BIO-STIMULATORY PROPERTIES OF A *Lupinus albus* L. SEED SUSPENSION

BY

ELMARIE VAN DER WATT

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Bloemfontein
South Africa

Promoter: Prof. J.C. Pretorius (Ph.D.)
Co-Promoter: Prof. A.J. van der Westhuizen (Ph.D.)

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CHAPTER 1

INTRODUCTION AND RATIONALE

During the World Summit of 1998, attention was drawn to the fact that about 480 million people on the planet were suffering from hunger at the time and more than 215 million children were experiencing growth problems because of malnutrition (Kohout, 1998). To add to this dilemma, it is estimated that the world population will increase by 70 to 80 million people per annum between now and 2020, that will lead to an increase in the current population of 6 billion by a third to reach almost 8 billion (Heidhues, 2001). Growth rates will be highest in Africa despite HIV/AIDS with its devastating effect on African economies and societies.

The coming decades will pose daunting challenges for policy makers and the international agricultural science community mandated to solve the complex problem of providing adequate food for an ever increasing world population (Heidhues, 2001). To produce and provide the food needed for the additional 2 billion people is possible, but difficult. There is general consensus that it cannot merely come from expanding the area under cultivation or under irrigation, as most of the arable land is already utilized, but rather from productivity increases (Penning de Vries, 2001) on available land. Without any doubt, the latter is the more difficult way of increasing food production. It will require increased agricultural research to generate a steady flow of technological innovations and adapting it to local ecological conditions.

One innovation that has recently come to the fore, due to the emphasis on organic farming systems, is the potential to apply natural plant extracts as either plant growth regulators or natural herbicides. A plant growth regulator is an organic compound, either natural or synthetic, that modifies or controls one or more specific physiological processes within a plant (Lemaux, 1999). If the compound is produced within the plant it is called a plant hormone e.g. auxins, gibberellins, cytokinins, abscisic acid and ethylene. A plant growth regulator is also defined by the EPA as any substance or mixture of substances that accelerates or retards the rate of growth or maturation, or otherwise alters the behaviour of plants or their produce through physiological action (Lemaux, 1999).

Many natural compounds contained in plant extracts, and which have an affect on the growth and development of plants, have been identified. These include compounds such as amino acids, caffeine, fatty acids, flavonoids, lactones, quinines, steroids and various sulphur containing compounds (Roberts and Hooley, 1988). For example extracts from *Cyperus esculentus* tubers and the foliage of immature *C. esculentus* plants inhibited the germination of lettuce (*Lactuca sativa*) seeds significantly (Reinhardt and Bezuidenhout, 2001). Aqueous extracts of *Dendrocalmus stictus* had a stimulatory effect on chlorophyll content, seed protein, nodulation and peroxidase activity in soybeans. Shoot and radicle growth of soybeans were increased by these aqueous extracts (Sadhna *et al*., 1998). Products developed from the alkaline extraction of coniferous tree periderm increased maize yield, in terms of cob production, by 25 % (Dumitrescu *et al*., 1998). A sea weed extract with growth promoting and yield increasing properties has been commercialized under the trade name "Kelpek" and is currently sold in many countries (Ferreira and Lourens, 2002). The potential, therefore, exists to apply a plant extract as a foliar spray in order to stimulate growth in crop plants and hence increase yields. A principal objective of the agricultural and horticultural industries (Roberts and Hooley, 1988) is to manipulate plant growth and development in such a way that the quantity or quality of a crop is enhanced. An elevated interest has been shown recently to identify natural plant compounds with the ability to manipulate plant growth and development over a short period, e.g. a growing season.

An additional consideration is that extracts from plants, which have bio-stimulatory properties, could directly serve as donor plants and sources of active compounds in the production of natural plant growth regulators. Even though plants are a rich source of biologically active natural products, the plant kingdom is still an underutilized source of phytochemicals. Hostettman *et al.,*1995, speculated that less than 10% of higher plant species have been screened for their biological activities, and most of them for only one activity. However, the exploitation of fragile plant communities and ecosystems for traditional and pharmaceutical purposes has been occurring at an accelerating rate in recent times. The destruction of natural vegetation due to the collection of wood from trees and shrubs for fuel, overgrazing by livestock, mining, damming river systems and urban sprawl (Kashem and Miah, 1996; Kaufman *et al*, 1999) contribute to the devastation of natural vegetation. From a scientific perspective cognizance must be taken of these facts and measures taken to rather turn the situation around than to exaggerate the problem (Kashem and

Islam, 1999). In this light, the development of alternative crops to serve as donor plants for whatever purposes should be high on the research agenda. The sustainable use of the available plants in the environment is necessary as well as the sustainable management of agricultural practices.

According to Gips (1986) agriculture may be judged as sustainable if it is (a) ecologically sound, (b) economically viable, (c) socially just, (d) humane, and (e) adaptable. Nowadays the sustainable issues, especially their implications in agriculture, have become the focus point of discussion due to the growing concerns over deforestation, pollution, desertification, over extraction of surface and ground water and inappropriate use of chemicals. Hence, the use of indigenous agricultural technologies is, of course, very important at least to save the environment from further deterioration and thereby to maintain the sustainability of existing agriculture.

From a technological development perspective, limitations of synthesized compounds necessitates the search for new effective alternatives, and this has led to renewed interest in natural product research. As plant concoctions are traditionally utilized as pharmaceuticals or even as pesticides in agriculture by the local community in South Africa, some plant species are disappearing due to overexploitation (van Wyk *et al*., 1997). This is probably a result of the fact that no measures have been taken in the past to avoid this tendency. To protect the environment against overexploitation the cultivation of natural indigenous plants, especially donor plants, will have to be considered in the future. This might further lead to the development of new crops, or the discovery of alternative uses for existing crops, with the added advantage of improving the local agricultural economy. In this study all of these aspects were considered and served as a rationale for the attempt to contribute in this regard.

As a result of a recent preliminary screening program in the department of Agronomy at the University of the Free State, South Africa, a seed suspension of *Lupinus albus* showed above average bio-stimulatory activity under laboratory conditions compared to other test plants. This prompted the investigation of the potential of a *L. albus* seed suspension, as well as extracts thereof, to be applied as a natural bio-stimulant in agriculture. The objectives of this study were:

1) to confirm the bio-stimulatory activity of a *L. albus* seed suspension on the respiration rate of monoculture yeast cells as well as seed germination and seedling growth of a number of vegetable crops under laboratory conditions (Chapter 3),
2) to determine the bio-stimulatory effect of a *L. albus* seed suspension on the yield of a number of agricultural and horticultural crops under field conditions in order to evaluate its application potential as a natural product in the agricultural and horticultural industries (Chapter 4),
3) to isolate and purify the active compounds(s) involved (Chapter 5),
4) to identify the active compound(s) as well as to elucidate its chemical structure(s) by means of Nuclear Magnetic Resonance (NMR) spectroscopy (Chapter 6) and
5) to postulate a possible mechanism of action of the active compound(s) isolated from the *L. albus* seed suspension (Chapter 7).

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Despite the impressive advances that have been made over the years in improving the yields of food crops, there is little reason to become complacent about the food supply, especially in the developing world. Between 70 and 80 million people will be added to the world's population every year between now and 2020, increasing the world's current population of 6 billion by a third to reach almost 8 billion (Heidhues, 2001). To produce and provide the food needed for the additional 2 billion people is possible but probably not without a special effort. To meet these demands, especially in light of the fact that the area under cultivation is expected to remain minimal or even decrease, increases in crop yields will have to be maintained at 2.5% per year over the next 30 years (Heidhues, 2001). In contrast, there is an overproduction of certain food commodities in many developed countries. This seems to cause a conflict of interest regarding the application of plant growth stimulants leading to increased yields. However, for the sake of the underlying investigation, the worst case scenario of possible future food shortage will be accepted as motivation.

The challenge is to increase both farm productivity and sustainability. However, the related requirements are sometimes conflicting at the physiological, agronomic and economic levels indicating that an exclusive focus on one aspect will not necessarily lead to the optimum solution (Penning de Vries, 2001). The question that needs to be answered is how this will be achieved? The general consensus is that it cannot merely come from expanding the cultivated area by simply removing forest to make more agricultural land available because of obvious secondary problems that might arise. Of these the effect on the ozone layer and global warming is probably the most important (Heidhues, 2001). However, other aspects such as the discovery of new soils and the need for comprehensive research to develop proper cultivation practices need to be considered. Theoretically, it is also possible to achieve higher yields or increase food production by increasing the land under irrigation. However, most of the world's irrigatable land is probably already in use and chances to expand are slim (Penning de Vries and van Keulen, 1995). It therefore seems that

future food production growth will have to come from productivity increases on already available land (Heidhues, 2001).

The current prognosis is that the production of food on available arable land will simply have to be increased by applying techniques that will not further deplete the natural resource. Based on a review of more than eighty case studies, including data from the 1980's, it seems that at least 16% of all agricultural land in developing countries is seriously degraded, implying that crops cannot be grown profitably in these areas (Scheer, 1999). Sustainable use of cultivated land can help to maintain nutrients in the soil and it is also the most effective way of restoring soil fertility and biodiversity. Examples include the planting of specific tree species in forests or degraded land that can effectively avoid nutrient loss in the soil and provide sustainable cropping systems (Garay, *et al*., 2004). The search for different plant species that could be used as alternatives in restoration of degraded soil still continues, making it no small challenge for agricultural research. But there are reasons to be optimistic by implementing other techniques to increase crop yields and quality.

Furthermore, genetic engineering and the development of transgenic crops that are more resistant to abiotic and biotic stress factors can be regarded as the technological breakthrough of the century. However, due to persistent resistance of consumers to genetically manipulated and inorganically cultivated crops, emphasis is currently placed on organic farming. Almost a decade ago Tyler and Miller (1996) stated that transgenic crops have not been around long enough to evaluate its short and long-term benefits and risks. One of the main arguments was that, without strict control, these crops can do great harm leading to the development of super organisms with unregulated alterations. They often refer to 'monster' crops that, on consumption, have the potential to alter the genetic characteristics of the animal or human consumer leading to unwanted mutations. Despite the fact that there is no scientific base for this argument, it seems that little progress has been made in educating the masses in this regard as some still see genetic engineering of crops as a threat.

However, consumer resistance towards transgenic crops is not more intense than the resistance towards the use of synthetic pesticides and even inorganic chemicals, including fertilizers. Slogans against transgenic crops and inorganic cultivation practices are often used by large outlets to promote the marketing of organically cultivated vegetable and fruit products placing more pressure

on traditional farming systems. According to Kohout (1998), natural bio-stimulants extracted from plants and applied in crop production systems either as foliar sprays or as a soil drench shows potential as an additional cultivation practice while complying with the standards set for organic farming. These include the use of non-toxic plant growth regulators that not only have the ability to increase yield and quality of crops, but also the potential to decrease the use of large amounts of inorganic chemicals. Although this alternative approach may be seen by some as a marketing gimmick, its scientific base as well as its economic benefits have been reported in the literature.

In the search for natural compounds with application potential in agriculture, it might be rewarding to be aware of the ways that plants interact within a given ecosystem so as to mimic certain natural processes controlled by natural compounds. According to Macías *et al*. (2001), certain natural compounds found in plants may have different target sites than traditional pesticides, herbicides and growth regulators. Understanding ecological mechanisms and environmental signaling (Langebartels and Kangasjävi, 2004) may lead to the discovery of natural plant compounds that show potential to be applied in agriculture towards increasing or inducing the resistance of crop plants to abiotic and biotic stress factors in monoculture crop production systems. As an example, jasmonate is known as the lead molecule of the jasmonate family of plant growth regulators involved in the defense of plants against insect parasites, bacterial and fungal pathogens as well as in wounding and desiccation responses (Raymond and Farmer, 1998; Howe and Schilmiller, 2002; Liechti and Farmer, 2002). Exogenous supply of jasmonate induces jasmonate-inducible proteins (JIP's) and various defense-related metabolites (Langebartels and Kangasjävi, 2004) confirming the application potential of plant compounds in manipulating plant metabolism.

However, although natural products are generally considered ideal from an environmental perspective due to their bio-degradability, they may have limitations (Dayan *et al.,* 1999). These may include a) natural compounds with complex chemical structures that are prone to loosing activity once isolated from donor plants, b) the lack of persistence due to an inadequate shelf life, c) a slow and uneconomic purification process, d) unknown mechanisms of action and e) toxicity to fish, birds or mammals. Although these limitations must be considered when attempting to develop natural products from plants for the agricultural industry, pollution of the environment by and hazardous effects of synthetic chemicals on non-target plants will remain in the minds of

consumers. This still supplies a rationale for scientists to seek alternative manipulation techniques including the search for effective, non-toxic and environmentally friendly natural plant products or their analogues (Cutler and Cutler, 1999).

Plants produce an array of secondary metabolites which, apart from physiological functions in the plant, most likely form part of the communication mechanism in the soil-plant-air continuum (Reigosa *et al*., 1999). Chemical interactions in the continuum are diverse and complex and probably provide the donor plant with a number of selective advantages. For example, these compounds seem to be connected with the ability of plants to survive in harsh environmental conditions while others die or become extinct. This has lead to the hypothesis that a plant's resistance to the environment is indirectly connected to the production of specific secondary metabolites (Nigg and Seigler, 1992). Interactions between plants and the environment are in many cases stressful to the plant leading to the production of a variety of secondary metabolites in an attempt to resist the stress condition, be it abiotic or biotic (Seigler, 1995). The potential to develop natural products from these wild type plants, of which the toxicology is known, exists. In this regard it is necessary to consider the fact that many wild type plant varieties may be lost as a result of overexploitation in the event that large scale utilization of the donor plants might be necessary during the production process. The need to preserve these plants may lead to the development of donor plants into alternative crops with an additional economic implication as a bonus to the agricultural industry.

In the following section the main aspects mentioned in the introduction will be elaborated in order to obtain a perspective on the place and need for the application of natural bio-stimulants in the agricultural industry.

2.2 Improvement of crops through genetic manipulation

2.2.1 The rationale for genetic manipulation

Generally, the issue of improving crop yields in Africa is one of the main challenges for the next century. Africa, more than any other continent, is urgently in need of biotechnology transfer, including the use of transgenic crops despite consumer resistance, in order to improve food security

for an ever growing population. Statistics show that crop production per unit land area in Africa is the lowest in the world today and will have to be doubled over the next 40 years to meet the needs (Amalu, 2004). The latter supplies the rationale for the application of existing as well as the development of new biotechnology techniques that may include not only genetic but also chemical manipulation techniques.

The DNA-recombinant technique has made it possible to transfer genetic traits from one species to another and achieve new genetic combinations in a much shorter time than via selection methods. This rapidly developing technology excites many scientists and investors who see it as a way to increase crop and livestock yields as well as to produce, patent and sell crop varieties with elevated nutritional value or with increased resistance to either abiotic or biotic stress factors or both compared to existing varieties (Pimentel, 1989; Amalu, 2004). With regard to the latter, successes include the development of crop cultivars expressing single insecticidal proteins, such as *Bt*-cotton and maize, leading to a reduction in the use of pesticides and, therefore, input costs that may be an important aspect for small-scale farmers common in Africa (Raghava and Haribabu, 2002).

2.2.2 Arguments for and against genetic manipulation

Critics are concerned that one of the most serious effects of the widespread application of genetic engineering biotechnology is a reduction in global biodiversity (Amalu, 2004). It is estimated that the 20 major food crops in the world have already become 70% less genetically diverse because a wide range of wild strains have been replaced by only a few varieties through cross-breeding with genetically manipulated (GM) varieties (Amalu, 2004). It may sound far fetched but, extended development of GM cultivars may not only potentially enhance the loss of biodiversity but also undermine the ability to produce new genetic combinations in future.

Two additional concerns are a) the health risk of consuming GM foods and b) the ecological risks of growing genetically modified plants (Amalu, 2004). With regard to a), it is believed that GM foods are not intrinsically good or bad for human health but, the contents of GM foods and the production process may need to be known for cultural or religious reasons or simply because consumers want to know. Concerning b), ecological issues including the spread of traits such as herbicide resistance

from GM plants to plants that are not modified, as well as the build-up of resistance in insect populations, have not yet been resolved (Amalu, 2004).

On the other hand and on a more positive note, future advances in genome elucidation of crops additional to that of *Arabidopsis*, promise the discovery of new genes and desirable characteristics that may fundamentally alter a crop's metabolic functions leading to either nutritional enhancement, resistance to stress factors and crop yield increases or all three (König *et al*., 2004). Examples include improving the effectiveness of the pollination process, especially in insect-pollinated crops, by the genetic engineering of floral scent leading to the broadening of pollinator attractiveness in crops that rely on a limited range of insect species for their pollination and therefore cannot be cultivated outside of their natural habitats (Dudareva and Negre, 2005). Further, genetic modification of the flavours and aromas of fruits, vegetables and herbs might have a significant impact on food quality. It is also possible that some allergenic volatile compounds could be eliminated from essential oils and food sources in this manner (Chaintreau *et al*., 2003). The latter is referred to as 'metabolic engineering' with the underlying aim to increase the production of endogenous metabolites or to achieve the synthesis of metabolites not normally produced by specific plants. Metabolic engineering has indeed produced remarkable and encouraging results e.g. increases in the levels of minor components such as vitamin A, vitamin E and essential oils as well as an alteration in the composition of major components such as fatty acids and starch (Shintani and Della Penna, 1998; Ye, 2000).

Realistically, however, metabolic engineering is rather expensive, is complex in several ways and needs a better understanding of metabolic regulation within the cell. Thousand or more metabolites are distributed among several compartments and, even though the metabolic routes are known, the concentrations of intermediates and the forces driving metabolic conversions (Chaintreau *et al*., 2003) as well as the modulation of metabolite concentration as a result of environmental influences, are difficult to predict (Morandini and Salamini, 2003). Nevertheless, the global area of commercial cultivation of genetically modified crops has risen to 58.7 million hectares in 2002. It is argued that, together with traditional plant breeding techniques, gene manipulation technology is here to stay (Konig *et al*., 2004).

In summary, although there are a lot of positive sides to genetic manipulation, it is very expensive (Konig *et al*., 2004). The main reason is that GM crops need to be monitored thoroughly to assess the safety of the finally harvested products and this is not only time consuming and labour intensive but compositional analysis of plant tissues is very expensive. The latter implies that product samples of GM crops have to be compared to that of parent crops in order to verify possible chemical differences and especially potential toxic residues (Konig *et al*., 2004).

A possible alternative to genetic manipulation in achieving the same goals, e.g. yield increases and the enhancement of resistance towards abiotic and biotic stress factors in agricultural crops is bio-manipulation using natural compounds extracted from plants. These may include specific secondary metabolites. A brief overview of secondary metabolites, their roles in plants and their application potential in agriculture is given in the following section.

2.3 Secondary metabolites and their role in plants

2.3.1 General overview

The compounds in living organisms may be divided into two major groups namely primary and secondary metabolites. Primary metabolites are those produced by and involved in primary metabolic processes such as respiration and photosynthesis whereas others, including many pathways clearly derived from primary metabolic pathways, are considered secondary (Seigler, 1995). Primary metabolites, which are virtually identical in most organisms, include ubiquitous small molecules such as sugars, amino acids, tri-carboxylic and fatty acids that act as metabolic intermediates. These universal building blocks and energy sources may differ in structural detail from one organism to another, but appear to have universal functions as enzymes, structural or hereditary materials. Nonetheless, differences in the pathways leading to these primary metabolites and the actual compounds involved in some fundamental processes differ in some instances (Haslam, 1986). In this regard compounds normally considered primary metabolites may accumulate in large amounts and behave in a manner usually associated with secondary metabolites.

Secondary metabolites, produced by pathways other than primary metabolic routes, are numerous and widespread, especially in higher plants. More than 20 000 were known in 1985 and at least 1000 additional compounds are described each year (Seigler, 1995). However, in practice the difference between primary and secondary metabolites is fuzzy. For instance plant hormones, initially considered secondary metabolites, were subsequently shown to be important intermediates in the formation of primary metabolites. These intermediates are necessary for the formation and production of secondary metabolites, by forming a bridge between these different metabolisms.

Numerous factors have been mentioned that play an important role in the production of secondary compounds found in plants while the amount of any given secondary compound at any given time is the result of an equilibrium that exists among synthesis, storage and degradation thereof. The onset of secondary metabolism is linked to the developmental stage of the organism and is often closely linked to morphological and cytological changes (Haslam, 1986). Thus, the ultimate origin of secondary compounds in all organisms is based on accumulation of changes (or mutations) of genetic materials originally associated with primary metabolic pathways. This accumulation of secondary metabolites observed in nature and the notion that these compounds arose from "errors" in primary metabolism, coupled with the apparent absence of excretory mechanisms in plants, led to the idea that secondary compounds arose and accumulated as "waste products" (Luckner, 1990). This was supported by the fact that secondary compounds are often leached from either aerial plant parts or exuded from the roots (Hrazdina and Jensen, 1992). The discovery of numerous functions of secondary metabolites over the past two decades in recent years suggested that the "waste product hypothesis" was not likely to hold.

These functions include regulation of plant growth and development, the repelling of harmful insects, pests and pathogens as well as the enhancement of the ‘reproductive fitness’ of the plant (Taiz and Zeiger, 1998). Specific secondary metabolites even serve as energy reserves, precursors of important organic compounds or sources of nitrogen and may be recycled within the plant (Rosenthal, 1982). Numerous secondary metabolites e.g. alkaloids accumulated in seeds have multiple functions, including a defensive role, but are mobilized and recycled during germination (Wink, 1987). Further, as plants exist in contact with a changing array of animals, plants, fungi and bacteria, secondary compounds (allelochemics) are involved in interactions with and between these

organisms (Nordland *et al*., 1981). Many appear to bestow protection to the plants that produce them (allomones). Others are involved in processes such as pollination and fruit/seed dispersal (synomones) and yet others are used by the interacting organism to locate the plant host (kairomones; Seigler, 1995).

Plants being unable to move to another place to escape from stress can, however, react to external influences by synthesizing specific chemical substances. They are able to adjust their metabolic system to adapt to changing environmental conditions and can spontaneously produce antibodies or activate defense mechanisms under control of specific enzymes (glucanase, chitinase, peroxidase) known as pathogenesis related-(PR)-proteins. This mechanism is referred to as "induced resistance" that has kept science and research busy for a long time. There are still some unknown aspects in this field of study including fine detail about the mechanisms of action as well as the defense compounds involved. It has, however, become clear that plant secondary metabolites are not just randomly produced compounds, but ones that have been shaped and optimized through natural selection during evolution (Wink, 2003).

Finally, the data provided by the study of plant secondary metabolites have proven important in linking various aspects of ecology, organic chemistry, biochemistry, both animal and plant systematics, mycology, phycology, plant physiology and evolutionary studies (Seigler, 1995). There are important implications of these data for food science, range science, nutrition, agronomy, horticulture and plant pathology.

2.3.2 The application potential of plant secondary compounds in the agricultural industry

It is not surprising that many secondary compounds are used by man as pharmaceuticals, spices, fragrances, pesticides, poisons, hallucinogens, stimulants or colouring agents (Luckner, 1990). Many of the compounds are important as medicines, pharmaceutical and industrial precursors, fuels, pesticides, flavourings, perfumery ingredients, plant hormones, adhesives and drugs of abuse. Despite these uses, natural compounds from plants are least applied in the agricultural and horticultural industries and this aspect partially prompted the underlying study.

Natural secondary compounds from plants that stimulate growth in crops have been reported in the past. Field tests over a number of years, demonstrated that wheat grain yields were increased appreciably when grown in mixed stands with corn cockle as compared to pure stands of wheat. One of the stimulatory substances isolated from the corn cockle was named agrostemmin which, when applied at the rate of 1.2 kg ha^{-1}, increased grain yields of wheat on both fertilized and unfertilized areas. Chopped alfalfa had a stimulatory effect on the growth of tomato, cucumber and lettuce. The active substance was identified as triacontanol (Putnam and Tang, 1986). p-Hydroxycinnamic acid was identified as a major compound in mungbean (*Vigna radiata* L.) plants and their rhizosphere soil. Crude mungbean saponins increased germination and enhanced growth of planted mungbeans, but the soil treatment did not increase yields (Chou *et al*., 1995).

From an ecological perspective and under natural conditions this phenomenon is known as allelopathy. Allelopathy (Delabays and Mermillod, 2002) is a chemical process whereby certain plants release natural compounds into the environment that can either stimulate or inhibit the growth and development of surrounding plants. In nature the latter is more likely, as a form of competition for growing space, where allelochemicals are released from the roots of one plant and absorbed by the roots of surrounding plants preventing it from growing in the same area. According to Delabays and Mermillod (2002) there are several ways in which an allelopathic plant can release its protective chemicals. These include (a) volatilization where a chemical is released in the form of a gas through the leaves and, on absorption, sensitive surrounding plants are stunted or die, (b) leaching of allelochemicals from decomposing abscised leaves preventing surrounding plants to establish in the same area and (c) exudation of allelochemicals into the soil through the roots preventing surrounding plants to prosper.

A study directed towards identifying bio-stimulatory properties in plant extracts was performed by Cruz *et al.* (2002a) by treating the roots of bean, maize and tomato with an aqueous leachate of *Callicarpa acuminata* and following the *in vitro* effects on radicle growth, protein expression, catalase activity, free radical production and membrane lipid peroxidation in the roots. The aqueous extract of *C. acuminata* inhibited the radicle growth of tomato but had no effect on root growth of maize or beans. However the expression of various proteins in the roots of all treated plants was observed. In treated bean roots the expression of an 11.3 kDa protein, showing a 99% similarity

with subunits of an alpha-amylase inhibitor found in other beans, was induced. In treated tomato an induced 27.5 kDa protein showed 95% similarity to glutathione-S-transferases of other Solanaceae. Spectrophotometric analysis and native gels revealed that catalase activity was increased two-fold in tomato roots and slightly in bean roots while no significant changes were observed in treated maize roots. Luminol chemiluminescence levels, a measure of free radicals, increased four-fold in treated tomato roots and two-fold in treated bean roots. Oxidative membrane damage in treated roots, measured by lipid peroxidation rates revealed almost a three-fold increase in peroxidation in tomato while no effect was observed in maize or beans (Cruz *et al.,* 2002a).

The significance of this study lies in the fact that various metabolic events can be manipulated in plants by treatment with certain plant extracts. What has to be established by researchers is whether these altered metabolic events contribute towards positive or negative physiological changes within the treated plants. The rationale for this type of research lies in the search for natural allelochemicals to be applied in sustainable crop production systems (Singh *et al.,* 2001). According to the authors, allelochemicals isolated from some plants show strong bio-herbicidal activity at high concentrations but at low concentrations these extracts can promote crop seed germination and seedling growth, hence showing a potential to be applied as bio-stimulatory agents or growth promoting substances in agriculture. It is therefore imperative that research in this regard should concentrate on both the inhibitory and stimulatory effect of plant extracts on seed germination, seedling growth and the physiology of other test plants in order to verify the action at hand (Khan *et al.*, 2001; Ameena and George, 2002; Chung *et al.*, 2002; Cruz *et al.*, 2002b; Duary, 2002; Obaid *et al.*, 2002).

Extensive research has been done in the past to study the inhibitory bio-activity of extracts from many plant species on weeds (Wu *et al.*, 2002). For example, extracts of *Wedelia chinensis* reduced seed germination, inhibited seedling growth, resulted in yellowing leaves and reduced resistance to disease in weeds such as *Cyperus difformis, Paspalum thunbergii, Alternanthera sessilis* and *Cynodon dactylon* at relative low concentration of 0.4 g fresh weight/ml water. This study confirmed the potential of *W. chinensis* extracts sprayed before crop emergence to control the germination of weed seeds (Nie *et al.,* 2002). Another study showed that a sorghum extract reduced seed germination and seedling growth of the weed *Trianthema portulacastrum* substantially at high

concentrations (75-100%) but, promoted shoot length of the weed at low concentrations (25%) (Randhawa *et al.,* 2002).

Similar contrasting results were reported for the effects of extracts from eight lucerne cultivars on seed germination as well as on root and hypocotyl development of lettuce seedlings (Tran and Tsuzuki, 2002). Extracts from some lucerne cultivars had a stimulatory effect in terms of seed germination as well as root and hypocotyl growth, whereas others showed the direct opposite effect, confirming that crop plants can also be affected by plant extracts aimed at controlling weed growth. Further, the sagebush, *Artemisia tridentate* ssp. *tridentate*, have well-documented allelopathic tendencies that have been ascribed to its most abundantly released secondary metabolites. It has been shown that the plant releases a highly biologically active substance, methyl jasmonate (MeJA), which is known to function as both a germination inhibitor and stimulator in laboratory studies (Preston *et al*., 2002).

Singh, *et al*. (2003) confirmed this phenomenon by showing that aqueous leaf leachates of *Eucalyptus citriodora* inhibited the germination and seedling growth of all test crops (*Vigna radiata, V. mungo* and *Arachis hypogaea*) investigated. Further, in their study of bio-activity of plant extracts, Deena *et al.* (2003) demonstrated the inhibitory effect of leaf, stem and root leachates from *Andrographis paniculata* on germination and seedling growth in rice. From this it became clear that the bio-activities of plant extracts are unpredictable and may give different and often contrasting results with regard to inhibition or promotion of growth and development in other plants. The reaction of crops to treatment with plant extracts may depend on the interaction between different types of plant species or even on the concentration of the extracts (Channal *et al.,* 2002a).

From an agricultural perspective, plant extracts containing growth promoting substances have always been of interest to the research community in terms of the role they could play in addressing future food security issues. The ideal break-through would be to identify a plant or plants that contain bio-stimulatory substances promoting growth, resistance to pathogens as well as yields in

agricultural and horticultural crops. Numerous plant species more or less adhering to this description of an "ideal candidate," were identified through research. Channal *et al.* (2002b) reported on the seed germination as well as seedling growth enhancement of sunflower and soybean by leaf extracts from three out of the seven tree species (*Tectona grandis, Tamarindus indica, and Samanea saman*) investigated. Similar effects were reported by Terefa (2002) for *Parthenium hysterophorus* extracts on tef (*Eragrostis tef*) and by Neelam *et al.* (2002) for *Leucaena leucocephala* extracts on wheat (*Triticum aestivum*). However, none of these studies revealed that treatment with the different plant extracts had any effect on the final yields of the crops under investigation.

In this regard, a report by Ferreira and Lourens (2002) demonstrating the effect of a liquid seaweed extract (now trading as a natural product under the name Kelpak) on improving the yield of canola, must be regarded as significant. Kelpak, applied singly or in combination with the herbicide clopyralid at various growth stages of canola (*Brassica napus*), cultivars Monty and Dunkeld, were assessed in a field experiment conducted in Langgewens and Tygerhoek, South Africa during 1998-99. Foliar application of 2 litres Kelpak/ha, applied at the four-leaf stage, significantly increased the yield of the crop in Langgewens. In Tygerhoek, application at the same rate significantly increased canola yields when applied at either the three- or five-leaf growth stage, but the highest yield was obtained by treatment with Kelpak at the three-leaf growth stage.

In this study *ComCat*®, a new natural bio-stimulant, was used as a positive control in all laboratory bio-tests and field trials. *ComCat*® is a unique family of natural products that are based upon a combination of bio-stimulants derived from plant materials. This product has demonstrated consistent plant growth enhancement and physiological efficiency in the treated plant's utilization of available nutrients. The name *ComCat*® has been derived from two allelopathic terms, communication and catalyzation, and the active substances identified as brassinosteroids, a new generation of phyto-hormones that act in synergism with auxin and other natural compounds including free amino acids and flavonoids (Schnabl *et al*., 2001). During the manufacturing process, active substances are obtained from natural donor plants multiplied in soils that are not treated with

any inorganic fertilizers or agrochemical aids. Moreover, donor plants are multiplied in virgin soils that are not treated with any inorganic fertilizers or agrochemical aids.

ComCat® is described and registered as a plant-strengthening agent by the European Union. Claims made by the manufacturers include the enhancement of vegetative growth, especially root growth, of many agricultural crops such as wheat, maize and some vegetables as well as yield enhancement due to improved utilization of soil nutrients, improved flower bud formation and fruit development (Hüster, 1999; personal communication)[1]. If applied at an early growth stage, *ComCat*® can be beneficial to the farmer providing that sufficient water and nutrients were applied. Other advantages of *ComCat*® include its ecologically friendly nature, induction of resistance against pathogens (Schnabl *et al*., 2001) and improvement of product quality without leaving residues in the crop (Hüster, 1999; personal communication)[2]. The product is not a fertilizer substitute but, instead, is a biological enhancer which stimulates the plant to more properly utilize available nutrients, it activates and induces allelopathy and disease resistance in the treated plant and stimulates greater production of sugars, which are the building blocks for cellulose and fruiting bodies. The result is a more productive, healthier plant with stronger plant stalks, better flowering and greater fruit biomass (Agraforum: Germany, 2002).

Large scale application of natural bio-stimulants under field conditions is a fairly new enterprise and has not been applied in agriculture as frequently as in the horticultural industry. Probably the best known growth stimulators are the phytohormones. Already in the nineteenth century the existence of growth stimulating substances in plants were suspected by Darwin and other researchers before him. It was a Danish researcher, Boysen-Jenson that obtained the first proof in 1910. His research led to the discovery of a chemical growth stimulant that was named auxin by Fritz Went in 1926. Other growth stimulants namely cytokinins, gibberellins and ethylene were discovered later. Brassinosteroids (BRs), a new plant hormone family, was fairly recently discovered in a variety of plant species and organs (Roth *et al*., 2000; Schnabl *et al*., 2001). These growth promoting substances are all referred to as plant hormones since they comply with the definition of a hormone, namely to be produced in one part of the plant while executing their effects elsewhere.

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[1] Hüster, T. Agraforum AG, Poststrasse 1, Bomlitz 29699, Germany
[2] Hüster, T. Agraforum AG, Poststrasse 1, Bomlitz 29699, Germany

2.4 Natural plant hormones as growth promoting substances

2.4.1 Auxins

The auxin-type plant growth regulators comprise some of the oldest compounds used in agriculture. The auxin, indole-3-acetic acid (IAA), was not found in itself to be useful in agriculture because it is rapidly broken down to inactive products by light and micro-organisms (Gianfagna, 1995). Nevertheless, a number of synthetic compounds were found to act similarly to IAA in the auxin bioassay tests. These synthetic compounds were found to increase root development, stimulate excessive, uncontrolled growth in broadleaf plants and increase fruit set in tomato (Gianfagna, 1995). IAA (Mauseth, 1991) is primarily synthesized from the amino acid tryptophan and the main areas of biosynthesis are the leaf primordia and developing seeds. From here it is transported to the areas where growth is stimulated in the plant by means of a concentration gradient in the cells. According to the Cholodny-Went hypothesis, IAA is translocated away from light to the shadow side of the plant where it stimulates growth depending on its concentration (Salisbury and Ross, 1992).

Other functions of auxins include induction of length growth, stimulation of root formation and the accompanying increase in the uptake of water and minerals, apical dominance and the inhibition of leave abscission. The hormone also influences the growth of stems in the direction of sunlight, ensuring that the plant receives optimal quantities of light in instances where it is overshadowed by other plants. It is thus obvious that this plant hormone or signal is perceived by plant cells and rapidly transduced into a wide variety of responses in growth and development (Leyser, 2001).

2.4.2 Cytokinins

The first cytokinin was discovered in the 1950's and was isolated from coconut milk. All cytokinins are adenine derivates. Two natural cytokinins, zeatine and isopentenyl adenine, were discovered later. In plants cytokinins are mainly produced in the root ends. The metabolic storage and transport of cytokinins is not yet clear, but it is suspected that transport takes place via the xylem from the roots to the aerial parts of the plant (Mauseth, 1991).

The main function of cytokinins is the stimulation of cell division in stems and the inhibition of cell division in the roots as well as the induction of cell enlargement in leaves. High concentrations of cytokinins are found in the endosperm that is involved in controlling the development and morphogenesis of the embryo and seed (Salisbury and Ross, 1992). In the agricultural and horticultural industry it plays and important role in subsequent growth and branching thus increasing lateral branching on carnations, roses and fruit trees (Davies, 1995).

2.4.3 Gibberellins

In Japan, the fungus *Gibberella fujikuroi* causes a disease in rice called 'bakanae'. Infected plants show nominal stem elongation in comparison to uninfected rice plants. In 1926 Kurosawa (Takahashi *et* al., 1990) proved that the reaction was repeatable when plants were treated with a filtrate of *Gibberella fujikuroi*. Subsequently, Japanese researchers concentrated on the identification and characterization of the active component that induced the elongation and in 1934 Yabuta (Takahashi *et* al., 1990) succeeded in isolating the compound which was named gibberellic acid (GA_3). Similar physiological effects by GA_3 on other plants showed that the same chemical had to be present in higher plants. Since then different gibberillllins have been isolated, purified, characterized and their metabolic effects studied. Today at least 62 different gibberellins are known and numbered in the order of their discovery (GA_3-GA_{62}). Every plant species contains 6 to 10 different gibberellins, of which some are biologically active and others inactive (Mauseth, 1991).

Gibberellins vary in their specific activity in different species and concentrations are influenced by different environmental signals (Salisbury and Ross, 1992). The areas of biosynthesis in the plants are the young tissue of seeds and the stem apex while roots are only considered a possible area for biosynthesis. Gibberellins are transported by both the xylem and phloem of the plant. Specific roles of gibberellins include the induction of seed germination, the promotion of hypocotyls and stem elongation, and the regulation of pollen development and flower initiation (Peng and Harberd, 2002). Seed germination is promoted by gibberellins in many plant species. Several GA signaling factors are known to induce the expression of genes encoding enzymes that mobilize food reserves, including starches, proteins and lipids, stored in the endosperm during seed germination (Peng and Harberd, 2002). Depending on the concentration of GA applied, it also has the potential to control

growth and flowering and induce earliness and out of season cropping in strawberry (Paroussi *et al.*, 2002).

2.4.4 Ethylene

Cousins, a ships captain, noticed the effect of ethylene in 1910 when he concluded that the presence of over ripe apples in a crate of unripe bananas accelerated the ripening process (Salisbury and Ross, 1992). In 1934 Gane proved that ripening fruit released ethylene gas. It was however, only in the 1950's that ethylene was accepted as a natural growth regulator. Ethylene is the only plant hormone known to exist in gas form and is now known to induce the ripening process in cells. It is transported in the apoplast via intercellular spaces and released during the fruit ripening process. The gas is used in the industry for artificially ripening climacteric fruit such as bananas, apples, mangoes and avocado's (Bennett *et al.*, 2001).

Auxins stimulate ethylene production in a target area that diffuse quickly to adjacent areas and cause a response much faster than what auxin is capable of producing on its own (Mauseth, 1991). It therefore seems that ethylene can act as final effector of auxins. Functions of ethylene include apical dominance or inhibition of branch elongation, stimulation of stem thickness growth, stimulation of leaf and fruit abscission (Salisbury and Ross, 1992) as well as regulation of cell metabolism in the plant.

2.4.5 Brassinosteroids (BRs)

A wide range of effects in plants have been attributed to brassinosteroid (BR) activity. A few include induction of resistance to microbial infection (Schnabl *et al.*, 2001), cell division and cell elongation (Krizek and Mandava, 1983), hypocotyl growth, increase in leaf lamina growth, increase in shoot apex fresh weight (Meudt, 1987) as well as pollen tube growth and proton pump activation (Asami *et al.,*2004). Although the BR mechanism of action has not been elucidated fully to date, similarities with that of other plant hormones namely cytokinins (Clouse, 1996) and auxins (Cao

and Chen, 1995) have been proposed. Strong synergism between BRs and auxins has also been found in a number of studies (Arteca *et al.*, 1988; Eun *et* al., 1989; Goda *et al*., 2002; Halliday, 2004). Both these hormones increase coleoptile growth, fresh weight and ethylene production. Brassinolide also interacted synergistically with auxin in the promotion of lateral expansion of the pea stem section, not only by a reduction in the expected elongation but also by enhancement of the expected lateral expansion (Sasse, 1989).

Although there is evidence that brassinosteroids and auxin can operate independently, other studies have demonstrated a link between brassinosteroids and auxin, indicating that some pathways are under dual control (Zurek *et al*., 1994; Clouse *et al*., 1993; Mussig *et al*., 2002). Halliday (2004) has recently demonstrated that there are strong links between brassinosteroid and auxin in the control of tissue elongation and gene regulation. It appears that brassinosteroid and auxin 'crosstalk' is important in the control of elongation growth, but genetic and physiological analysis suggests that their mode of interaction may be distinct in different tissues and possibly species-specific (Halliday, 2004). However, BRs cannot be classified as auxins, cytokinins or gibberellins but have the ability to increase the auxin sensitivity in plant tissue and influence endogenous hormone levels. Clouse *et al*. (1996) proved that auxins (IAA) and BRs differ on gene expression level. These studies suggest that the interactions between brassinosteroids and auxin are extensive and complex

Brassinolide (BL), castasterone (CS), teesterone (TE) and 6-deoxycastasterone (6-deoxyCS) are of the best known BRs isolated from plants and all belong to the C_{28}-BRs with a 24α-methyl group. Recently it was discovered that these BRs are produced though campesterol biosynthesis (Fujioka and Sakurai, 1997; Sakurai and Fujioka, 1997). Of all the named BRs, BL is biologically the most active. As a result of this BL is seen as the most important brassinosteroid in terms of its role in plant growth regulation.

As different plant parts (pollen, seeds, leaves, roots and flowers) contain BRs, it seems that the biosynthesis of BRs is not limited to a specific plant organ (Fujioka and Sakurai, 1997). BRs are also widespread in the plant kingdom and not confined to specific species. The highest BR concentration is found in pollen and unripe seed. The pollen of *Helianthus annuus*, for example,

contains more than 100 ng BR per gram fresh weight (Schmidt *et al*., 1997). In comparison to pollen and unripe seed, other plant parts only contain sub nanogram quantities of the BRs.

When the diverse variance of the chemical structures of the A-ring, B-ring and side chains of the BRs are taken into account, it highlights the possibility that more than a hundred different BRs could be present in the plant kingdom. It is expected that more BRs and BR related compounds will be discovered in future. The search for more unknown BRs in plants, as well as their physiological and biochemical roles in plants, remains a challenge.

2.4.6 Other compounds with growth promoting characteristics

A wide variety of substances with either growth promoting or growth inhibiting characteristics occur in virtually all plant parts. When these compounds are leached out during the decay of plant rests, they may inhibit seed germination or seedling growth of other plants in the vicinity of the parent plant. However, some natural compounds from plants, other than nitrogen released during the decay process, have been reported to promote seed germination and seedling growth. For example, flavanoids play major roles as internal physiological regulators and chemical messengers in plants by serving as antioxidants, enzyme inhibitors, stimulatorss of inhibitors of plant growth or directly as plant growth regulators. Flavanoids have also been reported to inhibit IAA oxidase (and enzyme which regulates the indole acetic acid (IAA) levels in plants) activity in peas (Seigler, 1998).

Mandava (1979) reported the growth regulating activity of cucurbitacins in plants while (+)-(S)-Abscissic acid (ABA) is involved in a number of hormonal roles in plants (Dinan *et al.*, 2001). ABA is found in all higher plants, acts as a growth inhibitor and is involved in bud and seed dormancy. Although few alkaloids have been tested for plant growth-regulatory activity, several are known to inhibit the growth of plants. For example lycoricidinol and lycoricidine from *Lycoris radiata* showed growth-inhibiting acitivty on *Avena* coleoptile sections (Mandava, 1979). Jasmonic acid is more effective than abscisic acid in promoting senescence by inducing the expression of a specific set of proteins (jips or jasmonate-induced proteins) in different plants (Sembdner and Parthier, 1993).

Many biological activities of lignans have been reviewed and they may be active in plants. A lignan isolated from the seed hulls of *Aegilops ovata* (Poaceae) is a potent germination inhibitor of lettuce seeds (Davin *et al*., 1992). Some fatty acids act as wound hormones in plants and is capable of inducing renewed cell division and cell extension activity in the parenchymatous cells of the mesocarp of bean pods. Modified fatty acids isolated from the alga *Eleocharis microcarpa* have been suggested to be involved in allelopathic interactions with other organisms (Seigler, 1998). This confirms that a wide range of structurally and functionally similar compounds have phenomenal stimulatory properties.

2.4.7 Closing remarks on plant growth regulators

It seems that extractable amounts of plant growth regulators are associated with the plant families Fabaceae and Caryophyllaceae (Schnabl *et al*., 2001). Subsequently, a screening program was conducted during 2002 (du Plessis, 2002) where extracts of different species from these two families were screened for their bio-stimulatory activity. Especially seed extracts or seed suspensions of most of the species tested showed above average bio-stimulatory potential. From the screening program it was concluded that species form the family Caryophyllaceae found in South Africa was either too small or did not produce sufficient amounts of seeds to justify large scale cultivation. However, in the case of seed suspensions from species belonging to the family Fabaceae the bio-stimulatory activity was at least just as significant as or better than that of the Caryophyllaceae.

During the preliminary screening program a seed suspension of *Lupinus albus* (Fabaceae) showed above average bio-stimulatory activity, in terms of the bio-tests applied, when compared to the other species tested. This prompted a full scale investigation in order to verify the application potential of this plant under *in vitro* and *in vivo* conditions using a number of different agricultural and horticultural crops.

2.5 Biology of *Lupinus albus*

2.5.1 Classification

Class: Magnoliopsida
Subclass: Rosidae
Order: Fabales
Family: Fabaceae
Genus: *Lupinus*
Species: *Lupinus albus*

The class Magnoliopsida, constitutes a much larger group than the monocots and is divided into six subclasses: (1) Magnoliidae, (2) Hamamelidae, (3) Caryophyliidae, (4) Dilleniidae, (5) Rosidae, and (6) Asteridae (Mauseth, 1991). They are more difficult to characterize than the monocots because of extreme diversity. *Lupinus albus* belongs to the subclass Rosidae that is the largest in terms of the number of families it contains. As a result of rather extreme diversity within the subclass, it is difficult to summarize a series of universal characteristics. However, one universal characteristic of the subclass Rosidae is the presence of pinnate compound leaves that is believed to have been the ancestral condition of the subclass. Rosidae contains 18 orders, 114 families and 58 000 species. Five of the orders contain almost 75% of the species with Fabales (14 000 species) as the largest one (Mauseth, 1991).

The family Fabaceae has economic value as it contains numerous ornamental as well as agricultural and horticultural cultivated genera that can be grown in temperate climates (Mauseth, 1991). Further importance of the Fabaceae is confined in the fact that species within the family are able to convert free air nitrogen, due to its symbiotic relationship with nitrogen binding soil micro-organisms, into a form that can be utilized by plants and animals. The importance of this characteristic extends beyond the basic need for food and fodder for all living things, whether they are economically important or purely ornamental and esthetic (Allen and Allen, 1981). Biological nitrogen fixation, particularly of the symbiotic type, plays a crucial ecological role in maintaining adequate nitrogen resources in the plant world. Quite distinctive in this respect are the numerous

members of the giant family Fabaceae which can thrive without any fixed nitrogen or with a minimal supply form the soil.

