GROWTH AND YIELD RESPONSE OF SELECTED CROPS TO TREATMENT WITH *ComCat*®

by

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DEDICATION

This dissertation is dedicated to my family, especially to my loving wife, Birthe Hüster, for the years that she sacrificed during my research and work looking after the family, and to our two sons, Maximilian and Jakob. Furthermore, to my parents who supported me during my school days and study time, and taking care of the whole family during my long absences from home.

DECLARATION

I declare that the dissertation submitted by me for the degree Philosophiae Doctor at the University of the Free State, South Africa is my own independent work and has not previously been submitted by me to another University. I furthermore concede copyright of the dissertation in favour of the University of the Free State.

Signed in Bloemfontein, South Africa.

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Thomas Hüster

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CHAPTER 1

INTRODUCTION AND RATIONALE

Despite the impressive advances that have been made over the years in improving yields of food crops, there is little reason to become complacent about food supply (Heidhues, 2001), especially in the light of current world population growth and the food insecurity in developing countries (Penning de Vries, 2001). To increase yields on limited land requires manipulation of agricultural crops by means of innovative techniques. Almost three decades ago, a need for this approach was expressed by Ting (1982), namely that it would be a giant step forward if technology to control plant growth could be developed to specifically minimize or maximize specific stages of crop development to the advantage of man. Roberts and Hooley (1988) added that the isolation, purification and identification of compounds with bio-stimulatory activities from plants, as well as information on their ability to increase the production of agricultural and horticultural crops over the short term, e.g. one growing season, must be regarded as extremely important from a food security perspective.

Since these statements were made the potential to manipulate crops and commercialize naturally occurring secondary metabolites involved in growth regulation in plants captured the imagination of plant biochemists and agronomists alike, and has lead to an unparalleled surge for information on these compounds and their functions in plants (Ramirez *et al*. 2000). This includes information on antibacterial (Rabe & van Staden, 1997), antifungal (Afolayan & Meyer, 1997), herbicidal (Kim *et al*., 1993), pesticidal (Richter & Koolman, 1991) and bio-stimulatory (Schnabl *et al*., 2001) properties of secondary metabolites. However, according to Hostettman & Wolfender (1997), less than 10% of the known higher plant species on the planet have been tested for bio-activity of any sort and in most cases only for a single activity.

Further, the current interest in organic farming worldwide, as a result of consumer resistance to the application of both inorganic and synthetic chemicals in the agricultural industry, supplied an additional rationale to take part in the search for natural plant extracts or existing natural products with the potential to serve as

alternatives in manipulating crops for increased productivity (Pretorius & van der Watt, 2011). Fairly recently a natural bio-stimulant, *ComCat*®, was developed from seeds of European plants belonging to the families Fabaceae and Caryophyllacea by a German company, Agraforum AG (www.agraforum.com), and registered under the German Plant Protection Act (Article 31(1)) as a plant strengthening agent. Further *ComCat*® is approved for the use in organic farming according to the EU regulations (EC) nº 889/2008, Annex I (European Union) and USDA/NOP-Final rule (USA) §205.203(c)(3). *ComCat*® can be regarded as one of the first commercialized natural plant growth regulators containing brassinosteroids (BRs) as active components, and this inspired the current study.

It is claimed by the production company that foliar application of *ComCat*® to plants at specific times during the vegetative growth phase has the potential to manipulate growth and development as well as to increase the yield and quality of agricultural crops. An additional claim is that foliar applications of *ComCat*® to agricultural crops can increase the resistance of plants towards abiotic and biotic stress factors. The compounds contained in *ComCat*®, identified to date by the production company from the main source, *Lychnis viscaria* (Volz, 2000), include two brassinosteroids 24-*epi*-castasterone and 24-*epi*-secasterone, as well as other compounds including flavanoids, phytosterols and free amino acids (Schnabl *et al*., 2001). Further, phytohormones such as auxins (indole-3-acetic acid), gibberellins (GA_3) and cytokinins (6-Benzylaminopyrine, Kinetin, trans-Zeatin) were identified by the producer, AgraForUm AG. However, according to Volz (2000) *ComCat*® contains a third unidentified brassinosteroid. It is believed that the phytohormones are collectively responsible for the bio-stimulatory activity of *ComCat*®, but that the brassinosteroids are probably the major active compounds.

Brassinosteroids (BRs) were discovered in 1979 (Grove *et al*., 1979) and are now recognized as a new class of steroid phytohormones (Zullo and Adam, 2002). Known attributes of BRs include the regulation of cell division and differentiation (Wang *et al*., 2006) as well as the ability to increase resistance towards abiotic and biotic stress factors (Fariduddin *et al*., 2009), to increase the yield of various crops (Fariduddin *et al*., 2008; Hasan *et al*., 2008) and to improve crop quality (Ali *et al*., 2008). Many of the claims made by the manufacturers of *ComCat*® coincide with research data on BRs published over the past three decades. Especially a statement by Ramraj *et al*. (1997) more than a decade ago, namely that further research

towards the application of purified BRs or BR-containing plant extracts in agriculture still needs to be conducted from an economic point of view, as well as the recent repetition of this statement by Janeczko *et al*. (2010), have supplied the rationale for pursuing the following objectives in this study:

1) To develop one or more fast bio-tests in order to measure the activity of *ComCat*® (Chapter 3) *in vitro*,
2) To quantify the germination response of seeds from selected crops, pre-treated with *ComCat*®, as well as subsequent seedling growth (Chapter 3),
3) To quantify the yield response of grain crops, maize and wheat, as well as vegetable crops, cabbage (leaf vegetable), carrot (root vegetable) and onion (bulb vegetable), to treatment with *ComCat*® *in vivo* under field conditions in order to validate *in vitro* results (Chapter 4)
4) To isolate, purify and identify the third unknown brassinosteroid referred to by Volz (2000) (Chapter 5).
5) In general the outcome of these objectives will collectively serve as parameters for assessing: 1) whether the properties of BRs can be exploited in organised agriculture, 2) whether a BR-containing natural product such as *ComCat*® can repeatedly be applied in agriculture in a sustainable fashion over seasons and 3) whether application of the product is acceptable from an economic perspective (Chapter 6).

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Chapter 2

LITERATURE REVIEW

2.1 Introduction

The use of natural products developed from wild plants is gaining interest and momentum throughout the world in both developed and developing countries. In developing countries the use of natural plant extracts is simply the result of the inability of subsistence farmers to afford commercial synthetic pesticides. However, in developed countries this is largely due to consumer resistance towards synthetic chemicals, including antimicrobial, herbicidal and plant growth regulators, believed to be potentially hazardous to the environment and human health (Pretorius & van der Watt, 2011).

Plant diseases cause large yield losses throughout the world and all important food crops are attacked with disastrous consequences for food security. In many cases, plant diseases may be successfully controlled with synthetic fungicides, but this is costly to African peasantry and often has disadvantages and side effects on the ecosystem (De Neergaard, 2001). It is, however, an established fact that the use of synthetic chemical pesticides provides many benefits to crop producers. These benefits include higher crop yields, improved crop quality and increased food production for an ever increasing world population. Despite the latter, synthetic pesticides may pose some hazards to the environment, especially when improperly used by farmers in developing countries who lack the technical skill of handling them, and who fail to adapt to this technology easily. This may result in undesirable residues left in food, water and the environment, toxicity to humans and animals, contamination of soils and ground water and may lead to the development of crop pest populations that are resistant to treatment with agrochemicals (Pretorius & van der Watt, 2011).

As a result of the problems outlined above, research towards seeking less hazardous and cheaper alternatives to conventional synthetic pesticides is rather high on the agenda (Dayan *et al*., 2009). One such alternative is the use of natural products from plants to either control plant diseases in crops via manipulation of the systemic acquired resistance (SAR) mechanisms or to increase yields by

manipulating the metabolism of plants (Pretorius & van der Watt, 2011). Plants have evolved highly specific chemical compounds that provide defence mechanisms against attack by disease causing organisms, including fungal attack, microbial invasion and viral infection (Cowan, 1999). These bioactive substances occur in plants as secondary metabolites, and have provided a rich source of biologically active compounds that may be used as novel crop-protecting agents (Cox, 1990). In nature some wild plants have the potential to survive both harsh biotic and abiotic environmental conditions. This has initiated the postulate that such plants might be utilized as sources for the development of natural products to be applied in agriculture by man as natural herbicides, bactericides, fungicides or products with bio-stimulatory properties in crude or semi-purified form. It is generally assumed that natural compounds from plants pose less risk to animals and humans and are more environmentally friendly than their synthetic counterparts (Johnson, 2001).

An aspect that has received a lot of interest lately from a research perspective is the potential to apply natural plant extracts as plant growth regulators. 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 (Salisbury & Ross, 1992). If the compound is produced within the plant it is called a plant hormone e.g. auxins, gibberellins, cytokinins, abscissic acid and ethylene.

More than two decades ago, Roberts & Hooley (1988) stated that the potential exists to apply a plant extract as a foliar spray in order to stimulate growth in crop plants and hence increase yields. According to the authors, a principal objective of the agricultural industry is to manipulate plant growth and development in such a way that the quantity or quality, or both, of a crop is increased. After the late eighties an elevated interest developed in terms of identifying natural plant compounds that possess the potential to manipulate plant growth and development over a short period, e.g. a growing season.

Reduction in the number of synthetic products due to more stringent pesticide registration procedures (Dayan *et al*., 2009), such as the Food Quality Protection Act of 1996 in the United States, has opened the door for the vigorous pursuit of natural products from plants over the past two decades. In the mean time, many natural compounds from wild plants have been isolated, purified, identified and patented but, only a few products are commercially available. Subsequently, special attention will *inter alia* be given in this chapter to the rationale for considering natural

plant growth regulators (PGRs) and its potential to be applied as agrochemicals in the agricultural industry. Emphasis will be placed on the PGR *ComCat*® that was investigated in this study and that was evaluated in terms of its current "natural product" status.

2.2 Rationale for considering natural compounds from wild plants to be developed as commercial products

Wild plants are a valuable source for the development of new natural products with the potential to be used in the crop production industry (Duke *et al.*, 1995). According to the authors, consumer resistance towards the use of synthetic chemicals has escalated, especially in developed countries, supplying a rationale for the application of natural product alternatives in the agricultural industry. Currently, in many developed countries, the tendency to shift to organic farming systems has evolved under consumer pressure in an attempt to reduce the risk of pesticide application. As a result, research on the possible utilization of biological resources and its application potential in agriculture has become very relevant. A promising approach is the use of natural plant products as an alternative to synthetic chemicals due to the apparent less negative impact on the environment (Ganesan & Krishnaraju, 1995; Ushiki *et al*., 1996).

