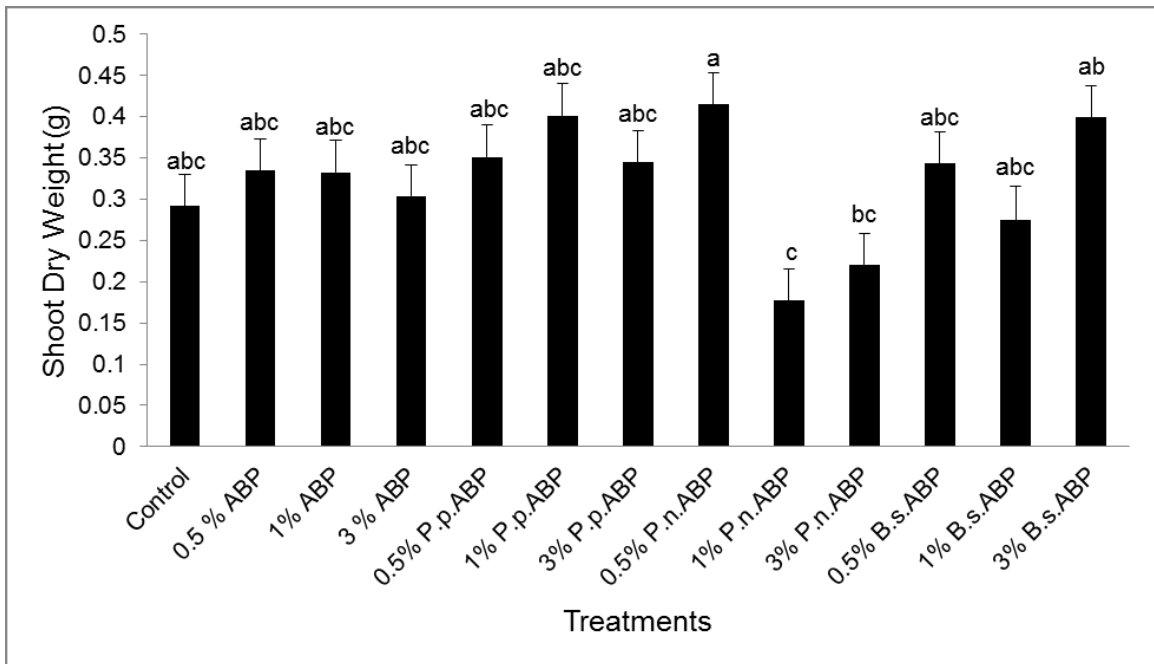


Figure 5.14: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum nodosum* by-product (ABP) on 3 weeks old healthy cucumber shoot dry weight ($P = 0.0002$). The error bars represent standard error of experiment. Different letters indicate significant difference with Tukey test at $\alpha = 0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.2.4 Effect of *Ascophyllum* byproduct on post emergence damping off disease

Data were analyzed using days as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and days was not significantly different ($P = 0.9997$) at 5% level of significance. However, the treatments were significantly different from each other ($P < 0.0001$) at 5% level of significance. So, the data were averaged over days to obtain means of each treatment. Multiple mean comparisons were conducted using Tukey's test. The days were also significantly different ($P < 0.0001$). Results indicate that the supplementation of either digested or undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, had almost no difference from control in number of cucumber seedling showing post emergence damping off symptom measured every 3rd day over a period of 2 weeks. The responses of treatments were similar to control (Figure 5.15). However, with the statistical analysis of only treatments, 1%ABP and 1% P.p. ABP supplementation in the cucumber growth media showed lowest number of post emergence damped seedlings as compared to all the treatments and control. (Figure 5.15).