One of the most important and utilized genuses is *Lupinus* L. The name had its origin from the Latin word "lupus", meaning “wolf,” in reference to the belief that the plant takes over the land and destroy or exhaust the fertility of the soil (Allen and Allen, 1981). As a result, lupines were subject of much discussion in the early literature of the Egyptians, Greeks and Romans. The genus is well adapted to a range of climate and soil types. In Australia 1,6 million tons of lupine seed are produced annually, representing 80 % of the total world production (Pollard *et al*., 2002). Internationally, the main use of lupine seed is for human consumption, although it has mainly been used as a stock feed in Australia where its market value is subject to the volatility of alternative sources of feedstuffs. The nutritional quality of wheat protein is lower than that of lupine seeds because of lower levels of amino acids (Kulp, 1988). Although the demand for wheat-based bakery products is increasing, it has been suggested that the nutritional quality of these products could be improved by supplementation with non-wheat proteins such as those from lupines which would improve the essential amino acid balance of the baked product (Huyghe, 1997). In this respect lupines might offer several advantages over soybeans. In particular, with regard to its digestibility, it contains less trypsin inhibitors that can interfere with digestive processes, as well as less phytic acid, which binds minerals such as calcium and zinc to reduce their bioavailability. Lupine seed also contain lower levels of saponins and lectins which can act as gastric irritants. The higher dietary fiber content of lupines is typically associated with cholesterol-lowering activity (Pollard *et al*., 2002). For lupine flour or proteins to achieve a market as a bread making additive, it is important to establish that dough mixing properties are not adversely affected. In this regard Dervas *et al*. (1999) reported that lupine flour can replace wheat flour up to 5% but, if defatted and concentrated, the replacement level can increase to 10%.

2.5.2 General uses of Lupinus species

White lupine has been cultivated for more than 3 000 years wherever Europeans have migrated, including Europe, Australia and Asia as well as North and South America (Grieve, 2000). It is probably of Egyptian or East Mediterranean origin, and has been cultivated since the days of the

ancient Egyptians. It is currently very extensively used in Italy and Sicily for winter and early spring grazing, forage, for ploughing-in to enrich the soil (green manure) and for its seeds. Of the more than 300 species contained in the genus *Lupinus*, only five are cultivated as many lupines have high levels of alkaloids (bitter tasting compounds) that make the seeds unpalatable and sometimes toxic. Besides its uses for land reclamation, flour produced from the seed was used for bread making, especially by very poor people during the late 19th century in areas of Spain, Corsica and Northern Italy, while the seeds were also utilized for medicinal purposes (Hill, 1991; Allen and Allen, 1981). The flour was prepared from cooked, dried and ground seeds from which the bitter principles had been rinsed out by boiling or steeping in water, as the fresh seeds are poisonous. *Lupinus albus* shows great potential as a bread additive because loaf height and structure are maintained while mixing time is usefully reduced and the nutritional quality is improved when lupine flour is added to traditional wheat flour (Pollard *et al*., 2002).

Although lupine alkaloids have traditionally been removed from the seed by soaking, German plant breeders produced the first selections of alkaloid-free or "sweet" lupin in the 1920s that is more suitable for direct consumption by humans or livestock (Golz and Aakri, 1993). The breeding process for introducing the newer "sweet" lupines to replace the "bitter" types is ongoing. For example, Chilean researchers have introduced sweet *Lupinus albus* varieties to southern Chile and have also developed low alkaloid *Lupinus mutabolis* varieties which may have applications in the high mountain areas where the traditional bitter *Lupinus mutabolis* types are grown (Von Baer, 1991). The most important center of alkaloid-free lupine production outside Europe is the southwest Cape, South-Africa, where lupines are grown for stubble grazing as mature standing crops during summer or for harvesting of seeds that are fed to livestock as protein concentrate.

In the past, major losses of domestic animals have been experienced from the ingestion of lupine foliage under range conditions as a result of poisoning or lupinosis. The following general conclusions were made from the voluminous literature on lupinosis: (1) not all *Lupinus* species are poisonous but, in general, use of these plants for forage and fodder merits caution as toxicity is variable. Moreover, taxonomic differentiation between harmless and poisonous species and varieties is not easy, (2) toxicity of a species varies seasonally and geographically. Plants in the pre-flowering stage are less likely to be toxic than thereafter. Further, all aerial parts of poisonous

species are toxic to some extent while most poisoning has resulted from ingestion of pods and seeds, (3) animals vary greatly in susceptibility to lupinosis. The greatest losses have occurred among sheep, while horses are less likely to be affected. Lupinosis is acute or chronic depending on the amount of toxic principles ingested, (4) different symptoms in a given type of livestock often result from ingestion of different lupine species and (5) the active toxic principle of Lupine species may be one of several of 25 to 30 quinolizidine alkaloids known to occur in about 75 members of the genus.

Lupine poisoning of cattle is attributed to quinolizidine alkaloids or their N-oxides. Five important toxins in lupines are d-lupanine, sparteine, lupinine, sparthulatine and hydroxylupanine. Effects of these alkaloids do not appear to be cumulative. Seeds of both strains are reported to contain trypsin inhibitors. Lupinosis is not clearly understood and may be due to metabolites (but not aflatoxins) from the fungus *Phomopsis*. Liver damage observed during lupinosis appears to be associated with abnormal iron uptake. Acute cases exhibit dullness, poor appetite and jaundice (Duke, 1981). Seeds especially developed for use as a winter cover and green manure crop has high alkaloid content that act as a natural herbicide when plant material is decomposing in the soil.

Medicinally lupine seed is used as deobstruent, a depurative, a diuretic and a tonic. A decoction of seeds increased the sugar tolerance in diabetic patients and the seeds, somewhat bitter in taste, can be used to destroy worms. Sometimes bruised seeds are soaked in water and applied to sores and an external application to ulcers. A mixture of lupine meal mixed with goat gall and lemon juice have also been used to form an ointment. However, a lupine seed decoction can produce toxic symptoms in humans including unpleasant sensations in the head and dimness of vision as well as laryngeal and pharyngeal constriction. The fresh seeds are especially poisonous, but prolonged treatment by boiling or steeping in water removes bitter alkaloids and makes them edible (Grieve, 2000).

Lupines can also play an important role in agriculture. During crop rotation lupine has been reported to decrease fungal infections in wheat from 36% to less than 1% (Reeves *et al,* 1984). In field experiments in Poland cucumber plants were sprayed with a 1.5% *Lupinus angustifolius* cv. Mirela extract at the beginning of flowering and fruiting. The extract increased the fruit yield by 15,4% compared to the untreated controls and had no effect on the P, K, Ca or Na content in fruit

(Barczak *et al*., 1998). Soybeans grown in a greenhouse were treated 30 days after establishment with 5, 15 and 25% concentrations of a *Lupinus termis* seed extract. Growth and yield of soybeans were increased by a 5% seed extract but decreased by a 25% extract (El-Daly and Soliman, 1997).

In this study a *Lupinus albus* seed suspension was screened *in vitro* (chapter 3) for possible bio-stimulatory activity and compared to *ComCat*®, used as a positive control, as well as an untreated negative control. Subsequently, both the seed suspension and a semi-purified extract were tested *in vivo* (chapter 4) for bio-stimulation of growth, development and yield of selected vegetable and other agricultural crops under field conditions. Finally, the active substance involved was isolated, purified and identified (chapters 5 and 6).

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CHAPTER 3

SCREENING OF A *Lupinus albus* SEED SUSPENSION FOR BIO-STIMULATORY PROPERTIES

3.1 ABSTRACT

A seed suspension of *Lupinus albus* L. was prepared by grinding seeds to a fine powder and treating it with an organic solvent to enhance its solubility in water. Three general laboratory bio-assays were employed to evaluate the plant growth-regulating effects of the seed suspension as an initial screening procedure. These included monitoring the effect on the respiration rate of monoculture yeast (*Saccharomyces cerivisiae*) cells, the germination rate of seeds from a number of crops and subsequent seedling development in terms of root and coleoptile growth. A commercially available bio-stimulant, *ComCat*®, was used as a positive control while distilled water served as a negative control in all bio-assays. The seed suspension was tested at four concentrations ranging from 0.05mg L^{-1} to 50.0 mg L^{-1}. A significant increase in the respiration rate of monoculture yeast cells treated with the seed suspension, compared to both the untreated and *ComCat*® treated controls was a strong indication of its bio-stimulatory activity. This was concentration dependent. Although seeds of different crops treated with the *L. albus* seed suspension showed no significant improvement in germination rate, the subsequent enhancement of especially root development in most of the test seedlings confirmed its plant growth regulatory potential. The latter was also concentration dependent.

Keywords: *Lupinus albus*, seed suspension, bio-stimulatory properties, seed germination, seedling growth

3.2 INTRODUCTION

The plant kingdom contains about 500 000 species and only a small portion have been investigated phytochemically for its possible application potential as donor plants in developing natural products for the agricultural industry. Since plants contain a wide range of naturally produced metabolic compounds, in many cases as a result of environmental signals in an attempt to resist abiotic and

biotic stress conditions, there is currently renewed interest in screening programs for plant-derived compounds as a means of identifying novel agents with, *inter alia*, anti-infective and bio-stimulatory application potential.

Moreover, two decades ago Blank (1985) remarked that the increased use of synthetic herbicides and pesticides lead to the introduction of many chemical compounds in modern society and that this may have been connected with the increase of pollutants as well as the decline of natural vegetation experienced back then. It is probably safe to say that this remark is still valid today. Many of the pollutants referred to by Blank (1985) decompose very slowly and consequently accumulate in nature. It seems, therefore, not far fetched to engage in screening programmes with the objective to identify natural phytochemicals from wild or even crop plants with the potential to serve as alternatives for synthetic pesticides on grounds of claims made in literature that natural compounds are more bio-degradable than their synthetic analogues. Additionally, wild plants that shows potential to act as donor plants for these phytochemicals in this respect, may have the added advantage to be developed as alternative crops. The latter is an aspect that has gained some attention in recent times.

Of all the known phytochemicals, especially in terms of their potential to be applied as natural products in the agricultural industry, plant growth regulators are probably the least studied. Although well known plant hormones such as auxins, gibberellins and cytokinins are applied in the horticultural industry on a rather small scale, it seems that most of the knowledge that has been collected over decades did not lead to large scale application in the agricultural industry. Economic viability might have been one of the reasons for this situation and accentuates the importance of keeping agricultural economics in mind when natural product development is considered; an aspect that will be dealt with later. More recently a new generation of phytohormones, namely brassinosteroids, was discovered (Schnabl *et al*., 2001). Despite the relative short time in which brassinosteroids received attention from the international research community, its application potential in the agricultural industry is rather well documented (Yopp *et al*., 1981; Takematsu and Takeuchi, 1989; Shen *et al*., 1990; Singh *et al*., 1993).

Further, other compounds that comply with the definition of secondary metabolites and that revealed plant growth regulatory activities have been identified. These include abscisic acid, sterols, cucurbitacins and the naphtaquinone juglone, to mention a few (Seigler, 1995). Undoubtedly more discoveries will follow. Sesquiterpene lactones were isolated from witch weed (*Striga hermonthica* [Del] Benth.), an economically important root parasitic weed of the Poaceae family in semi-arid Sub-Saharan Africa (Ejeta *et al*., 1993). These active substances are labile and active at extremely low (picomolar) concentrations and have been shown to stimulate seed germination. However, the compounds are extremely unstable in soils that limit their usefulness under practical field conditions (Yasuda *et al*., 2003). Understanding the structure-function relationships of these germination stimulants has also been hindered by their low rates of production, laborious isolation procedures and complex stereochemistry, emphasizing the thorough approach needed to evaluate the application potential of any given natural compound in the agricultural industry. In this regard it seems imperative that any claims made on the application potential of a specific natural compound, on grounds of *in vitro* results, should be supported by *in vivo* data.

Prior to the underlying study being undertaken, different organs of many South African wild plants as well as known economic crops were screened for their bio-stimulatory activity using a range of bio-assay procedures. Among these *Lupinus albus* seeds revealed the most potent bio-stimulatory activity prompting this thorough investigation. The *in vitro* bio-stimulatory activity of a *L. albus* seed suspension on the respiration rate of monoculture yeast cells, seed germination and subsequent seedling growth is reported in this chapter. In all bio-assays *ComCat*®, a commercially available natural plant strengthening agent registered in Germany (Agraforum, Germany) was used as a positive control. The registered name *ComCat*® represents a unique family of natural products based upon a combination of bio-active compounds derived from different plants and has consistently demonstrated the enhancement of growth and yield as well as physiological efficiency in treated crop plants.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

Lupinus albus seeds used in this study were bought from a local merchant, Free State Agricultural Products, during 2001.

3.3.2 Other materials

All chemicals used, were from Sigma (Germany) and of the purest grade available. Germination paper was provided and manufactured by Agricol (South Africa). Seeds from different crop plants used in the bio-assay procedure were locally purchased from SENWES, Stark Ayres or Mayford (South Africa). Dry instant baking yeast was also purchased locally.

3.4 METHODS

3.4.1 Preparation of a seed suspension

Five hundred grams of *L. albus* seeds were ground to a fine powder using a Kenwood coffee grinder followed by a Freutsch grinder manufactured in Germany. The powdered seeds were transferred to a plastic tray, spread out over the surface of the tray, covered with pure ethanol and left overnight to allow the ethanol to evaporate to dryness. The ethanol treatment was repeated twice over 48 h in an attempt to make the powdered seed more suspendable in water. Subsequently, the ethanol treated seed powder was suspended in water, hitherto referred to as the seed suspension (SS), and subjected to different bio-assays at different concentrations to determine its bio-stimulatory properties.

3.4.2 Screening of the *L. albus* seed suspension (SS) for bio-stimulatory activity

Two different bio-assays were employed to either initially determine or confirm the bio-stimulatory activity of the seed suspension. These included the effect of the seed suspension on the respiration rate of monoculture yeast (*Saccharomyces cerivisiae*) cells as well as on seed germination and subsequent seedling development in terms of coleoptile and root length growth.

3.4.2.1 The effect of a *L. albus* seed suspension (SS) on the respiration rate of monoculture yeast cells

A specially constructed glass respirometer (Plate 3.1) with a short bulged section (reservoir) to contain the yeast cells and a long calibrated tube, closed at the top end to collect CO_2 gas, was used

in determining the effect of different concentrations (0.05; 0.5; 5.0 and 50.0 mg L^{-1}) of the *L albus* seed suspension on the respiration rate of monoculture yeast cells. Dry baker's yeast (0.8 g) was placed in the reservoir of the respirometer. Subsequently, 70 ml of each of the SS concentrations containing 5 mg L^{-1} glucose to serve as respiratory substrate for the yeast cells was added to the respirometer. The apparatus was tilted sideways to release air bubbles trapped in the dry baker's yeast and placed in a water bath pre-heated to 29 ^{o}C. *ComCat*®, a commercial biostimulant, was used as a positive control at 0.5 mg L-1^{-1} (optimum concentration according to the manufacturers; Agraforum, Germany) and distilled water as a negative control. However, the same concentration range as was used for SS was also tested for *ComCat*® although the optimum concentration of the latter was previously determined at 0.5 mg L^{-1} by the manufacturers. Carbon dioxide release by the yeast cells was measured in ml at 30 minute intervals over a three hour incubation period by reading the released gas volume directly from the calibrated tube and replicated six times.

Plate 3.1: Specially constructed glass respirometer for screening the effect of an *L. albus* seed suspension on the respiration rate of monoculture yeast cells.

3.4.2.2 The effect of a *L. albus* seed suspension (SS) on seed germination and subsequent seedling growth

Two sheets of special germination paper (30 x 30 cm) were used to test the effect of each SS concentration on the germination of seeds from different crops as well as the subsequent seedling growth. A line, 10 cm from the top, was drawn on the one sheet and 20 seeds spaced evenly on the line. A second sheet of germination paper was placed on top of the first and moistened with either a specific SS concentration (see Method 1), distilled water (negative control) or different concentrations of *ComCat*® (positive control). Both sheets of paper were rolled up longitudinally and placed upright in Erlenmeyer flasks containing either SS, distilled water or the *ComCat*® solution and kept at 25 ^{o}C in a growing chamber in the dark. Seed germination as well as coleoptile and root lengths were determined at 24 h intervals over a 96 h incubation period and replicated six times.

3.4.3 Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using the NC: SAS Institute Inc., Dos statistical programme to identify differences between treatments. Tukey-Kramer's LSD (least significant difference) procedure for comparison of means (Steele and Torrie, 1980; Mason *et al.*, 1989) was applied to separate means ($P<0.05$). Treatments differing significantly were indicated either in figures or below tables as calculated LSD values and by using symbols for significant (*) and insignificant (ns) differences.

In the case where different concentrations of *CC* and *SS* were tested and compared, a separate untreated water control was used for each concentration. During the statistical analysis (ANOVA) all of the data were averaged but the different control data also pooled.

3.5 RESULTS

3.5.1 The effect of a *L. albus* seed suspension (SS) on the respiration rate of monoculture yeast cells

No significant differences in the respiration rate of monoculture yeast cells between SS treatments at concentrations of 0.05 mg L^{-1} (Figure 3.1A) and 0.5 mg L^{-1} (Figure 3.1B) and the *ComCat*® (*CC*) control were observed (see calculated LSD-values indicated in each graph). However, at a concentration of 5 mg L^{-1} (Figure 3.1C) SS increased the respiration rate of yeast cells significantly by 89% after 3 hours of incubation compared to the water control, while respiration rate enhancement calculated for the commercial bio-stimulant (*CC*) was also significant (46%). The same significant respiration rate enhancement in yeast cells by SS (84%) was observed at a concentration of 50 mg L^{-1} (Figure 3.1D) while that by *CC* was also significant, although the respiration rate enhancement was slightly less (63%).

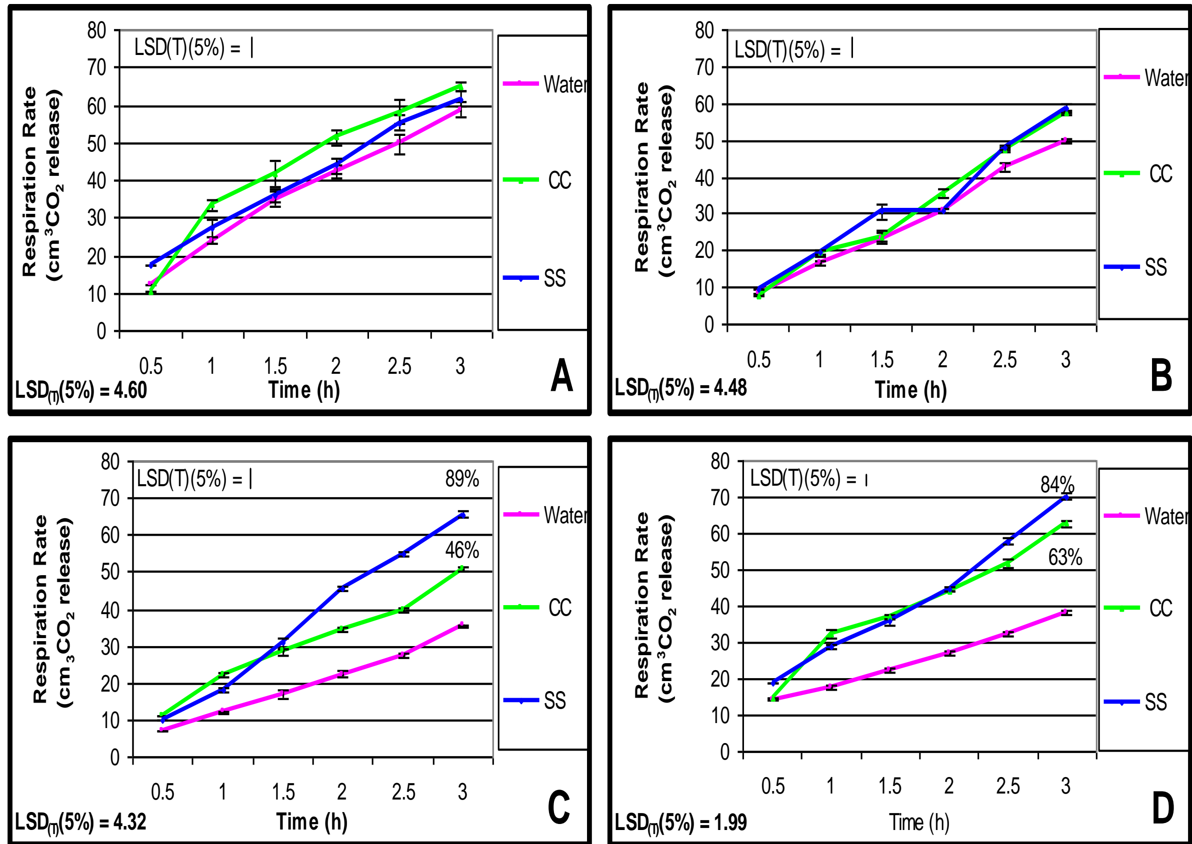

Figure 3.1: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [**A)** 0.05mg L^{-1}, **B)** 0.5 mg L^{-1}, **C)** 5mg L^{-1} and **D)** 50mg L^{-1} distilled water] on the respiration rate of monoculture yeast cells. A commercially available bio-stimulant, *ComCat®* ***(CC)***, was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in each graph separately as well as with a vertical bar.

Table 3.1: Statistical analysis of the interaction between pooled water control values and averaged treatment (*CC* and SS) values for the respiration rate of yeast cells after 3h of incubation as influenced by different concentrations

Concentration (mgL^{-1})	Treatments	
	CC	**SS**
0	38.3	38.3
0.05	65.3	61.6
0.5	56.6	58.6
5	51.0	65.6
50	62.6	70.3

Differences are significant at LSD $_{(T)}$ (5%) = 2.58; The ANOVA is attached as Table 3.1A in the Appendix.

An overall statistical analysis of pooled water control values and averaged treatment (*CC* and SS) values showed a significant interaction in terms of both concentration and treatment for the respiration rate of yeast cells after 3h of incubation (Table 3.1; see the calculated LSD-value presented below the table). This indicates that the increase in the respiration rate of yeast cells under the influence of these treatments was significantly different from the water control for all concentrations tested, although the *CC* and SS treatments were not necessarily significantly different from each other at specific concentrations (e.g. treatment at 0.05 and 0.5 mg L^{-1}). In order to verify whether the increase in the respiration rate of yeast cells was an indication of a positive or negative influence in terms of growth, the effect was tested on seed germination and subsequent seedling growth using seeds of different crop plants.

3.5.2 The effect of a *L. albus* seed suspension (SS) on the germination of Cress seeds

No significant differences in the percentage germination of Cress seeds were observed between the different treatments (CC, SS and the water control; Figure 3.2 A, B, C and D; see calculated LSD-values indicated in each graph). However, the overall statistical analysis of pooled values for the treatments at specific concentrations, as well as the water control, showed significant differences between the application concentrations (Table 3.2). The results revealed 0.5 mg L^{-1} (Figure 3.2B) and 5 mg L^{-1} (Figure 3.2C) as the best application concentrations for both *CC* and SS in terms of their stimulatory effect on seed germination. Subsequently, the effect of the different treatments on seedling growth, by quantifying coleoptile (Figure 3.3) and root growth (Figure 3.4), were investigated.

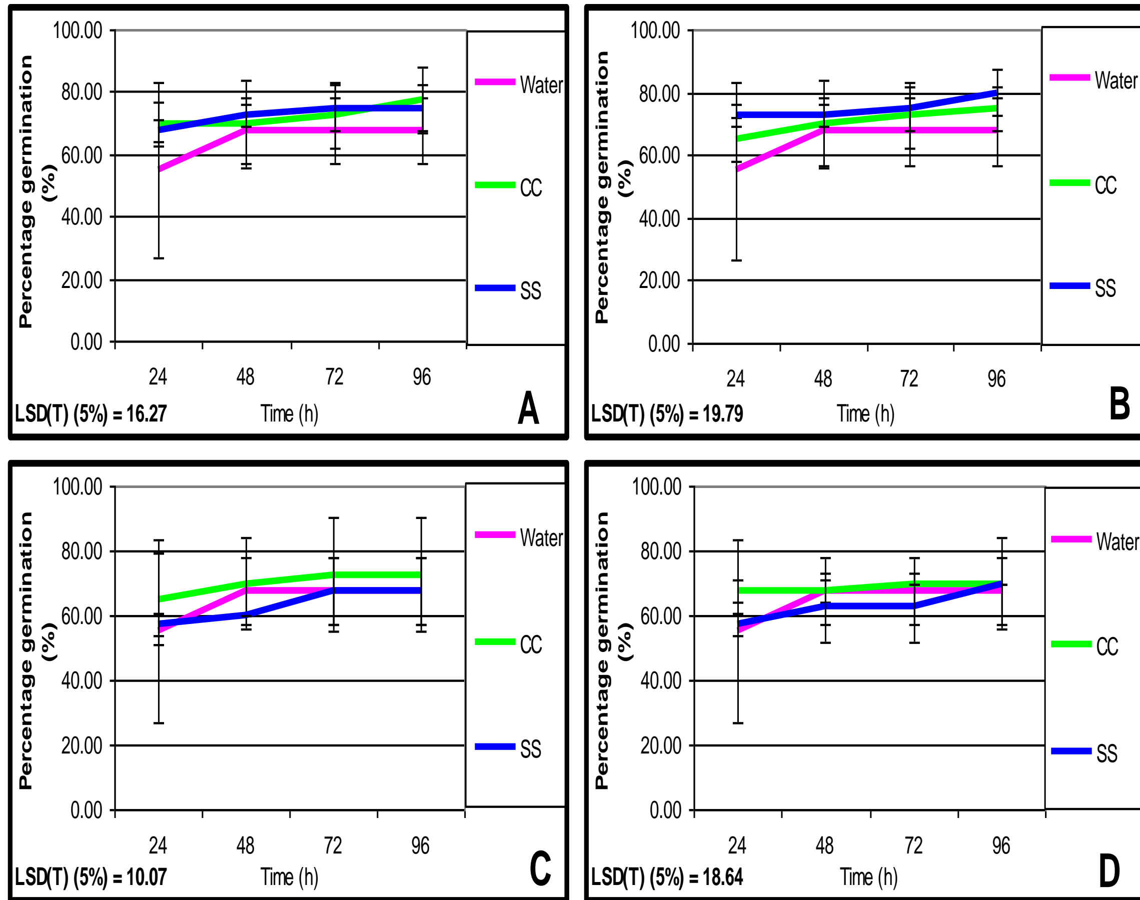

Figure 3.2: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [**A)** 0.05mg L^{-1}, **B)** 0.5 mg L^{-1}, **C)** 5mg L^{-1} and **D)** 50mg L^{-1} distilled water] on the percentage germination of Cress seeds. A commercially available bio-stimulant, *ComCat® (**CC**)*, was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in each graph separately.

Table 3.2: Statistical analysis of the averaged values for the effect of both (*CC* and SS) treatments on seed germination of Cress seeds after a 96 h incubation period as influenced by different concentrations and as compared to the water control

Concentration (mg L^{-1})	Pooled and averaged germination data for both treatments at different concentrations
0	62.5
0.05	70.0
0.5	73.8
5	71.3
50	68.8

Differences are significant at LSD $_{(T)}$ (5%) =3.51; The ANOVA is attached as Table 3.2A in the Appendix.

3.5.3 The effect of a *L. albus* seed suspension (SS) on the growth of Cress seedlings

Except for the 0.5 mg L^{-1} *ComCat*® concentration (Figure 3.3B) that had no significant effect on coleoptile growth of Cress seedlings, all other concentrations showed an inhibitory effect, although not significant in all cases (Figure 3.3A, C and D). In comparison, SS had no inhibitory effect on coleoptile growth but only the 50 mg L^{-1} concentration (Figure 3.3D) significantly enhanced coleoptile growth in Cress seedlings. Interactions between pooled treatment values of the two bio-stimulants as well as the application concentrations confirmed both the non-effect by *Comcat*® as well as the significant effect of SS on the coleoptile growth of Cress seedlings, but only at a concentration of 50 mg L^{-1} (Table 3.3). Similar measurements were made to quantify the effect of the bio-stimulants on root growth (Figure 3.4).

Compared to the water control, exactly the same tendency for the *ComCat*® effect on root growth (Figure 3.4A, B, C and D), as was seen for its effect on coleoptile growth, was observed except that all concentrations tested showed significant inhibition of root growth. Similarly, the SS treatment, at concentrations of 5 mg L^{-1} and 50 mg L^{-1} showed significant stimulation of root growth (Figure 3.4C and D) as was the case for coleoptile growth. The latter also differed significantly from the *CC* treatment in this regard. Overall the seed suspension showed the best root growth enhancement at a concentration of 5 mg L^{-1} (Figure 3.4C).

Statistical analysis of the pooled and averaged treatment values, for the 5 mg L^{-1} concentration only, revealed significant differences between *CC* and SS with regard to root growth enhancement in Cress seedlings (Table 3.4). It became clear that the bio-stimulatory effect of SS on both coleoptile and root growth was concentration dependant and that *CC* had either no effect on the growth of Cress seedlings or an inhibitory effect. Subsequently, the screening process was expanded to seeds and seedlings of five different crops. As the manufacturers of *CC* claimed the 0.5 mg L^{-1} concentration to be optimal for bio-stimulation of crop plants and the minimum stimulatory concentration (MSC) of SS was found to be 5.0 mg L^{-1}, using Cress seedlings as test organism, only these two concentrations were applied in the extended screening program.

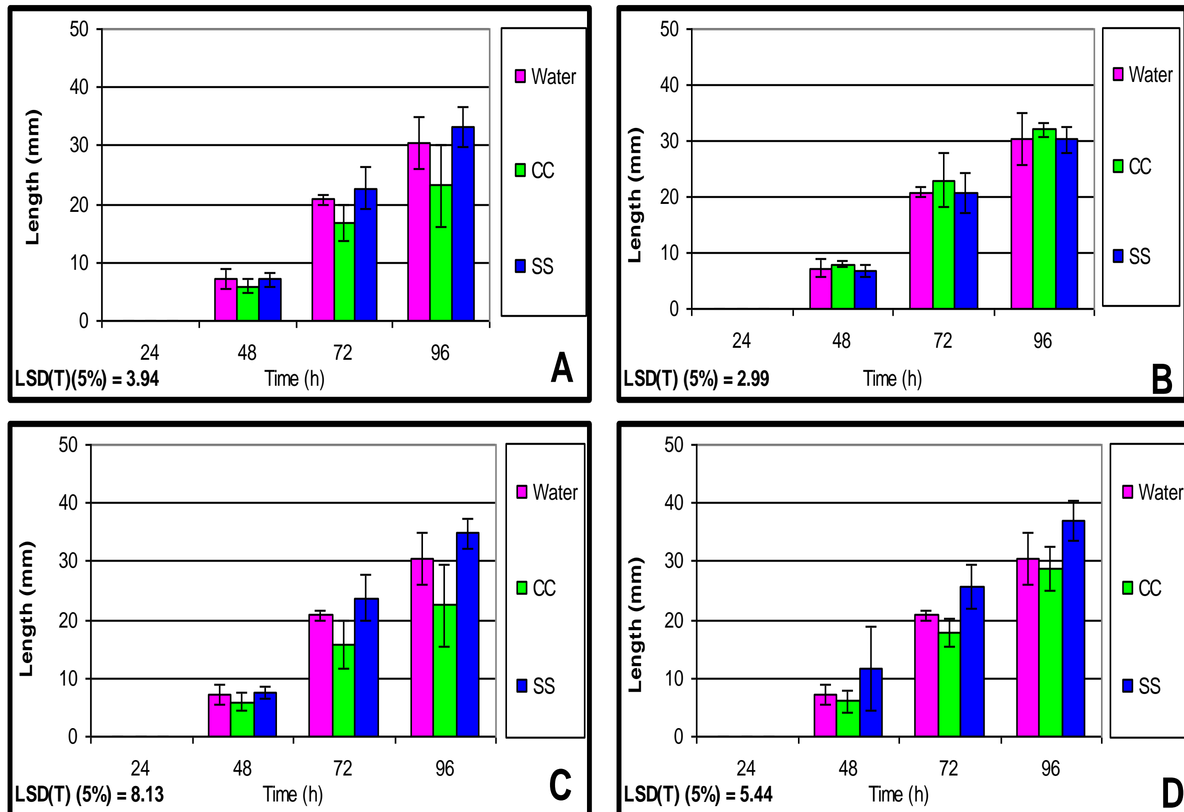

Figure 3.3: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [**A)** 0.05mg L^{-1}, **B)** 0.5 mg L^{-1}, **C)** 5mg L^{-1} and **D)** 50mg L^{-1} distilled water] on the coleoptile growth of Cress seedlings. A commercially available bio-stimulant, *ComCat® (**CC**)*, was used as a positive control and water as a negative control. Statistical significance is indicated by $LSD_{(T)}$ (5%) values in each graph separately.

Table 3.3: Statistical analysis of the interaction between pooled water control values and averaged treatment (*CC* and SS) values for coleoptile growth of Cress seeds after a 96h incubation period as influenced by different concentrations

Concentration	**Treatments**	
mg L^{-1}	***CC***	**SS**
0	30.7	30.7
0.05	23.1	33.3
0.5	32.1	30.2
5	22.5	34.9
50	28.7	37.1

Differences are significant at $LSD_{(T)}$ (5%) =5.58; The ANOVA is attached as Table 3.3A in the Appendix.

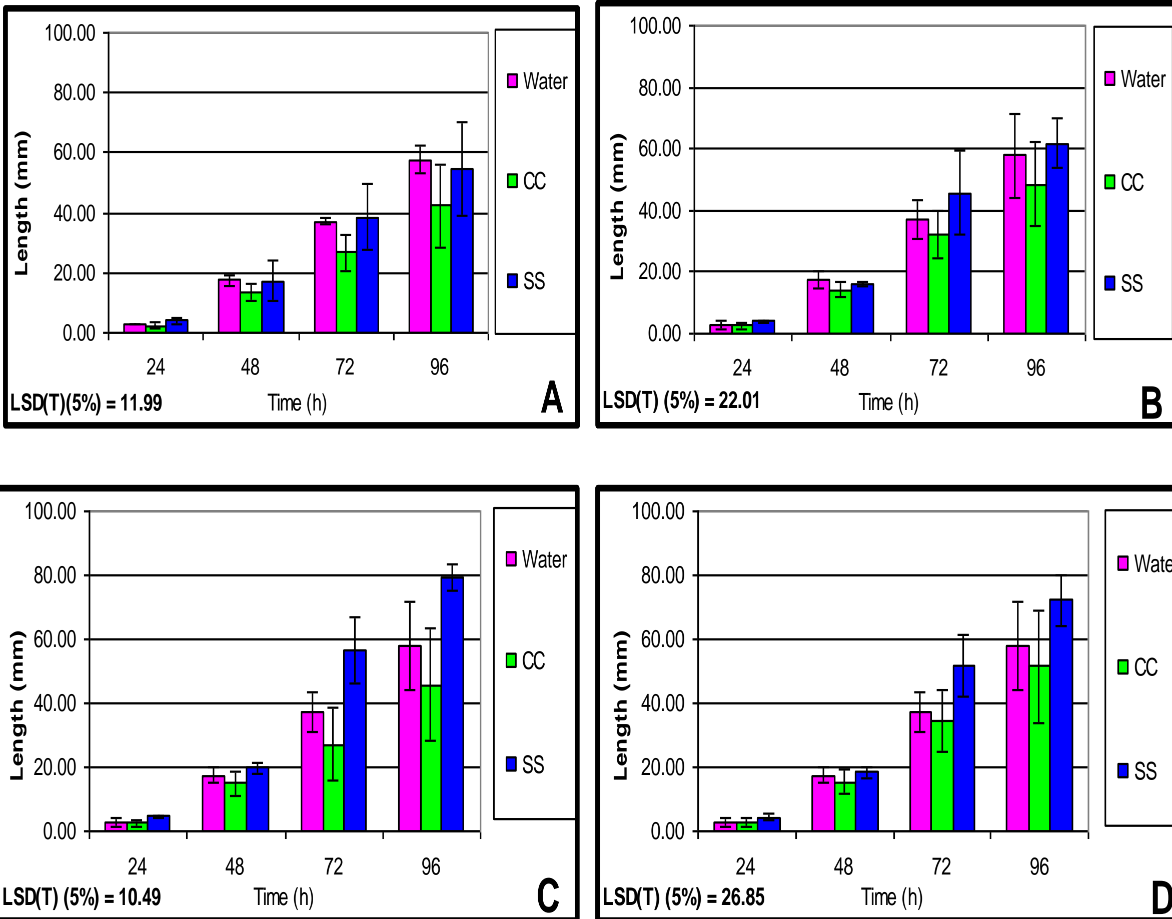

Figure 3.4: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [**A)** 0.05mg L^{-1}, **B)** 0.5 mg L^{-1}, **C)** 5mg L^{-1} and **D)** 50mg L^{-1} distilled water] on the root growth of Cress seedlings. A commercially available bio-stimulant, *ComCat® (**CC**)*, was used as a positive control and water as a negative control. Statistical significance is indicated by $LSD_{(T)}$ (5%) values in each graph separately.

Table 3.4: Statistical analysis of the averaged values for the effect of treatments (*CC* and SS) on the root growth of Cress seeds at 96 h as influenced by the 5 mg L^{-1} concentration only and as compared to the water control

Catalyst	Treatments
Water	54.65
CC	47.9
SS	64.7*

Differences are significant at $LSD_{(T)}$ (5%) =9.36; The ANOVA is attached as Table 3.4A in the Appendix.

3.5.4 The effect of a *L. albus* seed suspension (SS) on seed germination and seedling growth of different crops

3.5.4.1 Cauliflower

Significant differences in the percentage germination of cauliflower seeds were observed between the different treatments but only after 24 h of incubation (Figure 3.5, see calculated LSD-values indicated in the graph). Incubation at 48h, 72h and 96 h showed no significant differences between the treatments and the control.

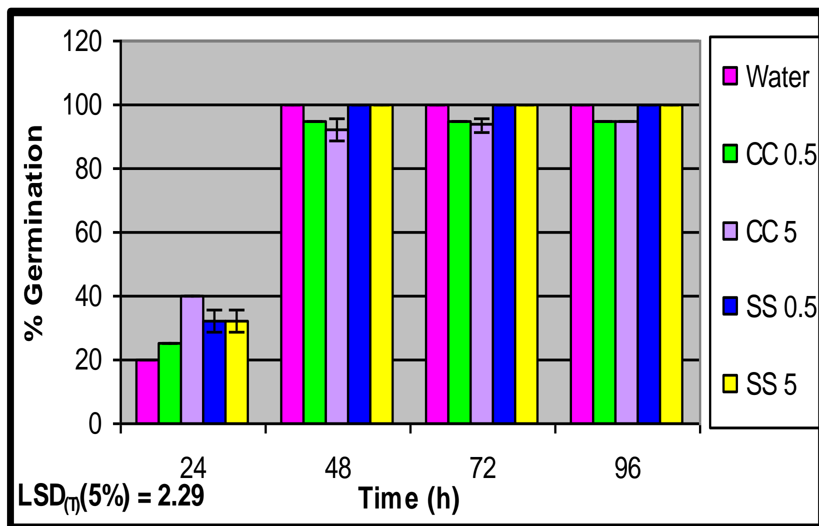

Figure 3.5: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the percentage germination of cauliflower seeds. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.5: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the percentage seed germination of cauliflower seeds at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control					
Time	**Effect on seed germination and interactions between treatments over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	20.0	25.0	40.0	32.2	32.8
48 h	100.0	95.0	92.2	100.0	100.0
72 h	100.0	95.0	95.0	100.0	100.0
96 h	100.0	95.0	100.0	100.0	100.0

Differences are significant at $LSD_{(T)}$ (5%) =2.29; The ANOVA is attached as Table 3.5A in the Appendix

Statistical analysis of the pooled and average values, revealed significant differences between *CC* and *SS* in terms of the percentage seed germination but again only at the 24 h incubation period (Table 3.5). *Comcat*®, at a concentration of 5 mg L^{-1}, had the most significant enhancing effect on the germination of cauliflower seeds, followed by *SS* at concentrations of 5mg L^{-1} and 0.5 mg L^{-1} respectively. However, neither treatment had any effect on seed germination during later incubation intervals compared to the water control. Subsequently, the effect of these treatments on the growth of cauliflower seedlings was followed.

Although not during early incubation intervals, both *CC* at 0.5 mg L^{-1} and *SS* at 5.0 mg L^{-1} had a significant enhancing effect on the coleoptile growth of cauliflower seedlings at 96 h of incubation compared to the untreated control (Figure 3.6). This was confirmed by the statistical analysis of pooled and averaged values for the 96 h incubation period (Table 3.6).

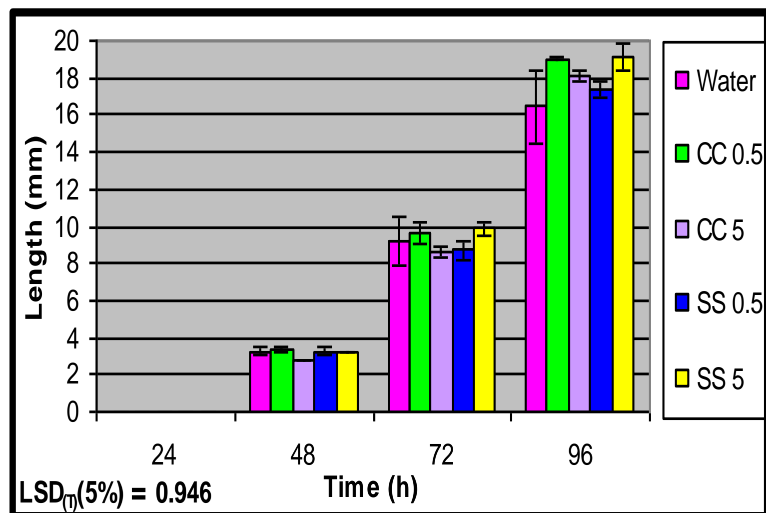

Figure 3.6: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the coleoptile growth of cauliflower seedlings. A commercially available bio-stimulant, *ComCat® (**CC**)*, was used as a positive control and water as a negative control. Statistical significance is indicated by $LSD_{(T)}$ (5%) values in the graph.

Table 3.6: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the coleoptile growth of cauliflower seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control

Time	Effect on coleoptile growth and interactions between treatments -over time				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	0	0
48 h	3.24	3.3	2.7	3.3	3.3
72 h	9.10	9.6	8.6	8.8	9.8
96 h	16.25	19.1	18.1	17.4	19.0

Differences are significant at $LSD_{(T)}$ (5%) =0.946; The ANOVA is attached as Table 3.6A in the Appendix

Both at the 72 h and 96 h incubation intervals *SS* at a concentration of 5 mg L^{-1} significantly enhanced root growth of cauliflower seedlings, compared to both the positive (*CC*) and negative (water) controls (Figure 3.7), and this was confirmed by the statistical analysis of pooled and averaged values (Table 3.7). However, *SS* at a concentration of 0.5 mg L^{-1} significantly inhibited root growth at all incubation intervals.

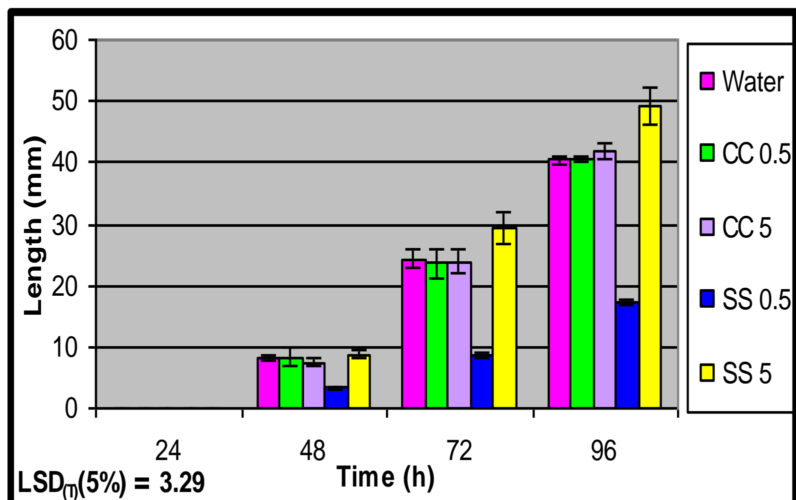

Figure 3.7: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the root growth of cauliflower seedlings. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.7: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the root growth of cauliflower seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control.

Time	**Effect on root growth and interactions between treatments over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	0	0
48 h	8.12	8.2	7.5	3.3	8.6
72 h	24.1	23.7	23.7	8.8	29.6
96 h	40.4	40.6	42.5	17.4	49.6

Differences are significant at LSD $_{(T)}$ (5%) =3.29; The ANOVA is attached as Table 3.7A in the Appendix

3.5.4.2 Cabbage

As shown in Figure 3.8, *SS* at a concentration of 0.5 mg L^{-1} was the only treatment that had a significant enhancing effect on the percentage germination of cabbage seeds over the full duration of the incubation period, as compared to both controls (also see the statistical analysis of pooled and averaged data in Table 3.8). However, *SS* at a ten-fold higher concentration of 5 mg L^{-1} had a

significant inhibiting effect on the germination of cabbage seeds, especially after 96 h of incubation, as compared to both controls. The commercial bio-stimulant, *CC,* had no significant effect.

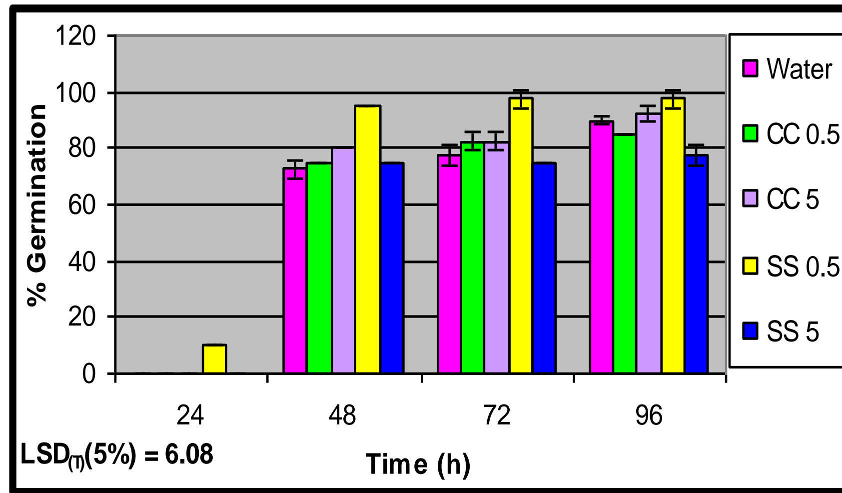

Figure 3.8: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the percentage germination of cabbage seeds. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.8: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the percentage seed germination of cabbage seeds at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control

Time	**Effect on seed germination and interactions between treatments over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	10.0	0
48 h	72.2	75.0	80.0	95.0	75.0
72 h	77.2	82.8	82.2	97.8	75.0
96 h	88.8	85.0	91.8	97.8	77.8

Differences are significant at LSD $_{(T)}$ (5%) =6.08; The ANOVA is attached as Table 3.8A in the Appendix

At 96 h of incubation the 0.5 mg L^{-1} *SS* treatment as well as treatment with both concentrations of *CC* significantly enhanced the coleoptile growth of cabbage seedlings while the 5.0 mg L^{-1} *SS* treatment significantly inhibited growth. (Figure 3.9; Table 3.9).