In this regard Dubey *et al*. (2010) stated that synthetic pesticides are generally persistent in nature and, upon entering the food chain, they destroy the microbial diversity and cause ecological imbalance. It must be accepted that the latter rather harsh statement will probably not apply for all synthetic chemicals currently used in the agricultural industry. However, it contributes to the sensitization of scientists working in the field of natural product development towards the potential environmental impact of newly developed products, whether synthetic or natural (Pretorius & van der Watt. 2011). Well known properties of some synthetic agro-chemicals include carcinogenicity, teratogenicity, high and acute residual toxicity, ability to create hormonal imbalance, spermatoxicity, long degradation period, environmental pollution and adverse effects on food leading to side effects on humans (Feng & Zeng, 2007). Additionally, the build-up of resistance in insects and disease causing micro-organisms after using synthetic agro-chemicals over a long period of time is becoming a great concern (Olufolaji, 2010).

In contrast to the grim picture outlined above with regard to synthetic chemicals, Dubey *et al*. (2010) referred to the general sentiment among natural product scientists namely that botanical, or natural, pesticides are bio-degradable and less hazardous to the environment while their use in agriculture is a practical sustainable alternative. This sentiment is largely based on the argument that donor plants from which natural products are manufactured have been in nature for millions of years and, upon decomposition in soil, had no catastrophic or adverse effects on the ecosystem.

Natural compounds are usually secondary metabolites and are synthesized in plants as a result of biotic and abiotic interactions (Waterman & Mole, 1989; Helmut *et al*., 1994). By means of bioassay guided screening a number of natural plant compounds have been isolated and progress has also been made towards the identification and structural elucidation of these bioactive compounds (Grayer & Harborne, 1994). Although extractable secondary metabolites have long been considered an important source of pharmaceuticals, evaluation of its application potential in agricultural crop production systems has been least studied in comparison.

However, a wide range of activities with both positive and negative effects, including plant growth regulation (Adam & Marquardt, 1986), the induction of plant resistance to various diseases (Daayf *et al*., 1995; Schmitt *et al.*, 1996) and promotion of beneficial micro-organisms in the soil rhizosphere (Williams, 1992) have been reported. Despite these efforts, the isolation of plant secondary metabolites has led to very few commercial successes in the agricultural industry and more specifically in crop management practices (Pretorius & van der Watt, 2011). Most importantly, it is envisaged that crude plant extracts might be more affordable to subsistence farmers as they are readily available and are probably cheaper to produce. Especially with regard to developing countries, consideration of applying natural plant products in its crude form should be high on the agenda. In this study the emphasis will be placed on natural products with bio-stimulatory properties.

2.3 Natural compounds from plants with bio-stimulatory properties

Allelochemicals found in plants are probably all secondary metabolites that are distinctive from primary metabolites in that they are generally non-essential for the

basic metabolic processes such as respiration and photosynthesis (Richard, 2001). They are numerous and widespread, especially in higher plants (Pillmoor, 1993), and often present in small quantities (1-5%) as compared to primary metabolites (carbohydrates, proteins and lipids). Approximately 88 000-100 000 secondary metabolites have been identified in all plant forms showing both structural and activity diversity (Verpoorte, 1998). Ecologically, these chemicals play essential roles in attracting pollinators, as adaptations to environmental stresses and serve as chemical defences against insects and higher predators as well as micro-organisms (Rechcigl & Rechcigl, 2000). Although the purpose of the production of secondary metabolites in plants has long been argued among researchers, it is now universally accepted that they are produced as a result of abiotic (Beart *et al*., 1985) and biotic (Bourgaud, *et al.*, 2001) stresses, probably as part of a plant defence arsenal. Besides the role secondary metabolites play in plant metabolism, the growth promotion or inhibitory properties of certain natural compounds from plants have been extensively researched (Wu *et al*., 2002). It therefore seems appropriate to consider the outcome of this research, or the current status of natural plant growth regulators (PGRs), in terms of their application potential in the agricultural industry from an economic and sustainability perspective.

Already at the end of the millennium two successful natural products developed in Moldavia (formerly part of the Soviet Union) are Moldstim™ and Pavstim™, extracted from hot peppers (*Capsicum annum* L.) and leaves of *Digitalis purpurea* L., respectively (Waller, 1999). Both products have been used on a large scale as plant growth regulators and for disease control. These developments are excellent examples of how natural plant resources can be exploited and applied in agriculture (Pretorius & van der Watt, 2011). According to the authors, 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. In their own endeavours to develop natural products from wild plants Pretorius & van der Watt (2011) have created the term ‘ideal break-through’ as a criterion to identify a plant or plants that contain bio-stimulatory substances promoting both growth and yield in agricultural crops. In applying this criterion the authors reported that extracts from numerous plant species, with bio-stimulatory properties, were identified and evaluated for its commercial potential. Some examples include a report by Channal *et al.* (2002) on seed germination as well as seedling growth enhancement of sunflower and soybean by leaf extracts from three tree species

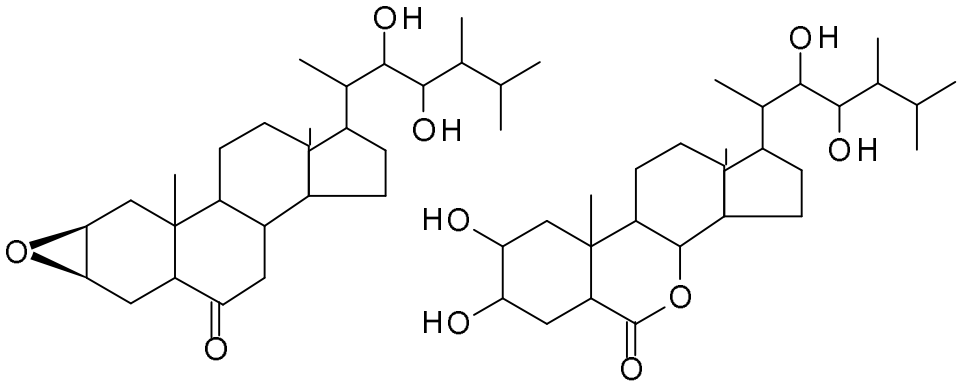
(*Tectona grano, Tamarindus indica* and *Samanea saman*). Tefera (2002) reported similar effects for *Parthenium hysterophorus* extracts on tef (*Eragrostis tef*) while Neelam *et al.* (2002) demonstrated similar effects for *Leucaena leucocephala* extracts on wheat (*Triticum aestivum*). However, none of these studies revealed that treatment with the different plant extracts had any effect on the final yields of the crops under investigation.

Ferreira & Lourens (2002) went a step further and demonstrated the effect of a liquid seaweed extract (now trading as a natural product under the name Kelpak™ (Pretorius & van der Watt, 2011) on improving the yield of canola. This was one of the first natural products developed after obtaining sufficient *in vivo* data by following standard agricultural practices. Kelpak™, containing auxins and cytokinin as active compounds, applied singly or in combination with the herbicide Clopyralid® at various growth stages of canola (*Brassica napus*) was assessed in a field experiment conducted in South Africa during 1998-99. Foliar application of 2 litres Kelpak™ ha^{-1} at the four-leaf growth stage, significantly increased the yield of the crop (Ferreira & Lourens (2002).

An aqueous leachate of *Callicarpa acuminata* was shown by Cruz *et al.* (2002a) to stimulate radicle growth in bean, maize and tomato. The authors also followed selected physiological events including protein synthesis, catalase activity, free radical production and membrane lipid peroxidation in roots treated with the *C. acuminata* extract. The significance of this study by Cruz *et al*. (2002a) lies in the fact that natural product researchers were sensitized towards the potential to manipulate various metabolic events in plants by treatment with plant extracts (Pretorius & van der Watt, 2011).

Probably the most effective compounds to enhance crop yield, crop efficiency and seed vigour has been identified as brassinosteroids (BR's; Mandava, 1979; 1988), first extracted from rape (*Brassica napus* L.) pollen (Adam & Marquard, 1986). Reports on a prototype bio-stimulatory natural product, *ComCat*®, developed from a BR-containing extract of *Lychnis viscaria* came from Friebe *et al*. (1999) and Roth *et al*. (2000). Two BRs have been identified as the main active components of *ComCat*® and these include 24-*epi*-secasterone and 24-epicastasterone (Volz, 2000; Figure 2.1). According to Volz (2000) a third BR remained unidentified. In 2003, after intensive research under laboratory, greenhouse and field conditions a product partially containing an extract of *Lychnis viscaria* was listed in Germany as a plant

strengthening agent under the trade name *ComCat*® and commercialized by a German company, Agraforum AG.



24-epi-secasterone 24-epicastasterone

Figure 2.1: Two brassinosteroids identified as active bio-stimulatory compounds of *ComCat*® (Volz, 2000).

Additional information on *ComCat*® was obtained from the manufacturer's website (www.agraforum.com). The product is manufactured from original and untouched wild plant species of which the genotypic and biochemical potential have not been altered by humans and is described as a natural plant strengthening agent. Attributes claimed by the manufacturers when agricultural crops are treated with *ComCat*® include induced root growth, flowering and resistance towards specific biotic and abiotic stress factors. The claim has also been made that, collectively, the latter can lead to improved growth and yield in agricultural crops.

Recently significant yield increases in tomato, pre-harvest treated with *ComCat*®, was reported by Workneh *et al*. (2009). The authors also claimed more than 70% shelf life extension and higher marketability in tomato fruit harvested from plants treated with *ComCat*® during the vegetative growth phase compared to the untreated control under ambient storage conditions. Preharvest *ComCat*® treated tomatoes also contained lower total soluble sugar levels at harvest and showed better keeping quality in terms of physiological weight loss and juice content compared to untreated controls. However, in the literature no information on the response of row crops such as maize and wheat to treatment with *ComCat*® could be

found. The latter prompted this study. Due to BRs being one of the main active compounds in *ComCat*®, a summary of this phytohormone group is provided.