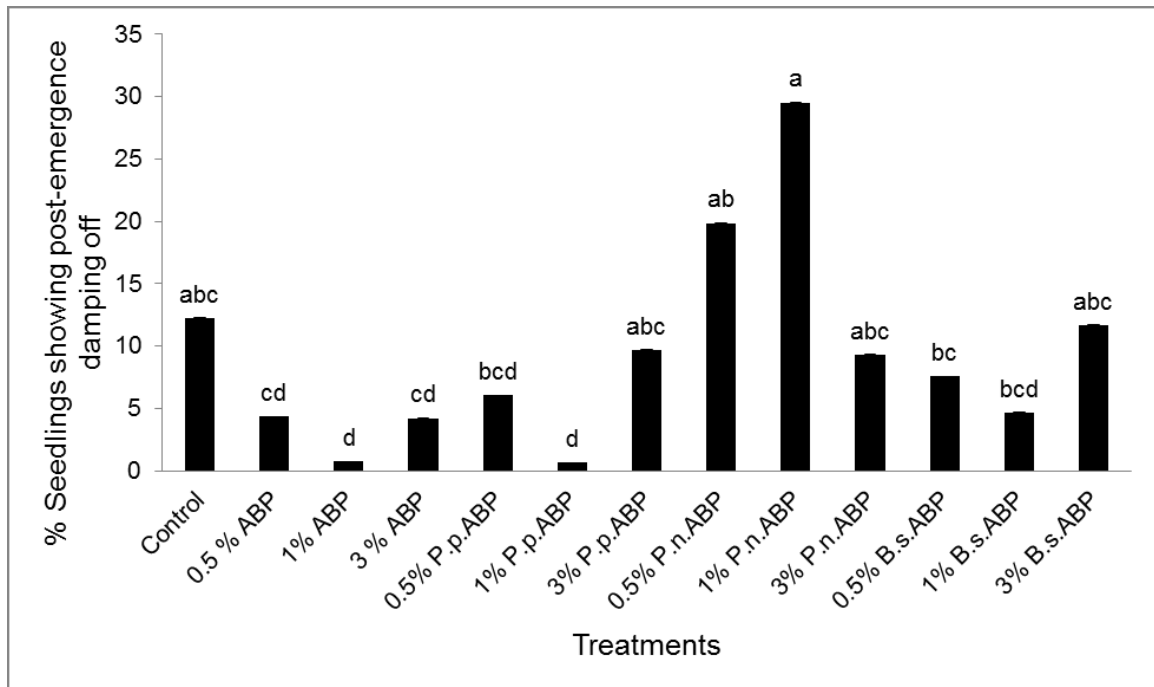


Figure 5.15: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum* byproduct (ABP) on number of cucumber seedlings showing post emergence damping off symptom at every 3 days over a period of 2 weeks. Means are an average of 2 weeks. Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.2.5 Effect of *Ascophyllum* byproduct on shoot fresh weight during post emergence damping off experiment

The responses of treatments were significantly different ($P < 0.0001$). Multiple mean comparisons were conducted using Tukey's test. Blocks were not significantly different ($P = 0.0171$). Results indicate that the supplementation of digested and undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, showed no significant difference in shoot fresh weight of 3 week old healthy cucumber seedlings as compared to control (Figure 5.16).

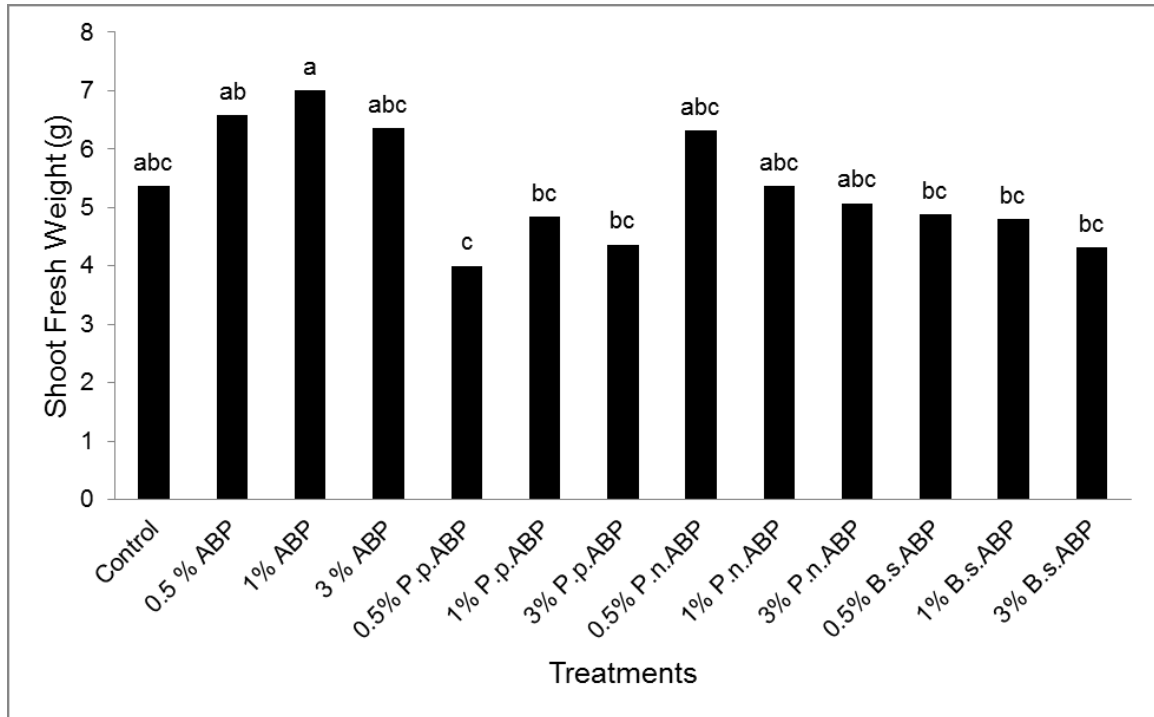


Figure 5.16: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum nodosum* by-product (ABP) on 3 weeks old healthy cucumber shoot fresh weight ($P < 0.0001$). Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.2.2.6 Effect of *Ascophyllum* byproduct on shoot dry weight during post emergence damping off experiment

The responses of treatments were significantly different ($P = 0.0002$). Multiple mean comparisons were conducted using Tukey's test. Blocks were not significantly different ($P < 0.0001$). Results indicate that the supplementation of digested and undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, showed no significant difference in shoot dry weight as compared to control in 3 weeks old healthy cucumber seedlings (Figure 5.17).