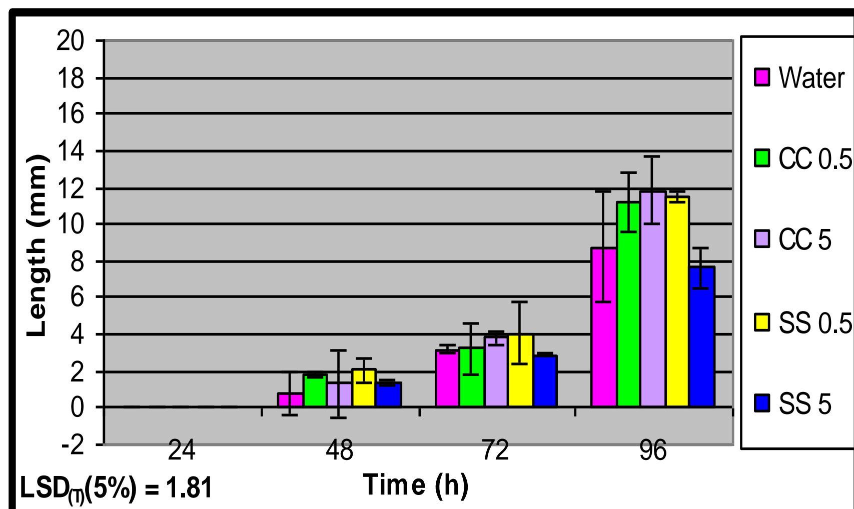

Figure 3.9: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the coleoptile growth of cabbage seedlings. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.9: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the coleoptile growth of cabbage seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control.

Time	Effect on coleoptile growth and interactions between treatments -over time				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	0	0
48 h	0.9	1.8	1.1	2.1	1.4
72 h	3.2	3.1	3.8	4.2	2.9
96 h	8.5	11.1	11.7	11.5	7.7

Differences are significant at LSD $_{(T)}$ (5%) =1.81; The ANOVA is attached as Table 3.9A in the Appendix

Exactly the same tendency of *SS* and *CC* treatments to significantly enhance root growth, as was seen for coleoptile growth, prevailed at the 96 h incubation interval except that both *SS* concentrations had the same stimulating effect (Figure 3.10). Further, these significant differences were already observed at the 72 h incubation interval compared to the untreated control. Statistical

analysis of pooled and averaged data confirmed that the 0.5 mg L^{-1} *SS* and *CC* treatments also differed significantly in favour of the former (Table 3.10).

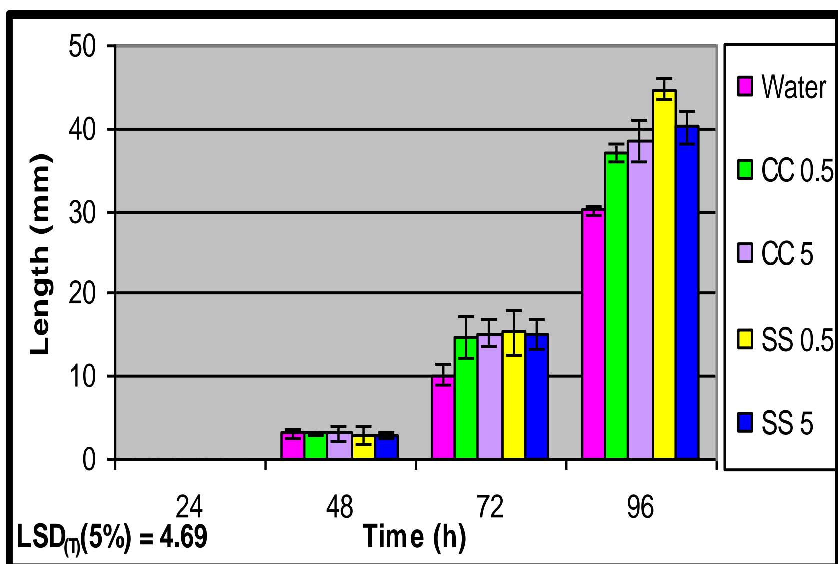

Figure 3.10: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the root growth of Cabbage seedlings. A commercially available bio-stimulant, *ComCat® (**CC**)*, was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.10: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the root growth of cabbage seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control.

Time	Effect on root growth and interactions between treatments -over time				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	0	0
48 h	3.0	3.1	3.2	3.0	3.0
72 h	10.3	14.6	15.4	15.6	14.9
96 h	30.2	36.9	38.2	45.6	39.7

Differences are significant at LSD $_{(T)}$ (5%) = 4.69; The ANOVA is attached as Table 3.10A in the Appendix

3.5.4.3 Lettuce

Only the commercial bio-stimulant *CC* , at a concentration of 0.5 mg L^{-1}, significantly enhanced the germination of lettuce seeds over the full duration of incubation while *SS,* applied at both 0.5 and 5.0 mg L^{-1}, slightly inhibited seed germination during the first 24 h of incubation (Figure 3.11; Table 3.11). The latter was not observed during later incubation intervals.

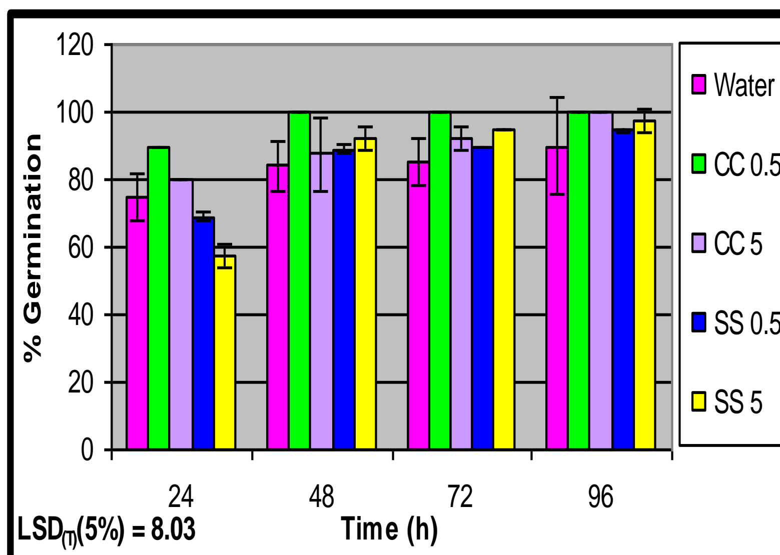

Figure 3.11: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the percentage germination of lettuce seeds. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.11: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the germination of lettuce seeds at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control.

Time	**Effect on seed germination and interactions between treatments -over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	74.4	90.0	80.0	69.1	57.8
48 h	84.4	100.0	86.6	89.1	92.2
72 h	85.6	100.0	92.2	90.0	95.0
96 h	91.3	100.0	100.0	94.6	97.8

Differences are significant at LSD $_{(T)}$ (5%) = 8.03; The ANOVA is attached as Table 3.11A in the Appendix

In agreement with previous results, different concentrations of *CC* and *SS* had varied effects on the coleoptile growth of lettuce seedlings. Both *CC* and *SS* applied at the higher concentrations of 5 mg L^{-1} tended to inhibit coleoptile growth at all incubation intervals while the lower concentration of 0.5 mg L^{-1} significantly enhanced coleoptile growth of lettuce seedlings in both cases especially at the 96 h incubation interval (Figure 3.12) as was confirmed by the statistical analysis of pooled and averaged data (Table 3.12).

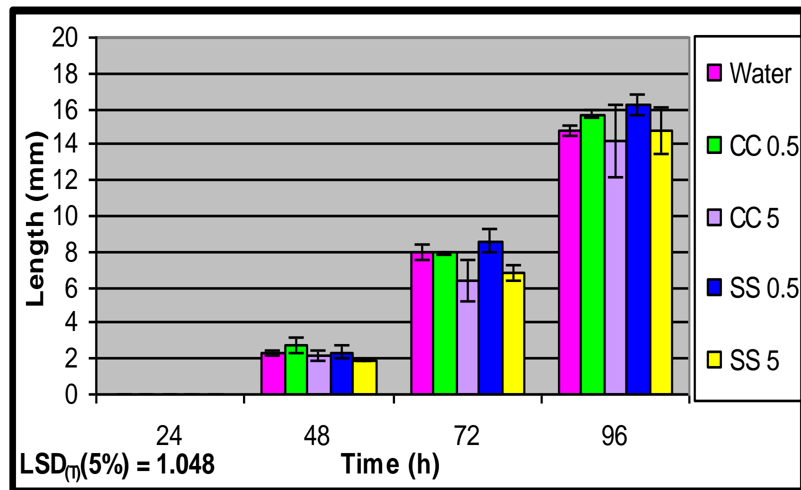

Figure 3.12: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the coleoptile growth of Lettuce seeds. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.12: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the coleoptile growth of lettuce seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control.

Time	Effect on coleoptile growth and interactions between treatments -over time				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	0	0
48 h	2.4	2.8	2.2	2.4	1.9
72 h	7.9	7.9	6.4	8.6	6.8
96 h	14.7	15.7	14.3	16.2	14.7

Differences are significant at LSD $_{(T)}$ (5%) = 1.048; The ANOVA is attached as Table 3.12A in the Appendix

Compared to the water control, both *CC* and SS significantly enhanced the root growth of lettuce seedlings at a concentration of 0.5 mg L^{-1} at 96 h of incubation (Figure 3.13) while the higher concentration either had no effect or the effect was non significant. Statistical analysis of the pooled and averaged values showed no significant differences between treatments at the earlier incubation intervals (Table 3.13) except at 72 h where *SS* was the only treatment that showed a significant enhancing effect on root growth of lettuce seedlings.

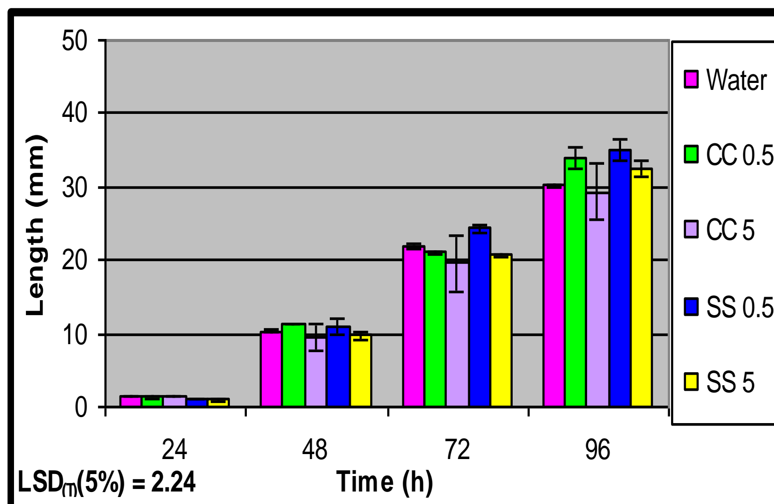

Figure 3.13: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the root growth of lettuce seedlings. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.13: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the root growth of lettuce seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control.

Time	**Effect on root growth and interactions between treatments -over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	1.4	1.5	1.6	1.1	1.0
48 h	10.4	11.4	9.8	11.1	9.7
72 h	21.9	21.0	19.9	24.3	20.7
96 h	30.3	33.9	29.7	35.0	32.2

Differences are significant at LSD $_{(T)}$ (5%) = 2.24; The ANOVA is attached as Table 3.13A in the Appendix

3.5.4.4 Beans

Only treatment with *SS* had a significant enhancing effect on the germination of bean seeds at 72 h and 96 h incubation intervals compared to the untreated control while the effect of the two concentration applications did not differ (Figure 3.14). Compared to the positive control, the 5 mg L^{-1} *SS* treatment differed significantly in terms of the germination of bean seeds at the 72 h incubation interval (Table 3.14).

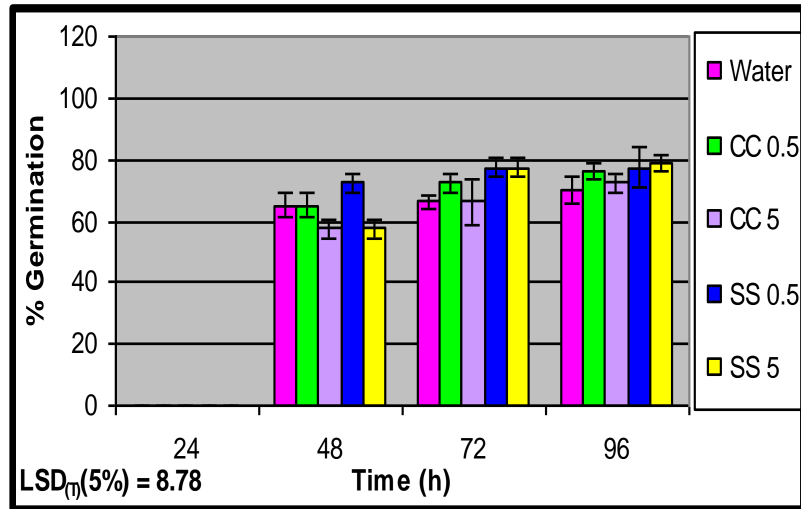

Figure 3.14: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the percentage germination of bean seeds. A commercially available bio-stimulant, *ComCat® (**CC**)*, was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.14: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the percentage germination of bean seeds at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control.

Time	**Effect on seed germination and interactions between treatments -over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	0	0
48 h	65.0	65.0	57.5	72.5	57.5
72 h	66.3	72.5	66.3	77.5	77.5
96 h	70.0	76.3	72.5	77.5	78.8

Differences are significant at LSD $_{(T)}$ (5%) = 8.78; The ANOVA is attached as Table 3.14A in the Appendix

Neither the *CC* nor the *SS* treatments had any significant effect on the coleoptile growth of bean seedlings (Figure 3.15; Table 3.15).

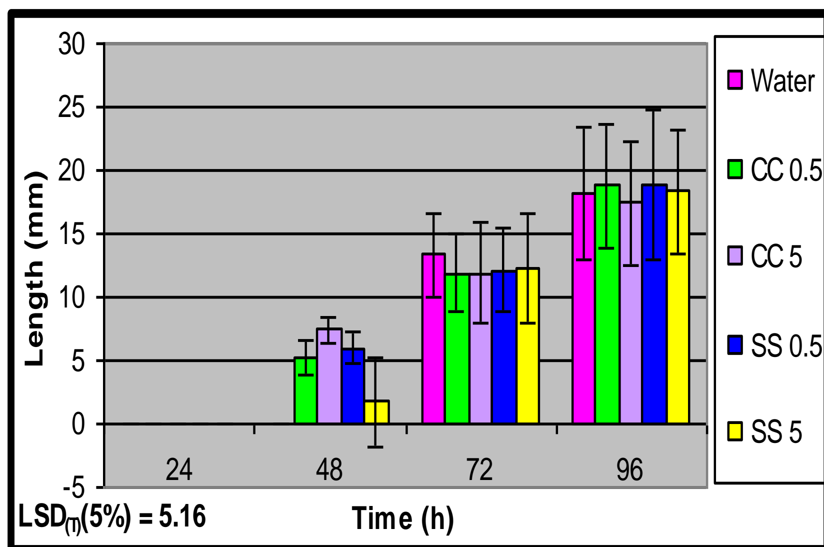

Figure 3.15: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the coleoptile growth of bean seedlings. A commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by $LSD_{(T)}$ (5%) values in the graph.

Table 3.15: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the coleoptile growth of bean seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control

Time	**Effect on coleoptile growth and interactions between treatments -over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0.0	0.0	0.0	0.0	0.0
48 h	0.0	5.2	7.0	5.8	2.1
72 h	12.9	11.0	10.5	14.4	12.5
96 h	16.9	19.6	17.8	19.1	19.9

Differences are significant at $LSD_{(T)}$ (5%) = 5.16; The ANOVA is attached as Table 3.15A in the Appendix

Only the 0.5 mg L^{-1} *CC* treatment had a significant enhancing effect on the root growth of bean seedlings, and also only at the 72 h incubation interval, while all other treatments either had no effect or tended to inhibit root growth (Figure 3.16; Table 3.16).

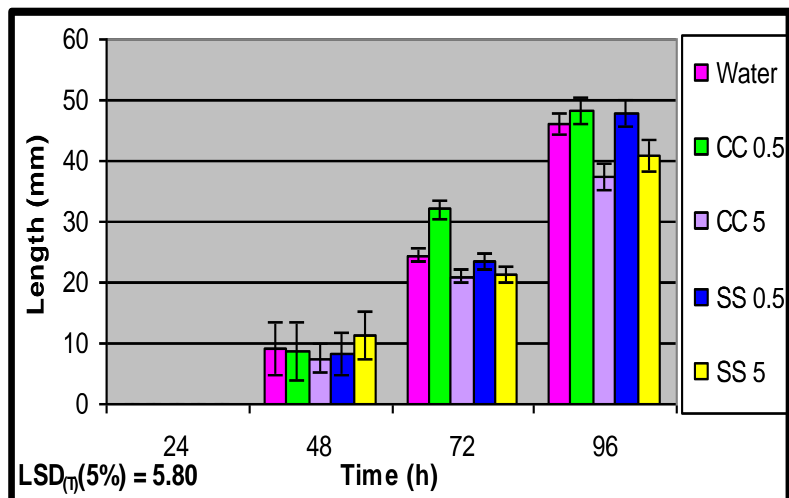

Figure 3.16: The effect of a solubilized seed suspension of *Lupinus albus* (**SS**) at different concentrations [0.5mg L^{-1} and 5mg L^{-1} distilled water] on the root growth of bean seedlings. A commercially available bio-stimulant, *ComCat® (**CC**)*, was used as a positive control and water as a negative control. Statistical significance is indicated by LSD $_{(T)}$ (5%) values in the graph.

Table 3.16: Statistical analysis of the interaction between averaged values for the effect of treatments (*CC* and SS) on the root growth of bean seedlings at different time intervals over a 96 h incubation period as influenced by different concentrations and as compared to the water control

Time	**Effect on root growth and interactions between treatments -over time**				
	Water	***CC* 0.5**	***CC* 5**	**SS 0.5**	**SS 5**
24 h	0	0	0	0	0
48 h	9.25	8.74	7.50	8.30	11.37
72 h	24.41	32.08	21.03	23.54	21.36
96 h	45.92	48.12	37.49	47.65	40.78

Differences are significant at LSD $_{(T)}$ (5%) = 5.80; ANOVA is attached as Table 3.16A in the Appendix.

Although beetroot, carrots, wheat and tomatoes were also tested the seed suspension had no significant effect on these seeds (Results not shown). According the results mentioned above it became clear that the application concentration seems to be effective at both 0.5 and 5 mg L^{-1}, depending on the bio-tests used and the type of organism applied to.

3.6 DISCUSSION

Bio-assays employed in a preliminary screening programme to test for bio-stimulatory properties of a finely ground and solubilized *Lupinus albus* seed suspension (SS) included its effect on the respiration rate of monoculture yeast cells as well as seed germination and seedling growth of a number of crops by comparing to an untreated water control. A commercial natural bio-stimulant, *ComCat*® (CC), was used as a positive control. As the active substance(s) of the *L. albus* seed suspension as well as its optimum concentration was unknown, a concentration range (0.05, 0.5, 5.0 and 50.0 mg L^{-1}) was tested under laboratory conditions. Although the optimum concentration of *CC* is 0.5 mg L^{-1} according to the manufacturers (Agraforum, Germany), it was also tested at the same concentration range as for SS.

Bakers yeast, *Saccharomyces cerivisiae*, was used in the respiration rate test because of its simple nutritional needs. Its usefulness is further based on its ability to convert sugars and other carbon sources into ethanol and carbon dioxide (Amthor, 1989) in the absence of oxygen (anaerobic) while the carbon dioxide released is stoichiometric with each molecule of glucose metabolized. This supplied a simple screening procedure to ascertain whether the active compounds contained in the *L. albus* seed suspension (SS) possessed the potential to manipulate respiratory metabolism in any way. Indeed respiration rate measurements on monoculture yeast cells revealed that both the 5 mg L^{-1} and 50 mg L^{-1} SS concentrations increased the rate significantly compared to the untreated control and also compared favourably with the positive control, CC, even at its optimum concentration of 0.5 mg L^{-1}.

The rationale behind this methodology is that, in the event of the respiration rate of a test organism being affected by an externally applied plant extract, either a stimulatory or an inhibitory growth response is possible (Thain and Hichman, 2000) and this had to be verified. For this purpose the effect of the extracts on both the germination of seeds and subsequent seedling growth were monitored. The results confirmed a stimulatory growth response as both root and coleoptile growth of Cress seedlings were enhanced over a period of 96 hours compared to the untreated control

Treatment of Cress, lettuce and bean seeds with a concentration range of the *L. albus* seed suspension indicated that 5 mg L^{-1} was the optimum concentration as the germination of seeds from

all of these crops was stimulated either markedly or significantly compared to the water control. Both elevated and lower concentrations than the optimum inhibited seed germination in these crops while germination of cabbage seeds was stimulated significantly by the rather low concentration of 0.5 mg L^{-1}. SS treatment had no significant effect on the percentage germination of beetroot, carrot, wheat, tomato and garden turnip seeds (results not shown) while the positive control, *CC*, had virtually no effect on the germination of seeds from any of the crops tested. The latter was in line with previous findings of the manufacturers (Hüster, personal communication)[1] namely that *CC* had little effect on seed germination but rather on seedling growth. Recently, Channal *et al.* (2002) obtained similar results when they tested the bio-stimulatory effects of fresh leaf extracts of seven tree species on seed germination and seedling growth of sunflower and soybean. None of the extracts showed significant effects on germination, but seedling growth was stimulated significantly.

The germination data strongly indicated that the seeds of different crops reacted differently to treatments with SS suggesting that different mechanisms of action might be involved; an aspect that needs further investigation. Subsequently, the influence of the *L. albus* seed suspension on the seedling growth of these crops were monitored in terms of both coleoptile and root growth. From the acquired data it became clear that different crops once again reacted differently to treatment with both SS and *CC*, and especially to different concentrations, in terms of coleoptile and root growth. For instance, a 5 mg L^{-1} SS concentration contributed to optimal coleoptile and root growth induction in Cress and cauliflower seedlings while a 0.5 mg L^{-1} concentration was optimal for cabbage and lettuce seedlings. Comparable results were obtained by Neelam *et al.* (2002) who showed that lower concentrations of aqueous extracts of fresh leaves, flowers and pods of *Leucaena leucocephala* stimulated the seed germination and seedling growth of *Triticum aestivum* while elevated concentrations had the opposite effect. Also Terefa (2002) demonstrated that only rather low concentrations of flower, root and stem extracts of *Parthenium hysterophorus*, a weed, had a stimulatory effect on seedling growth of tef (*Eragrostis tef*). Fruit and seed extracts from *P. juliflora* exhibited greater phytotoxicity than extracts from the other plant parts on seed germination and seedling growth of eight test plants (Noor *et* al., 1995). Moreover, it is interesting to note that neither of the two SS concentrations that had a stimulatory effect on the seedling growth of some of the test crops showed any effect on the growth of bean seedlings, the only other legume tested. This

[1] Hüster, T. Agraforum AG, Poststrasse 1, Bomlitz 29699, Germany

indicates that active substances extracted from plants might not have manipulatory or allelopathic effects on plants of the same species. From a practical application perspective, future research is needed to verify this aspect.

Although to different extents, seed treatment with SS had a more pronounced effect on both germination and subsequent seedling growth of the crops tested than did the positive control *ComCat*® (*CC*). The latter, for instance, inhibited both coleoptile and root growth in Cress seedlings even at the optimal concentration of 0.5 mg L^{-1} whereas the SS treatment either had no effect (beans) or stimulated seedling growth at either 0.5 or 5 mg L^{-1} concentrations in most of the test crops. Further, where *CC* stimulated coleoptile growth in cauliflower and cabbage, both belonging to the Brassicaceae family, it had virtually no effect on the growth of lettuce and bean seedlings. On the other hand *CC* stimulated root growth most in cabbage and lettuce while only slightly in bean seedlings. Studies on tomatoes revealed that the application concentration of brassinosteroids can have diverse effects on the growth behaviour of seedlings. Seeds soaked in a brassinosteroid concentration of 0.2 ppm resulted in the highest germination percentage, while seedlings from seeds soaked in 0.9 ppm exhibited the highest shoot and root length (Prakash *et* al., 2001). Further, Takeuchi *et al*. (1996) reported that BRs promoted germination of not only crop seeds but also the seeds of weeds and parasitic plants.

With the above the earlier observations regarding the different responses of different crops to seed treatment with natural bio-stimulants was confirmed. In this regard Aliotta *et al*. (1999) reported that an extract from *Ruta graveolens* contained a coumarin with a potent inhibiting effect on seed germination while Basile *et al*. (2000) demonstrated the inhibiting effect of a coumarin in a leaf extract of *Cucurbitus sativa* on the seed germination of some crops while stimulating both seed germination and seedling growth in other crops. At this point it is necessary to remark that seed scientists are aware of the fact that seed germination and seedling growth tests can be very demanding due to rather large standard deviations that can be acquired between replicates as well as the difficulty to arrive at conclusions. In this light, and although further research is necessary to test the reaction of a variety of crops to seed treatment with the natural bio-stimulants under scrutiny, the results obtained in this study supplied the rationale to test the response of crops to foliar sprays with these bio-stimulants under field conditions (chapter 4).

Natural compounds in plants are the result of either primary or secondary metabolism. During normal cell respiration not all of these compounds are oxidized completely to carbon dioxide and water, but many form part of an array of intermediates that act as building blocks of biosynthesis during anabolic metabolism (Amthor, 1989). Some of these compounds might even act as bio-active molecules within the plant itself or, on exudation, might affect other organisms in the environment allelopathically (Harborne, 1988). The phenomenon of allelopathy encompasses all types of chemical interactions among plants including growth inhibition or stimulation. Moreover, it has been shown that foliar sprays of plant extracts on other plants can have similar effects (Rizvi and Rizvi, 1992) confirming the manipulation potential of natural plant extracts as well as its potential to be developed as natural products for application in the agricultural and horticultural industries.

In conclusion, it was rather interesting to note that SS had a more pronounced effect on root growth than on coleoptile growth of seedlings following seed treatment of different crop plants. This is in agreement with the findings of Turk and Tawaha (2003) who reported that water extracts of some plants known for their allelopathic influence on the environment were more inclined to stimulate radicle than either coleoptile or shoot growth. Chung and Miller (1995) were of the opinion that the latter might be expected as roots are the first to come into contact with natural allelochemicals following absorption from the soil. Although the latter is not applicable to seed treatments, it seemed important to verify the possibility that translocation of the active substances might become an issue. For this reason, and since seedlings treated with these natural bio-stimulants theoretically have a better chance of either establishing on their own or being transplanted more successfully (Balestri and Bertini, 2003), the effect of foliar applications to recently transplanted seedlings on growth and yield were monitored (chapter 4).

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CHAPTER 4

INFLUENCE OF A *Lupinus albus* SEED SUSPENSION ON YIELD AND YIELD COMPONENTS OF DIFFERENT CROPS UNDER FIELD CONDITIONS

4.1 ABSTRACT

A seed suspension (SS) of *Lupinus albus*, was tested and compared to a commercially available bio-stimulant, *ComCat*®, in terms of its potential to increase the yield of different crops under field conditions. *ComCat*® served as a positive control while basic fertilizer served as a negative control in all field trials. In some cases *ComCat*® and SS were also tested in combination. All trials were laid out in a complete randomized block design while different yield parameters were measured. The results indicated that the effect of *SS* on yield was concentration dependent. Under field conditions a concentration of 5 mg L^{-1} was optimal for most vegetables, flowers, maize and wheat. Flower budding was significantly ($P<0.05$) enhanced in Gazanias but, although the SS treatment increased flowering in Impatience, this was non-significant. The dry kernel yield of maize under rain fed conditions as well as that of wheat under semi-irrigation was also enhanced by foliar spray treatment with SS, but this was only significant in the case of maize. The most significant yield enhancement, after treatment with SS, was observed in beetroot, lettuce and carrots under drip irrigation. Beetroot bulb, lettuce head and carrot yields were significantly ($P<0.05$) increased by 9.3, 20.0 and 24.3 ton ha^{-1} respectively. The results confirmed the bio-stimulatory potential of a *Lupinus albus* seed suspension (SS) initially observed under laboratory conditions.

Keywords: *Lupinus albus*, seed suspension, bio-stimulatory properties, yield, field trials

4.2 INTRODUCTION

Extracts from several crops have been reported to show strong allelopathic effects towards other crops and these include *Avena sativa, Brassica juncea, Glycine max* and *Medicago sativa* (Blum *et al.,* 1992). Various parts of these plants are known to synthesize compounds that are released into the environment mainly through leaching and decomposition of crop residues. As a result, an

elevated interest in the potential to manipulate crops by the exogenous application of extracts from plants with strong allelopathic characteristics has been shown over the past two decades.

Cruz-Ortega *et al*. (1988) reported that an ethanolic extract of corn pollen inhibited the growth of watermelon (*Citrullus lanatus*). Parasitic angiosperms of the genus *Orobanche* (commonly known as broomrapes) affect important dicotyledonous crops such as legumes, tomato, tobacco and sunflower, causing significant yield losses (Galindo *et al*., 2002). Allelopathy has also been experienced as a problem where either no or reduced tillage practices were applied as well as under stubble mulch farming where residues are left in the field after harvesting/thrashing in order to protect soil from wind and water erosion. Upon decomposition, these plant materials release allelochemicals that accumulate in the soil and that can have an effect on crop productivity via manipulation of metabolic events (Putnam, 1994). According to Singh *et al*. (2001) these allelochemicals can either inhibit or promote growth depending on the application concentration.

What has to be established through research is whether the application of extracts from these plants can be applied in the agricultural practice to manipulate metabolic events in crop plants, leading to either enhanced growth or yield or negative physiological changes within weed plants, leading to its bio-control. Einhellig (1996) reported that the amount of allelochemicals in plants is substantially elevated under biotic and abiotic stress conditions such as mineral deficiency, UV radiation and even herbicide treatment. The latter makes it difficult to manage the allelopathic manipulation of crop plants since critical concentrations of these allelochemicals can become an issue. Subsequently, the author suggested that, in agro-ecosystems, the effect of exogenously applied allelochemicals or their residues on crop plants can be minimized if: a) the traditional practice of crop rotation is revived with a new approach, b) crop residues are managed effectively in such a way that their effect on crops is minimized while harmful weeds are still suppressed selectively, c) crops are protected against the stress conditions so that the amount and extent of impact of allelochemicals is reduced, d) efforts are made to understand the mechanism of action of allelochemicals on other plants as well as their fate in soil and e) allelochemicals are applied in a controlled manner by using plant extracts developed as new natural products.

Although the suggestions of Einhellig (1996) is rather suppressing in terms of the potential to apply the allelopathy concept in the agricultural practice as a means to manipulate crops to the advantage of man, some promising results have been published in this regard. Ferreira and Lourens (2002) reported on the yield improving effect of a liquid seaweed extract (now trading as a natural product under the name Kelpak®) in canola, and this must be regarded as a significant contribution to the agricultural industry. Kelpak®, applied singly or in combination with the herbicide clopyralid at various growth stages of canola (*Brassica napus*; cultivars Monty and Dunkeld), were assessed in a field experiment conducted in Langgewens and Tygerhoek, South Africa during the 1998/1999 growing season. A foliar application of 2 litres ha^{-1} at the four-leaf growth stage significantly increased the yield of the crop at Langgewens. In Tygerhoek, application at the same rate significantly increased canola yield when applied at either the three or five-leaf growth stages, but the response was more consistent to treatment at the three-leaf growth stage.

Recently, a natural bio-stimulant, *ComCat*®, was developed and commercialized in Germany. The active substances of this natural product was identified as 24-episescasterone and 24-epi-castasterone, both belonging to the brassinosteroid (BS) phytohormone group discovered almost two decades ago and certified as plant growth stimulators (Khripach *et al*., 2000). Along with growth promotion, BS has an effect on plant development, in particular reproduction, maturation, senescence and seed as well as fruit yield. An important feature of BS is their ability to also increase the quality of crops (Prusakova *et al*., 1999a).

In light of an ever increasing world population and its possible effect on food security predicted for the future, a need to increase crop yields in future on available arable land (Heidhues, 2001) exists. The latter also prioritises and supplies a rationale for future natural product research, especially environmentally friendly natural bio-stimulants that have the potential to improve crop yields. As *ComCat*® is a product from seeds of European plants, this study was aimed at investigating seeds from South African plants for similar bio-stimulatory activities.

Preliminary screening of wild and domesticated South African plants for bio-stimulatory activity revealed the above average potential of a *Lupinus albus* seed suspension (*SS*) in this regard. In chapter 3 the potential of *SS* to promote seedling development in terms of coleoptile and root

growth was statistically confirmed. In this chapter the aim was to ascertain whether a foliar application of *SS* had the potential to enhance the growth and yield of economically important agricultural and horticultural crops under field conditions as a means to assess its application potential in the agricultural industry and to supply a rationale for the isolation and identification of the active substances involved. The commercial bio-stimulant, *ComCat*®, was used in comparison as positive control at the optimum concentration of 100 g ha^{-1} recommended by the manufacturers (Agraforum, Germany). *ComCat*® instead of the seaweed extract Kelpek® was used as a positive control in this study as the product was developed from seed extracts of land plants that compared directly to the seed suspension of *Lupinus albus* tested in this monograph.

4.3 MATERIALS AND METHODS

4.3.1 Plant material

Seeds of different crop plants [Gazanias (*Gazania krebsiana*), Impatiens (*Impatiens balsamina*), maize (*Zea mays*), wheat (*Triticum vulgare*), beetroot (*Beta vulgaris*), lettuce (*Lactuca sativa*), cabbage (*Brassica pekinensis*) and carrots (*Daucus carota*)] used in field trials were locally purchased from SENWES, Stark Ayres or Mayford (South Africa) during the 2002/2003 growing season.

4.3.2 Description of trial sites

All vegetable and flower trials were conducted on the experimental farm of the Department of Soil, Crop and Climate Sciences, University of the Free State, near Kenilworth in the Bloemfontein district (29°01’00”S, 26°08’50”E). Maize and carrot trials were conducted on a farm, 20 kilometres north, while that of wheat on a farm 40 kilometres west of the experimental farm.

4.3.3 Experimental design and trial layout

In all cases trials were laid out in a complete randomized block design. Plot sizes differed for the different crops according to the prescribed in row and between row spacing suggestions of the seed

merchant (Hygrotech) and the Agricultural Research Council (ARC, 2004). A summary of trial specifics is supplied for each crop in Tables 1, 2 and 3.

Table 1: Flower trial production guide

Flower	Gazanias	Impatience
Replications	3	3
Between row spacing	12.5 cm	12.5 cm
In row spacing	12.5 cm	12.5 cm
Plants/ha	40 000	40 000
Fertilzer/ha: N	40 kg ha^{-1}	40 kg ha^{-1}
P	20 kg ha^{-1}	20 kg ha^{-1}
K	0 kg ha^{-1}	0 kg ha^{-1}

Table 2: Grain trial production guide

Crop	Maize	Wheat
Cultivar	PAN 6043	PAN 3377
Replications	4	4
Between row spacing	2.4 m	-
In row spacing	27 cm	45 cm
Plants/ha	17 050	20-25kg seed
Yield potential	3.6 ton ha^{-1}	2.5 ton ha^{-1}
Fertilizer/ha: N	70 kg ha^{-1}	45-60 kg ha^{-1}
P	15-20 kg ha^{-1}	15 kg ha^{-1}
K	0 kg ha^{-1}	7-8 kg ha^{-1}

Table 3: Vegetable trial production guide

Vegetable	Beetroot	Lettuce	Cabbage	Carrots
Cultivar	Detroit dark red	Great Lakes	Drumhead	Snakpak
Replications	3	3	3	4
Between row spacing	30 cm	35 cm	65cm	20cm
In row spacing	10 cm	25 cm	45 cm	5 cm
Plants/ha	333 333	125 000	57 142	2 million
Yield potential	25-40 ton	25-30 ton	60 ton	70 tons
Fertilizer/ha: N	72.5 kg ha^{-1}	67.5 kg ha^{-1}	204 kg ha^{-1}	800 kg 2:3:2 ha^{-1}
P	12.5 kg ha^{-1}	8.5 kg ha^{-1}	36 kg ha^{-1}	-
K	172.5 kg ha^{-1}	121.5 kg ha^{-1}	216 kg ha^{-1}	-

4.3.4 Treatments

4.3.4.1 General

All crops were separately treated with a solubilized *Lupinus albus* seed suspension and a commercially available bio-stimulant *ComCat*® (positive control) by means of foliar applications while normally fertilized plots served as a negative control. In some cases *ComCat*® and *SS* were applied in combination. *ComCat*® was applied, according to the specifications of the manufacturers (Agraforum, Germany), at 100 g ha^{-1} and at approximately 600 L ha^{-1} for flowers and all vegetables while half the concentration rate, 50 g ha^{-1}, applied for maize. A slightly lower volume of water (approximately 500 L ha^{-1}) was used for foliar treatment of maize while a slightly higher volume (approximately 800 L ha^{-1}) applied for wheat.

Preliminary laboratory results (see Chapter 3) showed that a concentration of 5 mg L^{-1} was optimal for SS in enhancing seedling growth of a number of crops. Subsequently, SS was applied at 5 mg L^{-}

^{1} 10 m^{-2} as one liter covered approximately 10 m^2 in a fine spray mist. A thousand liters of water per hectare were used to apply SS as a foliar spray on all crops. In all cases a wetting agent, Solitaire, was added to the SS suspension in a ratio 1:1000 (v/v).

All plots received standard fertilizer at planting (see tables 1, 2 and 3) while standard disease and insect control measures were applied for each crop. Wheat was semi-irrigated using a sprinkler system, maize was cultivated under rain fed conditions while all vegetable crops as well as flowers were fully irrigated using a drip irrigation system.

4.3.4.2 Application specifications of treatments applied during the growing season for flowers and different crops

4.3.4.2.1 Flowers

Flowers were treated at the three leaf growth stage and additional treatments applied at four week intervals, with a maximum of four applications, for both Gazanias and Impatience. The application concentrations were the same for both flowers (see 4.2.4.1). For both flowers 1000 L water ha^{-1} was applied.

4.3.4.2.2 Maize and Wheat

Maize and wheat were treated only once at the three leaf growth stage. For optimum concentrations see 4.2.4.1. As a result of differences in plant stands the amount of water used to cover wheat and maize differed. For wheat 833 and for maize 509 L water ha^{-1} was applied.

4.3.4.2.3 Beetroot and carrots

Both beetroot and carrots were treated twice namely at the three to four leaf growth stage (stage 14 of principal growth stage 1; Meier, 1997) and at 30% root growth (stage 39 – 43 of principal growth stage 4; Meier, 1997). The latter stage is when the root has reached 30% of its expected growth or has grown in thickness to more than 0.5 cm. For both beetroot and carrots 600 L water ha^{-1} was applied.

4.3.4.2.4 Lettuce and cabbage

Both lettuce and cabbage received four foliar applications. The first was at the three leaf growth stage (stage 13 of principal growth stage 1; Meier, 1997) followed by three applications at two week intervals. For both lettuce and cabbage 600 L water ha^{-1} was applied.

4.3.5 Quantification of flower responses and crop yields

4.3.5.1 Flowers

The response of Gazanias and Impatience to treatments was quantified by counting the number of flowers produced in two-week intervals. During counting, dead buds as well as newly produced flowers were noted. Although each plot contained the same number of plants, the length of the flowering period differed between species. For this reason Gazanias were counted over a period of 24 weeks and Impatience over a 12 week period.

4.3.5.2 Wheat

Twenty ears per replicate were harvested at random, after completion of the drying cycle, from plants that did not form part of the area harvested to determine the total kernel yield, and the dry weights determined. Subsequently, dry kernels were removed from the ears, counted and weighed.

To obtain the total kernel yield, a 15 m^2 area (1.5 m x 10 m) of each replicate was harvested by means of a combine harvester. This area was chosen in the inner part of each plot to avoid any possible side effects. The final yield was expressed in ton ha^{-1}.

4.3.5.3 Maize

Maize cobs from twenty plants per row only from the middle three rows (60 cobs per replicate) were harvested by hand after completion of the drying cycle and when kernels contained 12% moisture. Subsequently, cobs were dehusked by means of a mechanical Sheller while care was taken that none of the kernels went astray. The dry kernel weight for each replicate was determined

separately and expressed as ton ha^{-1} on the basis of an average plant count of 17 400 plants per hectare.

4.3.5.4 Vegetables

4.3.5.4.1 Beetroot

Whole plants were removed by hand using a pitchfork and the bulbs separated from the foliage by means of a sharp knife. The fresh weight of bulbs and aerial parts were determined separately and expressed in ton ha^{-1}.

4.3.5.4.2 Lettuce and cabbage

Whole plants were removed by hand from each plot using a pitchfork and the heads separated from the foliage by means of a sharp knife. The fresh weight of heads and foliage were determined separately and expressed in ton ha^{-1}.

4.3.5.4.3 Carrots

Twenty carrots per replicate were selected at random from the small statistical trial and removed from the soil by hand. Subsequently, the root and foliage were separated, the fresh weights determined and the yield expressed in ton ha^{-1}. Additionally, the lengths of each carrot was measured and noted.

However, the plant stand of carrots per hectare is determined by the mass of seeds planted and usually runs into the millions. The use of only twenty carrots per plot can, therefore, be subject to criticism from a commercial farming perspective. For this reason a second large scale trial was conducted on a farm twenty kilometres North of Bloemfontein, where half a hectare was treated with SS while the other half served as an untreated control, in order to compare the result obtained with the smaller statistical trial. The bulk of carrots were harvested using a commercial harvester. The total root yield was determined using a commercial scale and the yield was expressed in ton ha^{-1}. In the latter instance no statistical analysis could be performed.

4.3.6 Statistical analysis

Analysis of variance (ANOVA) was performed on the data, using the NC: SAS Institute Inc., Dos Statistical Programme to identify differences between treatments. Tukey-Kramer's LSD (least significant difference) procedure for comparison of means (Steele and Torrie, 1980; Mason *et al*., 1989) was applied to separate means ($P<0.05$). Treatments differing significantly were indicated either in figures or below tables as calculated LSD values and by using symbols for significant (*) and insignificant (ns) differences.

4.4 RESULTS

4.4.1 The response of flowers to a foliar spray treatment with a *L. albus* seed suspension (SS) under irrigation

4.4.1.1 Gazanias

A direct comparison of the number of Gazania flowers counted on a two weekly basis, as a response to the different treatments, revealed statistically significant ($P<0.05$) differences between the treatments and the untreated control (Figure 4.1, LSD-value indicated in the graph). Over a 24 week period, two distinct flowering flurries reaching peaks at week 12 and week 20 were observed. The *CC* treatment showed a strong tendency to enhance flower formation up to the twelve week growth stage during the first flurry, while treatment with SS showed the same tendency only at later growth stages during the second flurry. Interestingly, the same tendency to increase the number of flowers up to the twelfth week as was observed for the *CC* treatment was observed where *CC* and SS were applied in combination but, this was not the case between weeks 16 and 20.

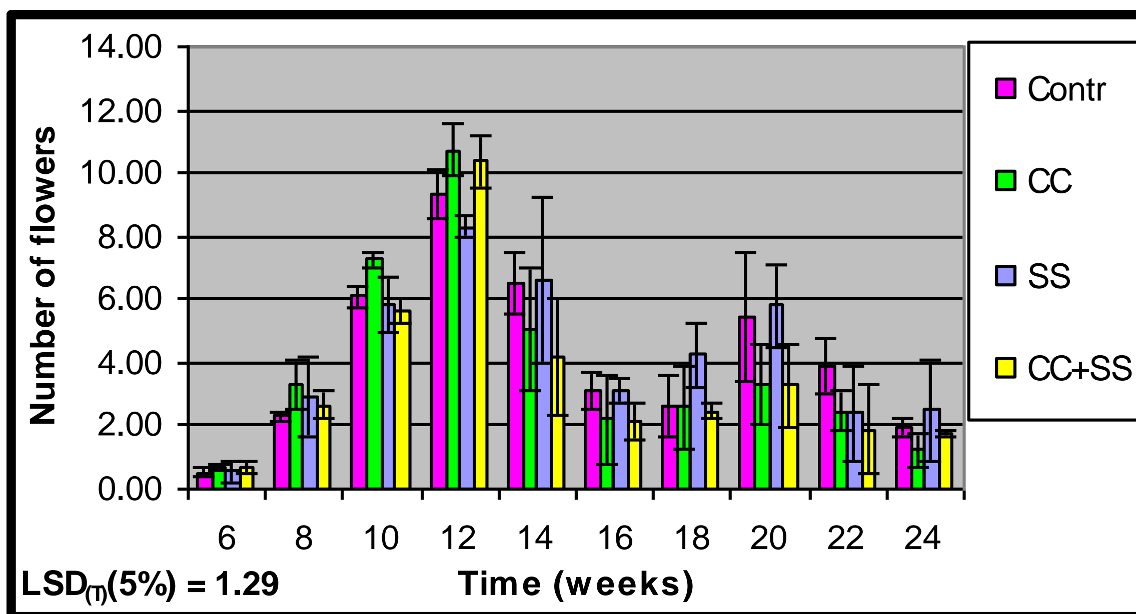

Figure 4.1: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray at a concentration of 5 mg L^{-1} on the flowering response of *Gazania*. A commercial bio-stimulant, *ComCat® (**CC**)*, was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.1A in the Appendix.

4.4.1.2 Impatience

In terms of the flowering response of Impatience to exogenously applied SS, and as compared to both the negative (untreated) and positive (*CC*) controls, marked but non-significant (P<0.05) differences were observed (Figure 4.2; LSD-value indicated in the graph). Foliar applications of SS consistently increased the number of flowers over the full duration of the growth cycle while both the *CC* and *CC*+SS combination treatments rather inhibited flower formation in Impatience. Calculated standard deviation values were rather high indicating large differences between replicates.

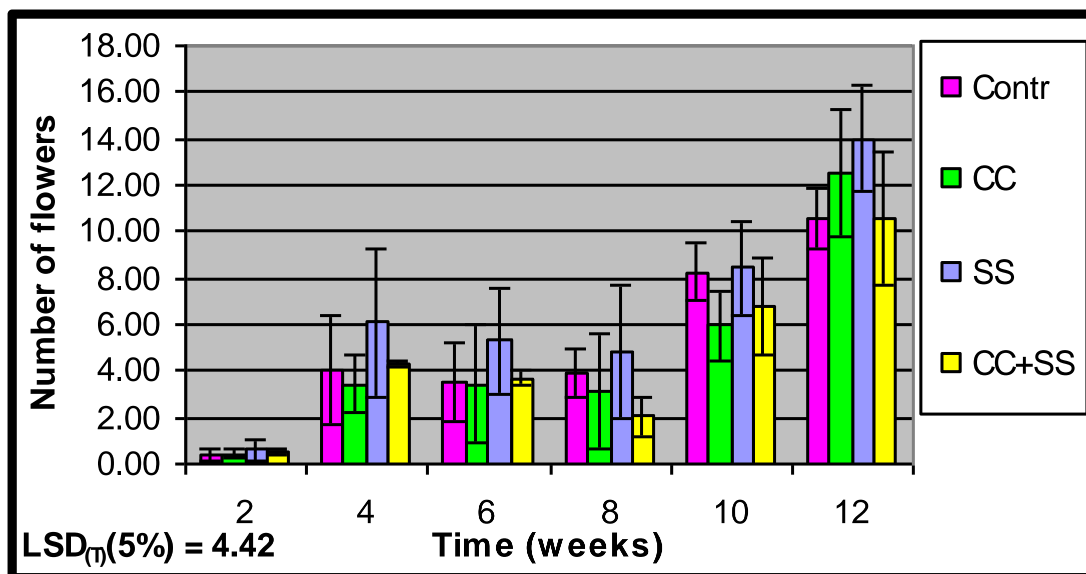

Figure 4.2: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray at a concentration of 5 mg L^{-1} on the flowering response of Impatience. A commercial bio-stimulant, *ComCat® (**CC**),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.2A in the Appendix.