2.4 Brassinosteroids (BRs)

2.4.1 The discovery of BRs

More than six decades ago researchers found that pollen extracts promote plant growth. Especially hexane extracts of maize pollen applied to the first internode of young bean seedlings contributed to marked elongation of the treated internode (Mitchell & Whitehead, 1941). From this it was hypothesized that maize pollen probably contained high concentrations of known plant hormones. Employing the bean internode response as a standard bio-assay, pollen extracts of many plant species were tested and compared with that of rape (*Brassica napus* L.) in terms of provoking a significant growth response. Only in 1978 were the active compounds in rape pollen identified as brassinosteroids (BRs; Grove *et al*., 1979). Futhermore, eight years later Suzuki *et al*. (1986) identified three different brassinosteroids in the pollen extracts of maize. Since then, the presence of BRs have been identified in 27 families of higher plants and three families of lower plants (Bajguz & Tretyn, 2003), and they occur in all parts of higher plants, including roots.

Today, more than 60 structurally and functionally related BRs have been identified from natural sources which are regarded as a new class of plant hormone (Rao *et al*., 2002) as it complies with the textbook definition for a plant hormone: *A plant hormone is an organic compound synthesized in one part of a plant and translocated to another part, where in very low concentration it causes a physiological response* (Salisbury & Ross, 1992).

Since the discovery of BRs, and already a decade ago, more than 1000 articles have been published on various aspects of their research, mainly by scientists from Japan, USA, Germany, China and the former Soviet Union (Khripach *et al*., 2000). Due to this wide interest in the newly discovered group and the research that followed, BRs were considered promising compounds for application in agriculture, because they showed various kinds of regulatory activity on growth and development of plants while their economic value as yield-promoting agents was predicted during the early 1990s (Cutler, 1991). Understandably, the vast number of publications on BRs cannot all be referred to in this monograph and, therefore, selected publications will be used to cover the aspects related to this study.

2.4.2 Transport of BRs in the light of potential foliar applications to crops

The question exists whether BRs are transported in plants and, therefore, whether foliar application of the compound can be used to manipulate crops? According to Nishikawa *et al*. (1994) long distance transport of exogenously applied BRs can occur in plants, particularly from root to shoot, although exogenous foliar applied 24-epibrassinolide is not efficiently exported from leaves. The latter has not been verified for all the different BR-species identified to date. It is accepted in the interim on a trial and error basis that the response of different crops to foliar applied pure BRs or BR-containing products, e.g. *ComCat*®, can serve as a crude screening method to determine whether BRs are transported from the leaves to other parts of the plant.

Rao *et al*. (2002), however, reported that BRs are highly mobile in the plant system. The authors based their perception on the fact that exogenously applied brassinolide to roots of intact young tomato and radish plants affected the hypocotyls and petioles while application to the bases of mung bean hypocotyls caused elongation of epicotyls. According to the authors, these studies clearly demonstrated the mobility of brassinosteroids in the plant system. Although there are reservations among scientists to retain the concept of 'translocation' in the definition of plant hormones, brassinosteroids still satisfy the 'translocation' property originally attributed to plant hormones (Rao *et al*., 2002). Consensus on this aspect is rather critical in the light of the application method of BRs followed to date namely foliar application or exogenous treatment of seeds with BRs.

2.4.3 Effects of BRs on seed germination

The germination response of seeds from different crops seems to be rather inconsistent. From the literature it is, therefore, difficult to come to a foregone conclusion on whether seed treatment with BRs has a promoting or inhibiting effect on seed germination. For example, Leubner–Metzger (2001) compared exogenously applied brassinolide and gibberellins to tobacco seed and observed different responses depending on the state of dormancy, or on whether imbibitions occurred in the dark or light. The author concluded that the two hormones acted in distinct pathways. He proposed that gibberellin and light act in a common pathway, whereas BR directly enhances the growth of the emerging embryo independent of gibberellin.

On the other hand, from the work of Ullah *et al*. (2002), it seems that BRs may act synergistically with other metabolites including auxin, ethylene, glucose, abscisic acid and/or gibberellins. Previous published results on this matter seem to indicate that seeds from different crops react differently to treatment with BRs.

Further, Rao *et al*. (2002) are of the opinion that the promotional effect of BRs on seed germination is well established. In a review article the authors referred to the following plant examples whose seed germination was improved by treatment with BRs: *Lepidium sativum* (Cress)*, Eucalyptus camaldulensis* (River Red Gum)*, Brassica napus* (rape), *Apios americanum* (groundnut), *Oryza sativa* (rice), *Triticum aestivum* (wheat), *Orabanchae minor* (common boomrape), *Solanum lycopersicum* (tomato) and *Nicotiana tabacum* (tobacco). Two years prior, using brassinolide, Chon *et al*. (2000) reported that 11 representative cultivars of rice had increased leaf sheath lengths and numbers when grown in light, whereas pretreatment of seeds with BR enhanced mesocotyl elongation in the dark. Additionally, inhibitory effects were observed at the highest concentration used confirming the typical hormonal action of BRs namely that a concentration below or above the optimum can contribute to opposite results.

One year later Fujii & Saka (2001) also showed with rice that time of application and length of exposure to brassinolide were important as shoot lengths of resulting seedlings were significantly promoted seven days after continuous and first day treatments of the seeds, but not after treatment on days 3 and 4 of germination. Further, root elongation varied dramatically from enhancement to inhibition. The significance of the authors' contribution is that whole plants, with their need for coordination between organs and their coordinated response to their environmental conditions, challenge our perception of control and complexity.

Further, wheat grown from seed treated with 28-homobrassinolide showed enhanced leaf numbers as well as fresh and dry weight (Hayat *et al*., 2001b). In another study with wheat, pretreatment of seeds with 24-epibrassinolide at concentrations ranging from 0.04 to 40 nM led to increased root length, with inhibition evident only after treatment with the highest concentration of the BR (Shakirova *et al*., 2002). Treatment of sorghum seedlings with 0.1–10 nM 24-epibrassinolide resulted in highly significant increases in shoot and root fresh weight, but only in plants treated with the highest concentration (10 nM).

However, from an agricultural perspective more research is needed to clarify the potential benefits of treating seeds with BRs. In this study emphasis will be placed on foliar application instead.

2.4.4 Effect of BRs on vegetative growth of crops

Experiments with whole plants following foliar treatment with BRs are more frequently found in literature and parameters used to measure their response to treatment with the hormone are rather divergent. These include growth and yield as well as multiple physiological, biochemical and genetic response parameters that will be dealt with separately.

With regard to growth and development, Hunter (2001) reported that treatment of soybean seedlings with 24-epibrassinolide at a concentration range between 0.1 and 10 nM contributed to inhibition of root and shoot length, dry weight and lateral root numbers. On the other hand, a decade earlier Schilling *et al*. (1991) reported enhanced root growth in the case of sugar beet treated with homobrassinolide again indicating that not all plants react positively to treatment with BRs.

From the abundant data available in literature on the promotional effect of BR treatment on vegetative growth in plants, the question arose whether this is a result of cell division or cell expansion. In this regard Bajguz (2000) demonstrated by means of synchronously dividing cultures of the alga *Chlorella vulgaris* that accelerated increases in cell number and marked increases in nucleic acid and protein levels followed BR treatment. The latter pointed strongly toward accelerated cell division. Two years later Fatkhutdinova *et al*. (2002) reported that the mitotic rate increased in roots of wheat after treatment with 24-epibrassinolide while volumes of nucleoli were also increased, similar to the plant's response to treatment with cytokinin.

On the other hand Yamamuro *et al*. (2000) demonstrated internodal expansion in rice upon treatment with gibberrellic acid and sensitivity for this response increased when BRs were applied simultaneously. The latter indicated a new possibility namely that BRs can synergistically be involved with other known growth hormones as part of the mechanism of action during vegetative growth. With regard to the latter Tanaka *et al*. (2003) concluded in their study on *Arabidopsis thaliana* that a synergistic

relationship exists between BRs and gibberellin (GA3) as well as indole-3-acetic acid (IAA) in terms of hypocotyl elongation.

The significance for this study from the examples referred to above lies in the fact that the concentration at which BRs are applied as well as the time of application seems to be critical. Further, different crops seem to react differently to treatment with BRs. All of these factors have been taken into account when the research protocol for this study was formulated and will be dealt with in the appropriate sections. In this literature review further attention was rather given to the response of plants to treatment with BRs in an attempt to identify aspects that needed to be considered in this study.

2.4.5 Effects of BRs on flowering

One of the most important events in the life cycle of flowering plants is the transition from vegetative to reproductive growth (Li *et al*., 2010). The authors studied the effect of BRs on flowering time in *Arabidopsis thaliana*. By using BR-deficient mutants they showed that endogenous BR-levels affect flowering time in this test plant. However, the effect of exogenously applied BRs was not included in their study. A recent comprehensive study by Holá *et al*. (2010) dealt with exogenously applied BRs to two field-grown inbred lines of maize (*Zea mays* L.). Their results revealed that application of 24-epibrassinolide at the early V3 growth stage delayed and treatment at the V6 stage advanced the dates of anthesis and silking.

It seems, therefore, that different plants react differently to BRs and that their effect depends on various internal and external factors (Li *et al*., 2010). Equally critical are plant species and genotype (Ali *et al.*, 2008), time of application (Sasse, 2003) and dosage (Fariduddin *et al*., 2003). It is clear that the phenomenon of flowering is a complex issue and dependant on numerous interacting endogenous and environmental signals (Boss *et al*., 2004). Much more research is needed to fully understand the involvement of BRs in flowering.

2.4.6 Effect of BRs on physiological activities in crops

2.4.6.1 Photosynthesis

The importance of radiant energy and spectral quality of light was emphasized for BR-induced growth in beans, which correlated with increased chlorophyll content and assimilation of photosynthate (Mandava, 1988). Treatment of mustard seedlings with 28-homobrassinolide increased chlorophyll levels and enhanced the net photosynthetic rate that correlated with increased yield (Hayat *et al*., 2000; Hayat *et al*., 2001a).