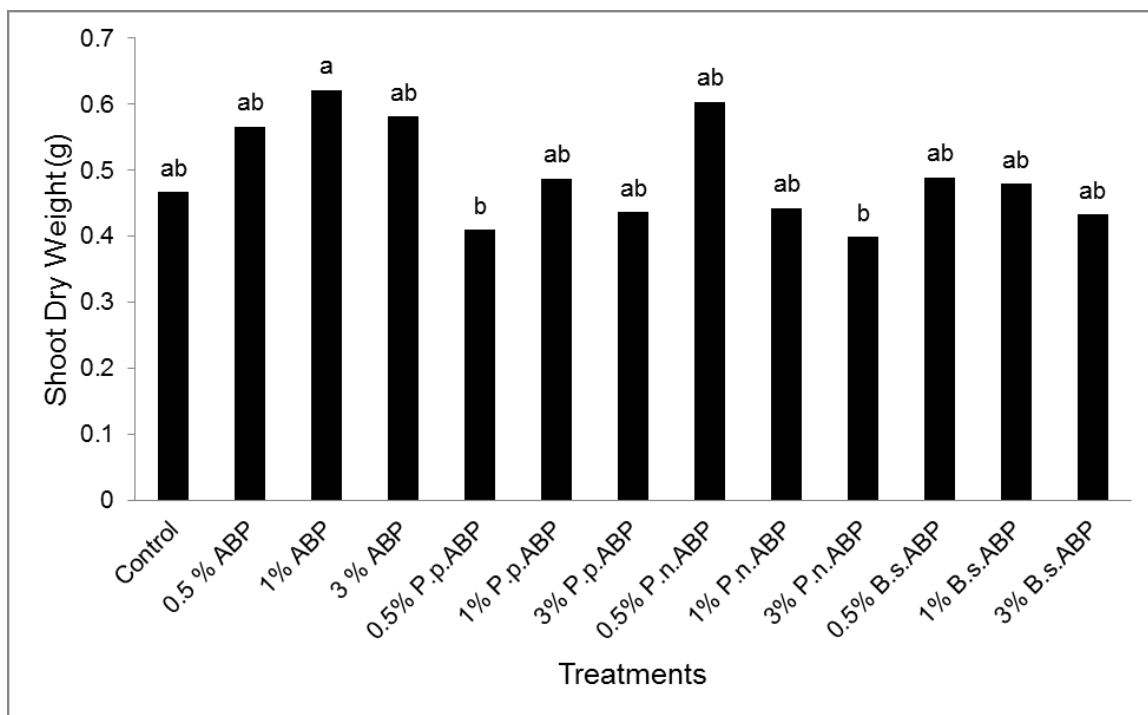


Figure 5.17: The effect of different concentrations of 3 weeks digested (P.p., P.n. and B.s. represents *Pseudomonas putida*, *Penicillium* and *Bacillus subtilis* respectively) and undigested *Ascophyllum nodosum* by-product (ABP) on 3 weeks old healthy cucumber shoot dry weight ($P = 0.0002$). Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 10 replications or blocks per treatment and the experiment was repeated 2 times.

5.3 Biochemical Analysis

To study the effect of the *Ascophyllum* by-product on defense response compounds enzymes, biochemical analyses of cucumber seedling tissue grown in treated Promix were conducted using little modifications from standard protocols.

5.3.1 Estimation of total phenol

Total Phenol content was measured using Folin Ciocalteu method with minor modifications in the standard protocol. Data were analyzed using time points as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and time points was not significantly different from each other ($P = 0.1775$) at

5% level of significance. The treatments were also not significantly different ($P = 0.1790$) at 5% level of significance. The time points were significantly different ($P < 0.0001$). Results indicate that all the treatments at each time points were not significantly different from one another. However, all the treatments were significantly different from one another between different time points. The total phenol content significantly increased in all treatments from 0 hours to 72 hours. At 72 h all the treatments showed high Phenolic content (Figure 5.18). Supplementation of either digested or undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, showed no significant difference in total phenol content measured at 3 time points.