4.4.2 The yield response of grain crops to a foliar spray treatment with a *L. albus* seed suspension (SS) under rain fed (maize) and semi-irrigation (wheat) conditions

4.4.2.1 The yield response of maize under rain fed conditions

Statistical analysis of dry kernel yield data in maize showed a significant difference between the *CC*+SS combination treatment and the untreated control at the 5% probability level (Figure 4.3; LSD value indicated in the graph). The latter treatment contributed to an increase in dry kernel yield of 680 kg ha^{-1} while the slight increases observed after single foliar spray treatments with both *CC* and SS individually were statistically non-significant.

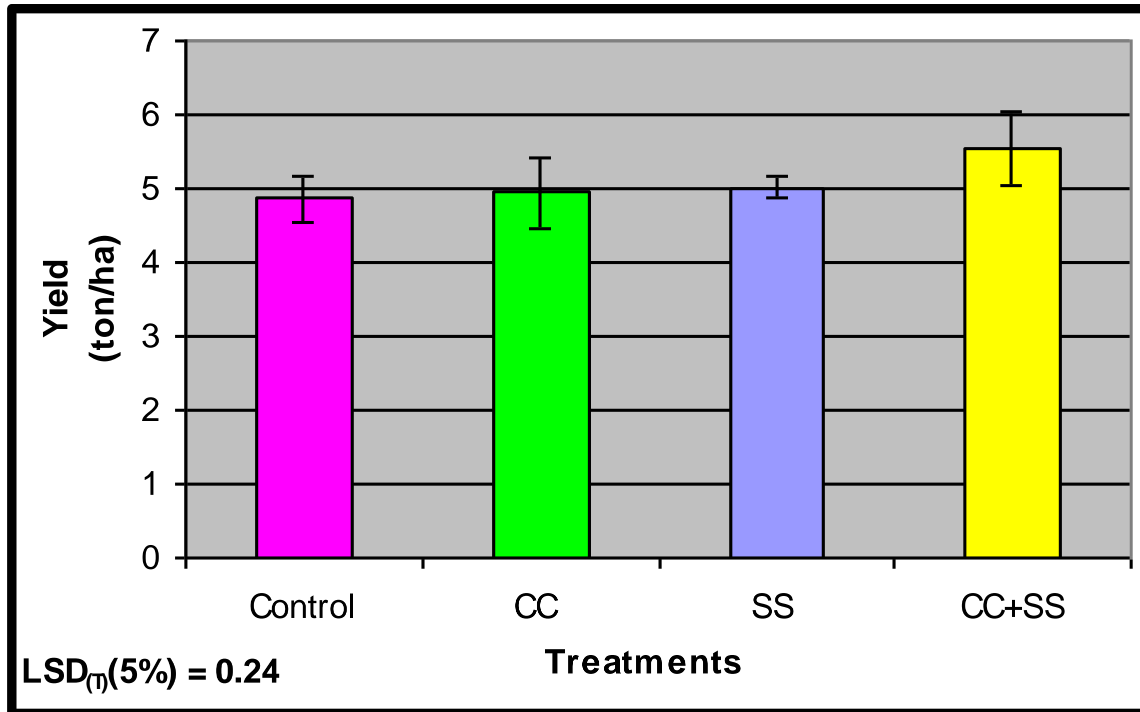

Figure 4.3: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray at a concentration of 5 mg L^{-1} on the dry kernel mass of maize under rain fed conditions. A commercial bio-stimulant, *ComCat® (**CC**),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}(5\%)$ value is indicated in the graph and the ANOVA attached as Table 4.3A in the Appendix.

4.4.2.2 The yield response of wheat under semi-irrigation conditions

Single foliar spray treatments of wheat at the three leaf growth stage with *CC* and SS, both separately and in combination, increased the dry kernel yield markedly compared to the untreated control (Figure 4.4; LSD value indicated in the graph). However, these differences were only significant ($P<0.05$) in the case of the SS and *CC*+SS combination treatments. Even though the yield response of wheat to these two treatments compared favourably to the commercial bio-stimulant applied separately as a positive control, the differences were non-significant.

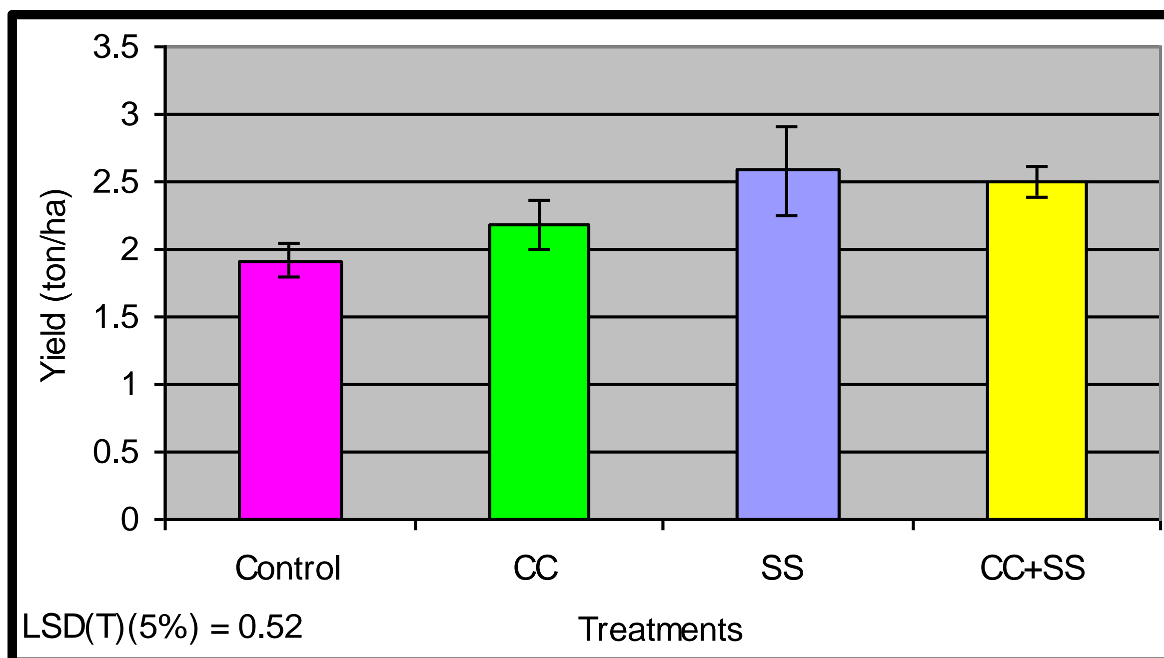

Figure 4.4: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the dry kernel mass of wheat under semi-irrigation conditions. A commercial bio-stimulant, *ComCat® (**CC**),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}(5\%)$ value is indicated in the graph and the ANOVA attached as Table 4.4A in the Appendix.

4.4.3 The yield response of vegetables to foliar spray treatments with a *L. albus* seed suspension (SS) under irrigation conditions

4.4.3.1 Beetroot

Compared to the untreated control, treatment of beetroot with SS at a concentration of 5 mg L^{-1}, first at the three leaf growth stage and followed by a second spray at 30% vegetative growth, increased the harvestable yield by 9.3 ton ha^{-1} (Figure 4.5; LSD value indicated in the graph). The latter yield increase was twice that obtained with the commercial bio-stimulant (*CC*). Where *CC* and SS were applied in combination, the yield increase was a staggering and highly significant ($P<0.05$) 21 ton ha^{-1}.

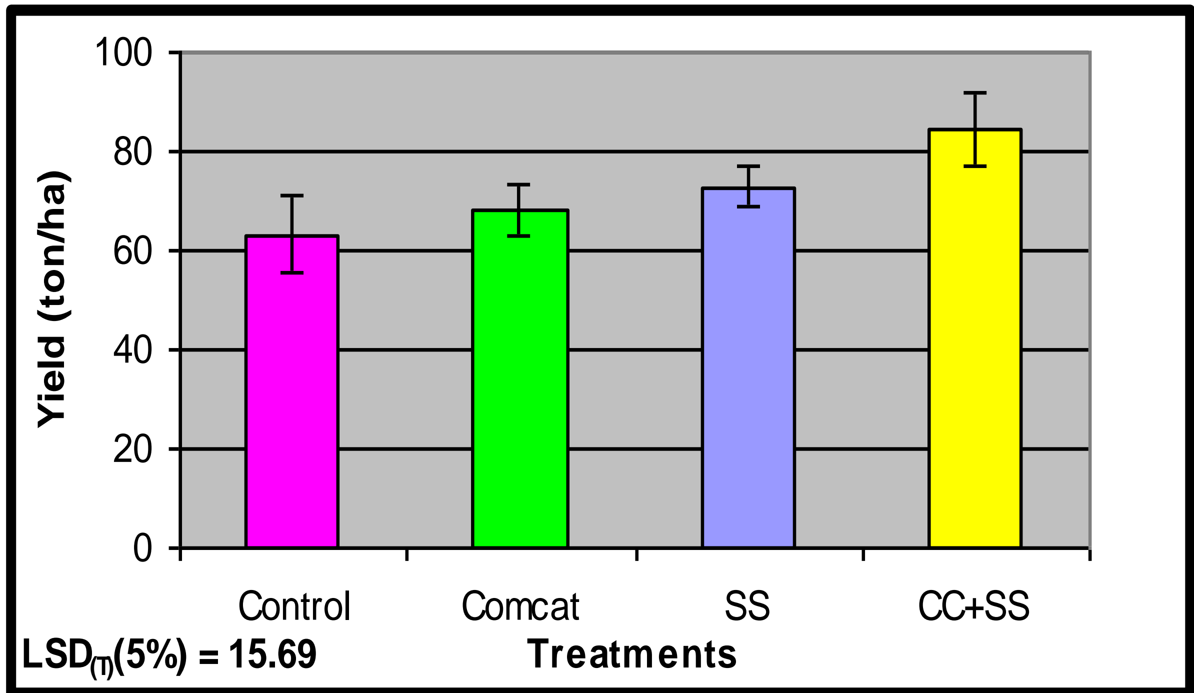

Figure 4.5: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the beetroot yield under irrigation conditions. A commercial bio-stimulant, *ComCat*® *(**CC**)*, was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.5A in the Appendix.

In order to verify whether the increase in bulb yield of beetroot was related to the growth of aerial parts, the effect of the different treatments on the foliage was additionally quantified (Figure 4.6; LSD value indicated in the graph). Although statistically non-significant, a decrease in the fresh foliage mass was observed in the case of the SS treatment when compared to the untreated control. However, as the *CC* treatment had no stimulating effect on foliage growth while the *CC*+SS treatment had a significant elevating effect, no clear tendency emerged in light of the fact that all three treatments increased the bulb yield.

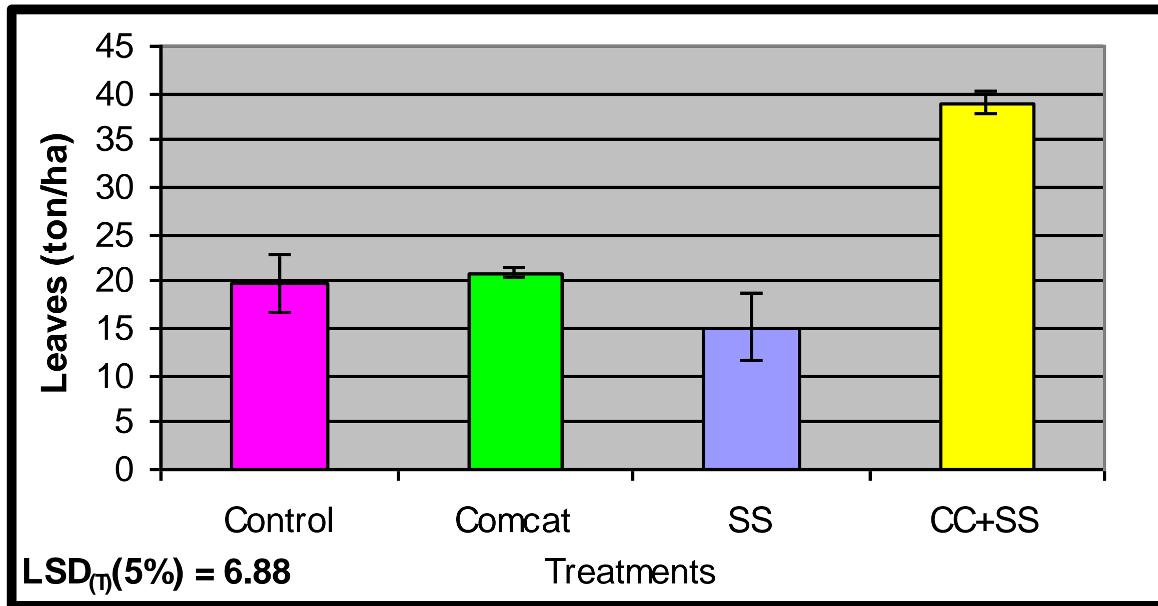

Figure 4.6: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the foliage fresh mass of beetroot under irrigation conditions. A commercial bio-stimulant, *ComCat*® *(**CC**),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.6A in the Appendix.

4.4.3.2 Lettuce

Compared to the untreated control, two foliar applications of SS resulted in a significant 20.4 ton ha^{-1} increase in the head mass of lettuce (Figure 4.7; LSD-value indicated in the graph). Although treatment with *CC* and *CC*:SS increased the head mass yield by 6.4 and 4.9 ton ha^{-1} respectively, this was not statistically significant. Neither of the treatments had a significant effect on the foliage growth of lettuce (Figure 4.8; LSD-value indicated in the graph).

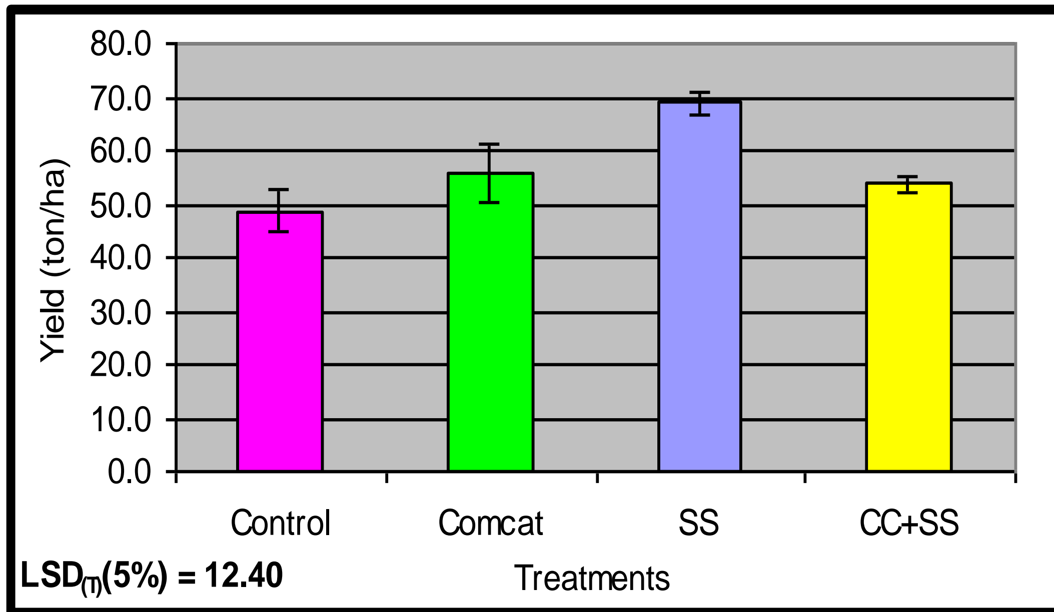

Figure 4.7: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the head fresh mass of lettuce under irrigation conditions. A commercial bio-stimulant, *ComCat® (CC),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.7A in the Appendix.

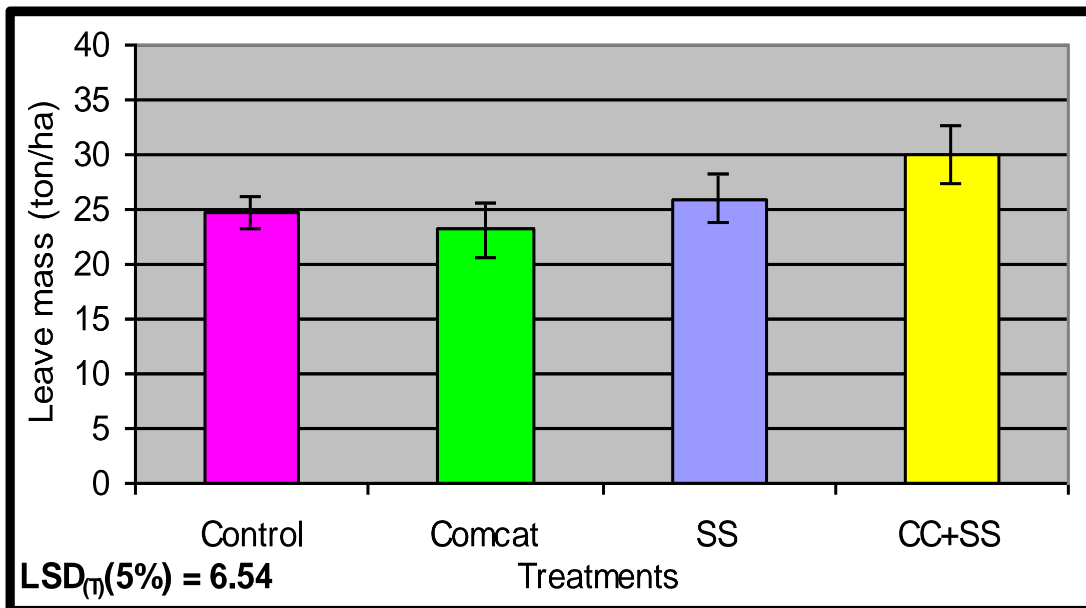

Figure 4.8: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the foliage fresh mass of lettuce under irrigation conditions. A commercial bio-stimulant, *ComCat® (CC),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.8 A in the Appendix.

4.4.3.3 Cabbage

Although all treatments had a slight enhancing effect on the foliage fresh mass of cabbage, none were statistically significant (Figure 4.9, LSD-value indicated in the graph).

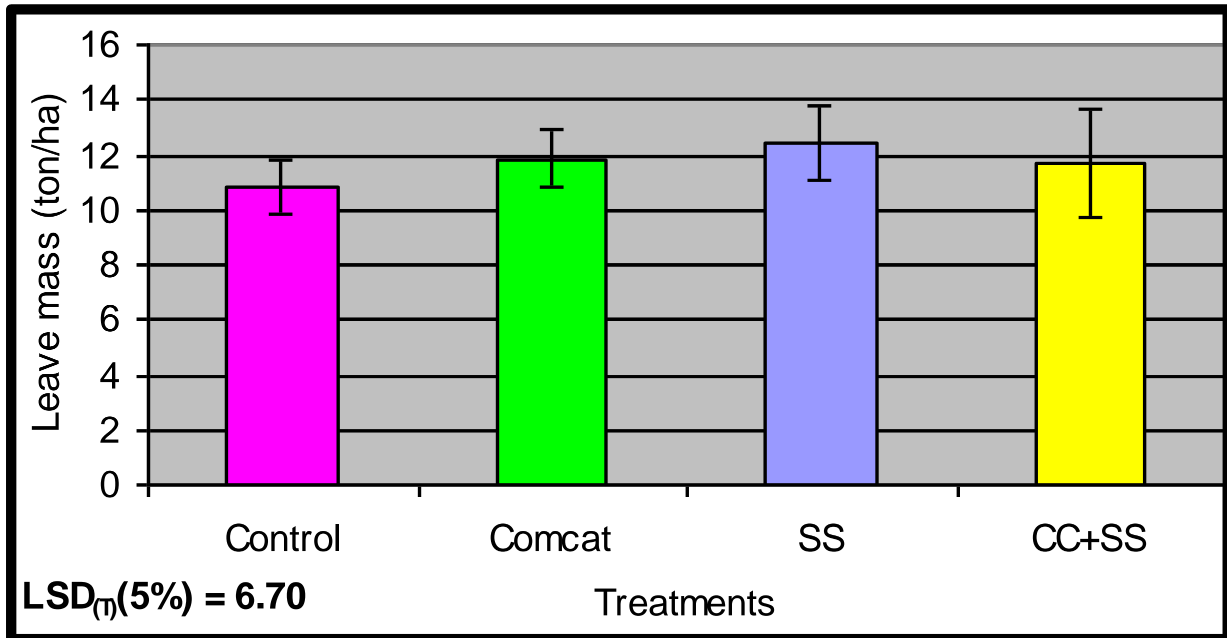

Figure 4.9: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the foliage fresh mass of cabbage under irrigation conditions. A commercial bio-stimulant, *ComCat® (**CC**),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.9A in the Appendix.

Except for treatment with *CC* that had a slight enhancing effect, SS applied separately and in combination with *CC* tended to decrease the head fresh mass in cabbage but neither of these were statistically significant (Figure 4.10; LSD value indicated in graph).

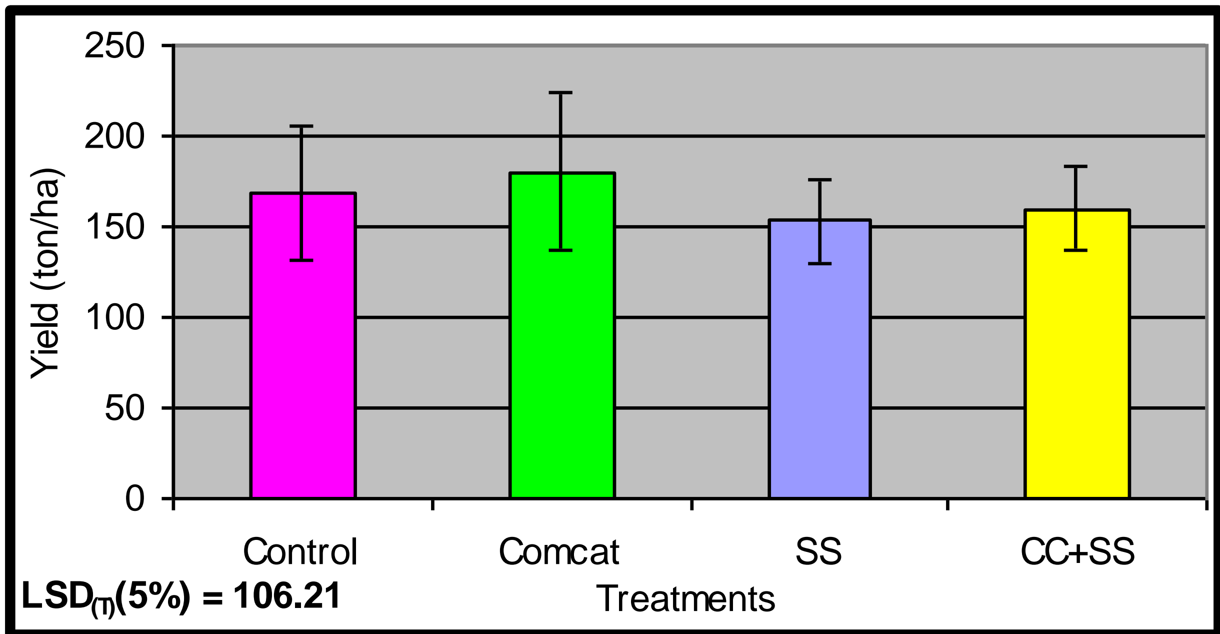

Figure 4.10: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the head fresh mass of cabbage under irrigation conditions. A commercial bio-stimulant, *ComCat® (**CC**),* was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}$(5%) value is indicated in the graph and the ANOVA attached as Table 4.10 A in the Appendix.

4.4.3.4 Carrots

Except for the *CC* treatment that had no effect, SS applied separately and in combination with *CC* significantly increased the length of carrots (Figure 4.11; LSD value indicated in graph), compared to the untreated control, while all three treatments tended to decrease the foliage fresh mass (Figure 4.12; LSD value indicated in graph), although not significantly.

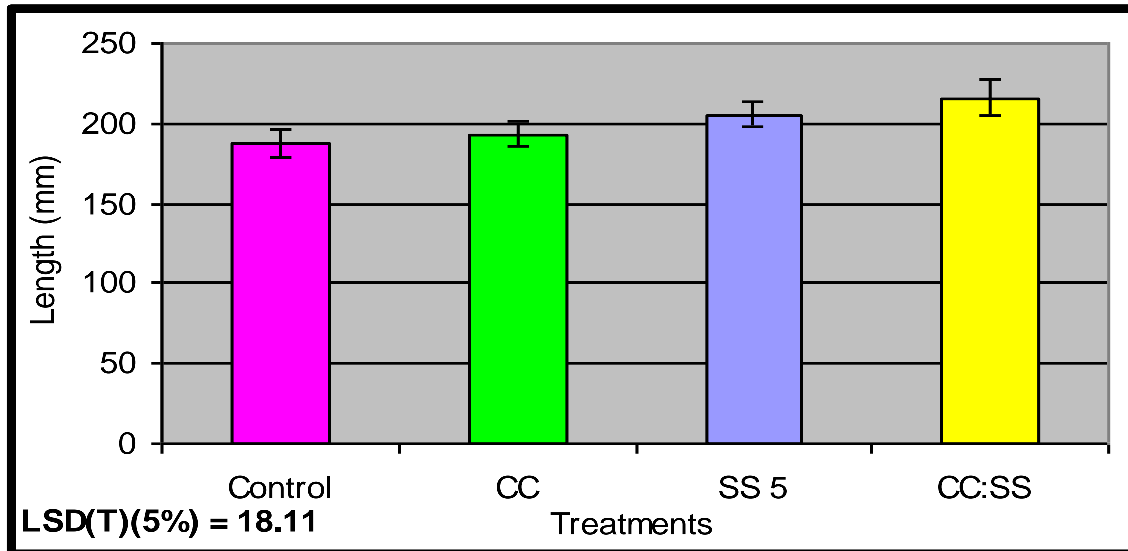

Figure 4.11: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the length of carrots under irrigation conditions. A commercial bio-stimulant, *ComCat®* *(**CC**)*, was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}(5\%)$ value is indicated in the graph and the ANOVA attached as Table 4.11 A in the Appendix.

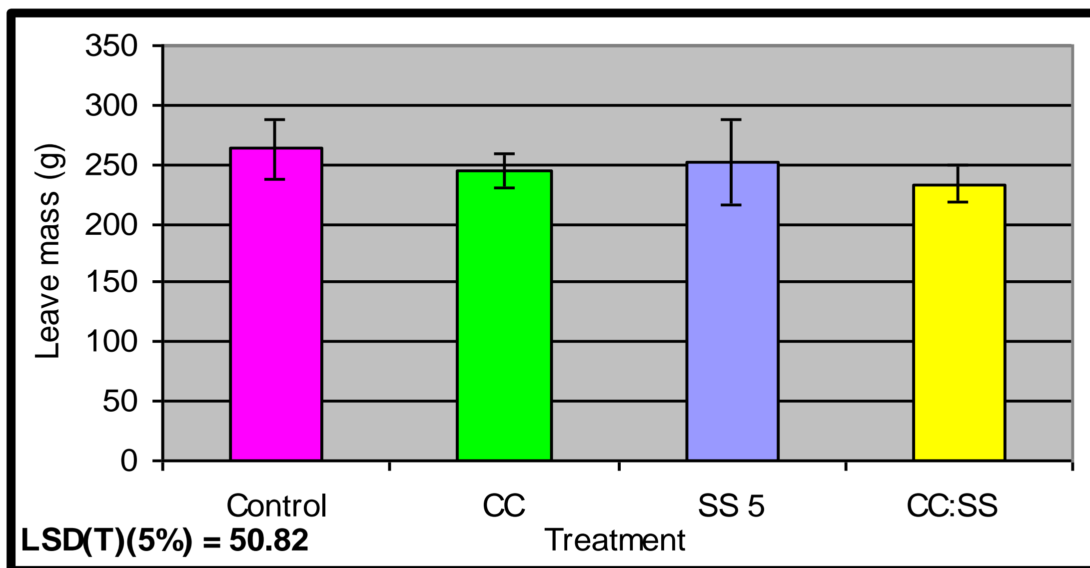

Figure 4.12: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the foliage fresh mass of carrots under irrigation conditions. A commercial bio-stimulant, *ComCat®* *(**CC**)*, was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}(5\%)$ value is indicated in the graph and the ANOVA attached as Table 4.12 A in the Appendix.

Compared to both the positive and negative controls, SS applied at 5 mg L^{-1} increased the carrot yield by an astonishing 24.3 ton ha^{-1} (Figure 4.13, LSD-value indicated in graph). Despite the fact that the commercial bio-stimulant, *ComCat*®, had no effect on the carrot yield, the treatment in combination with SS significantly increased the carrot yield by 18.0 ton ha^{-1} compared to the untreated control.

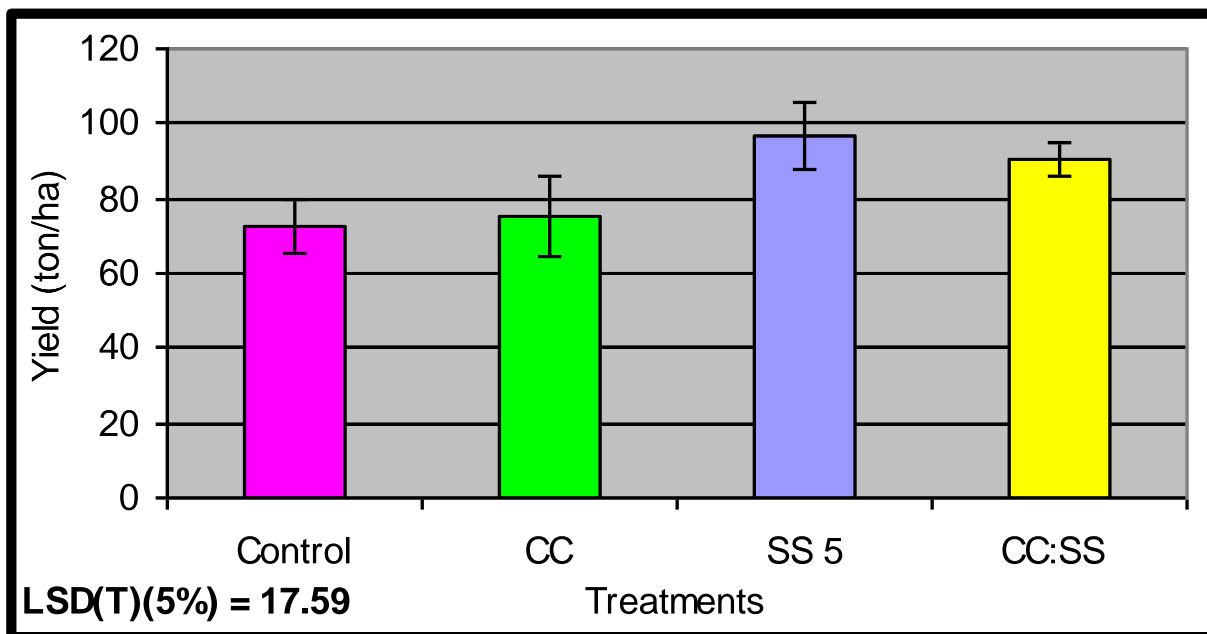

Figure 4.13: The effect of a solubilized *Lupinus albus* (**SS**) seed suspension applied as a foliar spray on the carrot yield under irrigation conditions. A commercial bio-stimulant, *ComCat*® *(**CC**)*, was used as a positive control both separately and in combination with SS. A standard fertilization treatment served as a negative control. The $LSD_{(T)}(5\%)$ value is indicated in the graph and the ANOVA attached as Table 4.13 A in the Appendix.

4.5 DISCUSSION

It must be acknowledged that natural bio-stimulants manufactured from either different plant organs or different plant species do not have the same growth or yield promoting effects on all agricultural and horticultural crops or even different cultivars of the same crop (Hüster, 2004; personal communication)[1]. This is understandable as the active substances involved most likely differ from product to product and there is no control over the circumstances under which the products are applied by farmers. Probably for these reasons, without disclosing names, certain commercial products have failed to provide consistent results in terms of promoting either crop growth or yield or both under practical farming conditions. A product that is robust enough to consistently perform under these conditions will without a doubt have an edge on its competitors. In this light, the development of a reliable plant growth regulator with the potential to be an economic asset to individual farmers and the agricultural industry alike remains a challenge.

Preliminary screening of a solubilized *Lupinus albus* seed suspension for bio-stimulatory activity using different bio-assay procedures (see chapter 3) supplied a strong indication of its potential to enhance seedling growth, especially root growth, upon seed treatment in a number of test crops, at an optimum concentration of 5 mg L^{-1}. From this it was reasoned that the seed suspension (SS) showed sufficient potential to contribute to stronger seedlings via enhanced root development that would improve the utilization of available water and nutrients from the soil under field conditions. Subsequently, field trials were conducted and SS applied as foliar sprays to four vegetable crops, two grain crops and two flowering plants in order to verify the preliminary results obtained under laboratory conditions. As yield increase is the principle objective for applying bio-stimulants from an agricultural perspective, emphasis was placed on this aspect. This was done as a means to a) assess the application potential of SS in the agricultural industry and b) to supply a rationale for the isolation and identification of the active substances involved. A commercial bio-stimulant, *ComCat*® (*CC*), was used as positive control at the optimum application of 100 g ha^{-1} recommended by the manufacturers (Agraforum, Germany) as a means to evaluate the bio-stimulatory potential of SS by comparison. Additionally, SS was applied in combination with *CC* to ascertain whether synergistic or antagonistic effects existed between the two extractants from different plant origins.

[1] Hüster, T. Agraforum AG, Poststrasse 1, Bomlitz 29699, Germany

Despite the use of lupine as a fodder crop, it has caught the attention of organic farmers as a rotational and nitrogen-fixing crop (Jensen *et al*., 2004). The authors reported that, on different soil types, lupine as a rotational crop has a more significant effect on the yield of winter barley compared to that of other legumes e.g. pea, probably due to its superior N-fixation properties leading to higher nitrogen residues remaining in the soil after rotation. Additional attributes of lupine as a rotational crop to the farmer include (i) deep-root penetration and better soil aeration, (ii) improvement of water penetration and conservation for follow-up crops and (iii) the ability of lupine to act as a 'cleaning crop' for root diseases that may affect subsequent crops (Takunov and Yagovenko, 1999). Additionally, Fuji, (2001) reported on the weed-suppressing ability of lupine via allelochemicals leached from the roots. Results obtained in this study under field conditions added to the repertoire of lupine attributes by confirming the above average potential of a seed suspension to add value to test crops as diverse as flowering plants, grain crops and vegetable crops.

Foliar treatments of two flowering plants, Gazanias and Impatience, with the commercial bio-stimulant, *ComCat*® (*CC*), at the three leaf growth stage followed up by three additional treatments applied at four week intervals contributed to a significant flowering response in terms of the number of flowers counted on a two weekly basis. In Gazanias this response was accentuated during the first of two naturally occurring flowering flurries between weeks 10 and 14 as well as between weeks 18 and 22. Interestingly, *CC* had an inhibiting effect on flowering of Gazanias during the second flurry. On the other hand, foliar treatments with the *L. albus* seed suspension (SS) showed exactly the opposite tendency by having no significant effect on flowering during the first flurry but significantly increased the number of flowers during the second flurry. Where *CC* and SS were applied in combination the flowering response of Gazanias followed the same pattern as it did where *CC* was applied on its own.

The difference in the flowering response of Gazanias treated with *CC* and SS both separately and in combination is difficult to explain at this stage but might be ascribed to the presence of different active compounds in SS and the commercial product. According to Sasse (1999) brassinosteroids (BR), the active compounds of *Comcat*®, have little effect on flower induction in *Perilla* sp. (short day plant) and *Raphanus* sp. (long day plant). However, flower number was increased significantly

in strawberry, a long day plant, by treatment with a BR analogue. From this it seems that the flowering response to treatment with *Comcat*® is rather species specific than day length dependent. Although Gazanias responded differently to treatment with SS in the sense that it responded at a later stage in the flowering cycle, indications are that the active substance in the *L. albus* seed suspension probably differs from that of *CC*. Further, as the flowering response of Gazanias to treatment with the *CC*:SS combination spray followed the same pattern as that of the *CC* treatment, it can be postulated that BR's in *Comcat*® might have a screening or overriding effect on the active substance contained in SS in this plant. More information is needed to verify this postulate.

The flowering response of Impatience to foliar spray applications of *CC* and SS at the same time intervals that applied for Gazanias, was altogether different than what was observed for the latter. Compared to the untreated control the commercial product (*CC*) had no effect on the number of flowers formed while treatment with SS consistently and markedly, although non-significantly, increased the number of Impatience flowers formed over a 12 week period. Upon treatment with the *CC*:SS combination treatment, Impatience followed the same flowering pattern as was observed where *CC* was applied on its own confirming the postulate stated earlier. Moreover, the fact that Impatience responded to the SS but not the *CC* treatment and also responded differently than Gazanias, emphasized that the flowering response might be species specific.

According to Groenewald and van der Westhuizen (2004) flowering in photoperiodic sensitive plants is considered to be brought about by transmissible signals which are produced in the leaves and which are either promotive or inhibitory during inductive or non-inductive photoperiods, respectively. The hypothetical flowering stimulus has been named florigen. However, the chemical nature of the elusive flowering stimulus has not been identified to date (Groenewald and van der Westhuizen, 2001). Recently the authors (Groenewald and van der Westhuizen, 2004) showed that arachidonic acid and prostaglandin as well as the steroids stigmasterol and cholesterol caused earlier flowering in *Pharbitis nil,* a model plant for flowering studies (Evans, 1975). Although reports on the flowering response to treatment with brassinosteroids (BRs), the active compound of *Comcat*®, are scarce in the literature, Padmapriya and Chezhiyan (2002) recently observed that, despite the cultivar, the flower diameter of chrysanthemum (*Dendranthema gandiflora*) was broadest after treatment with BRs compared to treatments with gibberellic acid and salicylic acid. Moreover, a

study by Plana-Llerena and Nunez-Vazquez (1999) showed that treatment of African violets with brassinosteroids increased the leaf number and flowering. Since BRs belong to the steroid group of compounds, more research is necessary on the flowering response mechanism of plants to exogenously applied BRs. The same goes for the response mechanism to treatment with a *L. albus* seed suspension once the active substance has been identified.

The effect of the different treatments on grain crops under either dry land or semi-irrigation conditions was also tested with the emphasis on the yield response. In maize, both *CC* and SS treatments had only a slight and non-significant increasing effect on the dry kernel yield of maize while the *CC:*SS combination treatment increased the yield significantly. The latter was contradictory to the response observed earlier in cut flowers indicating a possible synergistic effect in the case of maize. As the active compound of SS is not known at this stage, the observed synergism is difficult to explain. It is, however, well documented that exogenously applied BRs, the active compound of *CC* (*ComCat*®), has an enhancing effect on the yield of a number of grain crops (Schnabl *et al*., 2001). Recently Sivakumar *et al*. (2002) reported on the yield enhancing effect of BRs in pearl millet and attributed the yield response to a combination of factors that included increased chlorophyll content in leaves, improved nitrogen uptake and increased nitrate reductase activity in the roots leading to elevated protein and total sugar content in grains. Subsequently the yield response of wheat on treatment with SS and *ComCat*® was also tested under semi-irrigation conditions.

Compared to the untreated control a single foliar spray treatment with both SS and *CC* plus SS in combination at the 3-leaf growth stage significantly increased the dry kernel yield of wheat while *CC* applied on its own only had a slight but non-significant enhancing effect. As the combination treatment did not improve on the yield result obtained with the SS treatment alone, the synergistic effect between SS and *CC* observed in maize was not repeated indicating that generalization for grain crops in this regard is not possible at this stage. At least it was clear that no antagonism existed between the two treatments at the concentrations applied. Krishnan *et al*. (1999) demonstrated synergism in rice where BRs applied in combination with benzylaminopurine increased the number of fertile tillers, filled spikelets and the dry 1000-kernel weight significantly compared to the BR treatment alone.

All foliar applications of 28-homobrassinolide (HBR) at different concentrations and at different growth stages under both experimental conditions at research stations and in farmers fields in India between 1989 and 1995 significantly increased grain and seed yield in wheat, rice and mustard respectively (Ramraj *et al*., 1997). These results confirmed the positive effect of BRs on the quality and grade of grain crops. According to Ramraj *et al*. (1997) the increasing effect of BRs on grain phytomass can probably be attributed to increased sucrose translocation from the source to the sink as well as increased protein content in seeds. Further, these effects seem to be mediated through the promotion of fertilizer uptake and fructification via the promotion of germination and growth of pollen tubes which helps to overcome the effect of self-incompatibility (Lee *et al*., 1990). Moreover, the effects of BRs on seed- and fruit-setting may also be derived from a combination of preventative effects on flower and fruit drop, enhancement of photosynthetic capacity and translocation of photosynthate (Fuji and Saka, 1992). In this regard it has been shown that BRs induce changes in plasmalemma energization, carbohydrate translocation and assimilate uptake (Arteca, 1995) that can lead to yield increases in various crops.

Subsequently, the yield response of different vegetable crops on treatment with *ComCat*® and a *L. albus* seed suspension, both separately and together, was tested under field conditions. In the case of beetroot both *ComCat*® and SS applied separately had a marked but statistically non-significant increasing effect, of 4.0 and 9.3 ton ha^{-1} respectively, on the final yield. However, when the two products were applied in combination, the yield increasing effect was a highly significant 21 ton ha^{-1} indicating a possible synergistic effect. In lettuce exactly the same synergistic effect between *ComCat*® and SS was observed for the foliage fresh mass but not for the head mass that was significantly increased by almost twenty tons per hectare by the SS treatment. Furthermore, cabbage did not respond to either the separate *ComCat*® and SS or the combination foliar applications indicating that different crops react differently to treatment with these bio-stimulants. In the case of cabbage, belonging to the Brassica family from which brassinosteroids were originally isolated, it is possible that the additional supply of these phytohormones via *ComCat*® on top of the naturally occurring ones might have exceeded the optimum concentration contributing to an opposite effect well known for phytohormones. The latter was especially accentuated in the inhibitory effect observed for *ComCat*® on the foliar fresh mass of cabbage.

Enhanced yield as a response to treatment with the brassinosteroid 22,23,24-tri-epibrassinolide in lettuce cultivated in sub-optimally fertilized soil was reported by Meudt *et al*. (1983) confirming the response observed with *ComCat*®. According to Sasse and Sasse (1994) BRs qualitatively affect the morphogenesis of plants including the number of leaves, leaf area, fresh weight and dry weight of foliage as well as leaf senescence confirming the positive response of a leafy vegetable such as lettuce to treatment with *ComCat*®. However, the yield response of lettuce to treatment with SS by far exceeded that observed for *ComCat*® indicating that the active substance in the former might be different to that of the latter.

Budaj (2000) reported increased shoot production in carrots after treatment with BRs leading to stronger mature plants due to the larger leaves. However, in this study foliar treatment of carrots with neither *ComCat*® nor SS had any effect on the foliar fresh mass while SS increased the final carrot yield by an astonishing 24.3 ton ha^{-1} compared to the non-significant 2.5 ton ha^{-1} increase observed with *ComCat*®. Interestingly, the *CC*:SS combination treatment also significantly increased the final carrot yield indicating that the active substance contained in SS might have an overriding effect on that of *ComCat*® probably due to different mechanisms of action; an aspect that will be considered in chapter 7.

In conclusion, the bio-stimulatory effect of a *Lupinus albus* seed suspension (SS) leading to significant yield increases in a number of crops under field conditions was confirmed. This supplied the rationale to proceed with activity directed fractionation and semi-purification (Chapter 5) of the active substance(s) contained in the seed suspension as well as its isolation and chemical structure elucidation (Chapter 6).

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CHAPTER 5

ACTIVITY DIRECTED SEMI-PURIFICATION OF BIO-STIMULATORY COMPOUNDS FROM *Lupinus albus* L. SEEDS

5.1 ABSTRACT

In previous studies significant bio-stimulatory activity was confirmed in a solubilized *Lupinus albus* seed suspension by means of bio-assay screening procedures and field trials. These included an increase in the respiration rate of monoculture yeast cells, enhanced seedling growth and increased yields in a number of different crops. This supplied the rationale for the isolation and purification of active substances involved. The solubilized seed suspension was fractionated and semi-purified by means of activity directed chromatographic techniques including liquid-liquid extraction and column chromatography. Each semi-purified extraction or fraction was tested for bio-stimulatory activity by employing two *in vitro* bio-assay procedures. A commercial bio-stimulant, *ComCat*®, was used as a positive control while distilled water served as a negative control in all bio-assays. By means of liquid-liquid extraction of the seed suspension, the ethyl acetate fraction was identified as most active and further fractionated by means of column chromatography. A qualitative thin layer profile of every third fraction was obtained and those fractions that showed similar profiles were pooled. In this way twelve combined column chromatography fractions were obtained that were subjected to the bio-assay procedures in order to identify the active fractions. Of these, three combined column chromatography fractions showed significant bio-stimulatory activity.

Keywords: Liquid-liquid extraction, column chromatography, semi-purification, ethyl acetate fraction

5.2 INTRODUCTION

The confirmation of bio-stimulatory activity in a seed suspension of *Lupinus albus* L. during qualitative laboratory screening (Chapter 3) and field trials (Chapter 4) supplied the rationale for the isolation, purification and identification of the active compounds involved. As all aspects of the

plant's phytochemistry are unknown to science, different methods were employed to extract and isolate the active substances initially. Similarities in activities between the seed suspension and the commercial product *ComCat*® led to the suspicion that the active substances might also be similar and related to brassinosteroids (BRs), the active substance of *ComCat*®. This suspicion was further accentuated by the bio stimulatory activity observed for the *L. albus* seed suspension, in terms of its ability to enhance the respiration rate in monoculture yeast cells as well as seedling growth and yield of a number of agricultural crops, as these activities have been attributed to BR activity in the past (Yokota, 1999). The author isolated BRs from a large amount of immature *Phaseolus vulgaris* seeds and confirmed its association with the family Fabaceae. Wide occurrence of BRs in lower and higher plants indicates that BRs are commonly distributed in the plant kingdom and their role as essential growth regulators have been established (Yonemoto *et al*., 1993; Al-Humaid and Warrag, 1998; Yokota, 1999).

Research over years confirmed the presence of a wide range of master regulators that co-ordinate complex biochemical pathways involved in plant development as well as the expression of a large number of target genes (Memelink *et al*., 2000). The authors also reported that these master regulators can vary in their origin and can be regulated depending on the cell type or in response to environmental stimuli. These regulators can either be hormonal like BRs or might be totally different compounds but with hormonal effects. Further, despite the fact that various biochemical systems are similar in many organisms, it is not surprising that a large number of plant secondary compounds can be detrimental to the growth and development of many organisms. Elucidation of the roles of these compounds as chemical messengers has contributed to our understanding of many ecological problems and has led to the development of the disciplines allelopathy and chemical ecology.