24-Epibrassinolide application on *Cucumis sativus* leaves significantly increased the light-saturated net CO_2 assimilation rate from three hours to seven days after spraying with 0.1 mg ℓ^{-1} 24-epibrassinolide proving most effective (Yu *et al.,* 2004). Increased assimilation rate in 24-epibrassinolide-treated *Cucumis sativus* leaves was accompanied by increases in the maximum carboxylation rate of rubilose-1,5 bisphosphate carboxylase (Rubisco) as well as in the maximum rate of rubilose-1,5-bisphosphate (RuBP) regeneration. These treated leaves also had a higher quantum yield of PSII electron transport than the controls, which was mainly due to a significant increase in the photochemical quenching, with no change in the efficiency of energy capture by open PSII reaction centers. 24-Epibrassinolide did not influence photorespiration. Additionally, significant increases in the initial activity of Rubisco and in the sucrose, soluble sugars and starch contents were observed followed by substantial increases in sucrose phosphate synthase, sucrose synthase and acid invertase activities after 24-epibrassinolide treatment. It was concluded that 24-epibrassinolide increases the capacity of CO_2 assimilation in the Calvin cycle, which was mainly attributed to an increase in the initial activity of Rubisco (Yu et al., 2004).

2.4.6.2 Sucrose transolaction in plants

Petzold *et al*. (1992) reported that exogenous applied BRs in bean showed that BRs, as do auxin and gibberellins, enhance sink strength and phloem unloading while Nakajima & Toyama (1999) demonstrated that treatment of cucumber roots with 24-epibrassinolide promoted transport of ^{14}C-labeled sucrose from the primary leaf to the epicotyl.

According to Sasse (2003) extracellular invertases are important for the supply of carbohydrate to sink tissues. Treatment of tomato cell culture with three BRs led to specific enhancement of cell-wall-bound invertase activity that was concomitant with increased sucrose uptake.

2.4.6.3 Cell Expansion

Young vegetative tissue is particularly sensitive to BRs (Clouse & Sasse, 1998). Cell expansion, including elongation of hypocotyls, epicotyls and peduncles of dicots as well as coleoptiles and mesocotyls of monocots is induced when BRs are exogenously applied at extremely low concentrations of as little as 1 ppm (Mandava, 1988). Both BR and auxin promote cell elongation and synergism between the two plant hormones occur in many systems (Mandava, 1988). According to the author it is quite interesting to note that BRs do not cause any growth of *Avena* coleoptiles in dark, but in light coleoptiles respond to BR just as they do to auxin. They confirmed the sensitivity of BRs to light in soybean and mungbean tissue.

It has been shown that BRs are part of the phytochrome signalling pathway (Jackson & Thomas, 1997). The effect of BRs can be observed in R (red light), but not in B (blue light) or FR (far red light) as could be expected if BRs were part of the phytochrome signalling pathway. This together with the fact that brassinosteroids can affect many responses such as flowering time, leaf abscission, germination, senescence and stem elongation, which are often controlled by photoperiod, indicates that BRs could play an important role in the response of the plant to photoperiod.

2.4.6.4 Pollen and reproductive biology

Pollen is a rich source of endogenous BRs and *in vitro* studies have shown that pollen tube elongation could depend in part on BRs (Hewitt *et al*., 1985) while male sterility of BR-insensitive mutants support this (Clouse *et al*., 1996). From this it seems that BRs also play a physiological role in the fertilization of plants.

2.4.6.5 BRs and stress tolerance in plants

Evidence exists that treatment with BRs can induce resistance towards various abiotic and biotic stresses in plants (Khripach *et al*., 2000). Abiotic stress factors *inter alia* include drought, waterlogging, high salt and temperature extremes while biotic stress comes mainly from living organisms such as pathogenic viruses, bacteria and fungi, but also insects.

For a crop to survive abiotic stress, morphological, physiological, biochemical and molecular adaptations are part of a resistance strategy (Bajguz & Hayat, 2009). As a consequence, environmental stresses often activate cellular responses that include the development of new metabolic pathways, the accumulation of low molecular weight metabolites, the synthesis of special proteins, detoxification mechanisms and changes in phytohormone levels. Molecular studies have shown that synergism between BRs and other phytohormones exists that can be part of the resistance mechanism employed to survive specific stress conditions. In this section some published examples will be provided to confirm the involvement of BRs in reducing the effect of a given stress condition in plants without expanding on the possible mechanism(s) involved.

2.4.6.5.1 BRs and abiotic stress

In terms of abiotic stresses it was reported that BR-treated tomato and rice plants grew better than control plants under low-temperature conditions (Kamuro & Takatsuto, 1991). BRs also improved the tolerance of maize and cucumber (Khripach *et al*., 1999) seedlings against low-temperature stress. The ability of 28-homobrassinolide to confer resistance to moisture stress and high temperature in wheat was also established (Sairam, 1994). Kamuro & Takatsuto (1999), who were impressed by the ability of brassinosteroids to confer resistance of plants against a wide variety of environmental stresses stated: ‘The role of brassinosteroids in protecting the plants against environmental stresses will be an important research theme in future and may contribute greatly to the usage of brassinosteroids in agricultural production’. The authors emphasized the importance of the induction of antioxidant enzymes to protect cells that directly points towards membrane protection.

In general, drought, salinity and freeze-induced dehydration constitute direct osmotic stresses; whereas chilling and hypoxia can indirectly cause osmotic stress via effects on water uptake and loss (Bajguz & Hayat, 2009). According to the authors, plants have evolved a high capacity to synthesize and accumulate non-toxic solutes (osmoprotectants), e.g. proline, glycine betaine, mannitol and abscisic acid (ABA). Exogenously applied BRs might be involved in elevating some of these osmoprotectants in plants under abiotic stress.

Exogenous application of BRs also resulted in sustainment of seedling growth in three varieties of sorghum (*Sorghum vulgare*), viz. CSH-14 and ICSV-745 (susceptible to water stress) and M-35-1 (resistant to water stress), under osmotic stress conditions (Vardhini & Rao, 2003). Interestingly, osmotic stress considerably reduced protein content in all three sorghum varieties while the application of BRs not only restored, but also increased the protein level as well as that of free proline.

Similar results were reported by Sairam (1994) on the protective role of homobrassinolide on the growth of drought tolerant and drought-susceptible wheat (*Triticum aestivum*) varieties under drought stress conditions. Application of the BR contributed towards elevated nitrate reductase activity, chlorophyll content and photosynthesis while also improving membrane stability. The author regarded the latter as probably the most important factor that led to lower injury in the drought stressed plants.

Evidence that BRs were also effective towards protecting old rice leaves against cold stress was reported by Hotta *et al*. (1998) as well as Fujii & Saka (2001). Similarly, the ability of exogenously applied BRs to improve seed germination and seedling growth of cucumber (*Cucumis sativus*; Khripach *et al*., 1999) under chilling stress has been early reported. These results confirm that BRs increase the stress tolerance of plants towards abiotic stress conditions (Wilen *et al*., 1995).

Plants have a remarkable ability to take up and accumulate heavy metals from their environment. High concentrations of all metals can exert toxic effects on the metabolic pathways of plants, including those essential for growth and metabolism (Sharma & Bhardwaj, 2007). The authors found BRs to improve growth of *Brassica napus* under Cu metal stress as treatment with 24-epibrassinolide blocked copper metal uptake and accumulation in the plants. In *Brassica juncea*, treatment of plants with 28-homobrassinolide resulted in partially neutralizing the toxic effect of nickle

(Alam *et al*., 2007). Janeczko *et al*. (2005) found that BRs also reduced the content of cadmium in the seedlings of winter rape. The application of 24-epibrassinolide and 28-homobrassinolide alleviated the toxic effect of the heavy metal and increased the percentage of seed germination and seedling growth (Anuradha & Rao, 2007). The accumulation of heavy metals (cadmium, copper, lead and zinc) under the influence of BRs has been studied for different agricultural plants such as barley, tomato, radish and sugar beet. It was found that the application of 24-epibrassinolide significantly reduced the metal absorption; for example, the content of lead in beet roots was more than 50% lower than in the control culture (Khripach *et al*., 1999).

2.4.6.5.2 BRs and biotic stress

There is renewed interest in the protective effects of BR on plants under pathogen attack (Sasse, 2003). Results from cucumber plants treated with BRs and infected with fungal pathogens showed that synthesis of pathogenesis-related (PR) proteins was induced (Schnabl *et al*., 2001). The data suggest that BR-induced resistance can be distinguished from systemic acquired resistance and that it might provide added protection (Nakashita *et al*., 2003).

Plant immunity is based on a complex response that is highly flexible in its capacity to recognize and counteract different invaders (Bajguz & Hayat, 2009) According to the authors, invasion of plants by microbial pathogens activates either pre-existing physical and chemical barriers (systemic acquired resistance; SAR) or inducible defence mechanisms (systemic induced resistance; SIR). SIR defence responses are regulated by a network of interconnecting signal transduction pathways in which BRs can be involved.

Highly applicable to the underlying study was a report by Roth *et al*. (2000) on the foliar application of low concentrations of a BR-containing (24-epicastasterone and 24-episecasterone) extract of *Lychnis viscaria* seeds, one of the donor plants from which *ComCat*® was developed (Friebe *et al*., 1999). Roth *et al*. (2000) reported increased resistance of tobacco, cucumber and tomato towards viral (tobacco mosaic virus; *Sphaerotheca fuliginea*) and fungal pathogen (*Botrytis cinerea*) infection respectively. The activation of the plant's defence status was correlated with a stimulation of three pathogenesis-related (PR) proteins, including chitinase, β-1,3-glucanase and peroxidase, which are molecular markers of SAR. Induction of PR-

proteins has also been demonstrated in *Arabidopsis thaliana* (Szekeres *et al*., 1996). Additionally, Ikekawa & Zhao (1991) observed a reduction of leaf wilt disease in wheat after epibrassinolide application in field trials in China. Treatment of potato plants with brassinolide promoted potato tuber development and increased resistance to infections by *Phytophthora infestans* and *Fusarium sulfureum* (Kazakova *et al*., 1991).

In short, BRs can act efficiently in plants as immunomodulators when applied at the appropriate concentration and at the correct stage of plant development. The involvement of this phytohormone group in plant responses to abiotic and biotic environmental stresses has been scientifically confirmed opening up new approaches to protect crops against hazardous environmental conditions (Bajguz & Hayat, 2009).

2.4.7 Prospective uses of BRs in agriculture

2.4.7.1 General overview

Experiments to investigate the potential of BRs for use in agriculture began in the 1970's in the USA and showed beneficial effects (Maugh, 1981). In the early eighties, studies on BRs in Japan and the USSR confirmed their usefulness as agricultural chemicals (Takeuchi, 1992). Since then, numerous reports from all over the world have appeared and many potential practical uses have been patented.