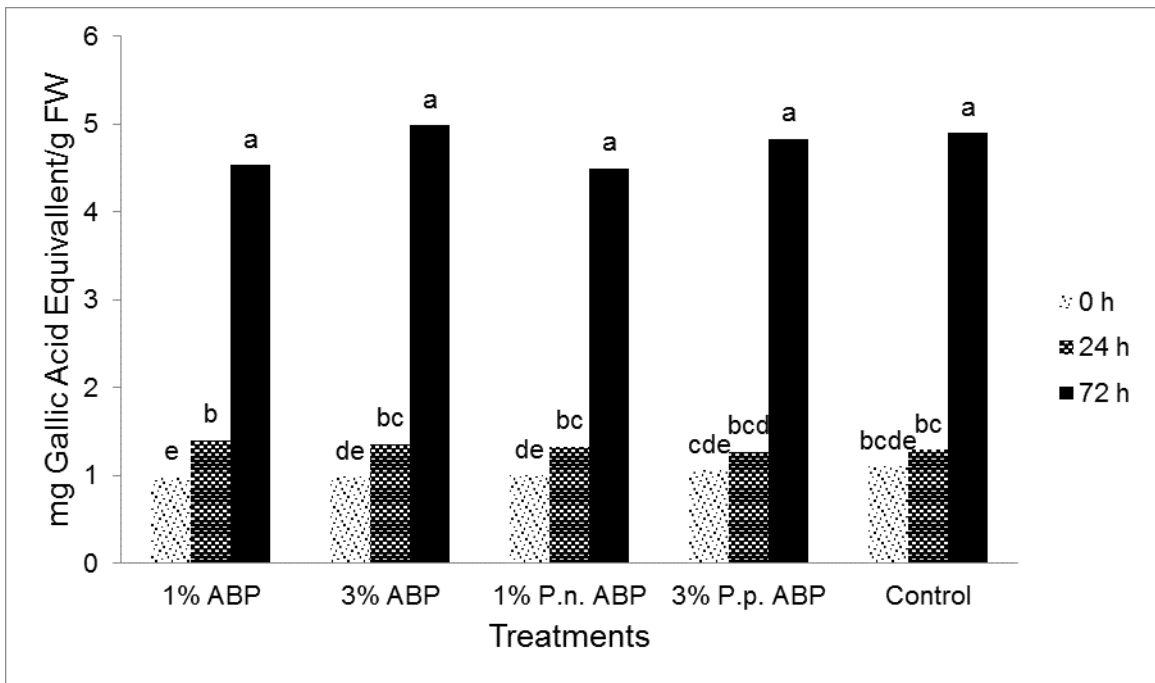


Figure 5.18: The effect of different concentrations of 3 weeks digested (P.p. and P.n. represents *Pseudomonas putida*, and *Penicillium* respectively) and undigested *Ascophyllum nodosum* by-product (ABP) on total phenol content in infected cucumber seedlings at 3 time points (0, 24, 72 hours after inoculation). Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 5 replications or blocks per treatment and the experiment was repeated 2 times.

5.3.2 Estimation of flavonoid content

Data were analyzed using time points as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and time points was not significantly different ($P = 0.4552$) at 5% level of significance. The responses of treatments were also not significantly different ($P = 0.0953$) at 5% level of significance. The time points were significantly different ($P < 0.0001$). 24h and 72 h time points tends to show higher flavanoid content as compared to 0 h in the treatments.

Results indicate that the responses of treatments were not significantly different from one another measured at 0, 24 and 72 hours after inoculation. Supplementation of either digested or undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, showed no significant difference in flavanoid content measured at 3 time points (Figure 5.19).

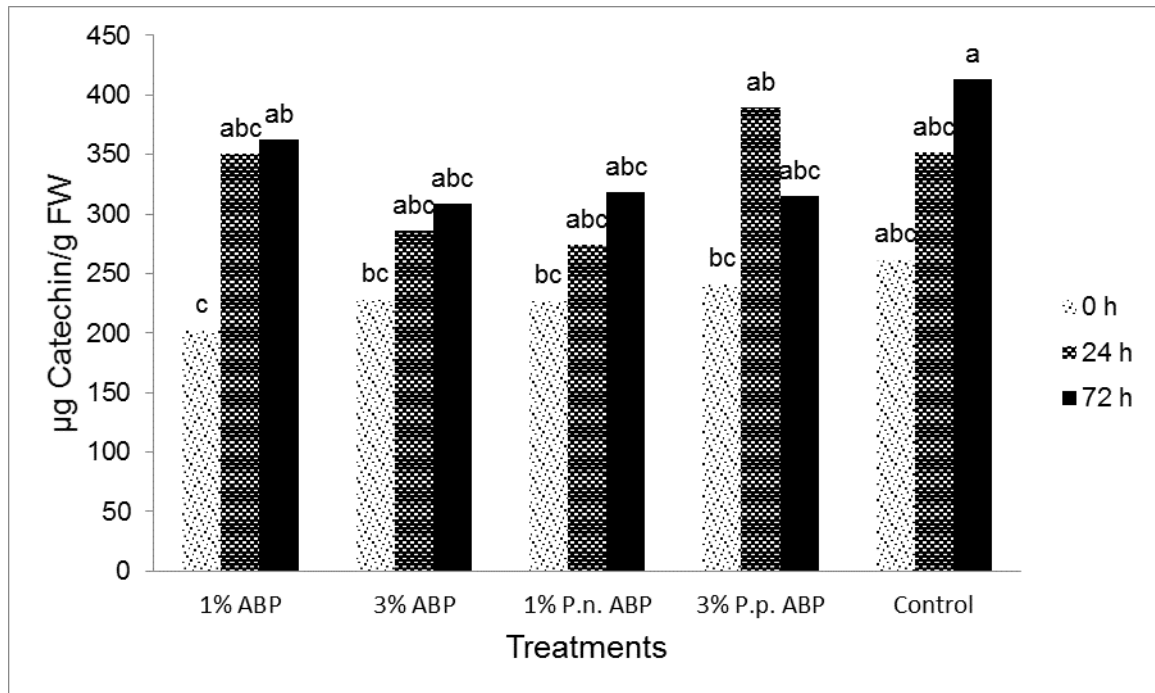


Figure 5.19: The effect of different concentrations of 3 weeks digested (P.p. and P.n. represents *Pseudomonas putida*, and *Penicillium* respectively) and undigested *Ascophyllum nodosum* byproduct (ABP) on flavanoid content in infected cucumber seedlings at 3 time points (0, 24, 72 hours after inoculation). Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 5 replications or blocks per treatment and the experiment was repeated 2 times.