Plant secondary metabolites are not just randomly produced compounds, but ones that have been shaped and optimized during evolution (Aerts *et al*., 1994). Since secondary metabolites have evolved as compounds that are important for the fitness of the organisms producing them, many of them interfere with the pharmacological and agricultural targets which make them interesting for biotechnological applications (Wink, 1999). As a consequence of these various applications, a

world market for plant extracts and isolated secondary metabolites exists, which exceeds 10 billion US dollars annually (Balandrin *et al*., 1985). Therefore, it is challenging for biotechnologists to find ways to produce these compounds in sufficient quantity and quality. The main and traditional way is to grow the respective plants in the field or in greenhouses and to extract the products from them. Confirmation of bio-stimulatory activity in a *Lupinus albus* seed suspension under laboratory and field conditions was compelling in isolating and purifying the active substances involved.

5.3 MATERIALS AND METHODS

5.3.1 MATERIALS

5.3.1.1 Plant material

Seeds from different crop plants used in the bio-assay procedure were purchased from the local merchants SENWES, Stark Ayres or Mayford (South Africa), during the 2002/2003 growing season.

5.3.1.2 Other Chemicals

Aluminium thin-layer chromatography plates (silica gel 60 F 254; 20x20 cm) were purchased from Merck (Germany), and preparative thin-layer chromatography plates (silica gel + indicator, 1mm, G 1510/LS 254; 20 x 20 cm) were from Schleicher and Schuell (Germany) or Sigma (Germany). Most of the chemicals used, namely methanol, hexane, ethyl acetate, sulphuric acid, hydrochloric acid, ethanol, chloroform and sodium hydrogen carbonate were purchased from Merck (Germany) and were of the purest grade available. Packing material for column chromatography (Kieselgel 60; particle size 0.063-0,200 mm; 70-230 mesh ASTM) was purchased from Merck (Germany).

5.3.2 METHODS

5.3.2.1 Preparation of a methanol crude extract and activity directed liquid-liquid extraction of active compounds

A liquid-liquid extraction procedure developed by Gamoh *et al*. (1989) to specifically fractionate brassinosteroids was followed as outlined in Figure 5.1. This was done in order to separate any possible brassinosteroid or related compounds from any other possible active substances in the crude *L. albus* seed extract. Numbers corresponding to the fractionation steps in figure 5.1 are indicated in the text. Five hundred grams of ground *Lupinus albus* seeds were initially extracted with three litres of 100% methanol over a period of one week. Every 24 h the methanol crude extract was decanted, pooled and replaced with fresh methanol **(1)**. Subsequently, the same plant material was extracted with three litres of an ethyl acetate:methanol mixture (1:1; v/v) for another week by following the same procedure as explained above **(2)**. The methanol and ethyl acetate:methanol extractants were combined **(3)**, mixed and filtered under vacuum through a double layer of Whatman No. 1 filter paper using a Buchner funnel fitted to a side arm Erlenmeyr flask connected to a suction pump

The combined filtrate was vacuum distilled at 35°C using a Buchi Rotavapor (Bibby Sterlin LTD, England) equipped with a cooled Liebig condenser in order to remove the bulk organic solvent. The combined concentrated extract was fractionated between ethyl acetate and water (2:1; v/v) by means of liquid-liquid extraction and the procedure repeated three times **(4).** Both the ethyl acetate and water fractions were concentrated **(5)** by means of vacuum distillation and the water fraction further fractionated twice between hexane and 90% methanol (1:1; v/v). The aqueous methanol fraction was subsequently concentrated by means of vacuum distillation at 35°C **(7)** and fractionated twice between ethyl acetate and a saturated sodium bicarbonate solution (1:1; v/v) **(8)**.

According to the prescribed method of Gamoh *et al*. (1989) for extracting brassinosteroids (BRs) the second ethyl acetate fraction (see Figure 5.1) contains the BRs. Nevertheless, in this study all of the collected SS fractions were assumed to be active and therefore all fractions were tested for bio-stimulatory activity by means of two bio-tests (see chapter 3; 3.3.2). Only the most active fractions

were further purified using different chromatographic techniques in order to isolate, identify and elucidate the chemical structure of the active compound(s) (see chapter 6).

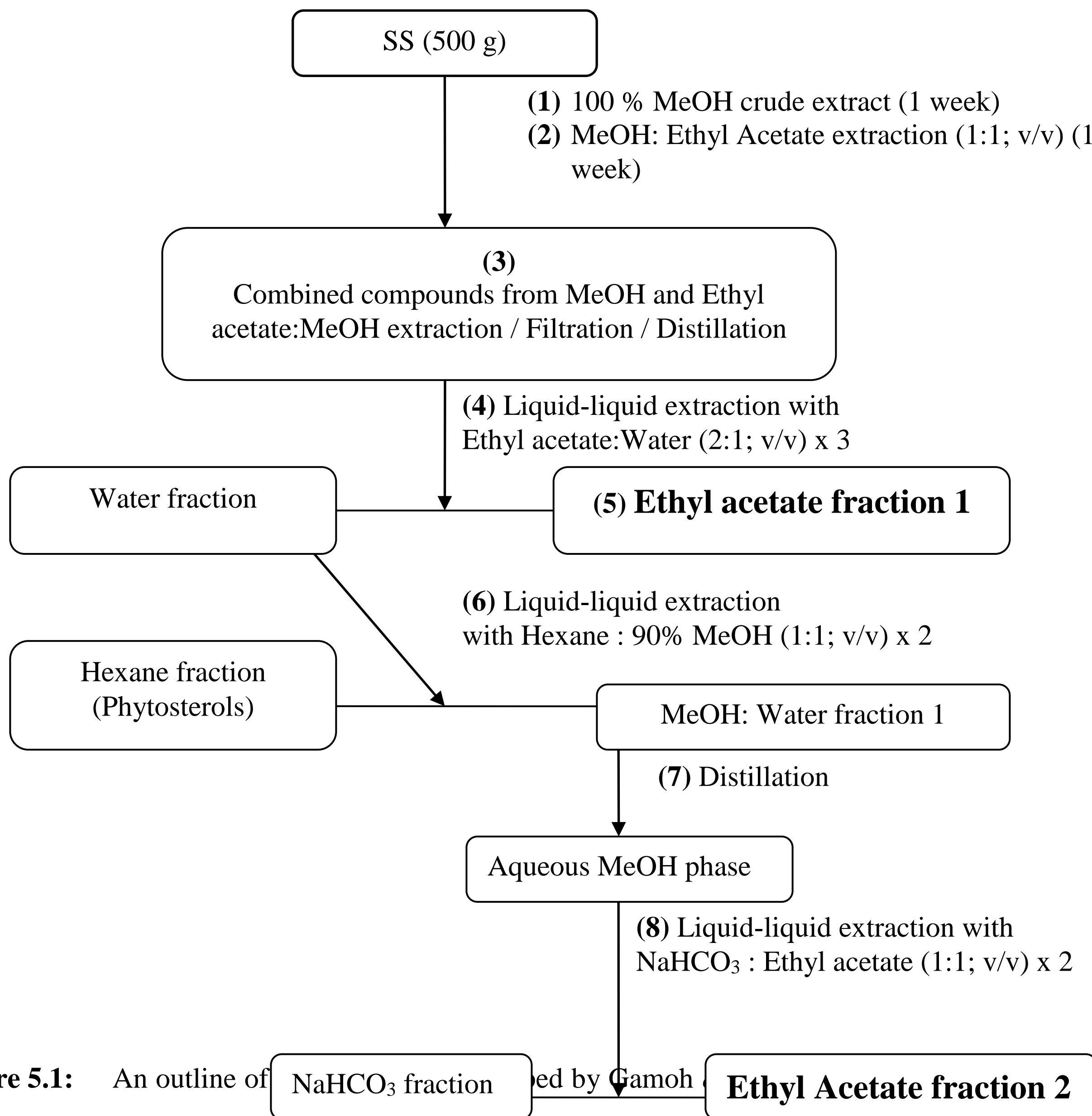

Figure 5.1: An outline of ... ed by Gamoh ... t and fractionate brassinosteroids.

5.3.2.2 Determination of the concentration of the crude extract as well as semi-purified fractions

Two cm^3 of the crude extract or semi-purified fractions were placed in a pre-weighed Petri dish and the mass determined. After drying in an oven at 70^0C the Petri dish was weighed again and the mass of the crude extract or semi-purified fractions determined by subtracting the mass of the Petri dish from that of the total mass. Known masses and volumes were constantly used and the concentrations expressed as mg cm^{-3} or µg cm^{-3}.

5.3.2.3 Qualitative thin layer chromatography of liquid-liquid extraction fractions

Using qualitative analytical thin layer chromatography (Q-TLC), all the liquid-liquid extraction fractions obtained as outlined above were spotted on a silica gel 60 F_{254}-aluminum backed and pre-coated plate (Mikes and Chalmers, 1979) in order to obtain a Q-TLC-profile of each fraction. Ten to 15 µg of each sample was loaded onto the plates at the baseline and developed in a saturated chamber using chloroform: methanol (95:5; v/v; Gamoh *et al*., 1989) and 1 ml of glacial acetic acid as solvent system. Glacial acetic acid was added to the mobile phase to prevent the fractions from streaking on the plate. After drying the plates in a stream of air, using a hair dryer, compounds were first detected under UV-light at 254 and 365 nm, stained with 10 % (v/v) ethanolic sulphuric acid and placed in an oven at 100°C for 20 minutes (Wagner and Bladt, 1996).

Only the most active liquid-liquid extraction fraction (see 5.2.2.2) was concentrated to dryness *in vacuo* and subjected to column chromatography for further fractionation of the active compounds.

5.3.2.4 Adsorption column chromatography of the most active liquid-liquid extraction fraction

By means of the activity directed liquid-liquid extraction procedure of Gamoh *et al*. (1989) ethyl acetate fraction 1 showed the highest bio-stimulatory activity *in vitro* and was further purified by means of adsorption column chromatography using Silica gel 60 (Merck, Germany) as stationary phase. One hundred grams silica gel 60 (70-230 mesh) was mixed with 300 cm^3 of the mobile phase (chloroform: methanol; 98:2; v/v) to form a slurry that was poured into a glass column (200 mm x 10 mm) and allowed to settle for an hour under gravitation. Five ml of the active ethyl acetate fraction at a concentration of 516 mg ml^{-1} was loaded onto the column and eluded stepwise

with 50 ml of a gradient solvent system of 100% chloroform followed by 50 ml each of different chloroform: methanol mixtures (v/v; 98:2; 95:5, 90:10, 85:15 and 80:20) at a flow rate of 1 ml min^{-1}. A total of 85 five ml fractions were collected. To ensure that all active substances were removed from the column it was finally cleaned with two bed volumes of 100% chloroform followed by two bed volumes of 100% acetone and collected separately in bulk.

5.3.2.5 Qualitative thin layer chromatography of column fractions

After vacuum distillation, 10 µg of every third column fraction as well as the bulk chloroform and acetone fractions obtained after cleaning the column was spotted on a pre-coated silica gel TLC-plate and the compounds contained separated as outlined in 5.2.2.3 using chloroform: methanol (10:1; v/v) as mobile phase. After developing the TLC-plates with 10% (v/v) ethanolic sulphuric acid at 100 ºC those column chromatography fractions with similar Q-TLC profiles were combined. In this way twelve combined fractions were obtained that were subsequently concentrated *in vacuo* and subjected to different bio-assay procedures (see chapter 3; 3.3.2) to determine which fractions contained the bio-active compound(s).

5.3.2.6 Statistical analysis

Analysis of variance (ANOVA) was performed on the bio-assay data, using the NC: SAS Institute Inc., Dos Statistical Program to identify differences between treatments. Tukey-Kramer's LSD (least significant difference) procedure for comparison of means (Steele and Torrie, 1980; Mason *et al.*, 1989) was applied to separate means ($P<0.05$). Treatments differing significantly were indicated either in figures or below tables as calculated LSD values or by using symbols for significant (*) and non-significant (ns) differences

5.4 RESULTS

5.4.1 A qualitative thin layer chromatography profile of the liquid-liquid extraction fractions obtained from a *L. albus* seed extract

Four different semi-purified fractions were obtained from the seeds of *Lupinus albus* by means of the brassinosteroid extraction procedure of Gamoh *et al*. (1989), their Q-TLC profiles illustrated (Plate 5.1) and the quantities that was recovered determined (Table 5.1). The TLC profiles of the different liquid-liquid extraction fractions showed some similarities but also distinct differences on grounds of differences in Rf-values of compounds. Ethyl acetate 1 (lane 1; Plate 5.1), Hexane (lane 2; plate 5.1) and ethyl acetate 2 (lane 4; Plate 5.1) all contained compound 1 in relative large quantities while lower quantities of compounds 2, 3 4 and 5 were only detected in the latter two fractions. Compound 6 was very specifically associated with only the ethyl acetate 1 fraction (lane 1; Plate 5.1) while compound 7 was only detected in this fraction and the hexane fraction (lane 2; Plate 5.1). Compared to compounds 1 to 5, the other two compounds were clearly detected in much lower quantities. Interestingly, none of these compounds were detected in the sodium bicarbonate fraction either by failing to migrate from the baseline under the influence of the mobile phase due to a totally different composition or due to the quantities of these compounds being too low to detect. Measured by the high concentration of compounds recovered in the sodium bicarbonate fraction the former seems more likely. An extremely low concentration of compounds remained in the original crude extract indicating that the extraction procedure was highly successful

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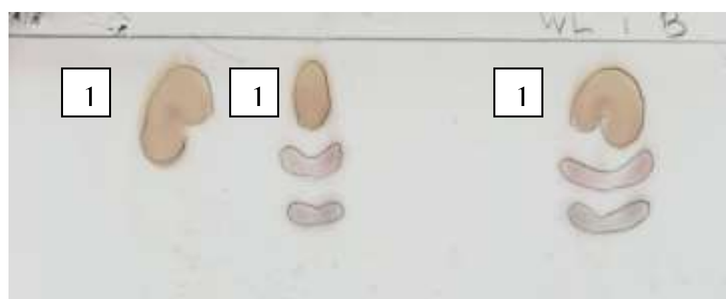

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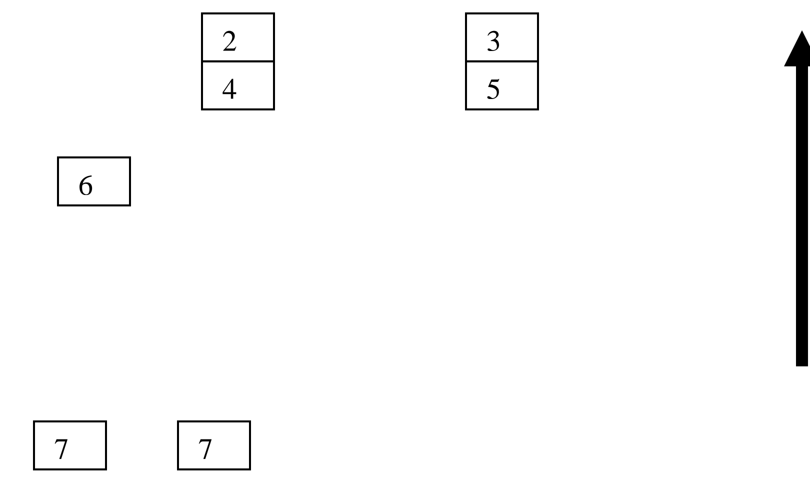

Plate 5.1: A qualitative TLC-profile of compounds contained in a *Lupinus albus* L. seed suspension (**SS**) fractionated by means of the liquid-liquid extraction procedure of Gamoh *et al*. (1989). (1= Ethyl acetate 1; 2 = Hexane; 3 = $NaHCO_3$ and 4 = Ethyl acetate 2). Mobile phase: Chloroform: methanol (95:5) + 1 ml glacial acetic acid. Stationary phase: Silica gel 60. The plate was stained with 10% (v/v) ethanolic sulphuric acid.

Table 5.1: **Concentrations of compounds recovered in the liquid-liquid extraction fractions as well as the remaining crude extract of *Lupinus albus* L. seeds using the brassinosteroid extraction procedure of Gamoh *et al*. (1989).**

Liquid-liquid fractions	Concentration in mg cm^{-3}
Crude extract	1.06
Ethyl acetate 1	752
Hexane	250
Sodium hydrogen carbonate	678
Ethyl acetate 2	516

Subsequently, all four liquid-liquid extraction fractions were tested for bio-stimulatory activity using different bio-assay procedures (see Chapter 3; 3.3.2).

5.4.2 Bio-stimulatory activity of different liquid-liquid extraction fractions obtained from a *L. albus* seed extract in terms of its effect on the respiration rate of monoculture yeast cells

The effect of the different liquid-liquid extraction fractions on the respiration rate of monoculture yeast cells was tested at a ten fold lower concentration (0.5 mg L^{-1}) than the original seed suspension and at 30 minute intervals over a period of three hours. Compared to the water control, only ethyl acetate fraction 1 and the hexane fraction significantly ($P<0.05$) increased the respiration rate of monoculture yeast cells and compared favourably to the commercial product *ComCat*® (*CC*) used as positive control (Figure 5.1 and Table 5.2; calculated LSD-value indicated in the graph).

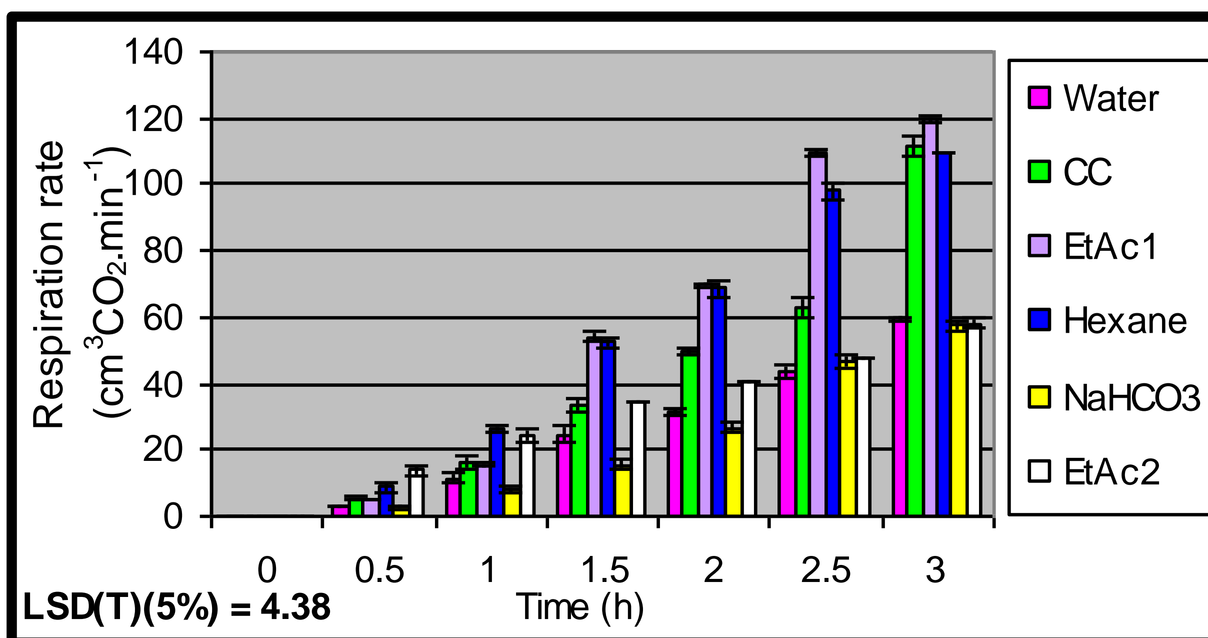

Figure 5.2: The effect of different liquid-liquid extraction fractions of *Lupinus albus* seeds at a concentration of 0.5 mg L^{-1} on the respiration rate of monoculture yeast cells. Water served as a negative control and a commercially available bio-stimulant, *ComCat*® ***(CC)***, was used as a positive control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph.

Table 5.2: Statistical analysis of the averaged and pooled respiration rate values of monoculture yeast cells as influenced by different liquid-liquid extraction fractions obtained from a *L. albus* seed extract.

Treatments	Respiration rate of different treatments after 3h of incubation (cm^3CO_2 min^{-1})
Control	59.6
CC	111.6
EtAc 1	119.6

Hexane	110.0
$NaHCO_3$	57.3
EtAc 2	58.0

Differences are significant at LSD $_{(T)}$ (5%) = 4.38; The ANOVA is attached as Table 5.2A in the Appendix.

In order to verify the activity detected in ethyl acetate 1 and the hexane fraction in terms of respiration rate enhancement in monoculture yeast cells the effect of all fractions was tested on Cress seed germination and subsequent seedling growth.

5.4.3 Bio-stimulatory activity of different liquid-liquid extraction fractions obtained from a *L. albus* seed extract in terms of its effect on the germination of Cress seeds

Compared to the water control, as was observed with the original *L. albus* seed suspension (see chapter 3; 3.3.3), none of the liquid-liquid fractions had a significant effect on and rather inhibited the germination of Cress seeds (Figure 5.2 and Table 5.3). However, after 96 h of incubation the ethyl acetate 1 fraction had a slight but non-significant enhancing effect.

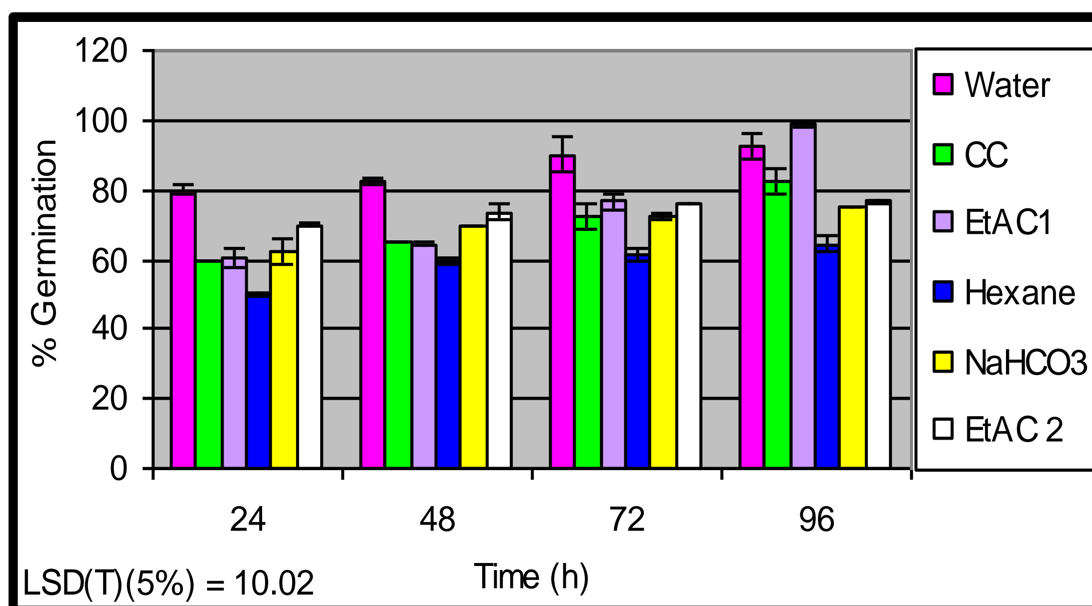

Figure 5.3: The effect of different liquid-liquid extraction fractions of *Lupinus albus* seeds at a concentration of 0.5 mg L^{-1} on the germination of Cress seeds. Water served as a negative control

and a commercially available bio-stimulant, *ComCat® (CC),* was used as a positive control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph.

Table 5.3: Statistical analysis of the averaged and pooled percentage germination values of Cress seeds as influenced by different liquid-liquid extraction fractions obtained from a *L. albus* seed extract.

Time	Effect on seed germination and interactions between treatments over time					
	Water	***CC***	**EtAc1**	**Hexane**	**$NaHCO_3$**	**EtAc2**
24 h	80.0	60.0	60.30	51.0	61.6	69.2
48 h	83.3	65.0	64.6	59.6	70.0	73.8
72 h	88.3	72.3	76.6	61.6	71.6	76.0
96 h	91.6	83.0	99.0	61.6	75.0	77.2

Differences are significant at LSD $_{(T)}$ (5%) =10.02; The ANOVA is attached as Table 5.3A in the Appendix

Subsequently, the effect of the liquid-liquid extraction fractions of a *L. albus* seed extract on Cress seedling growth was tested.

5.4.4 Bio-stimulatory activity of liquid-liquid extraction fractions obtained from a *L. albus* seed extract in terms of its effect on the growth of Cress seedlings

After 96 h of incubation only the two ethyl acetate fractions had a significant ($P<0.05$) effect on the coleoptile growth of Cress seedlings (Figure 5.3 and Table 5.4; the calculated LSD-value is indicated in the graph).

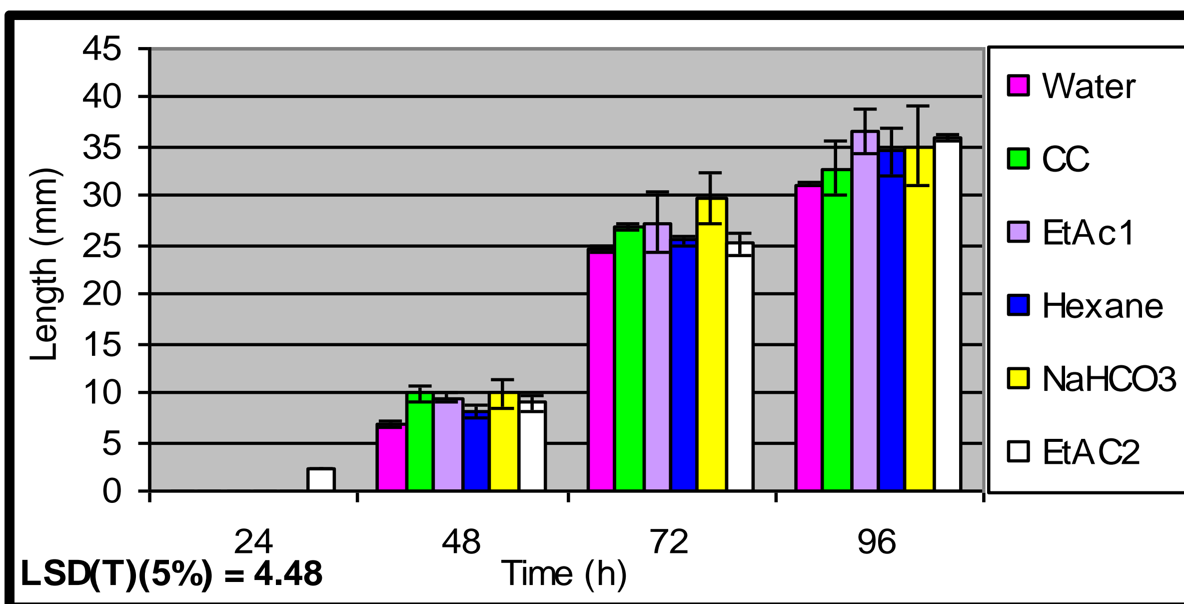

Figure 5.4: The effect of different liquid-liquid extraction fractions of *Lupinus albus* seeds at a concentration of 0.5 mg L^{-1} on the coleoptile growth of Cress seedlings. Water served as a negative control and a commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph.

Table 5.4: Statistical analysis of the averaged and pooled coleoptile length values of Cress seedlings as influenced by different liquid-liquid extraction fractions obtained from a *L. albus* seed extract.

Time	**Effect on coleoptile growth and interactions between treatments -over time**					
	Water	***CC***	**EtAc1**	**Hexane**	**$NaHCO_3$**	**EtAc2**
24 h	0	0	0	0	0	0.7
48 h	6.7	9.9	9.3	8.0	9.9	8.9
72 h	24.7	26.7	27.0	25.4	29.3	25.5
96 h	31.1	32.6	36.6	34.3	35.3	36.9

Differences are significant at LSD $_{(T)}$ (5%) = 4.48; The ANOVA is attached as Table 5.4A in the Appendix

Similarly, compared to the water control, the liquid-liquid ethyl acetate 1 fraction significantly increased the root growth of Cress seedlings (Figure 5.4 and Table 5.5; the calculated LSD value is indicated in the graph) but this was not observed for the ethyl acetate 2 fraction as was the case for coleoptile growth. From this it was concluded that most of the bio-stimulatory activity initially observed in a *L. albus* seed suspension (see Chapter 3) was contained in ethyl acetate fraction 1. Subsequently, only this fraction was purified further by means of column chromatography (see 5.2.5).

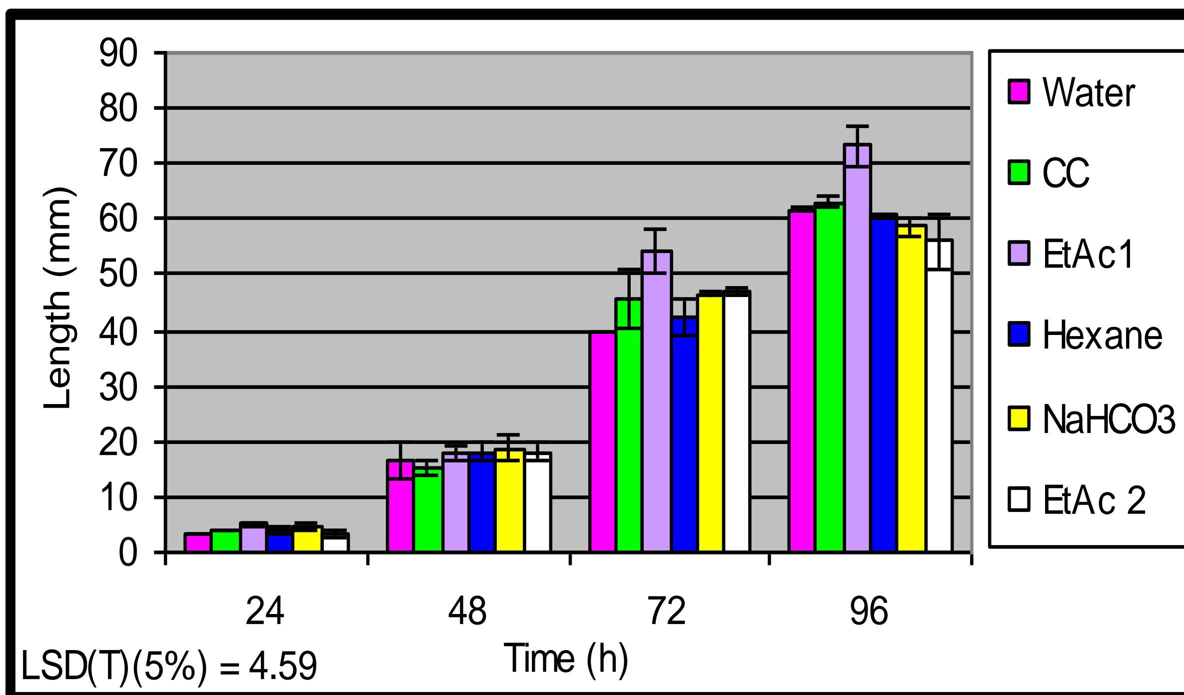

Figure 5.5: The effect of different liquid-liquid extraction fractions of *Lupinus albus* seeds at a concentration of 0.5 mg L^{-1} on the root growth of Cress seedlings. Water served as a negative control and a commercially available bio-stimulant, *ComCat*® ***(CC)***, was used as a positive control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph.

Table 5.5: Statistical analysis of the averaged and pooled root length values (mm) of Cress seedlings as influenced by different liquid-liquid extraction fractions obtained from a *L. albus* seed extract.

Time	Effect on root growth and interactions between treatments -over time					
	Water	***CC***	**EtAc1**	**Hexane**	**$NaHCO_3$**	**EtAc2**
24 h	3.5	4.0	5.1	3.9	4.4	3.3
48 h	16.3	15.6	17.9	18.1	18.6	18.3

72 h	39.8	46.2	53.9	42.3	46.5	46.2
96 h	61.7	63.3	73.5	60.4	59.2	55.9

Differences are significant at LSD $_{(T)}$ (5%) = 4.59; The ANOVA is attached as Table 5.5A in the Appendix

5.4.5 A qualitative thin layer chromatography profile of twelve combined column chromatography fractions obtained from the ethyl acetate 1 liquid-liquid extractant

A gradient solvent system was used to elute all active compounds from the highly active ethyl acetate 1 fraction in a total of 85 x 5 ml column fractions and those with similar Q-TLC-profiles were pooled in 12 combined column fractions (Plate 5.2). The TLC-profiles of compounds contained in the first four combined column fractions showed great similarity and seemingly represented only one compound but in different quantities. In combined column fractions 5 and 6 two distinct compounds was observed of which one or both may or may not have been similar to that contained in the first four fractions. The TLC-profiles of all of the other combined column fractions were distinctly different from the first six fractions indicating that the column chromatographic fractionation protocol followed successfully distinguished between different compounds.

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Plate 2: A qualitative TLC-profile of compounds contained in the twelve combined column chromatography fractions after fractionating the highly active ethyl acetate fraction 1 obtained from a *Lupinus albus* L seed extract. Mobile phase: Chloroform: methanol (95:5) + 1 ml glacial acetic acid (Gamoh *et al*., 1989). Stationary phase: Silica gel 60. The plate was stained with 10% (v/v) ethanolic sulphuric acid.

In order to distinguish between active and non-active compounds the effect of the twelve combined column fractions on the germination of Cress seeds as well as subsequent seedling growth was tested at a concentration of 0.5 mg L^{-1}.

5.4.6 Bio-stimulatory activity of twelve combined column chromatography fractions obtained from the ethyl acetate 1 liquid-liquid extractant in terms of its effect on the germination of Cress seeds and subsequent seedling growth

As was previously encountered, and compared to the water control, none of the combined column fractions had a stimulating effect on the germination of Cress seeds (Figure 5.5 and Table 5.6) but rather tended to inhibit germination.

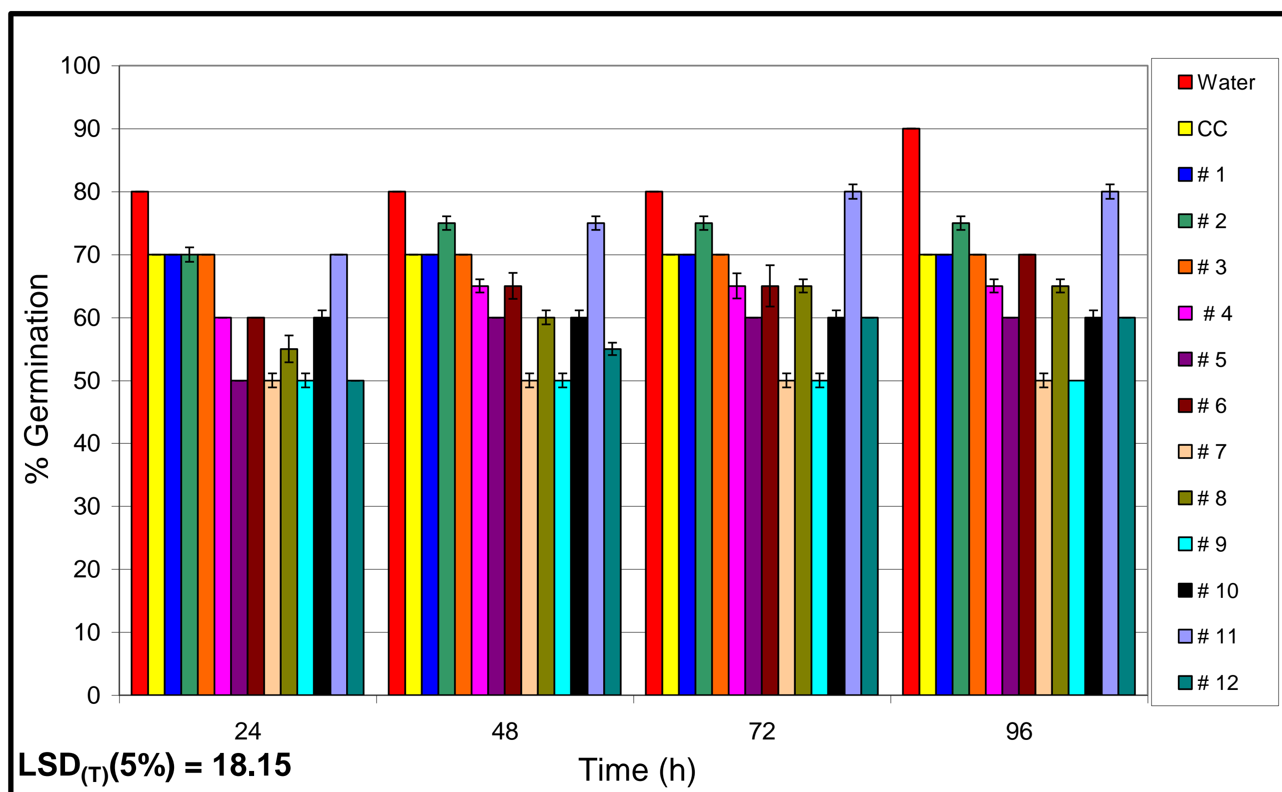

Figure 5.6: The effect of twelve combined column fractions obtained from a *Lupinus albus* seed extract on the germination of Cress seeds at a concentration of 0.5 mg L^{-1}. Water served as a negative control and a commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph.

Table 5.6: Statistical analysis of the averaged and pooled percentage germination values of Cress seeds as influenced by twelve combined column fractions obtained from a *L. albus* seed extract.	
Concentration ($mg\ L^{-1}$)	**Percentage germination at a concentration of 0.5 $mg\ L^{-1}$**
Water	90
CC	70
# 1	70
# 2	75
# 3	70
# 4	65
# 5	60
# 6	70
# 7	50
# 8	65
# 9	50
# 10	60
# 11	80
# 12	60

Differences are significant at $LSD_{(T)}$ (5%) = 18.15 ; The ANOVA is attached as Table 5.6A in the Appendix

However, the first four combined column fractions were the only ones that significantly ($P<0.05$) enhanced the coleoptile growth of Cress seedlings (Figure 5.6 and Table 5.7) while fractions 7 and 9 showed slight but non-significant activity.

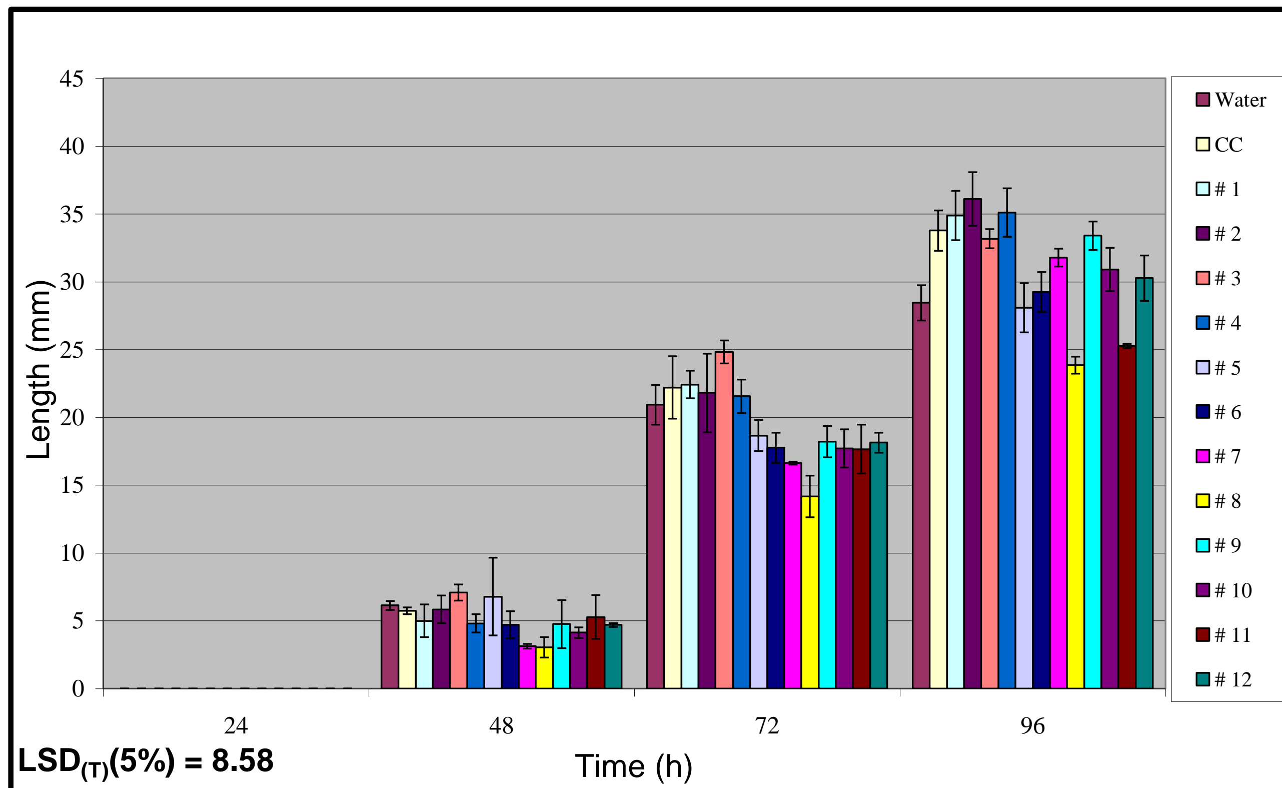

Figure 5.7: The effect of twelve combined column fractions obtained from a *Lupinus albus* seed extract on the coleoptile growth of Cress seedlings at a concentration of 0.5 mg L^{-1}. Water served as a negative control and a commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control. Statistical significance is indicated by the LSD $_{(T)}$ (5%) value in the graph.

Table 5.7: Statistical analysis of the averaged and pooled coleoptile length values of Cress seedlings as influenced by twelve combined column fractions obtained from a *L. albus* seed extract.	
Concentration ($mg\ L^{-1}$)	**Coleoptile length as influenced by treatments at a concentration of 0.5 $mg\ L^{-1}$**
Water	28.44
CC	33.77
# 1	34.88
# 2	36.10
# 3	33.16
# 4	35.10
# 5	28.08
# 6	29.25
# 7	31.77
# 8	23.84
# 9	33.40
# 10	30.88
# 11	25.25
# 12	30.26

Differences are significant at $LSD_{(T)}$ (5%) = 8.58; The ANOVA is attached as Table 5.7A in the Appendix

Similarly, the first three column fractions showed significant bio-stimulatory activity in terms of the root growth of Cress seedlings while fractions 4, 7, 9 and 10 showed marked to significant activity (Figure 5.7 and Table 5.8). In summary, the results indicated that the same compound or group of compounds was distributed over the first four column fractions in rather large quantities and these were also more active compared to other fractions.

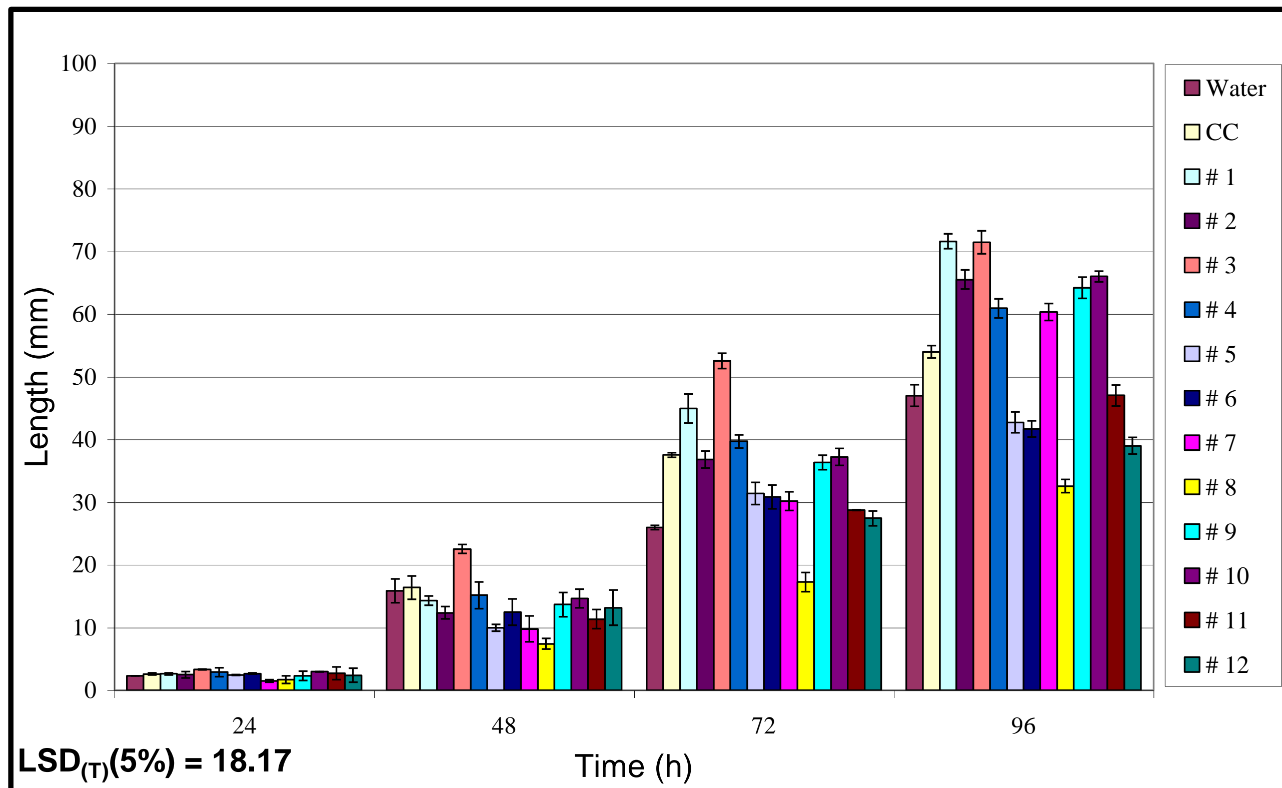

Figure 5.8: The effect of twelve combined column fractions obtained from a *Lupinus albus* seed extract on the root growth of Cress seedlings at a concentration of 0.5 mg L^{-1}. Water served as a negative control and a commercially available bio-stimulant, *ComCat® (**CC**),* was used as a positive control. Statistical significance is indicated by the LSD $_{(T)}$ (5%) value in the graph.

Table 5.8: Statistical analysis of the averaged and pooled root length values of Cress seedlings as influenced by twelve combined column fractions obtained from a *L. albus* seed extract.	
Concentration ($mg\ L^{-1}$)	**Root length as influenced by treatments at a concentration of 0.5 $mg\ L^{-1}$**
Water	46.99
CC	53.99
# 1	71.62
# 2	65.52
# 3	71.47
# 4	60.93
# 5	42.74
# 6	41.71
# 7	60.33
# 8	32.57
# 9	64.20
# 10	66.01
# 11	47.22
# 12	38.99

Differences are significant at $LSD_{(T)}$ (5%) = 18.17 ; The ANOVA is attached as Table 5.8A in the Appendix

In Table 5.9 the dry mass of compounds contained in twelve combined column chromatography fractions, after fractionation of the highly bio-active ethyl acetate 1 liquid-liquid extractant of *L. albus* seeds and drying at 35 ^{o}C, is shown. In comparison, the first four column fractions showed the highest bio-stimulatory activity (Table 5.9; shown in **bold typing**) and were also recovered in the highest amounts. The Q-TLC profile of the twelve column fractions (see Plate 2) strongly indicated that the first four fractions contained only one compound suggesting that the compound was the same in all. Fractions 7, 9 and 10 also showed bio-stimulatory activity (Table 5.9; shown in **bold typing**) but differed from the first four fractions in terms of the higher number of compounds contained in each fraction as well as the extremely low amounts recovered.