In the light of the special properties assigned to BRs and factors that threaten future food security, including an increasing world population, soil salinity, drought, global warming, pollution and pathogen attack, it seems obvious that exploration of the application potential of BRs in agriculture should continue. Zullo & Adam (2002) outlined the prospective agricultural uses of BR's. The assumption of its application potential was made from data collected over the past three decades and only a few are presented here. The possible attributes of BRs in agriculture include 1) its enhancing effect on plant growth (Rao *et al*., 2002) and especially root growth (Müssig *et al*., 2003), 2) its ability to increase resistance in crops towards abiotic (Kamuro & Takatsuto, 1999) and biotic (Kazakova *et al*., 1991) stress conditions and 3) its ability to increase yields in a variety of crops (Schnabl *et al*., 2001). All of these aspects will be considered in this section from a practical agricultural perspective.

Khripach *et al*. (2000) considered BRs as promising compounds for application in agriculture, because they showed various kinds of regulatory activity on growth and development in plants (Table 2.1).

Table 2.1. Physiological effects of brassinosteroids in plants according to Khripach *et al*. (2000).

Cell level	Whole plant level
Stimulation elongation and fission	Growth promotion
Effect on hormonal balance	Increase in the success of fertilization
Effect on enzyme activity; H-pump activation	Shortening the period of vegetative growth
Activation of protein and nucleic acid synthesis	Size and quantity of fruits increase
Effect on the protein spectrum and on the amino acid composition of proteins	Effect on the content of nutritive components and fruit quality
Effect on the fatty acids composition and on the properties of membranes	Increased resistance to unfavourable environmental factors, stress and diseases
Enhancement of the photosynthesis capacity and of translocation of product	Crop yield increase

2.4.7.2 Seedling establishment

For any crop producer seedling establishment remains a critical issue as it eventually determines the final plant stand and the production potential of a given area of land. Despite some controversy still surrounding the inquiry about the potential of BRs to promote seed germination (Leubner–Metzger, 2001; Rao *et al*., 2002), Fujii and Saka (2001) reported that treatment of rice seed contributed to a significant increase in shoot and root growth of seedlings. This was confirmed for wheat by Hayat *et al*. (2001b) and for sorghum by Shakirova *et al*. (2002). However, from an agricultural perspective more research is needed to clarify the potential benefits of treating seeds with BRs. In this study emphasis will be placed on foliar application instead.

Since the beginning of the research on isolated BRs from plant sources, the hormone group proved to be involved with either the promotion of plant growth when

applied foliar (Mitchell & Gregory, 1972) or acceleration thereof (Gregory, 1981; Braun & Wild, 1984). It was also reported to increase not only plant growth rate and root size but also root and stem dry weight indicating involvement on the physiological level. Almost two decades ago Singh *et al*. (1993) reported a 25% root growth increase in chick-pea after treatment of seedlings with 24-epibrassinolide while Rönsch *et al*. (1993) demonstrated that pretreatment with 28-homobrassinolide induced rooting and rooting quality in cuttings taken from mature Norway spruce donor plants while improving their viability. BRs also increased root growth in the case of transplanted *Pinus radiate* seedlings (Sasse, 2003).

From these examples it can be deduced that seedling growth stimulation is considered a proven physiological role of BRs in plants that holds promise from an agricultural perspective.

2.4.7.3 Synergism with fertilizer application

The future of commercialized bio-stimulants seems positive in the light of the elevated costs of fertilizer. In this regard research in terms of the use of bio-stimulants in combination with fertilizer levels lower than the recommended standard for different crops seems to be important in an attempt to lower input costs, that has become a gloomy issue for farmers lately. In this regard some of the manufacturers of natural bio-stimulants currently applied in agriculture claim increased production, profit increases and cutting of operating costs via reduction of fertilizer application as a benefit of its use (Chen *et al.,* 2002).

Although this is a sensitive issue and falls outside the scope of this study, elevated operating costs is currently a threat to the sustainable production of food from plants. In this regard it might be worthwhile to pursue the statement made by Chen *et al*. (2002) and determine whether the use of plant bio-stimulants together with reduced fertilizer application has a role in terms of sustainable crop production.

2.4.7.4 Stress management including biotic and abiotic stress factors

The potential applications of BRs in agriculture and horticulture are based not only on their ability to increase crop yield, but also to stimulate physiological processes involved with tolerance towards environmental stress factors (Khiripach *et al*., 2000).

According to the authors, increase in plant resistance towards phytopathogens can be used as a substitute for some traditional pesticides. Although the stress-protective properties of BRs have been known for some time, systematic investigations into their potential enhance plant resistance to diseases have only been undertaken fairly recently (Prusakova *et al*., 1995). Among the results obtained to date, most data are related to the influence of BRs on fungal phytopathogenesis. A number of examples have been supplied in section 2.4.6.5.2.

Farming, and especially crop production, is associated with risk due to not only biotic but also abiotic stress conditions such as drought, heat, chilling, salinity and waterlogging that can have devastating effects on crop yields. A number of examples that cover most of these abiotic stress factors have been supplied in section 2.4.6.5.1. All of these attributes confirm a potential place for BR application in the agricultural industry.

However, whether the application of BRs in a crop production system can replace or even partially replace the use of standard fungicides remains to be proven through research. In terms of abiotic stress there is not much a farmer can do to elude its influence except to adhere to recommended sowing dates in an attempt to avoid extreme environmental conditions. With regard to abiotic stress conditions BR-containing products seem to fill this gap. Foliar application of BRs at a rather young developmental stage have been shown to increase the tolerance of a variety of crops towards abiotic stressors and, in this regard, can play a pivotal role in reducing the risk associated with crop production practices.

2.4.7.5 Potential of BRs to increase crop yields

Foliar application of BRs have been shown to increase the yield of a variety of crops and numerous reports are available in the literature. Only a few examples, selected from different crop types, are referred to in this section.

In considering staple food row crops these include a 22% increase in fresh kernel weight of rice (cv. Taebaik; Lim, 1987), an increase in barley (cv. Nosovsky) seed weight (Prusakova *et al*., 1995), an 11% increase in maize (cv. Kwangok) kernel dry weight (Lim & Han, 1988) and a significant increase in wheat seed weight (Takematsu *et al*., 1988).

In terms of vegetables numerous reports on the yield increasing effect of BRs can be found in literature. Some examples include a 25% increase in the leaf weight of two different lettuce varieties (Meudt *et al*., 1983) and an increase in tomato fruit setting by 43-111% (Mori *et al*., 1986).

In terms of fruit crops, and in a patent description by Kuraishi *et al*. (1991), foliar applied brassinolide on orange trees during flowering significantly increased fruit setting while decreasing fruit drop causing an increased number of fruits per plant accompanied by an increase in the average fruit weight. The patent holders observed a similar decrease in fruit dropping in lemon, peach, pear, persimmon and apple.

Many other examples of brassinosteroid use for increasing crop yield can be found in the literature (Lim & Han, 1988; Sairam, 1994; Ramraj *et al*., 1997; Kamuro & Takatsuto, 1999; Khripach *et al*., 1999; Hayat, *et al*., 2000)

2.4.8 Application methods of BRs

Based on approaches found in the literature it seems that there are three possible application methods for BRs that can be considered from an agricultural perspective. On grounds of the fact that BRs can be absorbed by both plant roots and leaves, these include 1) seed treatment, 2) foliar application and 3) soil application. In this study seed treatment will be used as a bio-assay method under laboratory conditions especially with the objective to follow bio-activity during the isolation of BRs from *ComCat*®. Foliar application will be employed under field conditions to quantify the possible effect of *ComCat*® on growth and yield of selected crops. Soil application will not be used in this study.

2.4.9 Future research on BRs

According to Zullo & Adam (2002), although many BR's are commercially available and employed in some countries, more accurate studies on 1) dosage concentration, 2) dosage method, 3) time of application and 4) cultivar specificity are needed since many of the results were obtained by experiments performed in greenhouses or small fields. Especially dosage concentration and method need to be ascertained before a

natural product from wild plants can be applied in the agricultural practice with any hope of success. Concentration has been shown to be particularly critical as BR-containing plant extracts can have a strong growth promoting effect on plants at low concentrations but at high concentrations can inhibit growth (Zullo & Adam, 2002). This phenomenon is known as hormesis (Belz *et al.,* 2008).

There is general consensus amongst scientists 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 test crops in order to verify the action at hand (Khan *et al.*, 2001; Ameena & George, 2002; Cruz *et al.*, 2002b; Duary, 2002; Obaid & Qasem, 2002).

In terms of the manipulation of metabolic events by plant extracts, e.g. the rate of cell respiration, cognisance must be taken of the fact that this could be an indicator of either a positive or a negative influence on the final yield produced by a crop (Pretorius & van der Watt, 2011). In essence this means that *in vitro* results obtained in a laboratory should be verified *in vivo* under field conditions. Consequently, all three of these aspects were addressed in this study.

In chapter 3 the bio-stimulatory effect of *ComCat*® on the respiration rate of monoculture yeast cells and seeds was quantified as well as seed germination and seedling growth of selected crops as an indication of its mechanism of action. In chapter 4 the yield response of different crops to foliar applications of *ComCat*® at different concentrations under field conditions over two seasons is reported. In chapter 5 the bio activity of different *ComCat*® fractions were quantified by using the identified bio-tests from Chapter 3 and the third unknown brassinosteroid referred to by Volz (2000) is isolated, purified and identified using standard chromatography techniques.