5.3.3 Estimation of Phenylalanine ammonia lyase (PAL) activity

Data were analyzed using time points as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and time points was significantly different from each other ($P = 0.0004$) at 5% level of significance. However, the treatments were not significantly different ($P = 0.1760$) at 5% level of significance. Multiple mean comparisons were conducted using Tukey's test. The time points were also significantly different ($P < 0.0001$). Results indicate that the responses from all the treatments at each time points were not significantly different from one another. However, all the treatment responses were significantly different from one another between different time points especially with 24 hours PAL activity (Figure 5.20). PAL

activity shown by the undigested ABP was significantly higher in all treatments at 0 hours and 72 hours than 24 h. However, there was no significant difference in undigested ABP treatments between 0 and 72 hour (Figure 5.20). PAL activity shown by 1% P.n. ABP had no significant difference among each time point. Supplementation of both digested and undigested *Ascophyllum* byproduct (ABP) in the plant growth media, showed significant differences in PAL activity measured at 3 time points. The treatments were similar to control at each time point.

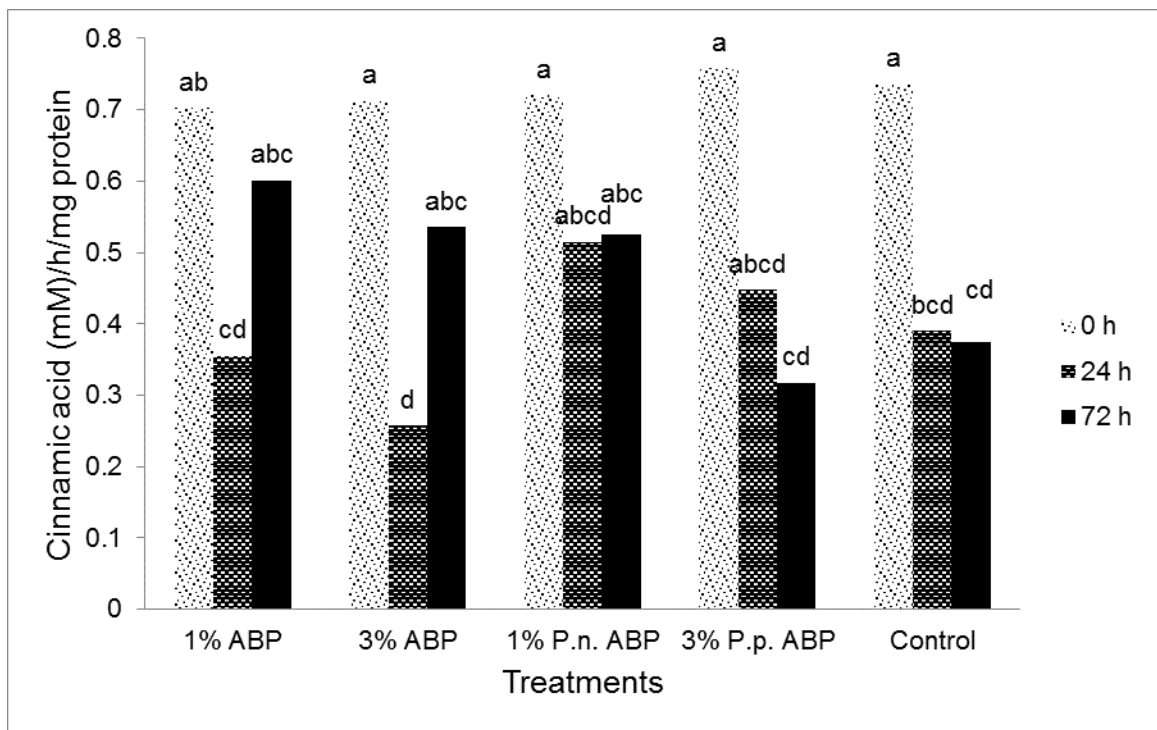


Figure 5.20: The effect of different concentrations of 3 weeks digested (P.p. and P.n. represents *Pseudomonas putida*, and *Penicillium* respectively) and undigested *Ascophyllum nodosum* byproduct (ABP) on Phenylalanine ammonia lyase activity in infected cucumber seedlings at 3 time points (0, 24, 72 hours after inoculation). Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 5 replications or blocks per treatment and the experiment was repeated 2 times.

5.3.4 Estimation of Polyphenol oxidase (PPO) activity

Data were analyzed using time points as repeated measures through Proc Mixed procedure in SAS 9.3 software. The interaction between treatments and time points was not significantly different ($P = 0.3227$) at 5% level of significance. The treatments were also not significantly different ($P = 0.1341$) at 5% level of significance. However, the time points were significantly different ($P < 0.0001$). Results indicate that all the treatments at individual time points were not significantly different from one another. PPO activity was significantly increased in all treatments from 0 hour to 72 hours (Figure 5.21). Supplementation of either digested or undigested *Ascophyllum nodosum* byproduct (ABP) in the plant growth media, showed no significant difference in PPO activity measured at 3 time points. The treatments were similar to control at each time point (Figure 5.21).