Table 5.9: Dry mass recovery of compounds contained in twelve combined column chromatography fractions after fractionation of the active ethyl acetate 1 liquid-liquid extractant of *L. albus* seeds. Fractions were dried at 35°C. Active fractions are indicated in **bold typing**.

Fractions	Dry Mass (mg)	Number of compounds detected in each fraction
1	**84.0**	**1**
2	**20.0**	**1**
3	**174.0**	**1**
4	36.7	1
5	23.9	2
6	84.7	3
7	**13.5**	**6**
8	21.5	6
9	**6.9**	**7**
10	**25.1**	**9**
11	25.2	10
12	14.4	9

5.5 DISCUSSION

The activity directed semi-purification of bio-stimulatory compounds contained in a *Lupinus albus* seed extract was based on liquid-liquid extraction and column chromatography. Bio-stimulatory activity in either liquid-liquid extractions or column chromatography fractions was followed using two bio-assay procedures namely the enhancement of the respiration rate in monoculture yeast (*Saccharomyces cerviseae*) cells as well as Cress seed germination and subsequent seedling growth as indicators of activity. The bio-assays included two controls namely water as a negative and a commercial bio-stimulant, *ComCat®*, as a positive control to evaluate the bio-activity contained in both liquid-liquid extractants and column chromatography fractions by comparison. Using the liquid-liquid extraction procedure of Gamoh *et al. (1989)* for specifically extracting brassinosteroids (BRs) from plant material, four fractions were collected namely two ethyl acetate fractions (numbered fractions 1 and 2), a hexane and a sodium bicarbonate fraction. According to Gamoh *et al*. (1989) ethyl acetate fraction 2 contains the brassinosteroids if present.

The bio-stimulatory activity of BRs is well established (Mussig *et al*., 2002; Halliday, 2004). However, in this study the ethyl acetate 2 fraction supposedly containing the BRs (Gamoh *et al*., 1989) showed no significant bio-stimulatory activity independent of the bio-assay procedure followed. The latter is indicative of BRs being either absent from or present in extremely low quantities in *L. albus* seeds or that a low concentration of the actual bio-active compound(s) contained in the highly active ethyl acetate fraction 1 might have remained in the second ethyl acetate fraction. Similarities in the qualitative thin layer chromatography profiles of these two fractions are in support of this postulate.

Of the four liquid-liquid extractants the first ethyl acetate fraction (numbered ethyl acetate 1) was the only one showing significant bio-stimulatory activity in terms of both bio-assay procedures followed while the hexane fraction showed signs of activity although very low and non-significant. As hexane is a non-polar solvent (dielectric constant (DC) = 1.9) compared to the more polar ethyl acetate (DC = 6.0; Loudon, 1988) one would have expected the TLC profiles of these two fractions to differ markedly but this was not the case. Similarities in the Q-TLC profiles of these two fractions might have been due to traces of the actual active compounds contained in the highly active ethyl acetate 1 fraction to have contaminated the hexane fraction and might explain the activity detected in the latter. As the concentration of compounds contained in the highly active ethyl acetate fraction was threefold higher than that of the hexane fraction, only the ethyl acetate fraction was purified further by means of column chromatography. A total of 85 fractions were collected from the column chromatographic separation procedure and those with similar Q-TLC-profiles were combined into twelve column fractions and tested for activity.

None of the twelve combined column fractions had any effect on the percentage germination of Cress seeds and this bio-assay procedure could not be used to distinguish between active and non-active column fractions. This is in agreement with a report of Leather and Einhellig (1985) that activity directed bio-assays determining seedling growth are usually more sensitive than that measuring percentage germination. Subsequently, the effect of the different combined column fractions on the growth of Cress seedlings was used for this purpose. Compared to the other column fractions, both coleoptile and root growth of Cress seedlings were enhanced most by fractions one to four. Further, similarities in the Q-TLC-profiles as well as RF-values of compounds contained in

these four fractions indicated that the active compound or group of compounds might be the same. This can only be confirmed by means of TLC fingerprinting using different spray reagents to characterise the compounds (Wagner and Bladt, 1996) as well as NMR spectroscopy (see chapter 6).

Although column fractions 7, 9 and 10 also showed significant bio-stimulatory activity in terms of coleoptile and root growth in Cress seedlings the quantities of compounds recovered from these fractions was tenfold lower than that recovered from the first four column fractions. In fact, the recovery of compounds in the latter three fractions was too low for further purification and subsequent elucidation of their chemical structures by means of NMR spectroscopy as at least 10 mg of a pure compound is needed for this. For this reason only column fractions one to four were combined and further purified (see chapter 6).

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CHAPTER 6

ISOLATION, PURIFICATION AND IDENTIFICATION OF BIO-STIMULATORY ACTIVE SUBSTANCES FROM *Lupinus albus* L. SEEDS

6.1 ABSTRACT

Activity directed fractionation of a methanol crude extract of ground *Lupinus albus* seeds by means of liquid-liquid extraction, using organic solvents of different polarities, resulted in an ethyl acetate extract containing most of the bio-stimulatory activity. Three general bio-assay procedures were employed to evaluate the plant growth stimulating properties of semi-purified fractions as well as pure compounds. These included monitoring the effect on the respiration rate of monoculture yeast (*Saccharomyces cerivisiae*) cells, the germination of cabbage seeds and subsequent seedling development in terms of root and coleoptile growth. A commercially available bio-stimulant, *ComCat*®, was used as a positive control while distilled water served as a negative control in all bio-assays. Subsequently, the highly active ethyl acetate extract was fractionated further by means of adsorption column chromatography resulting in eighty five fractions that were combined into twelve on grounds of similarities in thin layer chromatography profiles. Although significant bio-stimulatory activity was detected in six of these combined column fractions the dry mass recovery of active compounds allowed for only four fractions to be purified further. By means of preparative thin layer chromatography only one bio-active compound was isolated, purified and identified by means of nuclear magnetic resonance spectroscopy as a triglyceride, glycerol trilinoleate.

Keywords: Column and preparative thin layer chromatography, purification of active compounds, nuclear magnetic resonance (NMR) spectroscopy, bio-stimulatory activity, glycerol trilinoleate.

6.2 INTRODUCTION

The liquid-liquid extraction technique, using organic solvents with increasing polarities, is commonly used for fractionating bio-active compounds from crude plant extracts (Fourneau *et al*., 1996; Song and Oh, 1996; Amen *et al*., 1997; Benerle and Schwab, 1997; Silva *et al*., 1997).

Column and both qualitative and preparative thin layer chromatography techniques are currently still employed for isolating and purifying active compounds from plant extracts (Froytlog *et al*. 1998; Choung, *et al*. 2000; Tang, *et al*. 2000; Jang, *et al*. 2001; Shin, *et al*. 2001; Maciejewicz, 2001; Negi and Jayaprakasha, 2001; Zhu *et al*. 2001). Once active compounds are purified, nuclear magnetic resonance (NMR) and mass spectroscopy are commonly used to elucidate the chemical structures of compounds (Bonnlander and Winterhalter, 2000; Gil *et al*., 2000; Bilia *et al*., 2001; Lee *et al*., 2001; Pauli, 2001; Ratcliffe and Shacha, 2001; Takeoka *et al*., 2001).

In this study, significant bio-stimulatory activity has been confirmed in a *Lupinus albus* seed suspension under laboratory conditions (Chapter 3) using standard bio-assay procedures. These included monitoring the effect on the respiration rate of monoculture yeast cells, the germination of seeds and subsequent seedling development in terms of root and coleoptile growth. A commercially available bio-stimulant, *ComCat*®, was used as a positive control while distilled water served as a negative control in all bio-assays in order to evaluate the bio-stimulatory activity of the seed suspension by comparison. Subsequently, the yield improving properties of the *L. albus* seed suspension after foliar applications to a variety of agricultural crops was confirmed under field conditions (Chapter 4) by means of statistically designed field experiments. This supplied the rationale for proceeding in isolating and purifying the bio-active substance(s) involved as well as to elucidate their molecular structures by means of nuclear magnetic resonance (NMR) spectroscopy.

By means of activity directed liquid-liquid extraction using organic solvents in order of increasing polarity a highly active ethyl acetate extract was obtained and further fractionated by means of column chromatography (Chapter 5). Eighty five column fractions were combined into twelve on grounds of similarities in thin layer chromatography profiles of which the first four fractions were highly active and purified further.

6.3 MATERIALS AND METHODS

6.3.1 Materials

6.3.1.1 Plant material

See chapter 5 section 5.2.1.

6.3.1.2 Other material

See chapter 5, section 5.2.2

6.3.2 Methods

6.3.2.1 Purification of compounds contained in the first four combined column chromatography fractions by means of preparative thin-layer chromatography (P-TLC)

The first four of the twelve combined fractions obtained by means of column chromatography showed the highest bio-stimulatory activity (see chapter 5; 5.25 and 5.26) and was purified further by means of preparative thin layer chromatography (P-TLC). Fifty mg of each column fraction dissolved in 50 µl of the mobile phase was loaded in a band and in 10 µl increments onto a separate pre-coated silica plate (Merck Kieselgel 60 F_{254}; 20 X 20 cm^2, 1mm thickness) by streaking evenly over the full length of the baseline with the aid of a glass capillary tube. Using a fan, the plate was air dried between increments to concentrate the substances in as small an area as possible and subsequently developed in a saturated glass chromatography chamber using chloroform: methanol (10:1; v/v) as mobile phase. After observing the plate under UV light (254 and 365 nm) and marking the clearly defined boundaries of individual compounds, the silica containing the compounds were scraped off from the glass plates, using a spatula, and transferred to separate glass beakers. The compounds were recovered from the silica by elution with ethanol for compounds with RF-value above 0.5 and methanol for those with RF-values below 0.5 (Mikes and Chalmers 1979) followed by centrifugation for five minutes at 20 000 g. Only two compounds, of which the RF-values were almost identical, were identified in each column fraction and again tested for purity in an original analytical TLC system.

6.3.2.2 Determination of the purity of the two P-TLC compounds by means of qualitative thin layer chromatography (Q-TLC)

The two compounds isolated by means of P-TLC were initially prepared at a concentration of 1 mg ml^{-1} (1µg $µl^{-1}$). By drying between applications descending volumes (in µl) of each were spotted on different Silica gel 60 F_{254}-aluminium backed and pre-coated TLC plates in order to compare the Q-TLC profiles of different quantities (32, 16, 8, 4, 2, 1 and 0.5 µg) of each

component. A chloroform: methanol (10:1; v/v) solution was used as mobile phase. After observation under UV-light at 254 and 365 nm, the plate was coloured with 10% (v/v) ethanolic sulphuric acid and placed in an oven at 100°C for 20 minutes.

6.3.2.3 Preliminary phyto-chemical screening of the two isolated compounds using spray reagents

In order to identify the group of chemicals to which the two isolated compounds belonged, both were separated on silica gel 60 F_{254}-aluminium backed and pre-coated (0.1 mm) plates, cut to 10 x 3 cm, by using chloroform: methanol (10:1; v/v) as mobile phase in all cases. Tests for different chemical groups, including alkaloids, anthraglycosides, bitter principles, cardiac glycosides, coumarins, essential oils, flavonoids, phenolic compounds, saponins and steroids were performed on separate TLC plates using specific staining reagents according to the standard methods described by Wagner and Bladt (1996). Control plates were also sprayed with 5% ethanolic H_2SO_4 in order to detect all possible carbon containing compounds in the fraction. Both compounds isolated from *L. albus* seed was of the steroidal type. Subsequently, the two compounds were concentrated by evaporating the solvent under reduced pressure at 35°C in a rotary evaporator and the pure compounds further dried in a vacuum oven in preparation for structure elucidation by means of proton nuclear magnetic resonance spectroscopy (^{1}HNMR).

6.3.2.4 Elucidation of the structural formulas of the two purified compounds by means of mass spectrometry and nuclear magnetic resonance spectroscopy (NMR)

The isolated compounds were washed repeatedly with acetone to obtain an acceptable level of purity. Subsequently, the compounds were analyzed by spectroscopic and spectrometric methods [fast atom bombardment (FAB-MS)] and electron impact mass spectrometry (EI-MS)]. Nuclear magnetic resonance spectroscopy (NMR) was performed on a Bruker 300 MHz DRX 300 spectrometer at 296K (23°C) with tetramethylsilane ($Si(CH_3)_4$; TMS) as the internal standard. The solvents used were either deuteriochloroform ($CDCL_3$) or deuterio-acetone [$(CD_3)_2$ CO] as indicated. Chemical shifts were reported in parts per million (ppm) on the δ-scale and coupling constants are given in Hz. The following abbreviations are used:

s singlet

m multiplet

d	doublet
t	triplet
dd	doublet of doublets
br	broadened
DEPT:	distortionless enhancement by polarization transfer
COSY	correlation spectroscopy
NOESY	nuclear Overhauser effect spectroscopy
HMBC	heteronuclear multi-bond coherence
HMQC	heteronuclear multiple quantum coherence
^{13}C NMR	^{13}carbon nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
FAB-MS	fast atom bombardment
EI-MS	electron impact

All FAB mass spectra were recorded on a VG 70-70 E double-focusing mass spectrometer and mass determinations were obtained with a Kratos MS-80 mass spectrometer in the double focus electron impact (EI) mode. Structural elucidation was mainly achieved *via* NMR spectroscopy (1D and 2D) as well as chemical methods, e.g. hydrolysis. The 1D NMR experiments comprised the ^{1}HNMR, ^{13}CNMR and DEPT while the 2D included HMQC and HMBC.

6.3.2.5 Screening of the active compounds isolated from *L. albus* seeds for bio-stimulatory activity

The pure compounds were subjected to the same bio-assay procedures as described in Chapter 3, sections 3.3.2.1 and 3.3.2.2. Additionally, the wheat leaf unrolling bio-assay procedure described by Takatsuto, (1994) was employed to test for the effect of the pure compounds on wheat coleoptile growth at extremely low concentrations.

Wheat seedlings (*Triticum aestivum* L.) were grown in the dark at 26^{0}C for three days in vermiculite while watering every day with 20 ml distilled water. After three days the wheat seedlings were removed and the coleoptiles severed from the seeds using a sharp razor blade. Five coleoptile segments per replicate were placed on Whatman no 1 filter paper in separate sterile 90 mm Petri dish and moistened with either 10 ml sterile water (untreated control) or 0.1mg L^{-1} solutions of the purified compounds while a commercial bio-stimulant, *ComCat*®, was

used as a positive control at 0.5 mg L^{-1}. The coleoptile segments were incubated for 24 h at $30^{0}C$ in the dark and the length of each segment measured using a digital caliper. The bio-test was replicated three times.

6.3.2.6 Statistical analysis

Analysis of variance (ANOVA) was performed on the data, using the NC: SAS Institute Inc., Dos Statistical Program to identify differences between treatments. Tukey-Kramer's least significant difference (LSD) procedure for comparison of means (Steele and Torrie, 1980; Mason *et al*., 1989) was applied to separate means ($P<0.05$). Treatments differing significantly were indicated either in figures or below tables as calculated LSD values and by using symbols for significant (*) and insignificant (ns) differences.

6.4 Results

6.4.1 Determination of the purity of two compounds isolated from *L. albus* seed by means of column and preparative thin layer chromatography (P-TLC) using qualitative thin layer chromatography (Q-TLC)

By means of activity directed column chromatography followed by activity directed preparative thin layer chromatography (P-TLC) two bio-active compounds were isolated from *L. albus* seed. Their state of purity was tested by means of qualitative thin layer chromatography (Q-TLC) by spotting the two compounds on separate plates in a linear range of descending volumes in order to compare the Q-TLC profiles of different quantities of each component. Although the visual appearance of compound 1 (Plate 6.1) and compound 2 (Plate 6.2) was different on the Q-TLC plates the RF-values were almost identical. The results in plates 6.1 and 6.2 confirmed that both compounds were pure as only single spots were observed at all concentrations.

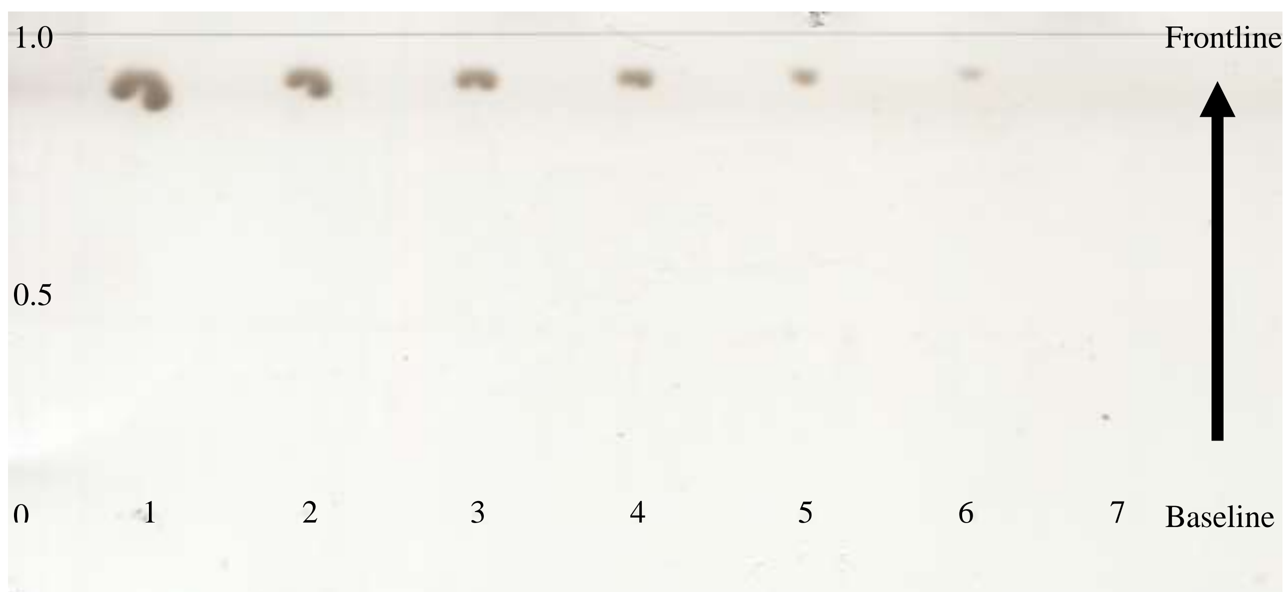


Plate 6.1: A qualitative TLC-profile of compound 1 isolated from *Lupinus albus* L. seed and spotted in a concentration range. (1= 32 µg μl^{-1}; 2 = 16 µg μl^{-1}; 3 = 8 µg μl^{-1}; 4 = 4 µg μl^{-1}; 5 = 2 µg μl^{-1} and 6 = 1 µg μl^{-1}). Mobile phase: Chloroform: methanol (95:5; v/v) + 1 ml glacial acetic acid. Stationary phase: Silica gel 60. The plate was stained with 10% ethanolic sulphuric acid.

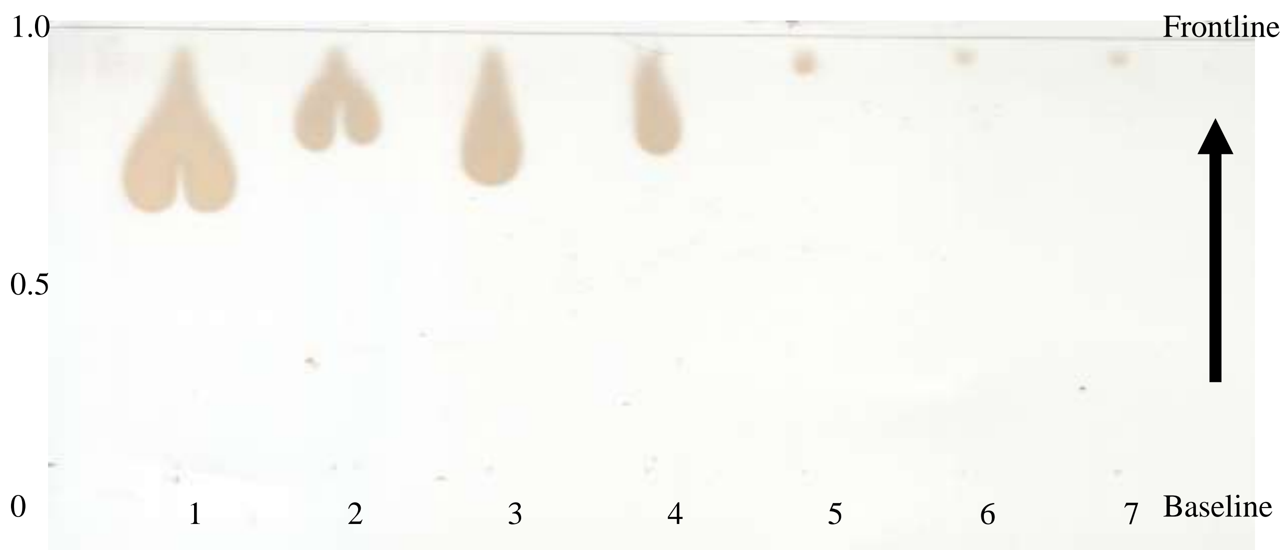


Plate 6.2: A qualitative TLC-profile of compound 2 isolated from *Lupinus albus* L. seed and spotted in a concentration range. (1= 32 µg μl^{-1}; 2 = 16 µg μl^{-1}; 3 = 8 µg μl^{-1}; 4 = 4 µg μl^{-1}; 5 = 2 µg μl^{-1} ; 6 = 1 µg μl^{-1} and 7 = 0.5 µg μl^{-1}). Mobile phase: Chloroform: methanol (95:5; v/v) + 1 ml glacial acetic acid. Stationary phase: Silica gel 60. The plate was stained with 10% ethanolic sulphuric acid.

In order to verify the similarities in RF-values obtained with the two compounds, a fingerprinting technique (Wagner and Bladt, 1996) was employed where both were spotted on a series of Q-TLC plates at a concentration of 64 µg $µl^{-1}$ and stained with different spray reagents to ascertain whether the two compounds belonged to different chemical groups. Positive results were only obtained with one of the plates (Plate 6.3) that were stained with 10% aqueous phosphoric acid confirming both compounds to be of a steroidal type (Wagner and Bladt, 1996). From this profile it seemed that the two compounds were identical which was later confirmed by means of NMR spectroscopy (see 6.3.3). Subsequently, the two compounds were combined and tested for bio-stimulatory activity using different bio-assay techniques.

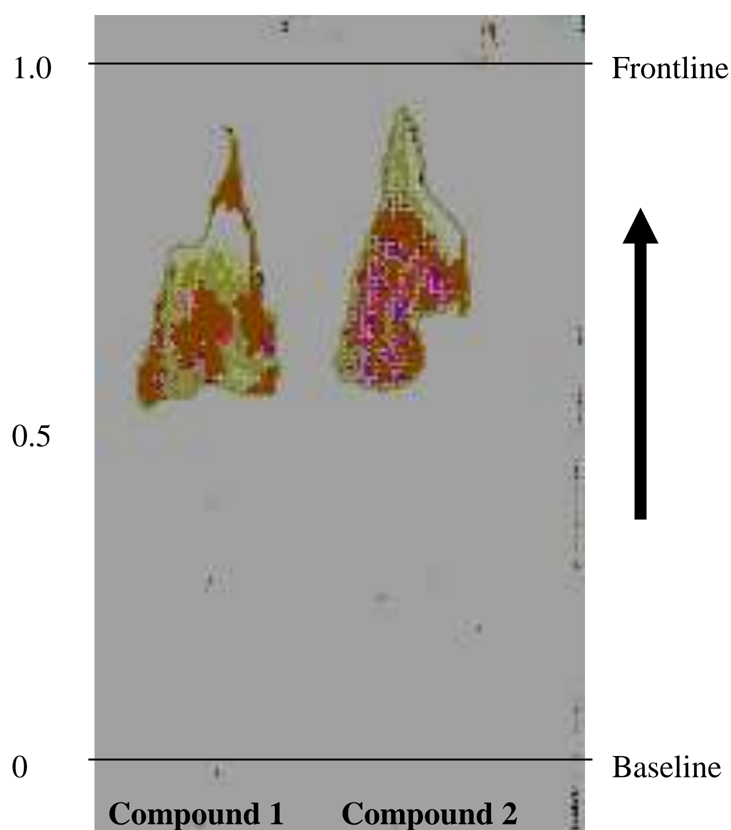


Plate 6.3: A qualitative TLC-profile of compound 1 and compound 2 isolated from *Lupinus albus* L. seed. Mobile phase: Chloroform: methanol (95:5; v/v) + 1 ml glacial acetic acid. Stationary phase: Silica gel 60. The plate was stained with 10% (v/v) aqueous phosphoric acid.

6.4.2 Bio-assaying of the compound purified from *L. albus* L. seeds for bio-stimulatory activity by means of different techniques

6.4.2.1 Effect on the respiration rate of monoculture yeast cells

The purified active compound, tested at a concentration of 0.5 mg L^{-1}, had a significant enhancing effect on the respiration rate of monoculture yeast cells over an incubation period of 90 minutes. After 40 minutes of incubation the pure compound already significantly ($P<0.05$; LSD = 23.19) increased the respiration rate by 115 % compared to the water control and compared favorably with the commercial bio-stimulant, *ComCat*® (Figure 6.1). After 70 minutes of incubation the pure compound reached its maximum capacity to increase the respiration rate of yeast cells compared to the positive control that only reached its maximum capacity after 90 minutes.

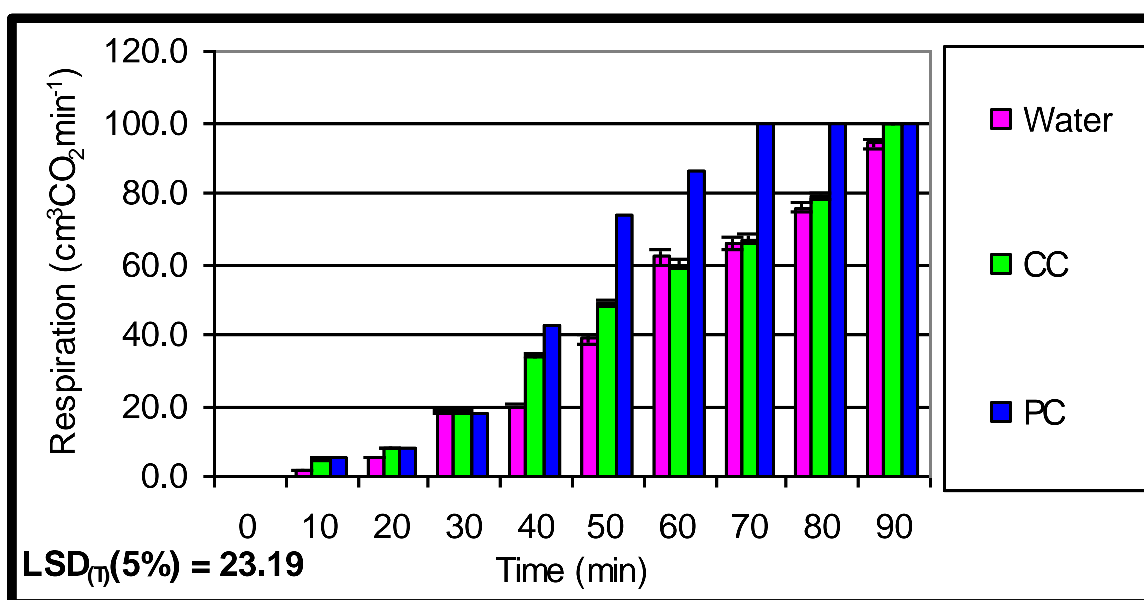


Figure 6.1: The effect of a compound purified from *Lupinus albus* seeds at a concentration of 0.5 mg L^{-1} on the respiration rate of monoculture yeast cells. Water served as a negative control and a commercial bio-stimulant, *ComCat® (CC),* as a positive control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph and the ANOVA attached as Table 6.1A in the Appendix. PC = Purified compound

In order to verify whether the increase in the respiration rate of yeast cells was an indication of a positive or negative influence in terms of growth, the effect was tested on cabbage seed germination and subsequent seedling growth.

6.4.2.2 The effect of a compound purified from *L. albus* seeds on the germination of cabbage seeds

Over the first 72 h of incubation the pure active compound had a significant ($P<0.05$; LSD = 4.87) effect on the percentage germination of cabbage seeds compared to both the water control and the commercial bio-stimulant (Figure 6.2). It seemed that the commercial product, *ComCat*® rather inhibited the germination of cabbage seeds. Subsequently, the effect of the compound on seedling growth was tested in terms of coleoptile and root growth.

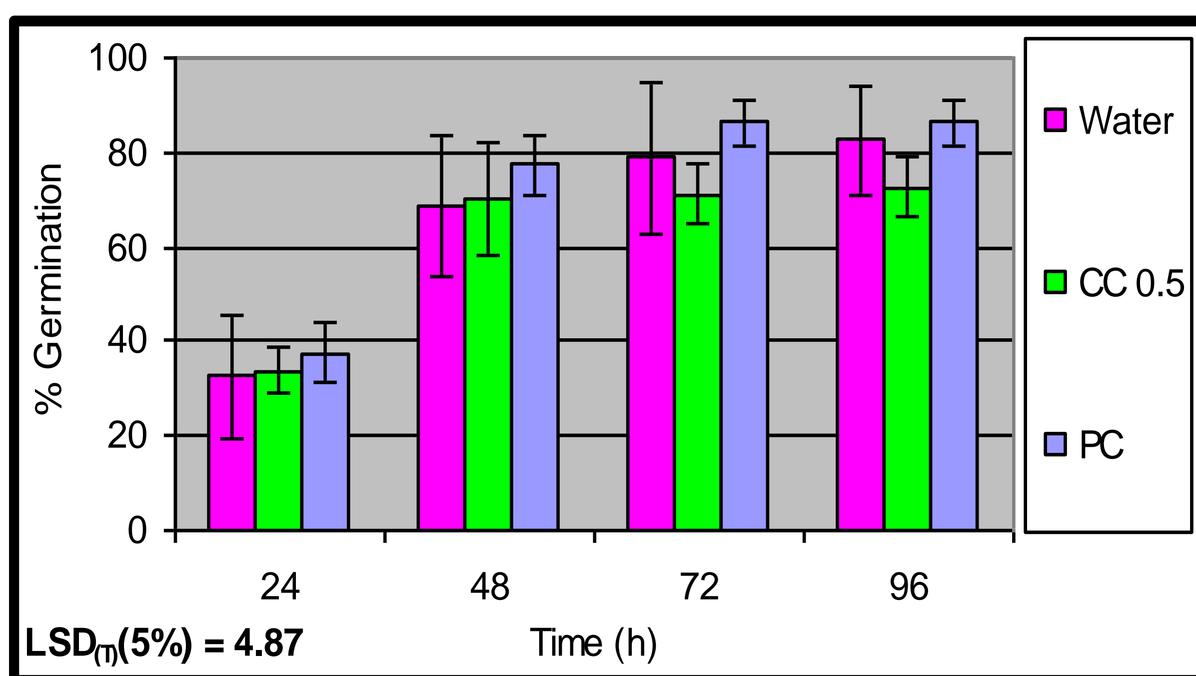


Figure 6.2: The effect of a compound purified from *Lupinus albus* (**SS**) seeds at a concentration of 0.5mg L^{-1} on the percentage germination of cabbage seeds. A commercial bio-stimulant, *ComCat® (**CC**),* was used as a positive control and water as a negative control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph and the ANOVA attached as Table 6.2A in the Appendix. PC = Purified compound

6.4.2.3 The effect of a compound purified from *L. albus* seeds on the growth of cabbage seedlings

Over the full duration of the incubation period the purified compound significantly ($P<0.05$) increased both the coleoptile (Figure 6.3; ; LSD = 1.16) and root (Figure 6.4; ; LSD = 2.88) growth of cabbage seedlings.

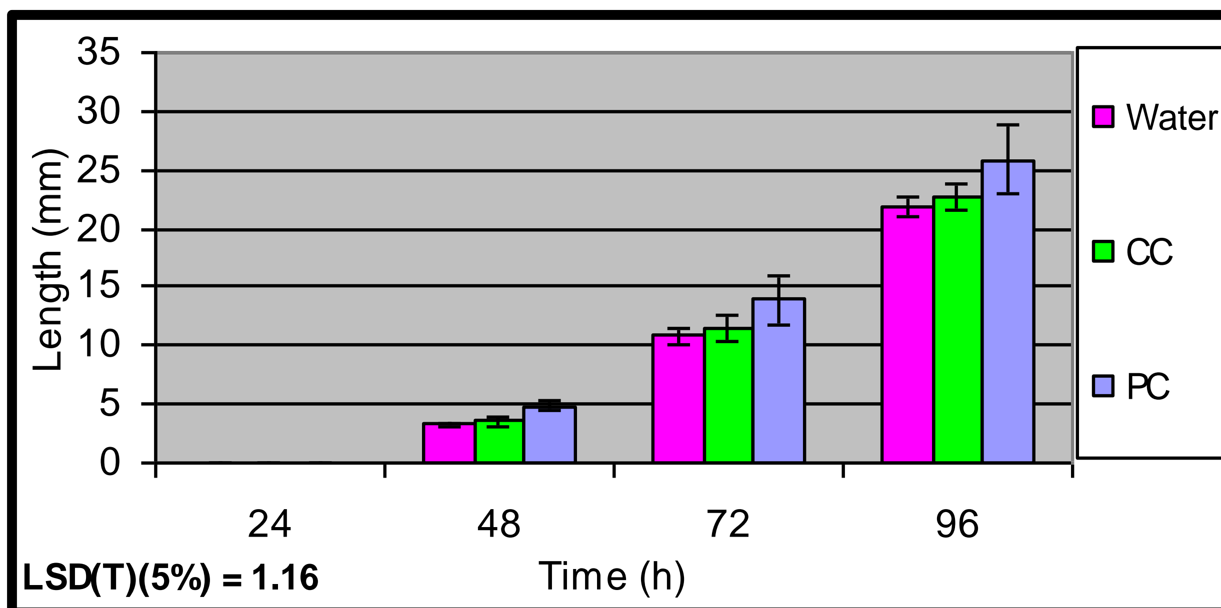


Figure 6.3: The effect of a compound purified from *Lupinus albus* L. seeds at a concentration of 0.5mg L^{-1} on the coleoptile growth of cabbage seedlings. A commercial bio-stimulant, *ComCat® (CC),* was used as a positive control and water as a negative control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph and the ANOVA attached as Table 6.3A in the Appendix. PC = Purified compound

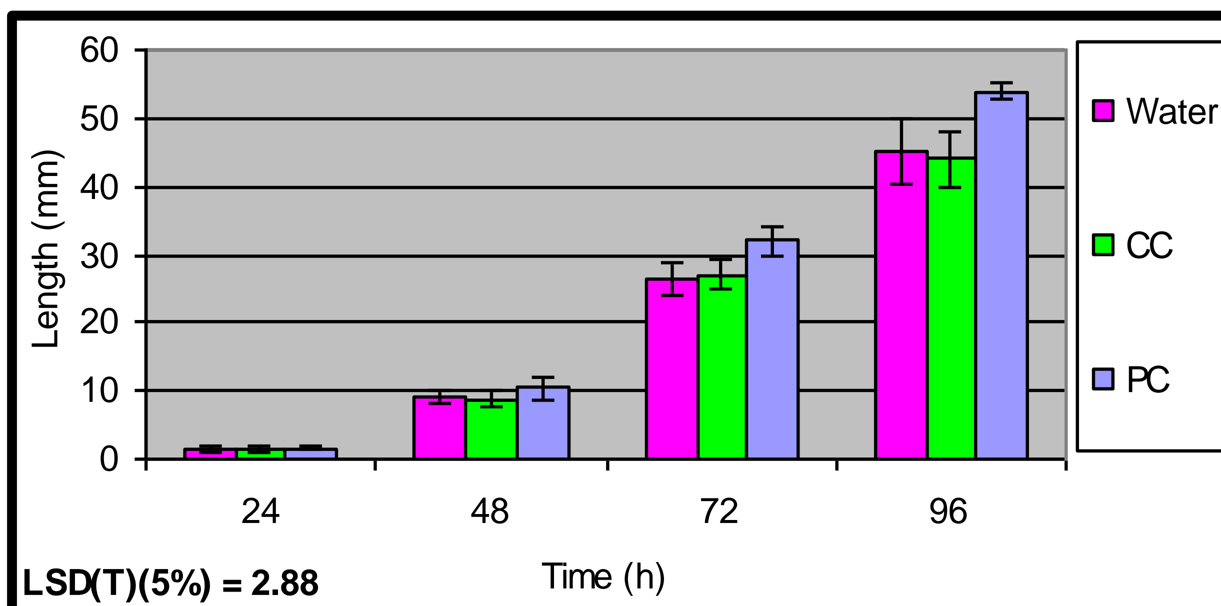


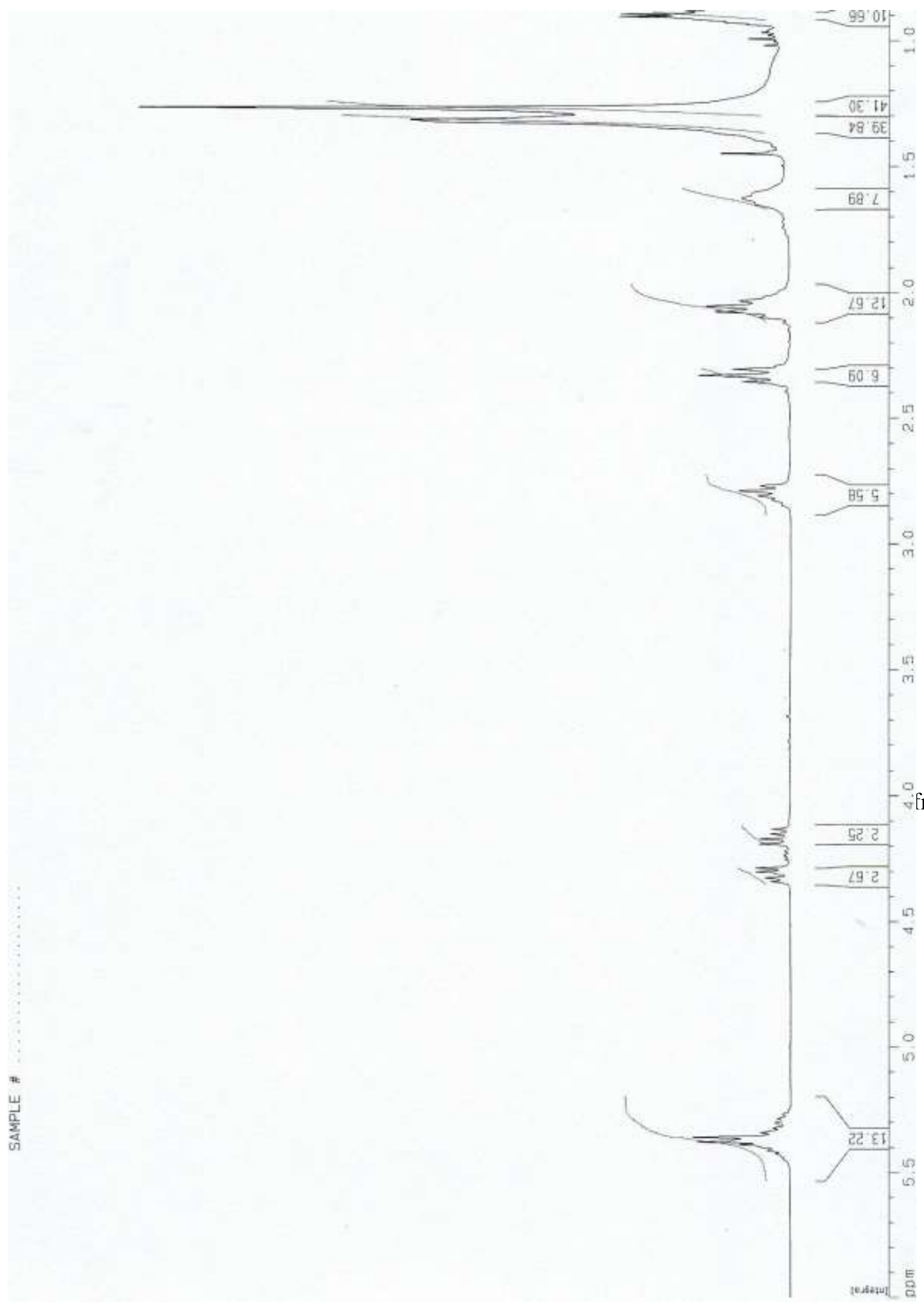
Figure 6.4: The effect of a compound purified from *Lupinus albus* L. seeds at a concentration of 0.5mg L^{-1} on the root growth of cabbage seedlings. A commercial bio-stimulant, *ComCat® (CC),* was used as a positive control and water as a negative control. Statistical significance is indicated by calculated LSD $_{(T)}$ (5%) values in the graph and the ANOVA attached as Table 6.4A in the Appendix. PC = Purified compound**.**

6.4.3 Identification of a bio-stimulatory compound purified from *Lupinus albus* L. seeds by means of Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry

The compound purified from an ethyl acetate extract of *L albus* seeds was obtained as yellow oil. The ^{1}H and ^{13}C NMR spectra (Plate 6.4 and Plate 6.5) and Table 6.1 (^{1}H NMR and ^{13}C NMR resonances) of the purified compound (Figure 6.1) did not reveal aromatic protons. However, 98 aliphatic protons were observed in the ^{1}H NMR spectrum (Plate 6.4). Assignment of the protons was aided by ^{13}C NMR, HMQC, HMBC and DEPT experiments.

Proton(s)	**$CDCl_3$ 298 K**	**Carbon**	**ppm (298 K)**
		1	173-
H-2	2.30(t)	2	34.5
H-3	1.60 (m)	3	26.5
H-4-6	1.29 (m)	4-6	22.6-34.7
H-7	1.30 (m)	7	22.6-34.7
H-8	2.00 (m)	8	27.5
H-9	5.35 (m)	9	128.2
H-10	5.35 (m)	10	130.3
H-11	2.80 (m)	11	26.0
H-12	5.35 (m)	12	128.4
H-13	5.35 (m)	13	130.6
H-14	2.00 (m)	14	27.5
H-15	1.30 (overlapped)	15	22.6-34.7
H-16-17	1.29 (overlapped)	16-17	22.6-34.7
CH_3	0.9 (m)	18	14.5
H-1'/3'	4.1-4.3 (dd)	1'/3'	62.5
H-2'	5.30 (m)	2'	69.3

Table 6.1: ^{1}H NMR and ^{13}C NMR data of a bio-stimulatory compound (1) purified from *Lupinus albus* L. seeds



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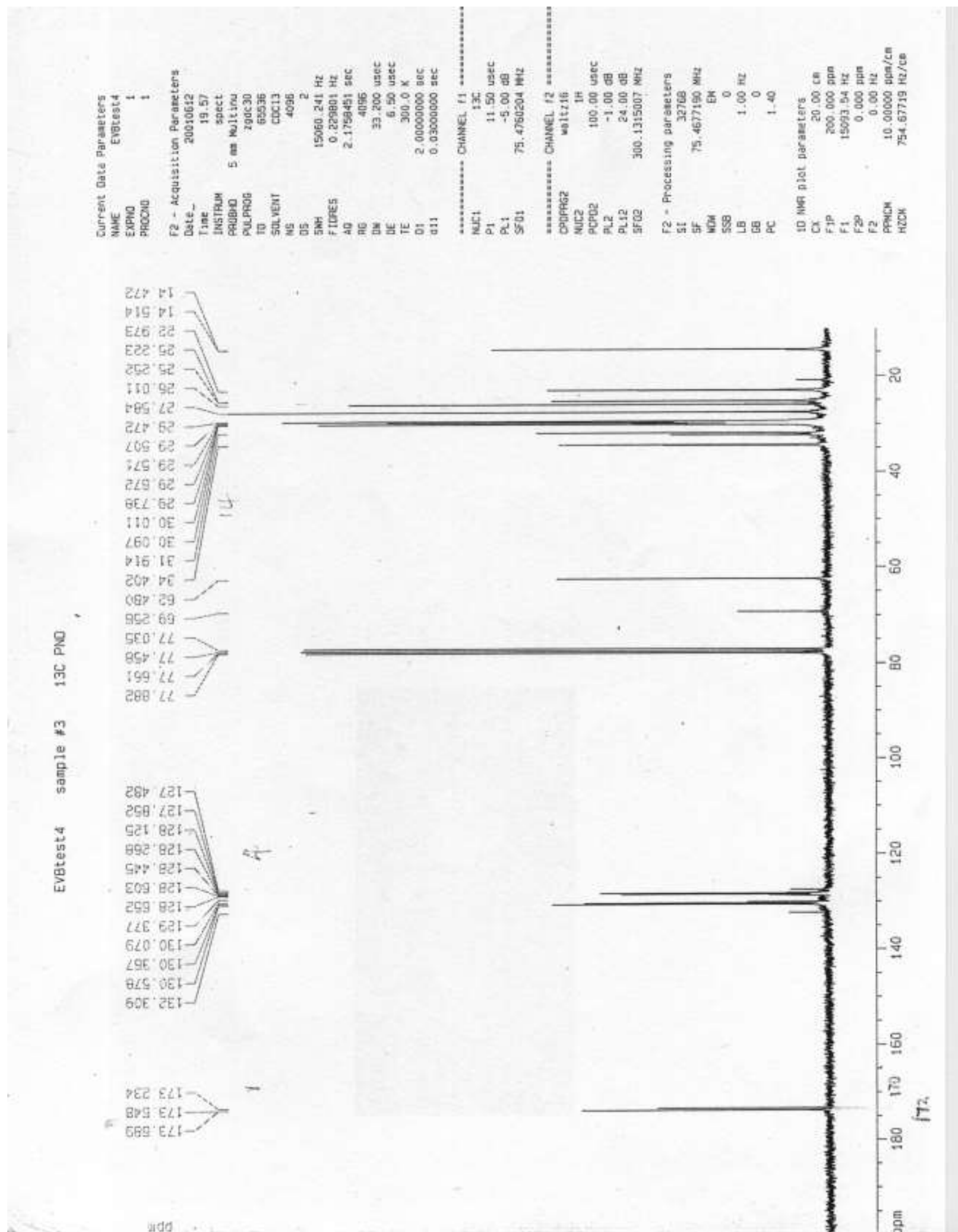
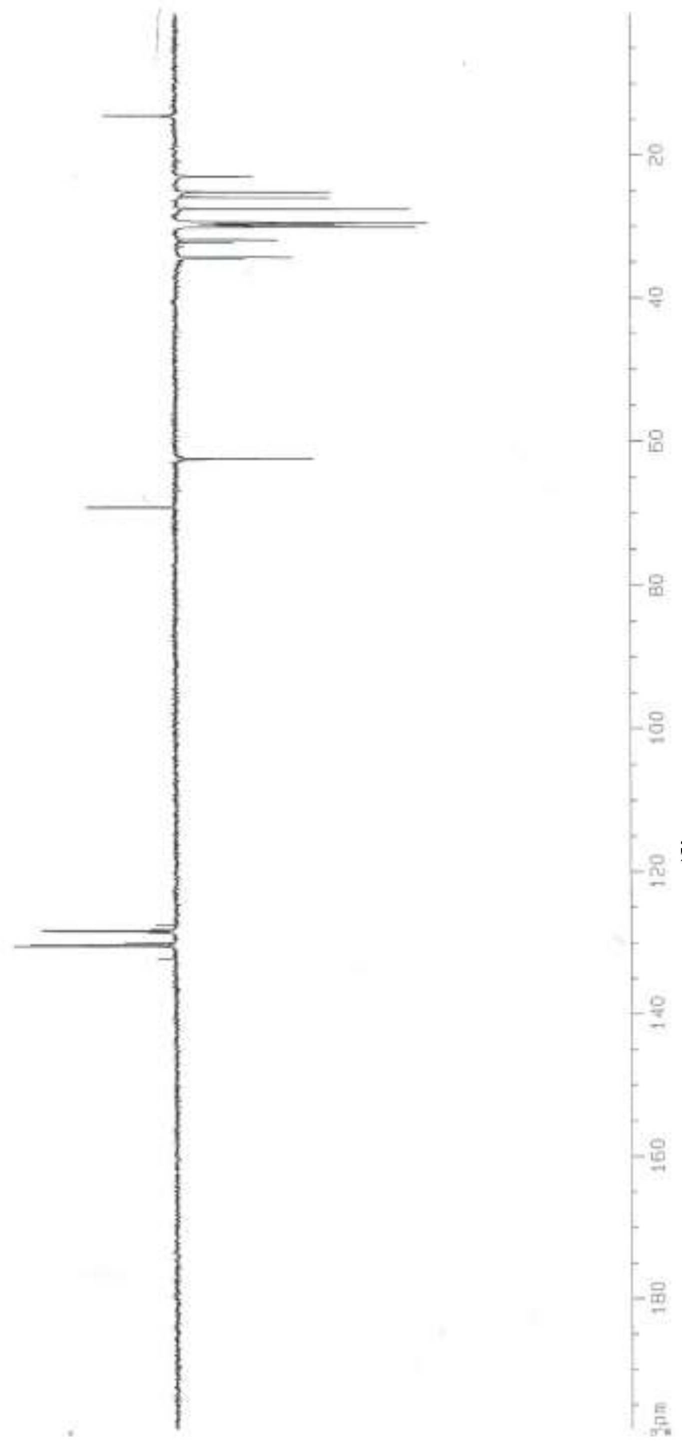


Plate 6.5: ^{13}C NMR spectrum of a bio-stimulatory compound (1) purified from *Lupinus albus* L. seeds before hydrolyzation.