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Chapter 3

SCREENING OF *ComCat*® FOR BIO-STIMULATORY ACTIVITY UNDER LABORATORY AND GLASSHOUSE CONDITIONS

Abstract

In this study seven different biotests were employed to quantify the bio-stimulatory activity of *ComCat*®, a natural and recently commercialized plant growth regulator. These included the respiration rates of monoculture yeast cells and pea seeds as well as seed germination, subsequent seedling growth and algal growth as indicators of its ability to either stimulate or inhibit plant growth. In most cases *ComCat*® applied at 0.5 mg ℓ^{-1} emerged as the optimum treatment as it significantly enhanced the respiration rates of monoculture yeast cells and pea seeds. Since respiration is affected during either growth stimulation or inhibition, this aspect was verified by means of seedling growth tests. The latter revealed that *ComCat*® had no significant effect on seed germination in any of the crops tested, but seedling growth in terms of especially root growth was significantly enhanced in all of the vegetable crops tested under laboratory conditions. These included radish, cabbage and peas where 0.5 mg ℓ^{-1} again emerged as the optimum concentration by inducing the most pronounced and significant response. Wheat seedlings, however, did not respond to treatment with the product when applied as a seed treatment, but when applied as either a soil drench before emergence or as a foliar application later in the growth cycle under glasshouse conditions, root growth was significantly enhanced. Under glasshouse conditions two vegetable test crops, lettuce and beetroot, responded very similar to treatment with *ComCat*® in terms of enhanced seedling growth. It was concluded that most test crops responded positively to treatment with *ComCat*® in terms of enhanced seedling growth, depending on the application rate and time of application, but also that different crops responded differently to treatment with *ComCat*®. Finally, the growth rate of an algal colony was also significantly increased by treatment with *ComCat*® at the optimum concentration of 0.5 mg ℓ^{-1}. This indicated that other single cell organisms, e.g. soil organisms involved with nutrient recycling, might also benefit from a soil drench treatment with the product under scrutiny.

Key words: *ComCat*®, bio-stimulatory activity, respiration, seedling growth, algal growth

3.1 Introduction

A bio-catalyst is traditionally defined as a compound that increases the rate of a chemical reaction by decreasing the activation energy needed to start the reaction and this is applicable to enzyme controlled chemical reactions (Bohinski, 1987). On the other hand, hormones are also active in plants and are traditionally defined as chemical compounds synthesized in a specific part of the plant that is translocated to other parts of the plant where it is involved in regulating growth, development and metabolism at a low concentration (Bieleski *et al*., 1974). However, specific non-hormonal intermediate metabolites, e.g. 2-ethyl-hexyl phthalate (Pretorius *et al*., 2008) and fatty acids (van der Watt & Pretorius, 2011), also contain bio-catalytic properties that can either stimulate or inhibit growth, development and metabolism depending on the concentration applied. Further, other secondary compounds from plants that show plant growth regulatory activities include abscissic acid, sterols, cucurbitacins and the naphtaquinone juglone, to mention only a few (Seigler, 1995). The latter makes it difficult to define the term “bio-catalytic“ and, therefore, the term “plant growth regulation“ is preferred due to its more specific reference to “bio-stimulatory“ regulation of growth and development (Bidwell, 1979). The latter two terms, “bio-stimulatory“ and “plant growth regulation“ are therefore used in this monograph as it applies to natural compounds.

Auxins, gibberellins, cytokinins and ethylene are plant growth regulating hormones known to man for a long time. Even today these hormones are applied on a rather small scale in the horticultural (e.g. nurseries) and agricultural (e.g. table grape or tomato) industries in a pure form (Pretorius & van der Watt, 2011). One of the reasons might be the cost of either extracting these hormones from plant material or synthesizing them. From this it must be accepted that not all scientific discoveries will lead to commercialized agricultural products. However, the product under scrutiny in this study, *ComCat*®, is produced from natural plant material (AgraForUm AG, Germany) and contains three of the known plant hormones, auxins, gibberellins and cytokinins as well as brassinosteroids (BRs) as active compounds. *ComCat*® has been commercialized by a German company (AgraForUm AG) and is currently traded in many countries.

BRs, discovered almost three decades ago (Grove *et al*., 1979), has in the meantime received phytohormone status and is now recognized by scientists as the fifth plant hormone group (Schnabl *et al*., 2001). Despite the relative short time in

which BRs received attention from the international research community, its application potential in the agricultural industry is rather well documented (Yopp *et al*., 1981; Takematsu & Takeuchi, 1989; Shen *et al*., 1990; Singh *et al*., 1993; Zullo & Adam, 2002; Holá *et al*., 2010). It has been shown that BRs not only promote vegetative growth in crops and induce resistance towards biotic and abiotic stress factors, but also enhance yields (Schnabl *et al*., 2001). However, in a recent report Holá *et al*. (2010) maintain that several interactions of BRs with plants rather hinder their wider introduction into the agricultural practice. This is in concert with a statement by Janeczko *et al*. (2010) that also confirms a statement made by Ramraj *et al*. (1997) more than a decade ago, which supplied the rationale for investigating *ComCat*® as a new class of BR-containing plant growth regulating natural products. From the two recent statements (Holá *et al*., 2010; Janeczko *et al*., 2010) it is clear that the scientific community is still not convinced that BRs have the potential to be applied in organized agriculture in a sustainable and economic fashion.

A positive answer to the question on the application potential and economic viability of any given natural compound remains decisive in efforts to commercialize a natural product. For example, sesquiterpene lactones that stimulate seed germination were isolated from witch weed (*Striga hermonthica*; Ejeta *et al*., 1993) but, although these compounds are labile and active at extremely low (pico molar) concentrations, they 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, especially from an economic perspective. In this regard it also 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. In this study attention was given to these aspects.

In this chapter seven different biotests were used to quantify the bio-stimulatory activity of *ComCat*® claimed by the manufacturers. Specific biotests were employed to ascertain whether the product could influence the respiration rates of monoculture yeast cells and seeds (Thain & Hichman, 2000) as an indication of its metabolic manipulation ability as well as seed germination, subsequent seedling growth and algal growth as indicators of its ability to either stimulate or inhibit plant growth.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 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). A mixture of two algal strains (*Scenedesmus obliquus*; 276-3a and 211-8b), used in one of the bio-tests, was obtained from the Department of Plant Sciences, University of the Free State, South Africa. A *Lupinus albus* seed suspension (SS) used in algal growth tests as a positive control was obtained from the Department of Soil, Crop and Climate Sciences, University of the Free State.

3.2.1.2 Other materials

ComCat® was purchased from AgraForUm AG, Germany. All chemicals used were from Sigma (Germany) and of the purest grade available. Germination paper was provided and manufactured by Agricol (South Africa). Dry instant baking yeast was also purchased locally.

3.2.2 Methods

3.2.2.1 Biotest 1: Manometric method for determining the bio-stimulatory effect of different ComCat® concentrations on the respiration rate of monoculture yeast cells by using a specially constructed respirometer

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 (0.25, 0.5, 1.0, 2.5 and 5.0 mg ℓ^{-1}) of *ComCat*® 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 *ComCat*® concentrations containing 5 mg ml^{-1} glucose to serve as respiratory substrate for the yeast cells was added to the respirometer. The apparatus was tilted 180^{o} to release air bubbles trapped in the dry baker's yeast and placed in a water bath pre-heated to 29^{o}C. Distilled water

containing only the glucose was used as a control. Respiration rates were followed as CO_2 release over 15 minute intervals for two hours. A total of nine readings were noted and the experiment was replicated three times. Respiration rate was expressed as the collective cm^3 CO_2 release over 2 hours.

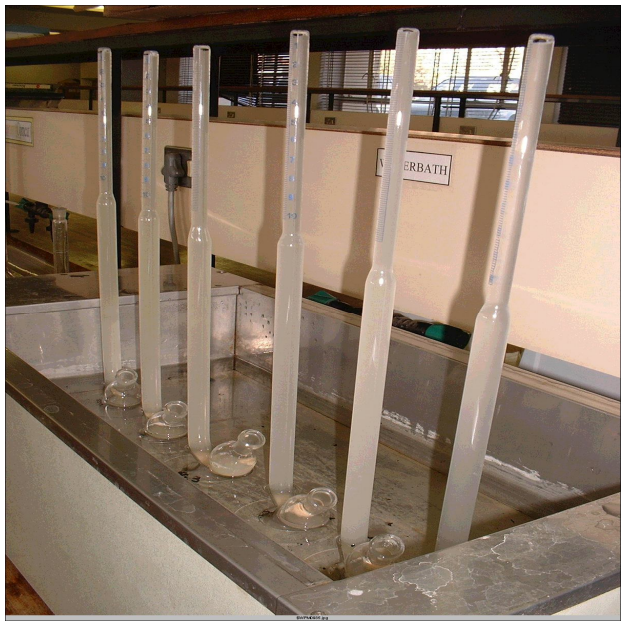

Plate 3.1: Specially constructed glass respirometer for screening the effect of different *ComCat*® concentrations on the respiration rate of monoculture yeast cells.

3.2.2.2 Biotest 2: Constant pressure manometric method for determining the effect of *ComCat*® on the respiration rate of pea seeds at a single concentration

For the determination of pea seed respiration rate by means of constant pressure manometry, a submersible differential Gilson respirometer was used. Fifteen cm^3 Warburg reaction vessels with centre wells and without sidearms were used.

Pea seeds were initially incubated for 24 hours in a 0.5 mg ℓ-1 *ComCat*® solution while control seeds were incubated in distilled water. Subsequently, 5 pea seeds per replicate were transferred to a Warburg reaction vessel and replicated four times. One cm^3 of distilled water was added to each flask. For measurement of O_2 consumption 300 µl 12% KOH was placed in the centre well and the absorption

surface enlarged by means of a folded piece of filter paper. The KOH removed the CO_2 produced during seed respiration according to the following reaction:

$$CO_2 + 2\ KOH \longrightarrow K_2CO_3 + H_2O.$$

Since the carbon dioxide is being removed, the change in the volume of gas in the respirometer is directly related to the amount of oxygen consumed.

In the case of net gas exchange determination, 300 µl distilled H_20 replaced the KOH in the centre well. Each reaction vessel was connected to the manometric U-tube by means of two steel springs and sealed with silicone gel. Each vessel was also connected to a reference vessel that did not contain seeds. Before any measurements were taken, the reference vessels were equilibrated at 29°C for 10 min. after which the atmospheric and manometric valves were closed.

Carbon dioxide (CO_2) liberation was deduced using the following equation:

Net gas exchange = CO_2 release + O_2 consumed

Gas exchange values were corrected according to the method of Gregory & Purvis (1965) by using the following equation:

$$X = \Delta Vg \times \frac{(T')(Pb - 3 - Pw)}{(T + 273)(P')}$$

Where:

X	=	total volume of the gas measured (mm^3) at STP
ΔVg	=	volume-change as determined with respirometer
T'	=	standard temperature, 273°K
T	=	temperature of waterbath, °C
Pb	=	prevailing atmosphere pressure, mm Hg
Pw	=	pour pressure of water at the prevailing temperature at which the experiment was conducted
P'	=	standard pressure, 760 mm Hg

Respiration rate was finally expressed as µl CO_2 release $seed^{-1}$ h^{-1}.