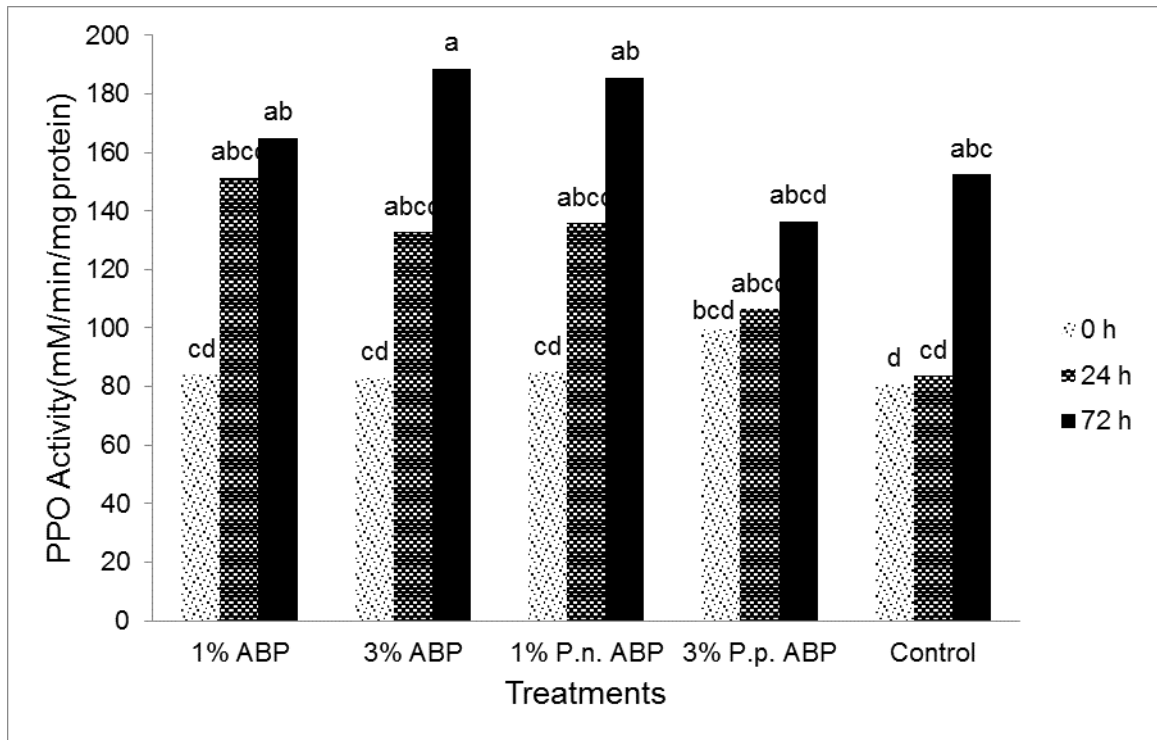


Figure 5.21: The effect of different concentrations of 3 weeks digested (P.p. and P.n. represents *Pseudomonas putida*, and *Penicillium* respectively) and undigested *Ascophyllum nodosum* by-product (ABP) on Polyphenol oxidase activity in infected cucumber seedlings at 3 time points (0, 24, 72 hours after inoculation). Different letters indicate significant difference with Tukey test at $\alpha=0.05$. There were 5 replications or blocks per treatment and the experiment was repeated 2 times.

CHAPTER 6 DISCUSSION

The increasing demand for food everyday has caused the demand to increase vegetable production in greenhouses. Greenhouse producers face serious economic losses due to damping off caused by *Pythium ultimum* (Gravel et al. 2007). This problem is especially serious in greenhouses where water is recirculated to prevent loss of nutrients (Stanghellini et al. 2000). Because of its fast multiplication and high rate of spread through soil, growth media, or water, it is one of the challenging pathogens to control (Ramamoorthy 2002). Fungicides show good results (Punja et al. 2003) but organic alternatives are in high demand for a sustainable and ecofriendly olericulture.

Ascophyllum nodosum, brown seaweed has shown many beneficial effects on plants. It not only serves as nutrient supplement (Khan et al. 2009) but application of its extracts as a spray has also shown to reduce pathogenic diseases such as downy mildew of grapes, *Phytophthora* infection in capsicum (Lizzy et al. 1998). Many extracts of this seaweed are commercially available to use in agriculture for various purposes. After the extraction of beneficial compounds from this seaweed, the left over material is referred as *Ascophyllum nodosum* by-product in this study. *Ascophyllum nodosum* is a rich source of complex polysaccharides which are not found in land plants, such as laminaran, fucoidan, and alginate (Khan et al. 2009). Hence the processed by-products obtained from this seaweed may also contain high concentrations of complex sugars and was thought to use it as an organic amendment to prevent damping off disease.

The first objective of this research was to evaluate the in vitro antimicrobial activity of *Ascophyllum nodosum* byproduct on both vegetative and reproductive growth of *Pythium ultimum*. Three experiments were conducted to investigate the effect of

different concentrations of ABP on *Pythium ultimum*. The liquid and solid culture methods were employed to test the effect of byproduct on changes in mycelial weight and diameter of *Pythium*. To test the reproductive ability, its sporangia were also counted. The results suggest that *Pythium* growth is enhanced both vegetatively and reproductively in the media containing higher concentrations of ABP as compared to control (Figure 5.1, 5.3). Iyayi and Losel, 2001 proved that different fungal pathogens are capable to convert the complex carbohydrates into simpler ones and that their growth increases with the time of solid state digestion of carbohydrate substrate. This study supports the results of all the antimicrobial assay of this research. The increase in *Pythium* mycelial growth at higher concentrations of *Ascophyllum nodosum* byproduct in the growth media, could be due to the fact that the product consists of high amount of complex carbohydrates mainly polysaccharides, which the oomycete can easily break down and utilize the nutrients for its own vegetative and reproductive growth.