The results of the ^{13}C NMR (**Plate 6.5**), DEPT (**Plate 6.6**) and HMQC (**Plate 6.7**) experiments revealed key points that were crucial in the elucidation of the structure. Importantly, the C-H protons resonated only between δ 5.3 and 5.4, the methyl groups at δ 0.9 and the rest of the peaks being methylene protons (**Plate 6.4**). The deshielded methylene protons (δ 4.1-4.4) and methine proton (δ 5.30), suggests protons attached to oxygen bearing carbons, implicating a glycerol ester type of structure. The olefinic complex multiplet resonating at δ 5.4 was assigned to the aliphatic double bonds (H-9/10 and 12/13), the doublet of doublets between δ 4.1 and 4.3 to H–1' (2H) and 3'(2H) and the deshielded multiplet at δ 5.3 to the tertiary oxomethine (H-2', C-69.3 ppm) (**Plate 6.5**). Couplings from the methylene protons (δ 4.1-4.3) to the deshielded methine (δ 5.3) as observed in the COSY (**Plate 6.9**) experiment and NOESY (**results not shown**) associations of the same protons confirmed the glycerol part of the structure. Strong HMBC of H-1'/3' to C-2' and C-1 (carbonyl carbon, C-173.6 ppm) and H-2' to C-1 unambiguously confirmed the presence of the glycerol ester in the structure (**Plate 6.10**). The aliphatic double bonds (δ5.35, C-128.2, 128.4, 130.3, and 130.6) showed a strong COSY correlation to the bis-allylic methylene protons (H-11) at δ2.75 and the mono-allylic protons (H-8 and 14) 2.0, confirming the H-9/10 and H-12/13 *cis* double bonds. The methylene protons (δ 2.3, triplet) were confirmed by the observed strong HMBC they showed to C-1 (**Plate 6.10**). The rest of the protons were established from the COSY, NOESY and HMBC spectra.

In order to confirm the ester linkage, the compound was hydrolyzed. Compound (1) was refluxed with 0.1M hydrochloric acid for 1 hour and the product extracted with ethyl acetate. With exception of the methylene (δ 4.1-4.3) and the methine protons (δ 5.3), the protons in the ^{1}H NMR spectrum (**Plate 6.8**) of the hydrolyzed product were identical to those in the ^{1}H NMR of the starting material (compound **1**). The results indicated that an ester linkage was cleaved and the fatty acid moiety was recovered. The ^{1}H NMR spectrum (**Plate 6.8**) of the product from the hydrolysis was identical to that of the authentic linoleic acid.



inus albus

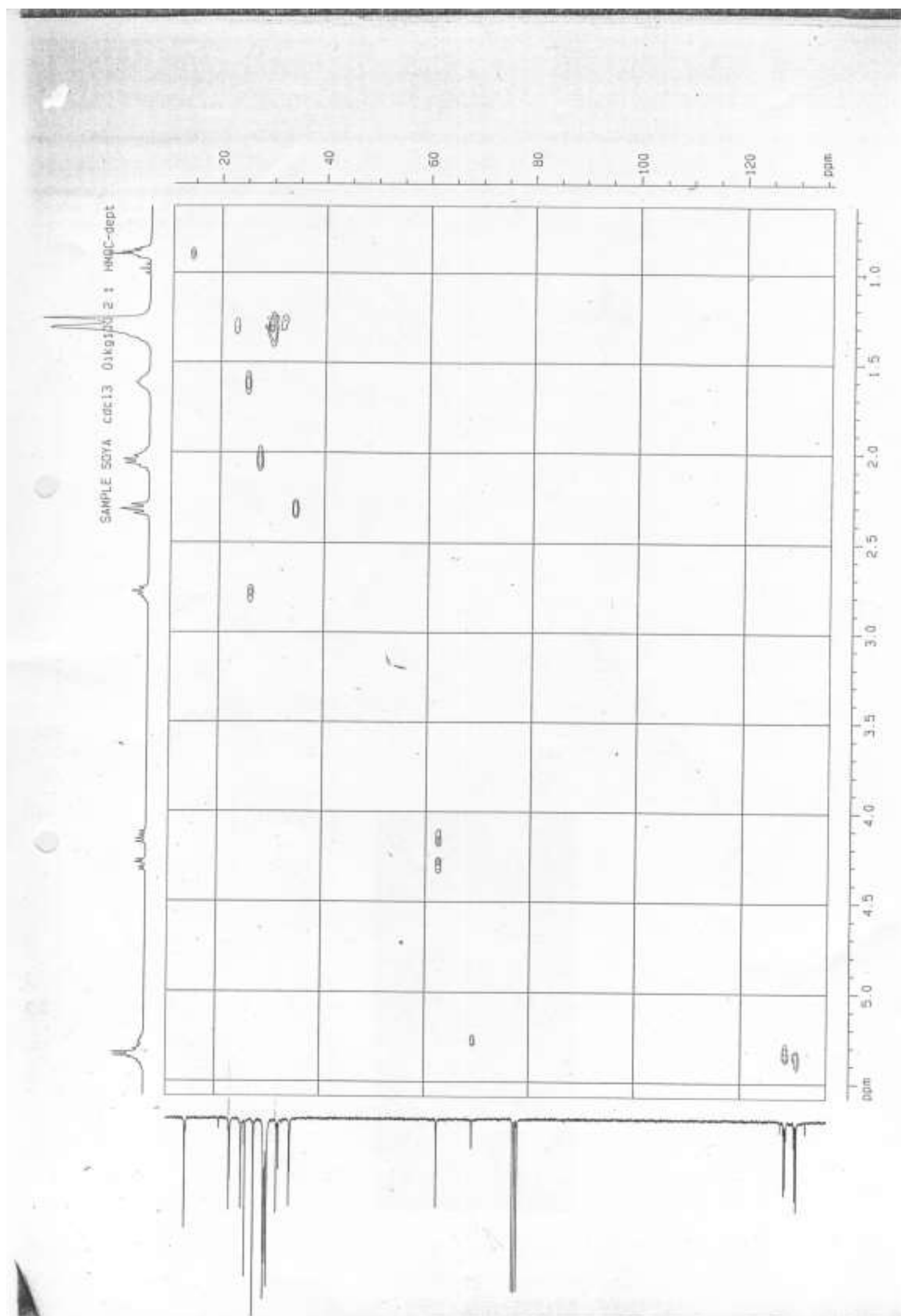


Plate 6.7: HMQC spectra of a bio-stimulatory compound (1) purified from *Lupinus albus* L. seeds

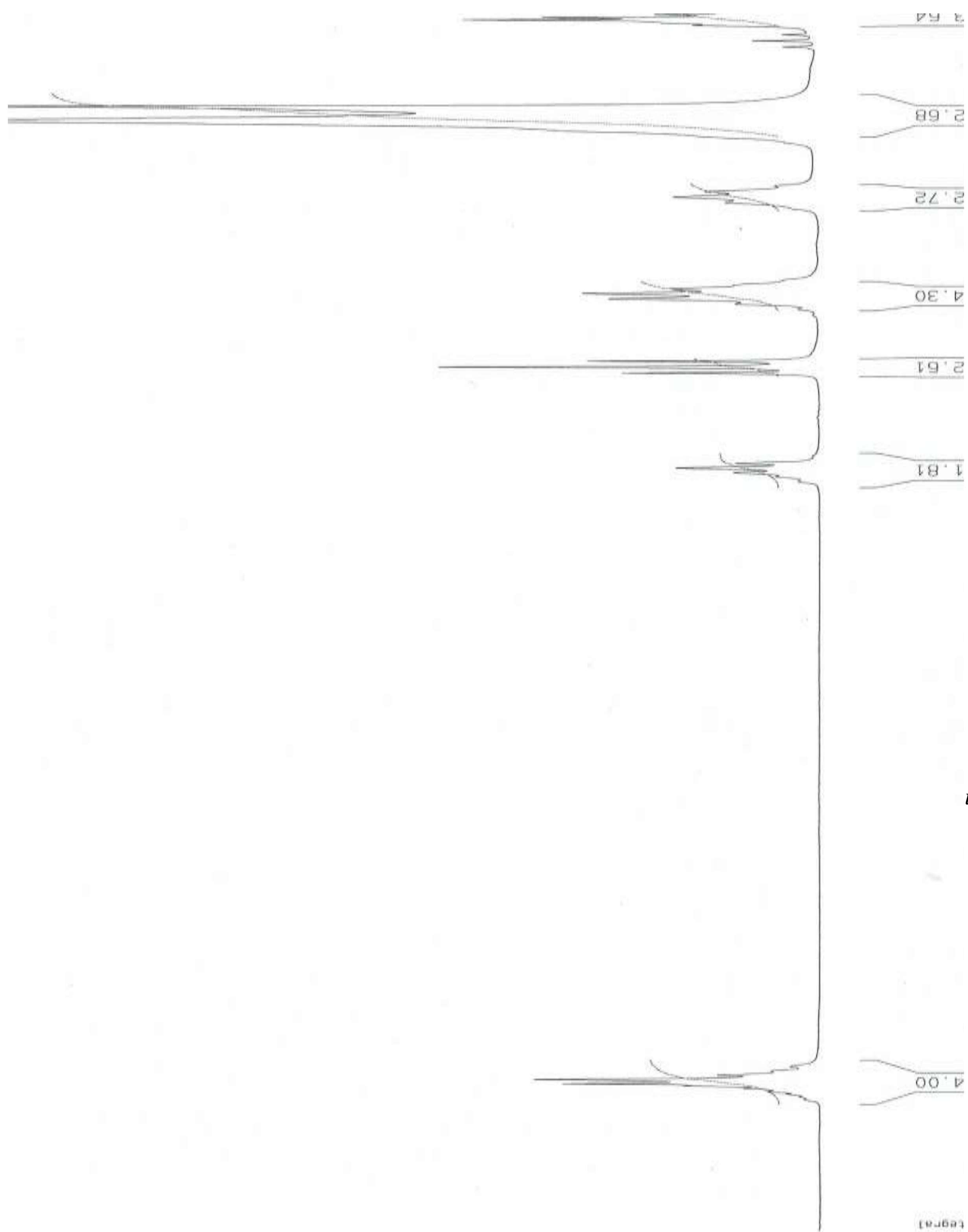


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Plate 6.9: COSY spectrum of a bio-stimulatory compound (1) purified from *Lupinus albus* L. seeds after hydrolyzation.

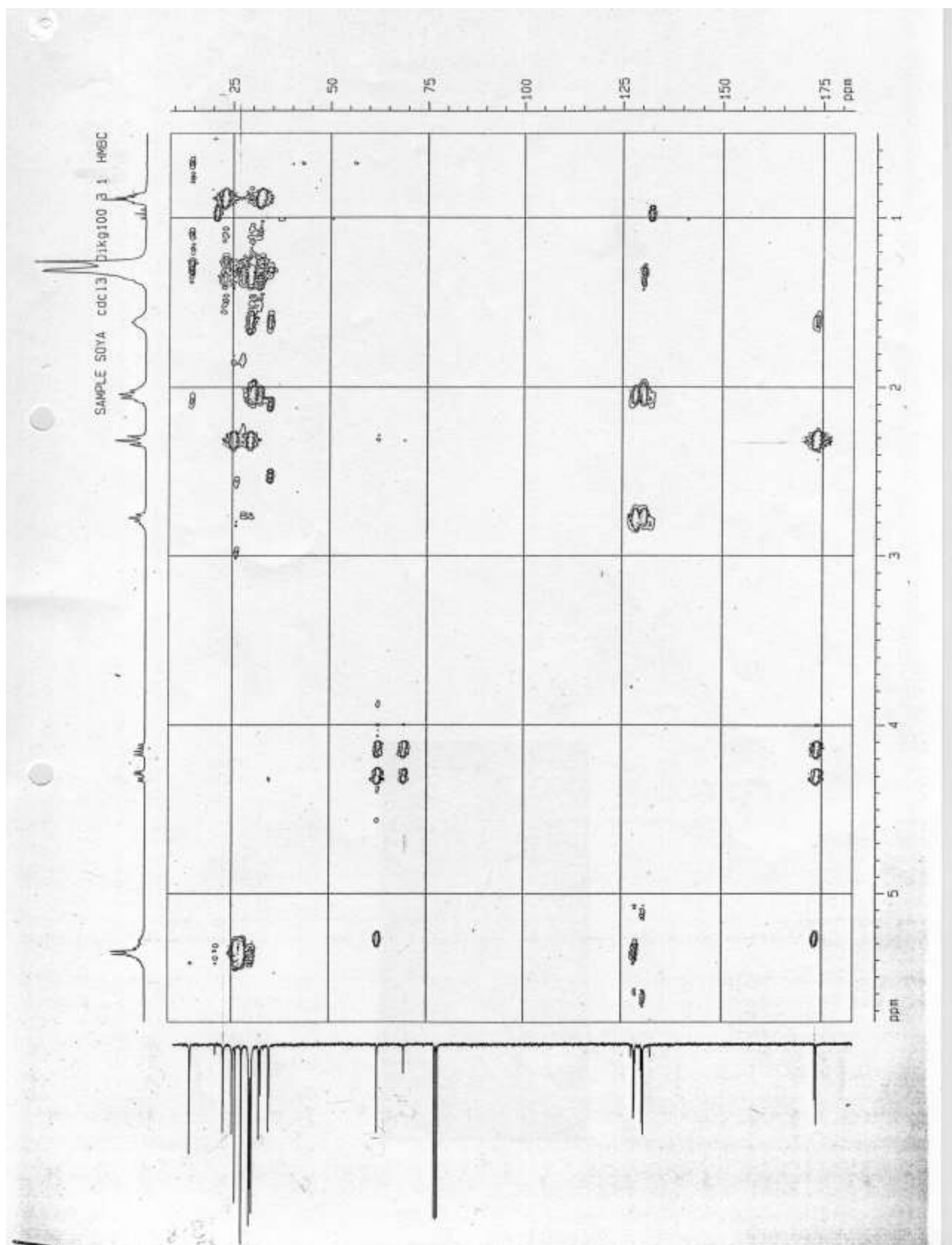
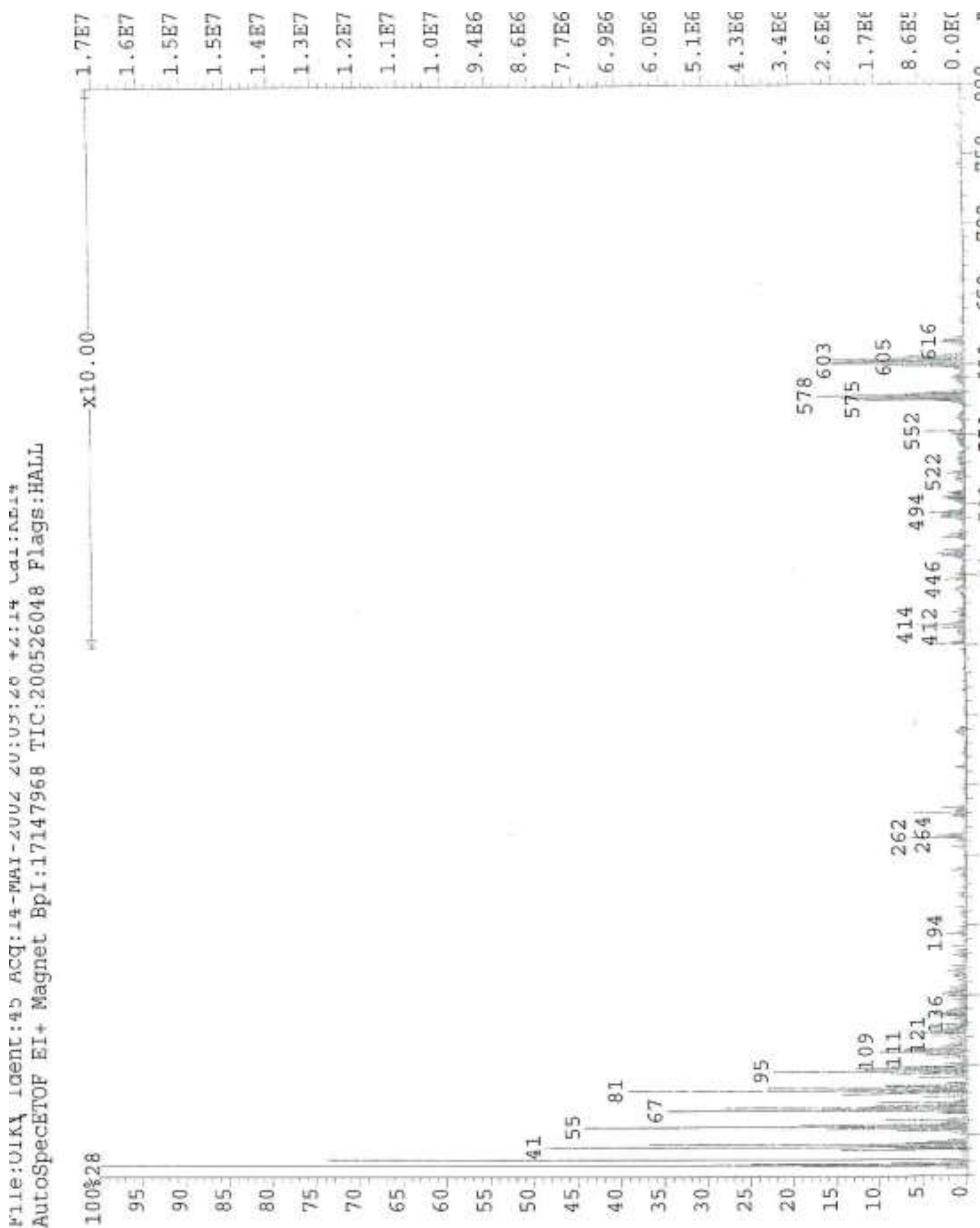
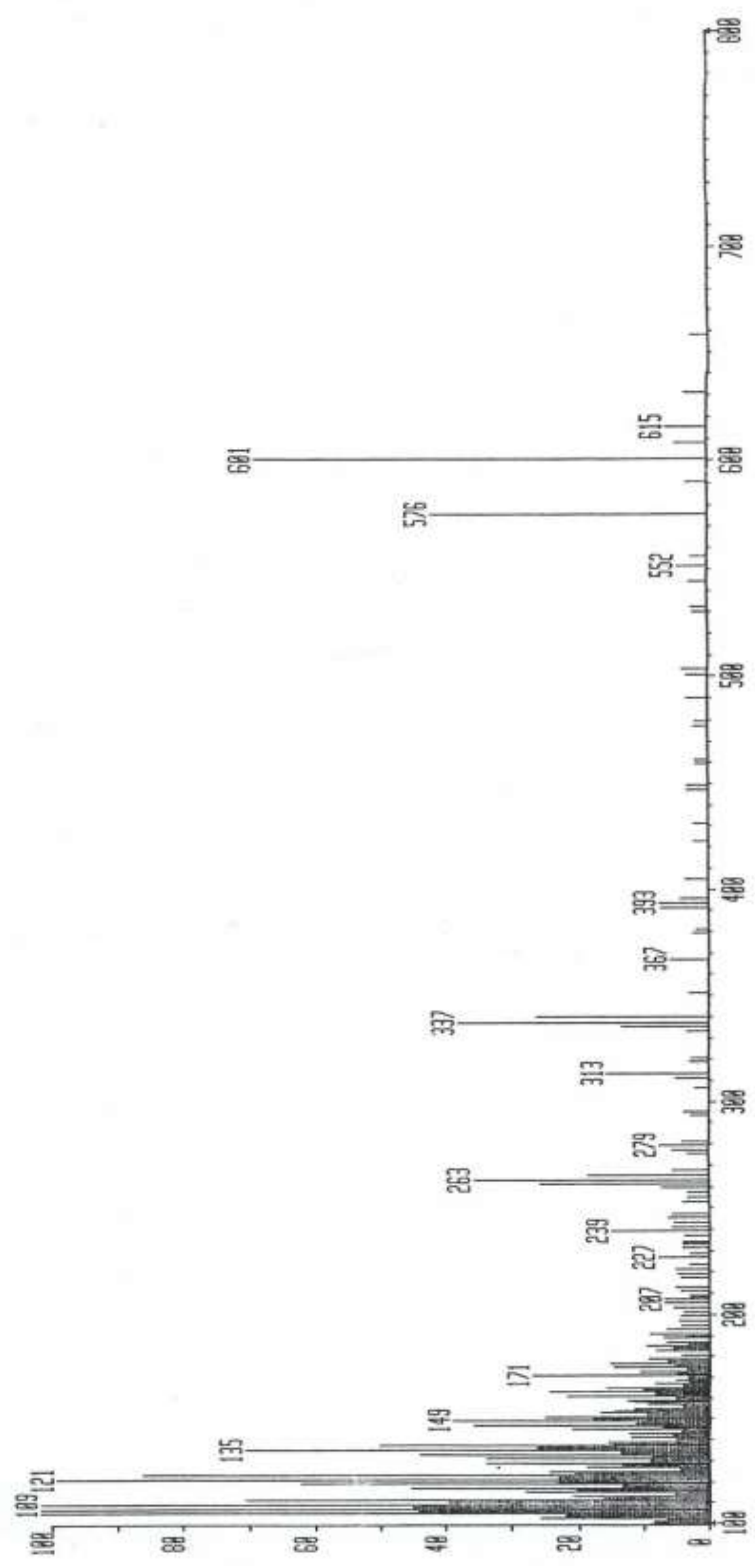


Plate 6.10: HMBC spectrum of a bio-stimulatory compound (1) purified from *Lupinus albus* L. seeds after hydrolyzation.

Although the expected M^+ (*m/z* 880) was not observed in both EI-MS and FAB-MS, the *m/z* 616 that was observed in their spectra (**Plate 6.11** and **6.12,** respectively) was in agreement with a glycerol dilinoleate indicating that one of the linoleates was cleaved. The molecular structure of $C_{57}H_{98}O_6$ which was in agreement with the calculated molecular mass of 879 confirmed the glycerol ester to be a known C-57 glycerol trilinoleate.



pinus albus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FAB/POS/NBA PT= 0° Cal:KF30
HMR: 632000
MASS: 10
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135
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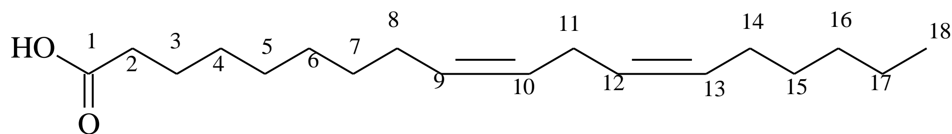

Figure 6.6: Structure of the fatty acid moiety of glycerol trilinoleate identified as linoleic acid

6.5 DISCUSSION

Column chromatographic fractionation of the active liquid-liquid ethyl acetate fraction 1 of a *Lupinus albus* L. seed extract, initially obtained by means of an extraction method described by Gamoh *et al*., (1989), resulted in four highly active combined column fractions. Similarities between the compounds in these fractions in terms of TLC-profiles and RF-values indicated the presence of two possible bio-active compounds. Following column chromatography fractionation, the active compounds were purified using preparative thin layer chromatography. However, subsequent qualitative thin layer chromatography profiles indicated similarities in their structural composition and strongly indicated that these two compounds could be of the same type.

Different spray reagents were additionally used to identify the chemical nature (Wagner and Bladt, 1996) of the compounds and revealed a steroidal type of structure for both. Together with the chromatographic results it was confirmed that these two compounds were identical, an aspect that was later verified during the nuclear magnetic resonance analysis of its chemical structure. As a result the two compounds were combined, treated as one and subjected to bio-tests in order to verify its bio-stimulatory activity. The compound showed significant bio-stimulatory activity in terms of increase in the respiration rate of monoculture yeast cells as well as the enhancement of coleoptile and root growth of cabbage seedlings. Subsequently, the pure active compound was subjected to nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry to elucidate its structure and to identify the compound by name. Different approaches of this technology assisted in identifying the active compound as glycerol trilinoleate, a tri-ester with three fatty acids moieties, identified as linoleic acid, attached to a glycerol “back bone”.

Edqvist and Farbos (2003) reported on the high levels of the epoxidated fatty acid vernolic acid in the endosperm of *Euphorbia lagascae* Spreng seeds that play an important role during

germination after being mobilized and oxidized. Panossian *et al.* (1983) isolated unsaturated poly-hydroxy fatty acids form the roots of *Bryonia alba* L. that showed prostaglandin-like activity in *in vitro* bio-assay tests. Further, a number of reports pointed towards plant growth regulatory activity contained in plant sterols and fatty acids (Seigler, 1998; Gong *et al.*, 1999; Sheng *et al.*, 2000; Padmapriya and Chezhiyan, 2002; Edqvist and Farbos, 2003). For example, the sterol estrone increases growth of root tips and pollen tubes of *Rumex tenuifolius* (Seigler, 1998) while β-sitosterol initiates flower bud formation in *Chrysanthemum* species (Padmapriya and Chezhiyan, 2002). Application of linoleic acid and linolenic acid to tissue slices of tomato fruits promoted ethylene production (Sheng *et al.*, 2000). Importantly, according to the authors, this effect was specific for unsaturated fatty acids which serve as substrates for lipoxygenase action and did not occur following similar treatments with saturated fatty acids. Oxylipins can also be produced from unsaturated fatty acids which serve as important switches or signals in plant metabolism e.g. defence reactions. Gong *et al.* (1999) reported that linoleic acid exogenously applied to barley seedlings during salt stress increased K^+ absorption and translocation, increased the phospholipid content in the tonoplast and increased the activities of H^+-adenosine triphosphatase (ATPase) and H^+-pyrophosphatase (PPase). The latter treatment also decreased the leakage of electrolytes and Na^+-absorption in turn influencing the translocation of Na^+ to the shoots.

Despite its growth promoting characteristics, fatty acids have also been shown to be involved with defense mechanisms in plants and to promote chlorophyll synthesis. According to Wink (2000) the application of systemin to plants as well as wounding resulted in increased linoleic and linolenic acid content, both known to be converted to dihydro-jasmonate and activate defense-related responses. Application of a 0.1% sucrose fatty acid ester (SFE) during flowering and pod filling increased the activity of superoxide dismutase by 41% and chlorophyll b content by 58% reaching its maximum 4-5 days after application (Ye *et al.*, 2000). SFE application also increased the leaf nitrogen content twofold, pod number by 70%, 100-kernel seed weight by 32% and seed protein content by 4.8% in soyabeans.

Fatty acids can also act as substrates for the lipoxygenase (LOX) enzyme in plants that catalyses its oxidation to hydroperoxide fatty acids which, in turn, act as precursors of jasmonic acid and related compounds (Kolomiets *et al.*, 2001). The authors reported that LOX is involved with physiological processes such as growth, senescence and stress-related responses and that

suppression of LOX activity resulted in reduced tuber yield, decreased tuber size and disruption of tuber formation in potatoes. Further, jasmonic acid (jasmonate) and traumatic acid, both fatty acid-derived chemical messengers, have been implicated as plant growth regulators (Boss and More, 1989). Jasmonate was initially described as a substance with growth inhibitory properties and, when exogenously applied, to promote senescence. Other effects of exogenously applied jasmonate to plants, including petiole abscission, tendril coiling and activation of ethylene synthesis as well as inhibition of root growth, chlorophyll production and pollen germination, were reported by Leon and Sanchez-Serrano (1998). Jasmonic acid also acts as defense stimuli in response to environmental stresses, such as mechanical wounding or pathogen attacks (Jose and Sanchez-Serrano, 1999). Traumatic acid, on the other hand, is capable of inducing renewed cell division and cell extension activity in the parenchymous cells of bean pod mesocarp (Seigler, 1998). Moreover, Eastmon *et al*. (2000) suggested that lipids can be used as a source of carbon for respiration in germinating oilseeds and that the product of fatty acid catabolism, Acetyl Co-enzyme A, can pass from the peroxisome to the mitochondrion independent of the glyoxylate cycle. However, according to the authors, an additional anaplerotic source of carbon is required during seedling establishment due to an increased demand and the glyoxylate cycle can act as this additional source.

In this study it was confirmed that glycerol trilinoleate, contained in a seed suspension of *L. albus* exogenously applied to a variety of crops, was the active substance responsible for induced seedling growth under laboratory conditions and yield increases under field conditions. However, elucidation of the mechanism of action did not fall within the scope of this study. The latter needs to be addressed in a future study as, currently, not much is known about the bio-activities induced in plants by either pure linoleic acid or glycerol trilinoleate when exogenously applied. In a future study it will also be necessary to verify whether the metabolic activities mentioned higher up form part of this mechanism of action. More is known about the mechanism of action of brassinosteroids (BRs) that were identified as the bio-stimulatory substances in the commercial product, *ComCat*®, used as a positive control in this study (Agraforum, Germany; personal communication). It has been shown that BRs promote root elongation and increase the coleoptile fresh weight in beans as well as ethylene production (Cao and Chen, 1995). In previous chapters a possible synergistic interaction between BRs and glycerol trilinoleate was demonstrated where the original *L. albus* seed suspension and the commercial bio-stimulant, *ComCat*®, were applied together. In some crops the combined

treatment resulted in higher yields than when the two were applied separately. In future research this aspect needs to be verified in terms of the mechanism of action.

In contrast, the presence of natural bio-inhibitors is widespread in the plant kingdom (Khan, 1982; Hedge and Miller, 1990; Noor *et al*., 1995). These studies showed that water extracts of fruit and seeds are most effective in inhibiting the germination and early seedling growth of a wide variety of plants. A more specific example is Sesbanimide, a cytotoxic alkaloid isolated from the seeds of a number of *Sesbania* species that significantly restricted seedling growth in a number of crops (Van Staden and Grobbelaar, 1995). Further, essential oils isolated from *Lactuca sativa* had little effect on the germination of seeds from other plants but significantly increased the number of unviable seedlings and also inhibited subsequent seedling growth (Robles *et al*., 1999). In this light the screening of plant extracts for potential bio-stimulatory activity by researchers interested in natural product development, of which this study is an example, can serve a dual purpose namely to simultaneously screen for herbicidal activity. In other words, the same bio-assay techniques as was used in this study can be employed for both purposes. In the event that seed germination or seedling growth is either stimulated or inhibited by a specific plant extract under laboratory or field conditions a rationale can be obtained for activity directed isolation of either bio-stimulatory or inhibitory active substances.

In summary, the efficacy of either glycerol trilinoleate or the linoleic acid moiety or both isolated from *L. albus* seeds in promoting growth and yield in a number of crops has been demonstrated in this study under laboratory and field conditions. Further, the possibility for developing a natural product with application potential in the agricultural and horticultural industries has been verified scientifically. However, although the action mechanisms of the active compound contained in a *L. albus* seed suspension (SS) as well as the synergism observed where SS was applied in combination with *ComCat*® need to be elucidated in a follow-up study, an attempt will be made in chapter 7 to postulate possible biochemical events that may form part of these mechanisms.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Reduction in environmental damage as a result of farming practices has been a subject of discussion over the past decade (Park and Seaton, 1996). The latter mainly deals with the concept of sustainability of natural resources and the role organic farming can play in improving the current situation as a new approach. The main reason for the focus on organic agriculture, and the rapid development of the organic sector in Europe and North America, is the banning of certain synthetic products that has been identified as unwanted. Of these, the banning of copper containing fungicides in Europe tops the list. This development has resulted in 2.2% of the agricultural land in the European Union already being set aside for organic farming only, while the figure exceeds 10% in countries such as Austria and Sweden (USDA, 2000). The latter is largely the result of consumer demand for environmentally friendly or "green" chemical-free food products and the many alternative approaches that have been developed with respect to issues of sustainability. These include integrated crop and pest management, low input sustainable agriculture, agro-ecology, perm culture, biodynamic farming and organic farming (Rigby and Caceres, 2001). Further, still very much part of the current debate over agricultural sustainability is issues such as soil health and structure, the exhaustible nature of artificial fertilisers and human health.

However, a decade ago Pretty (1995) argued that, although "organic agriculture is generally a form of sustainable agriculture", it can also have negative environmental effects. These include the leaching of nitrates from soil under legume cultivation, the volatilisation of ammonia from livestock waste and the accumulation of heavy metals in soils following the application of the "Bordeaux" mixture. The question is whether organic farming has the potential to compare favourably with conventional farming practices in terms of productivity. Despite some negative aspects pertaining to organic agriculture, an issue that cannot be overlooked is the need for alternative and biodegradable insecticides, bactericides, fungicides, herbicides and plant growth regulators as a result of consumer pressure towards the use of synthetic chemicals.

In this study the emphasis was placed on identifying a natural bio-stimulant or plant growth regulator with application potential in the agricultural industry and that has the potential to fit the requirements of organic farming practices. In an attempt to improve crop yields, plant growth regulators have been an important component of crop production research even prior to the discovery of plant hormones (Gianfagna, 1995). Two examples include tri-iodobenzoic acid (TIBA) and dinoseb. It was shown that TIBA reduced plant height and petiole length while stimulating branching and fruit set. It has since been registered as a yield-increasing product on soybean. Dinoseb, used primarily in maize fields as a pre-emergence herbicide, has been found to increase grain yield by 10-15 % (Gianfagna, 1995). Additionally, a water extract of sorghum has been shown to significantly increase the yield of wheat by 16-22%, compared to an untreated control, when applied as a foliar spray (Cheema *et al*,. 2002). Dumitrescu *et al*. (1998) reported that an extract of *Datura stramonium* increased the dry kernel yield of maize by 2-15% depending on the rate and time of application. To this short list can also be added the already commercialized seaweed extract, "Kelpek®" (Ferreira and Lourens, 2002).

The current use of plant growth regulators in high-value horticultural crops is not merely to increase crop yields directly, but also to increase the total biological yield or the harvest index (Chen *et* al., 2003). Moreover, the demand for organic farming is increasing because of manufacturer's claims that natural bio-stimulants applied in agriculture increase production and profits due to cutting of input costs (e.g. fertilizer costs) while it has no detrimental effect on the environment (Chen *et* al., 2003). With this background the underlying study aimed at: i) evaluating the bio-stimulatory potential of a *Lupinus albus* seed suspension on seed germination and subsequent seedling growth (chapter 3), ii) determining the effect of the seed suspension on the yield of different agricultural crops and vegetables under field conditions (Chapter 4) and iii) isolating and identifying the active compounds involved (Chapter 5 and 6).

The *in vitro* bio stimulatory effect of a *L. albus* seed suspension (SS) on the respiration rate of monoculture yeast cells compared favourably with that of a commercial bio-stimulant, *ComCat*®. As an increase in the respiration rate of cells can either indicate a negative (inhibitory) or a positive (stimulatory) response, it was necessary to verify this aspect with a second bio-test involving seed germination and seedling growth. The role of cell respiration in maintaining sufficient energy levels

for germination and seedling growth is indisputable (Amthor, 1989). During seed germination and the first few days of seedling growth the respiration rate is highest in meristematic tissue where the demand for energy and the biosynthesis of essential intermediates are greatest (Amthor, 1989). These intermediates form the building blocks necessary for growth. However, the stimulating effect of SS on cell respiration may be of secondary consequence during the later growth of seedlings. Although the maintenance of a sufficient respiration rate is important for supplying energy required by anabolic (endergonic) reactions during an early developmental stage (Amthor, 1989) the decrease in growth efficiency often observed at later stages of development in crops is also due to a decrease in respiration (Yamaguchi, 1978). Treatment of a variety of seeds with SS had no effect on germination, but significantly enhanced coleoptile and especially the root growth in seedlings. The latter confirmed that the initial respiration rate increase observed in yeast cells could be regarded as indicative of a positive rather than a negative effect of SS on cell metabolism and growth.

At this stage the active substance involved was not known. However, the literature study that followed revealed that a number of bio-stimulatory substances have been isolated from plants in the past. Wang *et al*. (1994) reported that brassinosteroids (BRs), the main component of *ComCat*® used as a positive control in this study, stimulated hypocotyl elongation by stimulating wall relaxation without concomitant changes in the properties of the cell wall. Zurek *et al*. (1994) demonstrated that BRs stimulate wall loosening in soybean epicotyl segments and contributed to increased growth. Potent growth-promoting properties were also observed in an acid extract from tomato waste (Suzuki, *et al.,* 2002). Although the compound was not identified, it was speculated to be an end product following the decomposition of polygalacturonic acid by pectinase that promoted the growth of lettuce seedlings (Suzuki *et al*., 2002). Yonemoto *et al*. (1993) reported that an alginate lyase-lysate (ALL) with growth-promoting properties had an effect on the elongation of above soil parts in several plants.

Since its discovery twenty years ago the mechanism of action of BRs (active substance of *ComCat*®) in promoting growth and development in plants, leading to increased yields, received special attention. In this regard Marquardt and Adam (1991) confirmed that treatment of field crops with BRs resulted in increased crop yields even under non-optimal growth conditions indicating that

its mechanism of action is not only to promote, for example, root growth. As the activity of BRs is affected by temperature and day length, leading to erratic results from season to season (Arteca, 1995; Kalituho *et al.,* 1997), this is a strong indication that phytochromes might be involved in BR activity. However, Asami *et al.* (2002) maintained that yield increases in field crops treated with BRs were the result of BRs acting as stimuli to increase proton pump activity in membranes. According to the authors experiments on the uptake by plant tissue of [^{14}C] sucrose, labelled in either the glucosyl or the fructosyl moiety, showed that sucrose is not modified during uptake. The hypothesis of Anderson and Beardall (1991) known as the sucrose/proton co-transport model, although seen as outdated by their peers, proposed that the plasma membrane of the sieve tube or an associated phloem cell contains an ATP-dependant proton pump that pumps protons out of the cell. The free energy of the resulting electrochemical gradient, in turn, actively allows the transport of sucrose into the phloem. Stimulation of cell respiration by BRs increases the activity of the proton pump leading to increased sucrose translocation, especially into storage tissue in stems of sugar cane and hypocotyls of beetroot leading to increased yield and quality of these crops (Anderson and Beardall, 1991). Further, groundnuts treated with BRs showed an increase in shoot and root growth that was associated with increased levels of DNA, RNA, soluble proteins and various carbohydrates, indirectly affecting the yield (Vardhini and Rao, 1998). According to Cutler *et al*., (1991), assimilation of uptake and enhancement of xylem differentiation in plants treated with BRs, serves as an explanation for the possible increase in root length and weight which also indirectly affects the yield outcome.

Brassinosteroids have also been shown to induce changes in plasmalemma energization and transport by acting on plasma membranes with receptor proteins. This in turn activates phospholipase c (PLC), increase levels of Ca^{2+} in the cytosol and activate a Ca-calmodulin complex that can activate certain enzymes, including several protein kinases. Some of these BRs can also be absorbed by the cells and move into the cytosol where they combine with receptor proteins. Subsequently, the steroid-receptor complex moves into the nucleus and activates the expression of specific genes involved with resistance towards biotic stress conditions or other important physiological processes that affect growth and yield of different plants (Salisbury and Ross, 1992). Schnabl *et al*., (2001) reported on the induction of pathogenesis related (PR) proteins in cucumber

after treatment with BRs. These PR-proteins include peroxidase, chitinase, β-1,3-glucanase and lypoxygenase that are involved in the resistance mechanism against fungal infection.

Although the effect of SS on seedling growth varied in terms of optimal concentration, it exceeded the efficacy of the commercial bio-stimulant, *ComCat®*, in most cases. In view of the high potential loss of seedlings after planting (Balestri and Bertini, 2003) these preliminary results looked promising and prompted a further investigation in an attempt to verify the potential of the seed suspension to act similarly under field conditions. Subsequently, final yield was taken as parameter to represent the collective effect of the seed suspension, applied as a foliar spray at an early vegetative growth stage, in a variety of crops. Treatment with *ComCat®* and SS, both separately and in combination, varied in their effects on the yield of different crops. At times the effects observed by means of small statistically laid out trials were non-significant (in the case of maize and cabbage) but in other cases the yield increases were highly significant, for example 0.7 ton ha^{-1} in wheat, 20.4 ton ha^{-1} in lettuce and 24.3 ton ha^{-1} in carrots. However, during the 2004 season the majority of these trials were repeated on a large scale on farms outside Bloemfontein and resulted in similar or better yield increases. Treatment with *ComCat®* also improved the yield of some crops but, in most cases, to a lesser extent than the SS treatment. When SS was applied in combination with *ComCat®* the yield of maize (680 kg ha^{-1}) and beetroot (21,0 ton ha^{-1}) was also significantly increased. Further, both *ComCat®* and SS had a significant effect on flower development in both Gazania and Impatience. On grounds of these results a rationale was established for isolating, purifying and identifying the bioactive substance in the *L. albus* seed suspension.

Activity directed fractionation (Gamoh *et al*., 1989), using three bioassay procedures, revealed that most of the bio-stimulatory activity was located in an ethyl acetate fraction. Further purification of the active compounds by means of column and preparative thin layer chromatography led to the isolation of one active compound of which the structure was elucidated by means of NMR spectroscopy and mass spectrometry. The compound was identified as glycerol trilinoleate, a triglyceride with three fatty acids (linoleic acid) attached to a glycerol moiety.

Although elucidation of the mechanism of action of SS, leading to increased yields, was outside the scope of this study, a literature study revealed that fatty acids have been shown to be involved in

defence mechanisms of plants and to promote chlorophyll synthesis (Wink, 2000). Fairly recently Seigler (1998) and recently Edqvist and Farbos (2003) reported on the plant growth regulatory activity of plant sterols and fatty acids. Despite these reports, very little information on the growth regulating action mechanism of either lipids or fatty acids was found in the literature and must therefore be regarded as highly speculative. For this reason an attempt was made to formulate two postulates with regard to the possible mechanism of action of the triglyceride contained in a *L. albus* seed suspension (SS) in promoting seedling growth as well as increasing yields in the field crops tested.

Postulate 1: Indirect bio-stimulatory action mechanism of trilinoleate via cell metabolism

In essence postulate 1 is based on the assumption proposed by Seigler (1998) that exogenously applied fatty acids might slightly increase the level of available fatty acids to be oxidized via α- and β-oxidation (Seigler, 1998). Most fatty acids occur in nature as esters of glycerol (commonly referred to as triglycerides or tri-acylglycerols) that either plays a role in energy supply or is stored as components of phospholipids, glycolipids and waxes. Some are converted to alcohols, aldehydes, olefins, hydrocarbons, acetylenic compounds and other secondary metabolites (Seigler, 1998). When a *L. albus* seed suspension (SS), containing trilinoleate as active substance, is applied to crop plants as a foliar spray, it is assumed that this increases the level of available fatty acids after being taken up by the leaves, its stimulatory effect might be via normal metabolism. Two possibilities are discussed.

Firstly, as it was shown that SS increased the respiration rate of monoculture yeast cells, it could be argued that young seedlings of all test crops that were sprayed at the three leaf growth stage, could utilize the fatty acids as respiratory substrate in the Krebs cycle (Anderson and Beardall, 1991) after being metabolized to acetyl co-enzyme A via β-oxidation, contributing to increased ATP production. This could lead to a higher energy status of the seedling. In order for this argument to hold and to test the postulate, it will be essential in follow-up studies to verify the amount of substrate that is taken up per gram of leaf tissue following a foliar application. However, this aspect is rather complicated by the difference in plant stand per hectare for different crops as well as

between irrigated and dry land crops. Individual maize plants cultivated under dry land conditions, for example, receives 1060 μg of the seed suspension of which 698 μg is active substance (triglyceride). Considering that many substrates in plant tissue is measured in nanograms the 698 000 ng pure triglyceride with which individual maize plants were treated, might be sufficient as respiratory substrate as postulated higher up.

It is well known that vegetative growth is slowed down in a young seedling shortly after being established and this may continue for approximately a week. Further, this coincides with the time when more energy is needed by the young seedling to establish systemic acquired resistance (SAR) towards abiotic and biotic environmental stress factors than for growth (Schnabl, 2001). It is interesting to note that significant yield increases were observed in certain crops such as maize and wheat that were sprayed only once at the three leaf growth stage. The latter indicates that the possible effect brought about by the foliar spray treatment with SS might have been via induced resistance against biotic and abiotic stress factors and this was sufficient to benefit the plant throughout the vegetative and reproductive growth cycle. Moreover, increased acetyl-CoA levels stimulate oxaloacetate (OAA) formation by regulating pyruvic acid carboxylase in an anaplerotic fashion (Anderson, 1989). The latter is important in the event that OAA is bled off the Krebs cycle for lipid and protein synthesis and to maintain Krebs cycle activity essential for growth (Bohinski, 1987; Anderson and Beardall, 1991). In a young developing seedling this metabolic mechanism would also be highly beneficial. However, it should be noted that, as yet, there is no full understanding of how the Krebs cycle is regulated in plants, although different compounds, including fatty acids, might be involved.