3.2.2.3 Biotest 3: The effect of *ComCat*® on radish seed germination and subsequent seedling growth under laboratory conditions and at different concentrations

Two sheets of special germination paper (30 x 30 cm) were used to test the effect of different *ComCat*® concentrations on the germination of radish, cabbage, pea and

wheat seeds as well as subsequent seedling growth. A line was drawn 10 cm from the top on the one sheet and 10 seeds spaced evenly on the line. A second sheet of germination paper was placed on top of the first and moistened with 0.25, 0.5, 1.0, 2.5 or 5.0 mg ℓ^{-1} of *ComCat*® in the case of radish while distilled water served as a control.

Both sheets of paper were rolled up longitudinally and placed upright in Erlenmeyer flasks containing either a *ComCat*® solution or distilled water and kept at 25°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 8 times. Seeds were taken as germinated when the radicle protruded the testa.

3.2.2.4 Biotest 4: The effect of *ComCat*® on cabbage, pea and wheat seed germination and subsequent seedling growth under laboratory conditions and at a single concentration

The same method as described in 3.2.2.3 was used for cabbage, pea and wheat except that *ComCat*® was tested at a single concentration (0.5 mg ℓ^{-1}).

3.2.2.5 Biotest 5: Effect of different *ComCat*® concentrations on wheat seedling growth over a 4-week period under glasshouse conditions

A Bainsvlei type light red loamy sand soil with a composition of 12% clay, 84% sand and 4% loam (Table 3.1) was used in a pot trial under glasshouse conditions. Soil analysis data is supplied in Table 3.1. The soil had a pH of 5.5 and cation exchange capacity (CEC) of 3.86. Each pot contained 3.3 kg of soil. Standard fertilizer, as recommended by the seed merchant, was applied to the soil: N @ 60, P @ 15 and K @ 7 kg ha^{-1}. Ammonium nitrate (NH_4NO_3) and potassium phosphate ($KHPO_4$) were used as sources of N, P and K respectively.

Fertilizer was placed broadcast on top of the soil and covered with a 5 cm soil layer. Ten wheat seeds (cv. Pannar 3377) were sown on top of the covering soil layer and pressed down to a depth of 2 cm. Pots were initially moistened thoroughly and afterwards spray irrigated every second day to maintain field capacity. Pots were kept in a glasshouse at 26°C day and 16°C night temperatures. Thinning was carried out

five days after germination and only four of the healthiest plants were left to grow in each pot. Early thinning was done in order to avoid possible future complications in separating root systems when root mass was determined. *ComCat*® was foliar applied at different concentrations (0.25, 0.5, 1.0, 2.5 and 5.0 mg ℓ^{-1}) at the 3-4 leaf growth stage. Each treatment was replicated four times. Distilled water served as a control. Four weeks after planting the total plant, root and aerial part fresh mass were determined.

Table 3.1: Soil analysis (ARC, Bethlehem, South Africa).

P (mg/kg)	**K** (mg/kg)	**Ca** (mg/kg)	**Mg** (mg/kg)	**Na** (mg/kg)	**pH** (KCl)	**Soil properties** Sand Loam	Clay	**CEC** ($cmol_c kg^{-1}$**)**
7.3	75.5	413	192.9	6.2	5.5	84% 4%	12%	3.86

CEC = Cation exchange capacity

3.2.2.6 Biotest 6: Effect of different *ComCat*® treatments applied at different growth stages and at a single concentration on lettuce, beetroot and wheat seedling growth under glasshouse conditions

Two seeds each of lettuce, beetroot or wheat were placed in 32 seed tray cells filled with Hygromix growing medium (Hygrotech, South Africa). This represented four replicates of eight treatments including the water control. The treatments were *ComCat*® applied at 0.5 mg ℓ^{-1} as I) a drench on day 0, II) a drench on day 1, III) a drench on day 3, IV) a drench on day 6, V) a drench and foliar spray on day 8, VI) a foliar spray on day 10 and VII) a foliar spray on day 13 after planting while viii) water only served as a control. Two litres of a *ComCat*® solution (0.5 mg ℓ^{-1}) were used to drench the seed tray cells on the first three days of the experiment, after which only one litre was used. When foliar sprayed after leaves were visible (V – VIII), a wetting agent (Tween 20) was added to the *ComCat*® solutions at 1 ml ℓ^{-1}. The seed trays were kept in a glasshouse at 26^{o}C day and 16^{o}C night temperatures. The plants were harvested four weeks after planting and the fresh root and leaf mass were determined separately.

Table 3.2: Growth stages (Meier, 1997) when test plants were foliar sprayed with *ComCat*®.

	Lettuce	Beet root	Wheat
Day 8 (Growth stage 13)	2-3 true leaves	3 true leaves	2 true leaves
Day 10 (Growth stage 14)	3 true leaves	4 true leaves	3 true leaves
Day 13 (Growth stage 15)	4 true leaves	5 true leaves	4 true leaves

3.2.2.7 Biotest 7: The effect of ComCat® on the in vitro growth of algae under controlled conditions

3.2.2.7.1 Preparation of a micronutrient solution

One component after the other was added to 500 ml distilled water until each one was completely dissolved and made up to a final volume of 1 ℓ as summarized in Table 3.3.

Table 3.3: Summary of a micronutrient solution that was used as part of the algal growth medium.

	Stock solution ($g\ 100ml^{-1}$)	Applied solution
$ZnSO_4.7\ H_2O$	0.1	1 ml
$MnSO_4.7\ H_2O$	0.1	2 ml
H_3BO_3	0.2	5 ml
$Co(NO_3)2\ .4H_2O$	0.02	5 ml
$Na_2MoO_4\ .4H_2O$	0.02	5 ml
$CuSO_4\ .7H_2O$	0.0005	1 ml
Distilled water		981 ml
$FeSO_4\ .7H_2O$		0.7 g
EDTA (Titriplex III, Merck)		0.8 g

3.2.2.7.2 Preparation of soil extract

For optimal maintenance of algal stock cultures this basal medium was modified by the addition of soil extract. The soil extract often helps to culture species which are otherwise often hard to culture. Five hundred cm^3 soil was transferred to a 2 litre Schott bottle, covered with one litre distilled water and shaken thoroughly on a mechanical shaker for 1 hour. The soil solution was divided into two equal parts, transferred to separate one litre Schott bottles, autoclaved for 10 minutes at 121°C and centrifuged for 10 minutes at 4000 rpm. The supernatant was transferred to a series of small 30 cm^3 containers and again autoclaved for 20min at 121°C. This served as stock solutions that were stored in a refrigerator until used.

3.2.2.7.3 Preparation of a basal growth medium

The colony of algae obtained for this bio-test was a mixture of two *Scenedesmus obliquus* strains, 276-3a and 211-8b. The basal growth medium for sustaining the algal colony during the biotest consisted of a mixture of different solutions as summarized in Table 3.4. The micronutrient solution composition is summarized in Table 3.3.

Table 3.4: Preparation of a basal growth medium for sustaining the *Scenedesmus obliquus* colony during growth studies.

	Stock solution (g 100 cm^{-3})	**Nutrient solution (cm^3)**
KNO_3	1.0	20
K_2HPO_4	0.1	20
$MgSO_4 .7\ H_2O$	0.1	20
*Soil extract		30
**Micronutrient solution		5
Distilled water		905

*Soil extract was prepared separately. **Micronutrient solution composition summarized in Table 3.3.

3.2.2.7.4 Methodology followed to quantify algal growth

A 250 cm^3 Erlenmeyer flask equipped with a side arm was filled with 100 cm^3 basal medium for each replicate separately. A foil cap was placed over the opening of the flask, shiny side down. The flask containing the basal medium was sterilized in an autoclave at 121°C for 20 min. and cooled down to room temperature. Ten cm^3 of *Scenedesmus* medium were added to the flask under sterile conditions in a laminar flow cabinet. Subsequently, *ComCat*® was added to the medium to a final concentration of 0.5 mg ℓ^{-1}. Growth medium only served as a negative control while a *Lupinus albus* seed suspension (SS; van der Watt & Pretorius, 2011) at 5.0 mg ℓ^{-1} (optimum concentration) served as a positive control. Each treatment was replicated four times.

The flasks were placed in a growth room at 25°C, optical density data collected daily using a Klett-Summerson Photo-electric Colorimetera and Klett-Meter units noted. Photosynthetic light in the growth room was determined at 100 micro-einsteins m^{-2} sec^{-1} with a LI-185B Quantum sensor attached to a Quantum radiometer/photometer (Li-Cor, Inc. Model L1 185B).

3.2.3 Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using the NCSS 2000 Statistical program, to identify differences between treatments. Tukey's mean significant difference (MSD) procedure for comparison of means (Steele & Torrie, 1980) was applied to separate means on a 5% (P<0.05) probability level. Although the response of seeds, seedlings, algae or yeast cells to treatment with *ComCat*® was measured at intervals over a specific time period, statistical analysis was only done at the end of the period.

3.3 Results

3.3.1 Biotest 1: The respiratory response of monoculture yeast cells to treatment with *ComCat*® at different concentrations

At a concentration of 0.5 mg ℓ^{-1} *ComCat*® significantly (P<0.05) enhanced the respiration rate of monoculture yeast cells by 24.3% over a 2 h incubation period

(Figure 3.1) compared to the water control. Although *ComCat*® at a lower (0.25 mg ℓ^{-1}) and slightly higher (1.0 mg ℓ^{-1}) concentration also contributed to significant increases in the respiration rate of yeast cells of 12.4% and 10.1%, respectively, compared to the control, the 0.5 mg ℓ^{-1} concentration was optimal. At the highest concentrations (2.5 and 5.0 mg ℓ^{-1}) a slight but non-significant inhibition of respiration in yeast cells was observed (Figure 3.1).