However, results also suggest that the *Pythium* growth in media containing 0.5g/l and 1g/l concentrations of ABP had no significant difference from control (Figure 5.2, 5.3). Hence, lower concentrations of ABP, do not enhance *Pythium* growth either vegetatively or reproductively. Therefore, these lower concentrations of ABP were used as soil amendment to test the second objective of this research.

The second objective of this research was to study the effect of *Ascophyllum nodosum* by-product on seedling growth and systemic resistance against damping off in cucumber caused by *Pythium ultimum* in vivo. Preliminary studies suggest that *Ascophyllum nodosum* byproduct when amended in the plant growth media decreased the growth of plants during 1st week. This may be again due to high concentration of

complex carbohydrates. In this research, *Ascophyllum nodosum* byproduct was digested by three beneficial microbes, *Bacillus subtilis*, *Pseudomonas putida* and *Penicillium*. This digestion was carried out in order to break down the complex polysaccharide and to help provide the carbohydrates in simpler form to the plants when amended in the plant growth media. The test plants used in this research were cucumber seedlings. To investigate the effect of different concentrations of all the microbe digested and undigested *Ascophyllum nodosum* byproduct on cucumber seedlings, an experiment was designed. In this experiment the 0.5%, 1% and 3% concentrations of all the digested and undigested *Ascophyllum nodosum* byproduct was amended in the twice autoclaved Promix, cucumber seedlings were raised for 3 weeks. The response measured were seedling height over a period of 3 weeks, and after 3 weeks seedling leaf area, shoot fresh and dry weight, root dry weight, root length, root area, number of leaves were measured. The experiment was repeated twice in the growth room on shelves in a randomized complete block design. Results clearly suggest that the increase in the undigested ABP decreases the growth (in terms of most of the responses measured) of cucumber seedlings (such as in Figure 5.5). Lower concentrations showed no significant differences (Figure 5.6). Clear visual and statistically significant observations were found that 3% (highest concentrations) undigested ABP decreased all the biometric measurements made on cucumber seedling (example Figure 5.7, 5.8). However, in case of digested ABP, the same concentration of 3% digested ABP showed significantly higher biometric measurements made on cucumber seedling as compared to 3% undigested ABP and were similar to control (such as in Figure 5.10). There was slight similarity between the lower concentrations of digested and undigested ABP (Figure 5.10).

It has long been known that, the plants grow faster when their growing medium is amended with bacteria, fungus and any other beneficial microbe, because the microbes break down of nutrients into simpler forms and plant available forms. Nitrogen and Phosphorus have especially been known to increase in the plants grown in bacteria or fungus amended media as compared to the plants grown in sterile conditions (Ingham et al. 1985).

As stated above, the increased growth of cucumber seedlings in the digested *Ascophyllum nodosum* byproduct amended with plant growth media could be due to the presence of beneficial microbes and the supply of nutrients in simpler forms, thereby making it easier for plants to take up nutrition from the growth media. On the other hand the undigested ABP amended with plant growth media decreased cucumber seedling growth due to the absence of beneficial microbes and the undigested ABP contains complex polysaccharides, which makes it difficult for the plants to take up nutrition. Such a high concentration of complex polysaccharides may make the growth media toxic for the plants. Hence, it can be concluded that the bacterial digested *Ascophyllum nodosum* byproduct can be used as soil amendment for growth of seedlings in nurseries.

Two experiments were conducted to investigate the effect of different concentrations of the three beneficial microbe digested and undigested *Ascophyllum nodosum* byproduct on pre and post emergence damping off of cucumber seedling. Similar to seedling health experiment as described previously, 0.5%, 1% and 3% concentrations of all the digested and undigested products were amended in the Pro-mix and were inoculated by *Pythium* sporangial suspension both pre and post emergence of cucumber seedlings depending on the type of experiment. The number of seedlings

showing damping off symptoms was recorded every third day from the day of inoculation. Shoot fresh and dry weights were also measured. The experiment was conducted for two weeks. Both the experiments were repeated twice in the growth room on shelves in a randomized complete block design. The pre-emergence damping off experiment results clearly suggests that there was no significant difference between the treatments in showing pre emergence damping off symptoms (Figure 5.12). Also the control was not significantly different than any of the treatments. However, *Penicillium* digested higher concentrations of ABP showed an increase in damping off symptoms although not significant. This could be due to the fact that all the fungus likes to grow on a slightly acidic medium, whereas the ABP medium was alkaline. It is thought that the *Penicillium* was not able to digest ABP equal to that of bacterial digestion. From the *in vitro* experiments, it was clear that *Pythium* likes to grow in undigested ABP supplemented media. So, this may be the reason that there was higher number of damping off symptoms caused in the *Penicillium* digested ABP supplementation, although not significantly different than control. The undigested ABP at any concentration were not significantly different from control but tended to decrease pre emergence damping off symptoms (Figure 5.12). Likewise, the undigested and digested ABP showed no significant difference among each other and also as compared to control in their shoot fresh and dry weight.