Secondly, fatty acids also act as precursors for the synthesis of secondary metabolites via the mevalonic acid pathway (Figure 7.1) in both the cytosol and plastids (Woeste *et al.*1999). Acetyl-CoA acts as primary substrate of the mevalonic acid pathway to produce isopentenyl pyrophosphate (IPP), the precursor of a large number of secondary metabolites including terpenoids, alkaloids, steroids, saponins, cardiac glycosides and abscisic acid (ABA) (Verpoorte and Alfermann, 2000). According to the authors some of these secondary metabolites, e.g. terpenoids, steroids and ABA, are known to act as elicitors or stimuli to activate defence reactions in plants. Scientists often assumed in the past that, because plants are sessile, they do not possess mechanisms for rapidly

responding to environmental or hormonal stimuli (Boss and Morrè, 1989). However, as technology progressed so that rapid measurements of small changes in cellular metabolites could be measured within minutes after a stimulus was applied; it became evident that plants do indeed show rapid metabolic changes in response to stimuli. What then became important was the elucidation of signalling transduction mechanisms involved in plants on perceival of an outside stimulus or signal that results in an amplified response in the cytosol.

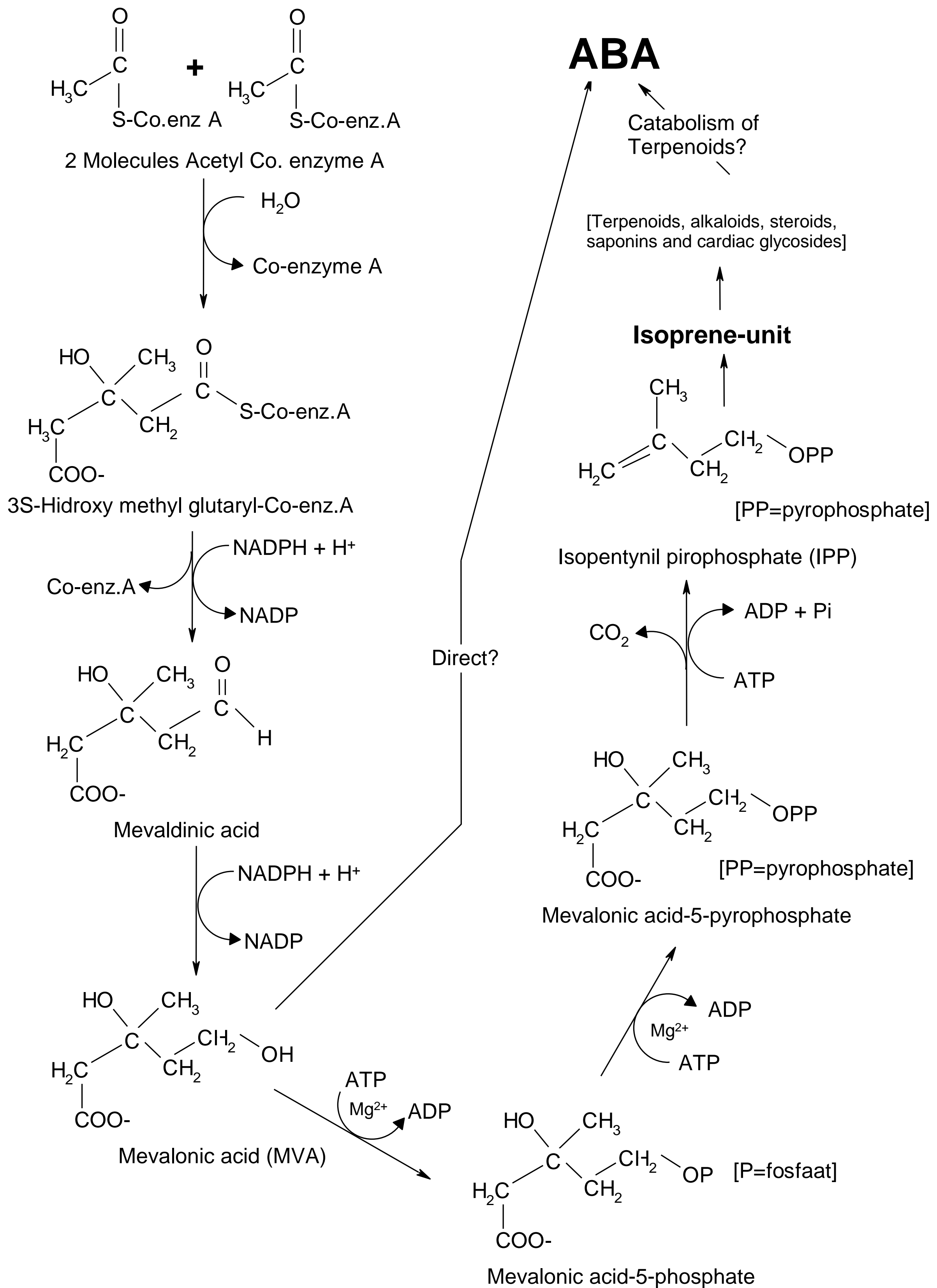


Figure 7.1: The mevalonic acid pathway (Goodwin and Mercer, 1983).

A possible mechanism is the effector (hormone)-responsive system that usually include at least three essential elements: 1) a receptor site to recognize and bind the extracellular elements; 2) some form of transduction mechanism that recognizes a change in configuration or conformation of the receptor and 3) an amplifier that translates the received message into an increase (or decrease) in the intracellular concentration of a chemical species capable of exerting control over intracellular processes (Boss and Morrè, 1989). Since the responsive systems of crop plants to linoleic acid after foliar treatment with SS is unknown at this stage, it is pure speculation. However, its significant bio-stimulatory effect under laboratory and field conditions confirmed in prior chapters strongly indicates that it might act as a stimulus that activates certain metabolic pathways, including either increased ATP or secondary metabolite production or both. The possibility further exists that certain secondary metabolites can induce resistance by acting as elicitors for the expression of specific genes, e.g. genes controlling the synthesis of PR-proteins involved in resistance towards fungal infection.

Lastly, as photosynthesis forms an extremely important part of anabolic metabolism in plant cells and is directly related to the production of assimilates translocated to harvestable parts of crops during the grain filling or fruit development stage, the possibility exists that available fatty acids might affect the process. Anderson and Beardall (1991) reported a marked change in fatty acid composition, including an increase in the amount of linolenic or linoleic acid, during chloroplast development. Recently, fatty acids have been shown to be crucial in the protection of the photosynthetic machinery under cold conditions (Sewon *et al*., 1997). During this time linolenic acid levels decreased while linoleic acid levels increased as a response to cold treatment leading to a hardened state characterized by the accumulation of chlorophylls. It is postulated that exogenously applied trilinoleate (SS) might have evoked the same increase in chlorophyll production in crop plants as was reported under cold stress conditions (Sewon *et al*., 1997). This could explain the increased yields observed in the study via an increased photosynthesis rate. However, this aspect was not investigated in this study and is merely mentioned for the sake of a comprehensive explanation of the metabolic postulate.

<u>Postulate 2</u>: Indirect bio-stimulatory action mechanism of trilinoleate via second messengers

Depending on the composition and turnover of lipids several developmental processes can be affected during the life cycle of a plant e.g. by increasing enzyme levels or by producing second messengers (Park *et al*., 2002). Compounds that have been identified as second messengers produced by this turnover include arachidonic acid, eicosapentaenoic acid, jasmonic acid (JA), its precursor 12-oxo-phytodienoic acid (OPDA), as well as traumatic acid and its methyl esters. In oil rich seeds this process of second messenger production is characterized by the mobilization of storage lipids that serve as a major carbon source during the growth of seedlings. However, it is postulated that in seeds with low lipid content exogenously applied SS (trilinoleate) might supply either a metabolic or a growth stimulus or both via second messengers as an action mechanism normally not in contention in these seeds. A similar stimulus in the vegetative part of crops after foliar application of SS is also postulated. The question is how this stimulus could possibly be activated? Before it is attempted to construct a simplified model for a possible mechanism of action, some background information concerning the metabolic involvement of lipids or lipid related compounds is supplied.

According to Feussner *et al*. (1997) storage lipids are degraded during early stages of seed germination and a new set of proteins, including a specific linoleate-13-lipoxygenase (LOX) enzymatic protein, becomes detectable in membranes. LOX (linoleate: oxygen oxidoreductase) constitutes a family of dioxygenases that is widely distributed in plants and animals. They catalyse the region- and stereo specific dioxygenation of saturated fatty acids as well as that of exogenously applied lipids. Interestingly, LOX-2 isolated from soybeans, has been shown to catalyse the *in vitro* cyclo-oxidation of arachidonic acid, yielding significant amounts of prostaglandins or other second messengers (Feussner *et al*., 1997).

Although the C20 unsaturated fatty acid arachidonic acid is the most common substrate of LOX in animals, higher plants might have lost the ability to synthesize arachidonic acid or may not need it. Even though Singh *et al*., 2005 and Hornung *et a*l., 2005 discovered the presence of low levels of

arachidonic acid in certain plants for example *Phytophthora megasperma*. In other plants substrates such as the 18-carbon fatty acids linoleic and linolenic acids may, however, act as substitute substrates to produce second messengers (Boss and Morrè, 1989). The authors have shown, for example, that both exogenously applied arachidonic acid and eicosapentaenoic acid can directly act as second messengers and specifically elicit the accumulation of phytoalexins in potato tubers. However, when the levels are too low, fatty acids might act as substrates. It has also been argued that eicosanoids can be synthesized from extracellular arachidonic acid supplied by invading organisms and that this mechanism is utilized by the plant to sense the invasion. Another possibility is that metabolites of linolenic and linoleic acid can serve the role of eicosanoids in plant tissues (Boss and Morrè, 1989).

There are two known pathways for metabolizing the hydroperoxides of linoleic and linolenic acid in plants [Figure 7.2, linoleic acid (18:2) and linolenic acid (18:3)]. The first pathway leads to the synthesis of jasmonic acid (JA) while the other to that of dihydrojasmonic acid (DH-JA). Recently Claeys *et al*. (1986) presented evidence that hydroperoxides can also undergo rearrangement to trihydroxy fatty acids so that 13-hydroperoxy linoleic acid would form 9,12,13-trihydroxy-10-octadecenoic acid with physiological effects in both plants and animals. One of these hydroxylated derivates of JA is tuberonic acid that can act as tuber-inducing signals isolated from potato (Beale and Ward, 1998). Recent studies also identified a dimer, 12-oxo-PDA, involved in the production of dihydro-jasmonates (DH-JA) that is related to jasmonic acid and is also highly active.

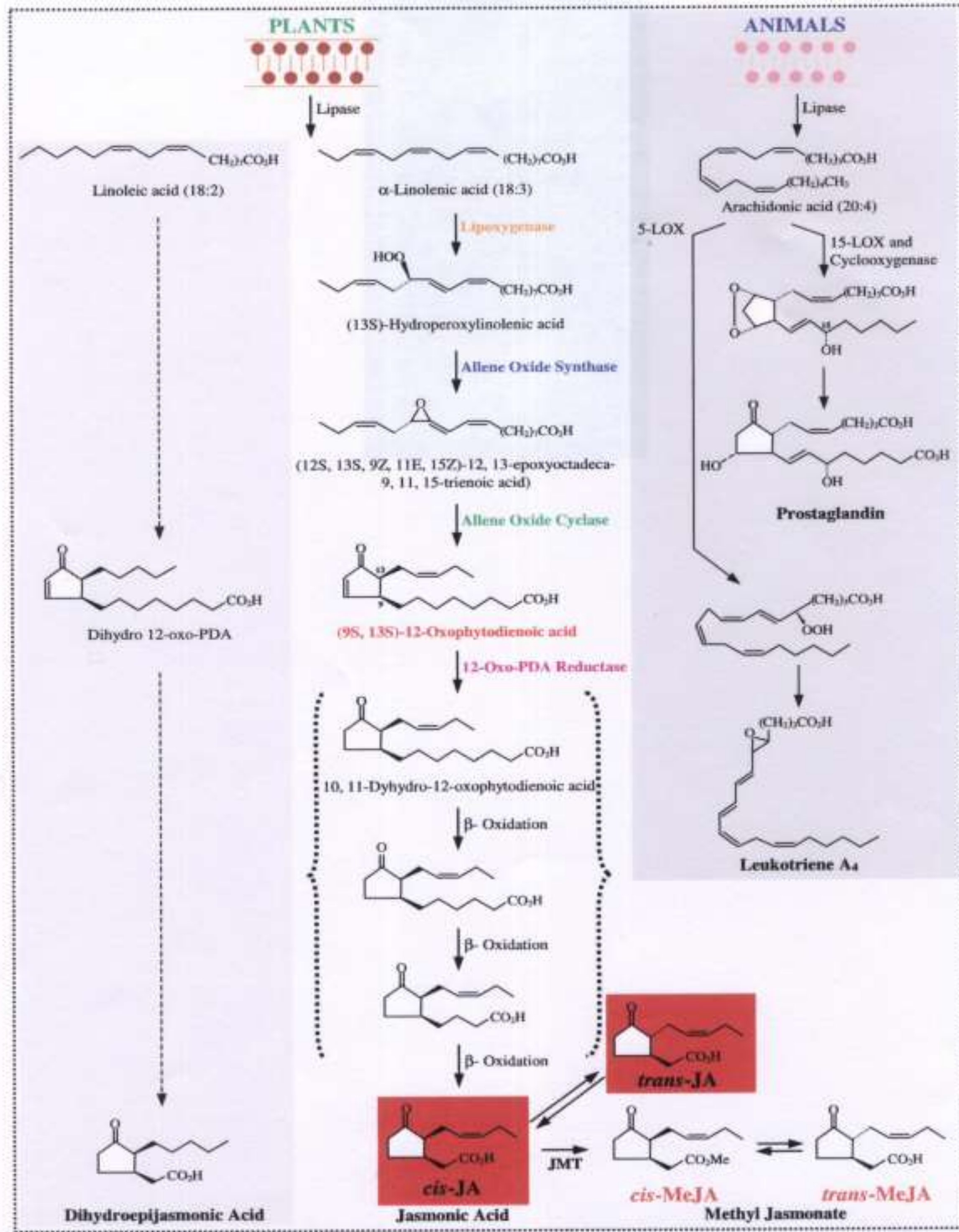


Figure 7.2: The octadecanoid pathway leading to jasmonic acid (JA) via 12-oxo-phytodienoic acid (OPDA) biosynthesis in plants. In parallel, the analogous pathway in animals leading to the prostaglandins and leukotriene synthesis has been presented. Synthesis of dihydroepijasmonic acid from linoleic acid is also presented (Agrawal, *et al*., 2004).

DH-JA could be synthesized via two different biosynthetic routes, either starting from linolenic acid followed by a reduction of the double bond, or directly from linoleic acid (Gundlach and Zenk, 1998). Studies revealed that precursors of DH-JA derived from linoleic acid seems to be more restricted in their occurrence and its ability to act on certain physiological processes, while JA seems to be ubiquitously distributed in a variety of plants. Although jasmonic acid (JA) is commonly produced from linolenic acid, studies confirmed that exogenously applied linoleic acid could be converted to linolenic acid to produce JA (Gundlach and Zenk, 1998).

The precursor to JA, 12-oxo-phytodienoic acid (12-oxo-PDA) with a structure quite similar to that of prostaglandins, is also regarded as a second messenger with a regulatory role distinct from that of JA (Zimmerman and Feng, 1978; Vick and Zimmerman, 1987). Roblin and Bonmort (1984) reported that 12-oxo-PDA can alter membrane permeability or ion transport and shorten the time of flowering of *Pharbitis nil*. Importantly, Zimmerman and Vick (1983) showed that 12-oxo-PDA could stepwise be metabolized to JA, including a reduction step catalyzed by the enzyme 12-oxo-PDA reductase (Figure 7.2). Additionally, Gundlach and Zenk (1998) reported that 12-oxo-PDA reductase is not strictly substrate specific, but can also convert DH-12-oxo-PDA to DH-JA as well as DH-JA to JA.

Jasmonic acid (JA) is probably the best characterized of the potential chemical messengers derived from the lipoxygenase-dependant oxidation of fatty acids. Perhaps one of the greatest surprises in recent years is the wide range of physiological activities induced in plants by JA (Seigler, 1998). One of the first physiological activities demonstrated for JA by Aldridge *et al*. (1971), after its isolation from culture filtrates of the fungus *Lasiodiplodia theobromae*, was that it inhibited plant growth, but only at high or toxic levels. The phytohormone effects of JA are similar to those of abscisic acid (ABA), an established phytohormone. As a result Sembdner and Parthier (1993) argued that JA should be recognized as a representative of a unique class of phytohormones. In this regard JA shares both chemical and biological similarities with the plant growth regulator abscisic acid (ABA) as both compounds inhibit growth and promote the senescence of detached leaves at high levels while low levels can have a stimulating effect. This confirms its typical hormone-like action.

According to Woeste *et al.* (1999), one of the main physiological activities of JA is to increase ethylene biosynthesis leading to increased leaf and flower senescence and fruit ripening (Mueller, 1998; Seigler, 1998). JA is also believed to induce the expression of a specific set of proteins (jips or jasmonate-induced proteins) in different plants (Sembdner and Parthier, 1993) and in some instances may be involved with the induction of the formation of seed storage proteins. Other examples of the physiological activity of JA include; a) the inhibition of the auxin and light-induced opening of pulvinules of *Mimosa pudica* L., b) the promotion of rooting of mung bean seedlings, c) the mediation of the elicitor-induced accumulation of secondary metabolites by acting as a second messenger (Vom Endt, *et al*., 2002) and d) the induction of tuber formation in potatoes (Hamberg and Gardner, 1992).

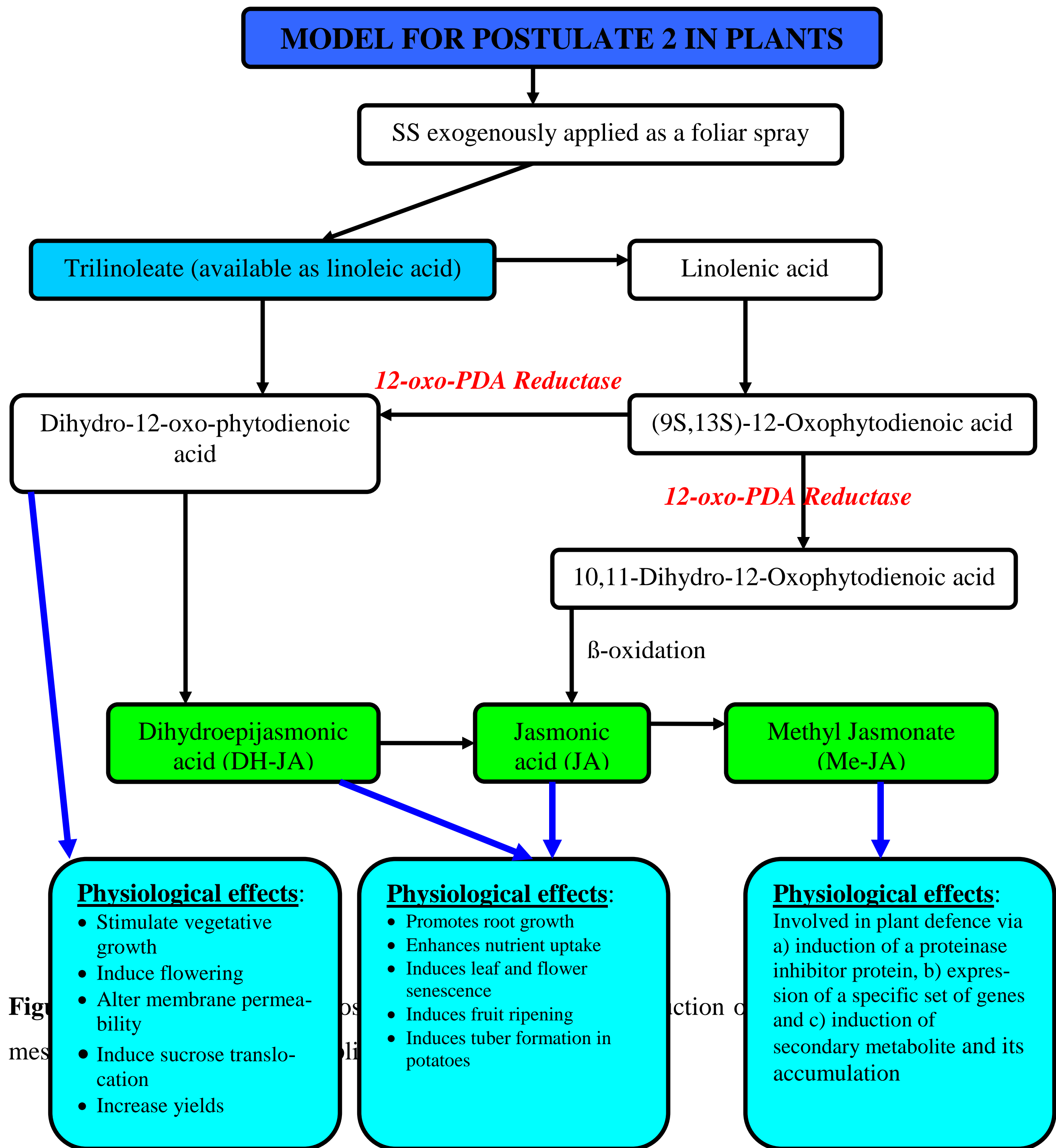
When a methyl ester of JA (Me-JA) was applied to the surface of tomato plants, the formation of a defensive proteinase inhibitor protein was induced, not only in the plant to which the application was made but also in nearby plants (Seigler, 1998). The significance of this observation probably lies in the fact that Me-JA can directly be involved in a plant defence mechanism, possibly as a second messenger, via inducing the expression of a specific set of genes in different plants. Just as significant is the role of Me-JA in inducing the accumulation of secondary metabolites in tissue cultures of 36 test plants reported by Gundlach *et al*. (1992) and the possibility that this could be part of the defence mechanism mentioned earlier.

Despite the available information on JA and Me-JA, Crombie and Mistry (1991) have shown that octadecanoid precursors of jasmonic acid (JA) were involved in activating the synthesis of wound-inducible proteinase inhibitors. The significance of this finding is the proposal of the authors that wounding activates the synthesis of systemin, perhaps by releasing this potent 18-amino acid peptide from an inactive propeptide, and that systemin could serve as a systemic signal that releases linolenic acid or other free fatty acid substrates from membranes after binding to a plasma membrane receptor. This alternatively could lead to the activation of specific lipoxygenases that, when acting on linolenic acid, would produce a rapid accumulation of JA or other C18-eicosanoid equivalents in plants (Davies, 1995).

Much less is known about the octadecanoids and their possible physiological activities than about the 20-carbon eicosanoids found in animals (Vick and Zimmerman, 1978). At present there is no report of any physiological activities in plant tissues associated with the trihydroxy octadecanoids. However, an unusual acetyl ester was isolated from avocado fruits, which inhibited IAA-induced growth of wheat coleoptiles and kinetin-supported growth of soybean callus at concentrations above 50 µM. Oxygenated 12-carbon fatty acids have been shown to have physiological activity in plants by acting as a gibberellin synergist that stimulated the growth of dwarf peas and maize. Gundlach and Zenk (1998) also showed that the oxygenated fatty acid, especially 12-oxo-PDA, often shows considerably higher biological activity than JA.

Other examples of second messengers produced by the oxygenation of fatty acids include traumatic acid (TA) that has also been implicated as a plant growth regulator with physiological connotations. Some of these physiological activities include the induction of renewed cell division and cell extension activity in the parenchymatous cells of the bean pod mesocarp by TA (Tanimoto and Harada, 1984). The authors also demonstrated that submicromolar concentrations of TA stimulated adventitious bud formation in the epidermis of *Torenia* stem segments

From the information summarized above, a simplified model is presented (Figure 7.3) in an attempt to illustrate postulate 2 and the possible involvement of trilinoleate, the active bio-stimulatory compound isolated from a *L. albus* seed suspension (SS), in enhancing seedling growth and increasing yields in agricultural crops. In this model it was calculated for the maize trial, that each plant received 15.7 µg active substance (trilinoleate). This is in agreement with the low dosages of known phytohormones such as IAA and GA applied in the horticultural industry. The 15.7 µg trilinoleate is available to be metabolized as illustrated in the model.



From the physiological effects listed for dihydro-12-oxo-phytodienoic acid, DH-JA, JA and Me-JA the following affects of a *L. albus* seed suspension, as observed in this study, can be explained as follows:

a) Enhanced seedling growth, especially in terms of root growth as observed in a number of crops (Chapter 3), coincides with the physiological activities reported for the above second messengers. This was observed under both laboratory and field conditions indicating that treated seedlings must have been in a better position to absorb nutrients from the environment which indirectly must have had an effect on vegetative growth and probably yield.

b) Yield is dependant on the successful translocation of stored photosynthate, in the form of sucrose in higher plants, from the source (storage tissue) to the sink (e.g. maize and wheat kernels) during the grain filling stage. As a matter of fact, the only way that crop yields could be enhanced in terms of increased phytomass of the harvestable parts is by increasing either the photosynthesis rate or the translocation of stored carbohydrates to these parts or both. Further, probably the only way to achieve the latter is to improve the permeability or translocation capacity of membranes. The JA precursor, dihydro-12-oxo-phytodienoic acid, has been reported to alter membrane permeability and induce sucrose translocation. The possibility exists that both DH-JA and JA can also be involved in a membrane energizing mechanism (see the effect of SS on respiration rate), but this will have to be tested in a follow-up study.

Importantly, significant seedling growth enhancement as well as yield increases observed in this study, especially in vegetable crops, strongly indicates that the active bio-stimulatory substance contained in a *L. albus* seed suspension, namely trilinoleate, may indirectly be involved in an action mechanism described as postulate 1 or postulate 2 or a combination of the two. A follow-up study will be needed to test both of these hypotheses and elucidate this mechanism of action. However, at this stage the active substance isolated from a *L. albus* seed suspension, trilinoloeate, as well as its isolation and application methodology, has been patented under the number 19803630.2 and the PTC number WO 99/38379 while the development of a natural product with commercialization potential for the agricultural and horticultural industries, is envisaged.

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SUMMARY

The sustainable increase in crop yields on available land, in light of an ever growing world population, remains an issue of importance for agronomists and plant physiologists alike. With this in mind, the underlying study was aimed at investigating the bio-stimulatory properties of a *Lupinus albus* L. seed suspension (SS) as well as its potential to be applied as a natural plant growth regulator in the horticultural and agricultural industries. Preliminary bio-tests, including the respiration rate of monoculture yeast cells, seed germination and seedling growth, strongly indicated that the rather crude seed suspension possessed the ability to manipulate these aspects when applied exogenously. SS significantly increased the respiration rate of monoculture yeast cells and, although it did not have a significant effect on seed germination, stimulated seedling growth in terms of coleoptile and root length in a number of crops at an optimal concentration of 5 mg L^{-1}. Subsequently, the effect of SS on the yields of a variety of vegetable crops, flowers and cash crops was tested under field conditions at a concentration of 5 mg L^{-1}. This concentration proved to be optimal for most vegetables, flowers, maize and wheat as either flower formation or yield of different crops were significantly enhanced. The most significant yield increases, after a foliar spray treatment with SS at an early growth stage, was observed in beetroot, lettuce and carrots under drip irrigation by increasing the yield with 9.3, 20.0 and 24.3 ton ha^{-1} respectively. This supplied the rationale for activity directed isolation and purification of the active bio-stimulatory compounds involved using standard chromatography techniques. By means of NMR spectroscopy and mass spectrometry the active compound was identified as a triglyceride, glycerol trilinoleate, and its structural formula elucidated. It was concluded from this study that the development of a natural product with growth regulating and yield enhancing properties should be considered strongly from a commercial perspective.

Keywords: *Lupinus albus* L.; seed suspension; bio-stimulatory properties; nuclear magnetic resonance (NMR) spectroscopy; glycerol trilinoleate; natural product.

OPSOMMING

Die volhoubare verhoging in die oesopbrengs van landbougewasse op beskikbare grond is ‘n prioriteit vir beide agronome en plantfisioloë in die lig van ‘n groeiende wêreldbevolking. Met dit in gedagte is die onderhewige studie onderneem met die doel om die bio-stimulerende eienskappe van ‘n *Lupinus albus* L. saad suspensie (SS) te ondersoek asook die potensiaal om dit as ‘n natuurlike plantgroeireguleerder in die akkerbou- en tuinbou-industrieë toe te pas. Voorlopige bio-toetse, insluitende die respirasietempo van monokultuurgisselle, saadkieming en saailinggroei, het sterk daarop gedui dat die relatiewe ru saadsuspensie oor die potensiaal beskik om genoemde aspekte te manipuleer wanneer dit uitwendig aangewend word. SS het die respirasietempo van gisselle betekenisvol verhoog en, alhoewel dit nie veel van ‘n effek op saadkieming gehad het nie, het dit saailinggroei in terme van koleoptiel en wortellengte betekenisvol gestimuleer teen ‘n optimum konsentrasie van 5 mg L^{-1}. Gevolglik is die invloed van SS op die oesopbrengs in ‘n verskeidenheid van groente-, blom- en kontantgewasse onder veldtoestande getoets teen 5 mg L^{-1}. Hierdie konsentrasie was ook optimaal vir die meeste gewasse aangesien blomvorming in blomgewasse of oesopbrengs in ander gewasse betekensvol verhoog is. Statisties was die oesopbrengsverhoging mees betekenisvol in drupbesproeide beet, slaai en wortels na blaarbespuitings met SS op ‘n vroeë ontwikkelingsfase gemeet aan oesopbrengsverhogings van 9.3, 20.0 en 24.3 ton ha^{-1} respektiewelik. Laasgenoemde het die rasionaal verskaf om voort te gaan met die aktiwiteitsgerigte isolasie en suiwering van die aktiewe komponente betrokke deur van standaard chromatografietegnieke gebruik te maak. Deur middel van KMR spektroskopie en massaspektrometrie is die aktiewe komponent geïdentifiseer as ‘n trigliseried gliseroltrilinoleaat, en die struktuurformule ontsyfer. Uit hierdie studie is tot die gevolgtrekking gekom dat die ontwikkeling van ‘n natuurlike produk met groeiregulerende en oesopbrengs verhogende eienskappe vanuit ‘n kommersiële perspektief, sterk oorweeg moet word.

Sleutelwoorde: *Lupinus albus* L.; saad suspensie; bio-stimulerende eienskappe; kernmagnetiese resonans (KMR) spektrometrie; gliseroltrilinoleaat; natuurlike produk.

APPENDICES

APPENDIX 1: **CHAPTER 3**

Table 3.1A: Analysis of variance of the interaction effect between different treatments on the respiration rate of monoculture yeast cells at 180 minutes

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	1	128.1	128.13	0.0000*
Concentration	4	2898	724.6	0.0000*
Treatment x Concentration	4	308.8	77.21	0.0000*
Error	20	16	0.8	
Total	29	3351.4		

* = Term significant [LSD $_{(T)}$ (5%) = 2.58]
ns = non-significant

Table 3.2A: Analysis of variance of the effect of different treatments on the % germination of Cress seeds at 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	1	2.5	2.5	0.874
Concentration	4	565	141.25	0.000897*
Treatment x Concentration	4	10	2.5	0.998673
Error	30	2950	98.33	
Total	39	3527.5		

* = Term significant [LSD $_{(T)}$ (5%) = 3.51]
ns = non-significant

Table 3.3A: Analysis of variance of the effect of different treatments on the coleoptile length of Cress seedlings at 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	1	335.241	335.241	0.000437*
Concentration	4	115.698	28.92451	0.275795
Treatment x Concentration	4	321.4613	80.36532	0.013742*
Error	30	644.3985	21.47995	
Total	39	1416.799		

* = Term significant [LSD $_{(T)}$ (5%) = 5.58]
ns = non-significant

Table 3.4A: Analysis of variance of the effect of different treatments on the root length of Cress seedlings at 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	1	2826.77	2826.77	0.000950*
Concentration	4	1664.198	416.0496	0.123535
Treatment x Concentration	4	922.6808	230.6702	0.376465
Error	30	6313.777	210.4592	
Total	39	11727.43		

* = Term significant [LSD $_{(T)}$(5%) = 9.36]
ns = non-significant

Table 3.5A: Analysis of variance of the effect of different treatments on the % germination of Cauliflower seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	364.8953	91.22383	0.000000*
Time	3	68752.55	22917.52	0.000000*
Treatment x Time	12	1088.311	90.69258	0.000000*
Error	60	45.9375	0.765625	
Total	79	70251.7		

* = Term significant [LSD $_{(T)}$ (5%) = 2.29]
ns = non-significant

Table 3.6A: Analysis of variance of the effect of different treatments on the coleoptile length of Cauliflower seedlings over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	10.47481	2.618703	0.000000*
Time	3	3742.59	1247.53	0.000000*
Treatment x Time	12	17.13222	1.427685	0.000000*
Error	60	7.838226	0.1306371	
Total	79	3778.035		

* = Term significant [LSD $_{(T)}$ (5%) = 0.946]
ns = non-significant

Table 3.7A: Analysis of variance of the effect of different treatments on the root length of Cauliflower seedlings over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	1952.492	488.123	0.000000*
Time	3	17099.09	5699.698	0.000000*
Treatment x Time	12	1463.805	121.9837	0.000000*
Error	60	95.0679	1.584465	
Total	79	20610.46		

* = Term significant [LSD $_{(T)}$ (5%) = 3.298]
ns = non-significant

Table 3.8A: Analysis of variance of the effect of different treatments on the % germination of Cabbage seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	3227.911	806.9777	0.000000*
Time	3	100547.6	33515.88	0.000000*
Treatment x Time	12	595.2328	49.60273	0.000000*
Error	60	323.2031	5.386719	
Total	79	104694		

* = Term significant [LSD $_{(T)}$(5%) = 6.08]
ns = non-significant

Table 3.9A: Analysis of variance of the effect of different treatments on the coleoptile length of Cabbage seedlings over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	26.40147	6.600368	0.000000*
Time	3	1191.092	397.0308	0.000000*
Treatment x Time	12	36.84115	3.070096	0.000000*
Error	60	28.80478	0.4800797	
Total	79	1283.14		

* = Term significant [LSD $_{(T)}$ (5%) = 1.81]
ns = non-significant

Table 3.10A: Analysis of variance of the effect of different treatments on the root length of Cabbage seedlings over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	225.657	56.41442	0.000000*
Time	3	17956.43	5985.476	0.000000*
Treatment x Time	12	341.7295	28.47746	0.000000*
Error	60	192.8719	3.214531	
Total	79	18716.69		

* = Term significant [LSD $_{(T)}$ (5%) = 4.69]
ns = non-significant

Table 3.11A: Analysis of variance of the effect of different treatments on the % germination of Lettuce seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	1619.023	404.7559	0.000000*
Time	3	6029.269	2009.756	0.000000*
Treatment x Time	12	1810.864	150.9053	0.000000*
Error	60	565.4688	9.424479	
Total	79	10024.63		

* = Term significant [LSD $_{(T)}$ (5%) = 8.03]
ns = non-significant

Table 3.12A: Analysis of variance of the effect of different treatments on the coleoptile length of Lettuce seedlings over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	13.73935	3.434837	0.000000*
Time	3	2698.368	899.456	0.000000*
Treatment x Time	12	10.59041	0.882534	0.000004*
Error	60	9.707134	0.1617856	
Total	79	2732.405		

* = Term significant [LSD $_{(T)}$ (5%) = 1.048]
ns = non-significant

Table 3.13A: Analysis of variance of the effect of different treatments on the root length of Lettuce seedlings over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	66.89865	16.72466	0.000000*
Time	3	10798.85	3599.618	0.000000*
Treatment x Time	12	70.69165	5.890971	0.000000*
Error	60	43.92062	0.7320104	
Total	79	10980.36		

* = Term significant [LSD $_{(T)}$ (5%) = 2.24]
ns = non-significant

Table 3.14A: Analysis of variance of the effect of different treatments on the % germination of Bean seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	598.75	149.6875	0.000000*
Time	3	75273.75	25091.25	0.000000*
Treatment x Time	12	751.25	62.60417	0.000000*
Error	60	675	11.25	
Total	79	77298.75		

* = Term significant [LSD $_{(T)}$ (5%) = 8.78]
ns = non-significant

Table 3.15A: Analysis of variance of the effect of different treatments on the coleoptile length of Bean seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	44.67883	11.16971	0.030352*
Time	3	4187.707	1395.902	0.000000*
Treatment x Time	12	150.7768	12.56473	0.001264*
Error	60	233.3043	3.888405	
Total	79	4616.466		

* = Term significant [LSD $_{(T)}$ (%) = 5.16]
ns = non-significant

Table 3.16A: Analysis of variance of the effect of different treatments on the root length of Bean seedlings over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	4	424.4872	106.1218	0.080502
Time	3	29684.88	9894.96	0.000000*
Treatment x Time	12	679.4545	56.62121	0.324918
Error	60	2903.431	48.39051	
Total	79	33692.25		

* = Term significant [LSD $_{(T)}$ (5%) = 5.80]
ns = non-significant

APPENDIX 2: CHAPTER 4

Table 4.1 A: Analysis of variance on the total number of flowers formed by Gazanias under field conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	21.15923	7.053077	0.002780*
Week	9	862.4836	95.83151	0.00000*
Treatment X Week	27	74.68848	2.76624	0.010894*
Replication	2	31.54905	15.77452	0.000053*
Treatment X Replication	6	13.08182	2.180303	0.153337(ns)
Week X Replication	18	53.49104	2.971724	0.011769*
Error	119	1128.128		
Total	120			

* = Term significant [LSD $_{(T)}$ (5%) = 1.29]
ns = non-significant

Table 4.2A: Analysis of variance on the total number of flowers formed by Impatience under field conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	134.3188	44.77292	0.160515(ns)
Week	5	1783.682	356.7365	0.000000*
Treatment x Week	15	461.0957	30.73971	0.281400(ns)
Error	48	1196.531	24.92774	
Total	71	3575.628		

* = Term significant [LSD $_{(T)}$ (5%) =4.42]
ns = non-significant

Table 4.3A: Analysis of variance on the dry kernel yield of maize under rain fed conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	0.955189	0.3183964	0.000108*
Replication	3	0.0113523	0.0037841	0.827563(ns)
Error	9	0.115105	0.01278934	
Total	15	1.081645		

* = Term significant [LSD $_{(T)}$ (5%) =0.24]
ns = non-significant

Table 4.4A: Analysis of variance on the dry kernel yield of wheat under semi-irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	5	1.269973	0.2539945	0.002176*
Replication	3	0.0625352	0.0284507	0.668908(ns)
Error	15	0.5906175	0.0393745	
Total	23	1.923125		

* = Term significant [LSD $_{(T)}$ (5%) =0.45]
ns = non-significant

Table 4.5A: Analysis of variance on the final yield of beetroot under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	747.0993	249.0331	0.015870 *
Replication	2	127.1326	63.56631	0.208861 (ns)
Error	6	185.4751	30.91251	
Total	11	1059.707		

* = Term significant [LSD $_{(T)}$ (5%) =15.69]; ns = non-significant

Table 4.6A: Analysis of variance on the foliage fresh mass of beetroot under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	966.5134	322.1711	0.000098*
Replication	2	13.43132	6.715661	0.383962(ns)
Error	6	35.73597	5.955996	
Total	11	1015.681		

* = Term significant [LSD $_{(T)}$ (5%) = 6.88]
ns = non-significant

Table 4.7A: Analysis of variance on the head fresh mass of lettuce under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	784.43	261.4767	0.004421*
Replication	2	22.60667	11.30333	0.585573(ns)
Error	6	115.76	19.29333	
Total	11	922.7967		

* = Term significant [LSD $_{(T)}$ (5%) = 12.40]
ns = non-significant

Table 4.8A: Analysis of variance on the foliage fresh mass of lettuce under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	76.99789	25.66596	0.049660*
Replication	2	7.96625	3.983125	0.515802(ns)
Error	6	32.26228	5.377047	
Total	11	117.2264		

* = Term significant [LSD $_{(T)}$ (5%) = 6.54]
ns = non-significant

Table 4.9A: Analysis of variance on the head fresh mass of cabbage under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	1257.227	419.0757	0.826886 (ns)
Replication	2	111.0527	55.52637	0.961694 (ns)
Error	6	8474.263	1412.377	
Total	11	9842.542		

* = Term significant [LSD $_{(T)}$ (5%) = 106.21]
ns = non-significant

Table 4.10A: Analysis of variance on the foliage fresh mass of cabbage under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	3	4.141358	1.380453	0.865881 (ns)
Replication	2	2.117517	1.058758	0.834055 (ns)
Error	6	33.96062	5.660103	
Total	11	40.21949		

* = Term significant [LSD $_{(T)}$ (5%) = 6.70]
ns = non-significant

Table 4.11A: Analysis of variance on carrot length under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	5	2935.658	587.1317	0.000779*
Replication	3	196.9043	65.63476	0.469423(ns)
Error	15	1107.737	73.84914	
Total	23	4240.3		

* = Term significant [LSD $_{(T)}$ (5%) = 19.72]
ns = non-significant

Table 4.12A: . Analysis of variance on the foliage fresh mass of carrots under irrigation conditions after treatment with CC and SS both separately and in combination

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	5	12012.94	2402.587	0.017912*
Replication	3	906.5946	302.1982	0.692400(ns)
Error	15	9192.498	612.8332	
Total	23	22115.03		

* = Term significant [LSD $_{(T)}$ (5%) = 56.81]
ns = non-significant

Table 4.13A: Analysis of variance on the final carrot yield under irrigation conditions after treatment with CC and SS both separately and in combination.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	5	1663.832	332.7665	0.025500*
Replication	3	72.50181	24.16727	0.854266(ns)
Error	15	1403.52	93.56802	
Total	23	3139.855		

* = Term significant [LSD $_{(T)}$ (5%) = 22.19]
ns = non-significant

APPENDIX 3: **CHAPTER 5**

Table 5.2A: Analysis of variance of the effect of different liquid liquid chromatography fractions on the respiration rate of monoculture yeast cells at 180 minutes

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	5	14002.28	2800.456	0.000000 *
Time	2	0.7777778	0.3888889	0.851976 (ns)
Error	10	23.88889	2.388889	
Total	17	14026.94		

* = Term significant [LSD $_{(T)}$(5%) = 4.38]
ns = non-significant

Table 5.3A: Analysis of variance of the interaction of different liquid liquid chromatography fractions on the percentage germination of Cress seeds over a period of 96 h

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	5	4524.796	904.9592	0.000000*
Time	3	3168.503	1056.167	0.000000*
Treatment X Time	15	1382.721	92.18143	0.000000*
Error	48	487.7083	10.16059	
Total	71	9563.729		

* = Term significant [LSD $_{(T)}$(5%) = 10.02]
ns = non-significant

Table 5.4: Analysis of variance of the effect of different liquid liquid chromatography fractions on the coleoptile length of Cress seeds at 96 h

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	5	68.697778	13.79556	0.000073 *
Time	3	13397.17	4465.723	0.000000 *
Treatment X Time	15	83.41222	5.560815	0.004116*
Error	48	97.37334	2.028611	
Total	71	13646.93		

* = Term significant [LSD $_{(T)}$(5%) = 4.48]
ns = non-significant

Table 5.5: Analysis of variance of the effect of different liquid liquid chromatography fractions on the root length of Cress seeds at 96 h

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	5	417.3907	83.47814	0.000000 *
Time	3	37842.67	12614.22	0.000000 *
Treatment X Time	15	495.1888	33.01258	0.000000*
Error	48	102.5267	2.135973	
Total	71	38857.78		

* = Term significant [LSD $_{(T)}$(5%) = 4.59]
ns = non-significant

Table 5.6: Analysis of variance of the effect of combined column chromatography fractions on the percentage germination of Cress seeds at 96 h

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	13	4591.071	353.1593	0.000001*
Replication	2	3.571429	1.785714	0.952215(ns)
Error	26	946.4286	36.4011	
Total	41	5541.071		

* = Term significant [LSD $_{(T)}$(5%) =18.15]
ns = non-significant

Table 5.7: Analysis of variance of the effect of combined column chromatography fractions on the coleoptile length of Cress seeds at 96 h

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	13	538.8148	41.44729	0.000209*
Replication	2	5.324432	2.662216	0.723902
Error	26	211.5792	8.137662	
Total	41	755.7184		

* = Term significant [LSD $_{(T)}$(5%) =8.58]
ns = non-significant

Table 5.8: Analysis of variance of the effect of combined column chromatography fractions on the root length of Cress seeds at 96 h

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares (SS)**	**MSS (Mean sum of squares)**	**Significance F**
Treatment	13	6420.348	493.8729	0.000000*
Replication	2	109.1385	54.56926	0.242830(ns)
Error	26	948.8274	36.49336	
Total	41	7478.313		

* = Term significant [LSD $_{(T)}$(5%) =18.17]
ns = non-significant

APPENDIX 4: **CHAPTER 6**

Table 6.1A: Analysis of variance of the interaction effect between different treatments on the respiration rate of monoculture yeast cells at 180 minutes

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	2	5230.691	2615.346	0.000000*
Replication	2	325.9506	162.9753	0.050602(ns)
Treatment x Replication	4	264.4938	66.12346	0.279808(ns)
Time	8	89094.0280	11174.25	0.000000*
Treatment x Time	16	3942.198	246.3873	0.000059*
Replication x Time	16	954.2716	59.64198	0.319324(ns)
Error	32	1589.951	49.68596	
Total	80	101701.6		

* = Term significant [LSD $_{(T)}$ (5%) = 23.19]
ns = non-significant

Table 6.2A: Analysis of variance of the effect of different treatments on the % germination of Cabbage seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	4	1604.375	401.0938	0.000000*
Time	3	26065.94	8688.646	0.000000*
Treatment x Time	12	448.125	37.34375	0.132179 (ns)
Replication	3	913.4375	304.4792	0.000006*
Treatment x Replication	12	2613.125	217.7604	0.000000*
Time x Replication	9	195.3125	21.70139	0.506399 (ns)
Error	36	834.375	23.17708	
Total	79	32674.69		

* = Term significant [LSD $_{(T)}$ (5%) = 4.87]
ns = non-significant

Table 6.3A: Analysis of variance of the effect of different treatments on the coleoptile growth of Cabbage seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	4	44.93202	11.233	0.000069*
Time	3	6263.276	2087.759	0.000000*
Treatment x Time	12	36.24743	3.020619	0.029656(ns)
Replication	3	38.93	12.97667	0.0000796*
Treatment x Replication	12	37.37915	3.114929	0.025150*
Time x Replication	9	29.08611	3.231791	0.029345*
Error	36	48.2037	1.338992	
Total	79	6498.055		

* = Term significant [LSD $_{(T)}$ (5%) = 1.16]
ns = non-significant

Table 6.4A: Analysis of variance of the effect of different treatments on the root growth of Cabbage seeds over a period of 96h.

ANOVA				
SOURCE	**Degrees of Freedom**	**Sum of squares**	**Mean sum of squares**	**Significance**
Treatment	4	179.7936	44.9484	0.001425*
Time	3	25089.92	8363.306	0.000000*
Treatment x Time	12	162.0387	13.50323	0.118572(ns)
Replication	3	30.68871	10.22957	0.0303671(ns)
Treatment x Replication	12	376.9095	31.40912	0.000807*
Time x Replication	9	20.08628	2.231809	0.977701(ns)
Error	36	292.981	8.138362	
Total	79	26152.41		

* = Term significant [LSD $_{(T)}$ (5%) = 2.88]
ns = non-significant