The post emergence damping off experiment results clearly suggests that 1% undigested and 1 % *Pseudomonas putida* digested ABP significantly decreased post emergence damping off symptoms as compared to control (Figure 5.15). However, 0.5% and 1% *Penicillium* digested ABP tended to show higher post emergence damping off

symptoms as compared to other treatments and control, although not significantly different than control (Figure 5.15). Likewise, the undigested ABP tended to show higher shoot fresh and dry weight as compared to other treatments, although not significantly different to control (Figure 5.16, 5.17). However the other digested ABP showed no significant difference in the shoot weights as compared to control.

So, overall, it can be said that *Penicillium* digested *Ascophyllum nodosum* byproducts supplementation tended to show higher damping off symptoms, although no significant difference was found as compared to control. The undigested *Ascophyllum nodosum* byproducts tended to show lower damping off symptoms, 1% concentration showed significant reduction in the post emergence damping off symptoms as compared to control. It can also be noted that the lower concentration of undigested *Ascophyllum nodosum* byproducts especially 1% showed no significant difference in the seedling health as compared to control (example Figure 5.10). Hence, the bacterial digested *Ascophyllum nodosum* byproduct can be used as soil amendment for growth of seedlings and slight prevention from damping off disease in nurseries beds.

Higher considerable damping off in the seedlings which were grown in the digested *Ascophyllum nodosum* product may also be correlated with the antimicrobial assay results, which the product allows pathogen to grow over it. Digestion might have provided simpler forms of nutrients to the pathogen also. This might have also provided a three way interaction between seedlings, pathogens and the nutrient release from products which needs to be further researched in detail.

The third objective of this research was to study the changes in defence response compounds (Total Phenolic content and Flavonoid content) and enzymes (Phenylalanine

ammonia lyase and Polyphenol oxidase) in cucumber in response to growing media amendment with *Ascophyllum nodosum* byproduct. Similar to seedling health experiment as described previously, 1% and 3% concentrations of *Pseudomonas putida* and *Penicillium* digested and undigested products were amended in the Pro-mix and were inoculated by *Pythium* sporangial suspension after emergence of cucumber seedlings. Plant tissue samples were collected at three different time points (0, 24 and 72 hours after inoculation). The collected tissue samples were flash frozen in liquid nitrogen and stored at -80°C for biochemical and enzyme analysis. 5 replications were maintained per treatment per time point. The experiments were repeated twice in the growth room on shelves in a randomized complete block design. Results clearly indicate that total phenolic content, flavanoid content, Phenylalanine ammonia lyase activity, and Polyphenol oxidase activity increases in seedlings with the increase in the age of seedlings, however, no significant difference was found between the treatments (Figure 5.18, 5.19, 5.21). At each time point, the responses of treatments were similar to control. However, 1% and 3% undigested product tended to show higher Polyphenol oxidase activity at 24 h and 72 h as compared to other treatments, although not significant (Figure 5.21). Since, with the increase in the seedling age, the total Phenolic content, flavanoid content and both the enzyme activity increases, so it can be said that if these biochemical and enzyme analysis were conducted on the older tissue samples then, a clear difference might have been observed between the treatments.

Future research needs to be conducted on biochemical changes of ABP after digestion with *Bacillus subtilis* and *Pseudomonas putida*. These bacteria are known to be plant pathogen antagonist, and their spray has shown to significantly reduce disease

severity (Punja and Yip 2003). But no significant antagonism was observed, when these bacterial digested ABP was used as growth media amendment to prevent Damping off disease caused by *Pythium ultimum*. Effects of ABP on *Bacillus subtilis* and *Pseudomonas putida* during digestion process should also be tested. There may be chances that the substances secreted by these beneficial microbes are neutralized by the compounds released by ABP during digestion. It can be recommended that cucumber seeds should be soaked in bacterial culture before seeding, so as to let bacteria grow along the roots while seed germination (Elad and Chet 1987). This may show significant prevention from damping off, if the bacteria are antagonistic to *Pythium ultimum*. *In vitro* test can also be conducted to confirm antagonism of bacteria against *Pythium ultimum*.

Finally, it can be concluded that higher concentration of *Ascophyllum nodosum* byproduct (ABP) significantly increased *Pythium ultimum* growth both vegetatively and reproductively indicating presence of many nutrients in ABP. *Ascophyllum nodosum* byproduct even at higher concentrations can be utilized as a soil amendment or as mulch after digesting it with beneficial microbes (*Bacillus subtilis*, *Pseudomonas putida* and *Penicillium*) instead of considering it as waste. 3% digested ABP showed significant improvement in most of the biometric measurements of 3 weeks old cucumber seedlings as compared to 3% undigested ABP, although not significantly different than control. The digested ABP supplementation showed similar biometric measurements of cucumber seedling health as that of control. Digested and undigested ABP did not show any significant reduction in damping off caused by *Pythium ultimum*, and hence there was no significant increase in biochemical compounds and defence enzymes activity in

cucumber leaf tissues as compared to control. The presence of complex polysaccharides in ABP can be exploited to use it as an elicitor of other bacterial disease in plants.

So, it can be finally concluded that ABP can be successfully used as a growing media for sub culturing, maintaining and increasing sporangia in microbial cultures such as *Pythium ultimum*. To obtain and harvest more sporangia for other laboratory research purposes from *Pythium*, ABP can be successfully used in growing media of *Pythium ultimum*. It can be successfully used to maintain and subculture many other beneficial microorganisms such as *Bacillus subtilis*, *Pseudomonas putida*, *Penicillium*.

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