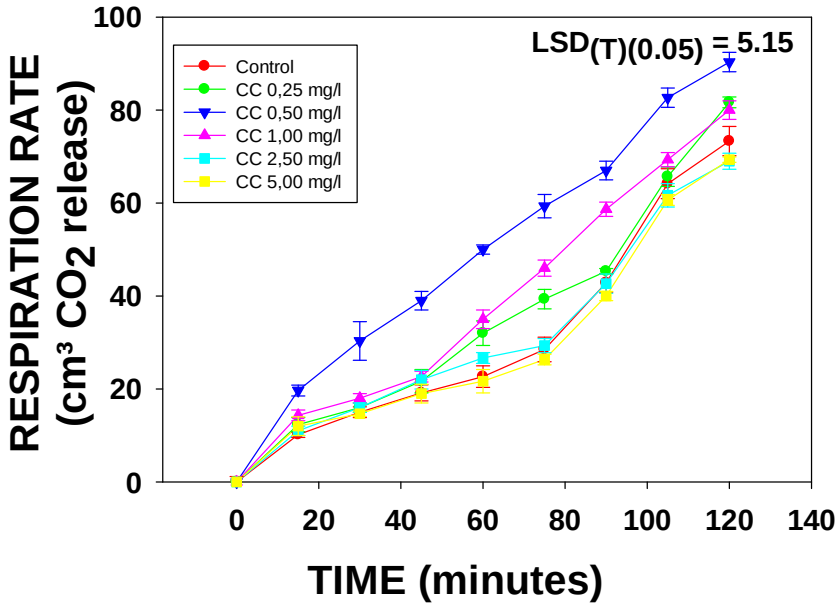

Figure 3.1: The respiratory response of monoculture yeast cells to treatment with *ComCat*® (CC) at different concentrations and at 15 minute intervals over a 2 hour incubation period. Statistical significance is indicated by LSD $_{(T)(0.05)}$ values at 120 min. Error bars = Standard deviation.

3.3.2 Biotest 2: The respiratory response of pea seeds to treatment with *ComCat*®

In order to ascertain whether the respiration rate induction observed in monoculture yeast cells after treatment with *ComCat*® was also applicable to plant material, pea seeds were used. The product was only tested at a concentration of 0.5 mg ℓ^{-1} as this was the optimum concentration indicated by biotest 1. Expression of respiration rate both as CO_2 release (Figure 3.2A) and net gas exchange (Figure 3.2C) rates showed that treatment with *ComCat*® at a concentration of 0.5 mg ℓ^{-1} contributed to statistically significant ($P<0.05$) enhancement of the respiration rate of pea seeds, compared to the untreated water control. This was more than twofold in both cases. However, no difference in O_2 consumption between treated and control seeds was

observed (Figure 3.2B). The latter strongly indicates that the seeds were in a state of anaerobic respiration for the full duration of the incubation period.

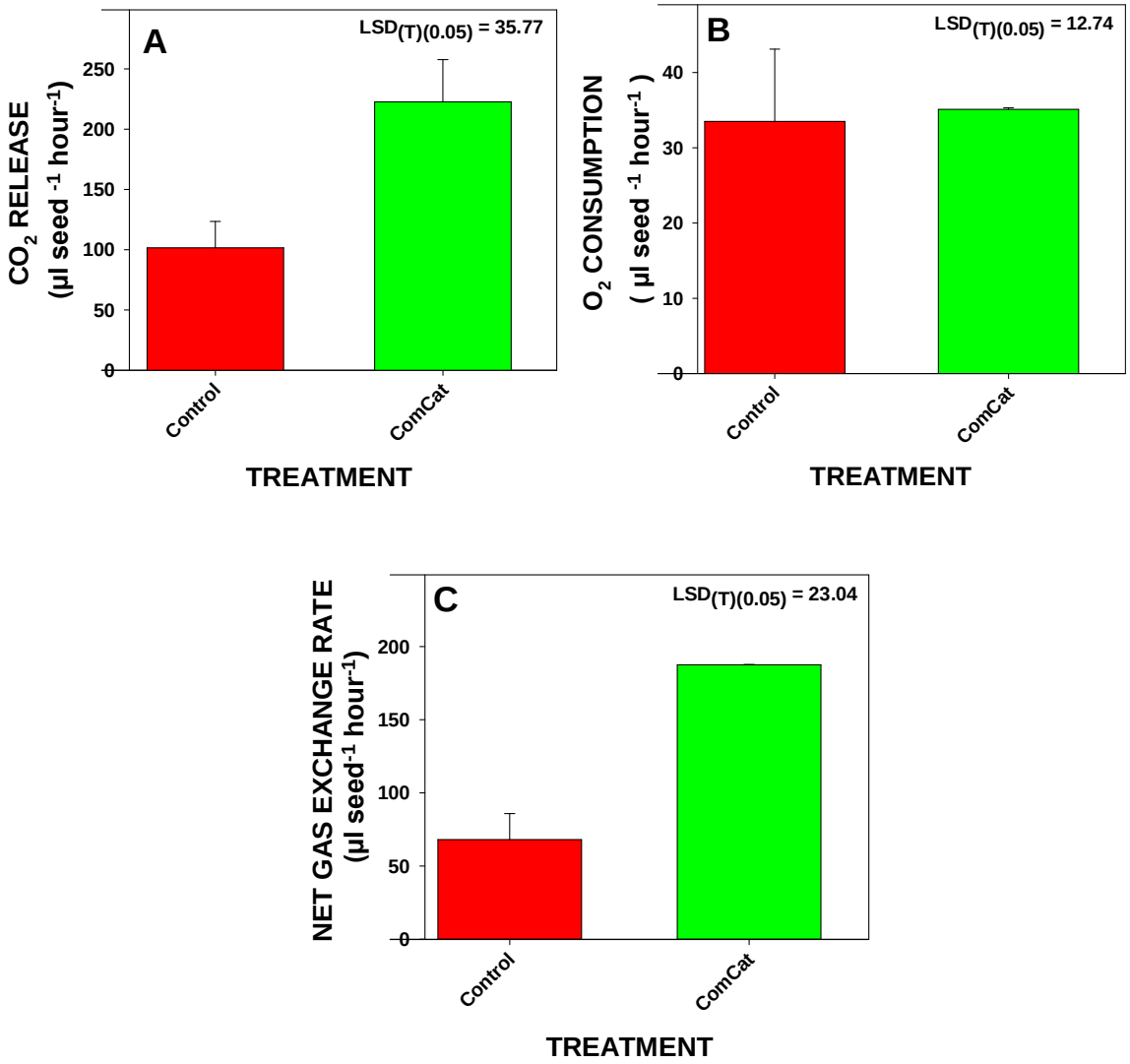

Figure 3.2: The respiratory response of pea seeds to treatment with *ComCat*® at a concentration of 0.5 mg ℓ^{-1}. Respiration rate is expressed as A) CO_2 release , B), O_2 consumption and C) net gas exchange rate (C). $LSD_{(T)(0.05)}$.values are indicated in each graph separately. Error bars = Standard deviation.

However, manipulation of the respiratory metabolism of living cells can have a positive or negative association as both bio-stimulants and growth inhibitors are known to enhance the rate of respiration. It was therefore imperative to verify whether the respiration rate increase observed in monoculture yeast cells and pea seeds was indeed related to growth stimulation or inhibition. To achieve this, a

standard procedure was followed namely to quantify the effect of *ComCat*® on seed germination and seedling growth.

3.3.3 Biotest 3: Germination of radish (*Raphanus sativus*) seed and subsequent seedling growth response to treatment with *ComCat*® at different concentrations

No significant differences in the germination rate of radish seeds were observed either between treatment of seeds with different *ComCat*® concentrations or the water control (Figure 3.3 A).

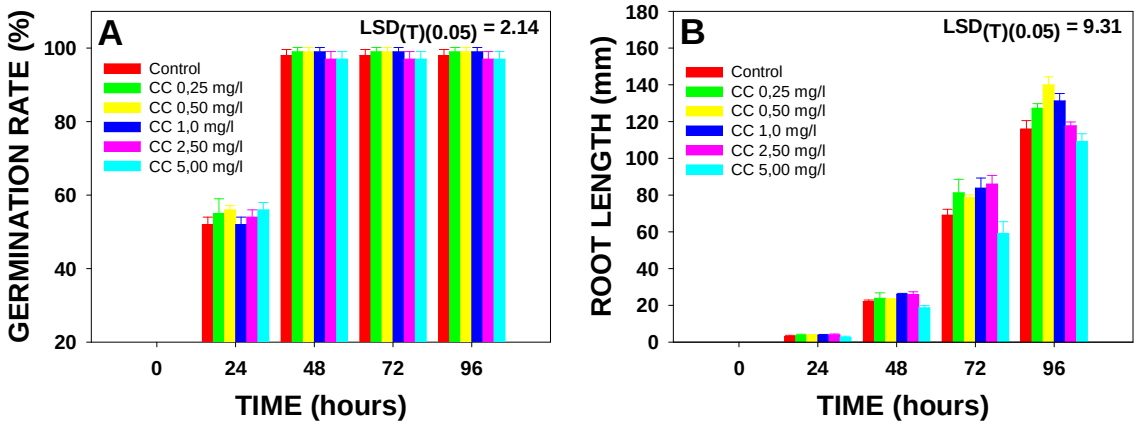

Figure 3.3: The effect of different *ComCat*® (CC) concentrations on A) the germination rate of radish seeds as well as subsequent B) root and C) coleoptile growth of seedlings at 24 hour intervals over a 96 hour incubation period. $LSD_{(T)\ (0.05)}$ values at 96 min. are supplied in graphs. Error bars = Standard deviation.

After 72 h of incubation, root length growth of radish seedlings was significantly ($P<0.05$) enhanced by all of the different *ComCat*® concentrations except the highest (5.0 mg ℓ^{-1}) which significantly inhibited growth (Figure 3.3B). The same tendency applied at 96 h of incubation except that the second highest *ComCat*® concentration (2.5 mg ℓ^{-1}) had no effect on root growth. Of all the *ComCat*® concentrations tested, it was once again the 0.5 mg ℓ^{-1} concentration that had the most significant enhancing effect on seedling root growth compared to especially the higher *ComCat*® concentrations tested as well as the water control.

Exactly the same tendency in terms of the effect of *ComCat*® on coleoptile growth of radish seedlings, as was seen for root growth, was observed (Figure 3.3C). Again the lower range (0.25, 0.5 and 1.0 mg ℓ^{-1}) *ComCat*® concentrations tended to

increase coleoptile growth from 48 h upwards to 96 h while the higher concentration range (2.5 and 5.0 mg ℓ^{-1}) inhibited growth. However, at 96 h of incubation none of the differences between treatments and the water control were statistically significant.

3.3.4 Biotest 4: Germination of cabbage, pea and wheat seeds seed and subsequent seedling growth response to treatment with *ComCat*® at a single concentration

3.3.4.1 Cabbage (*Brassica oleracea*)

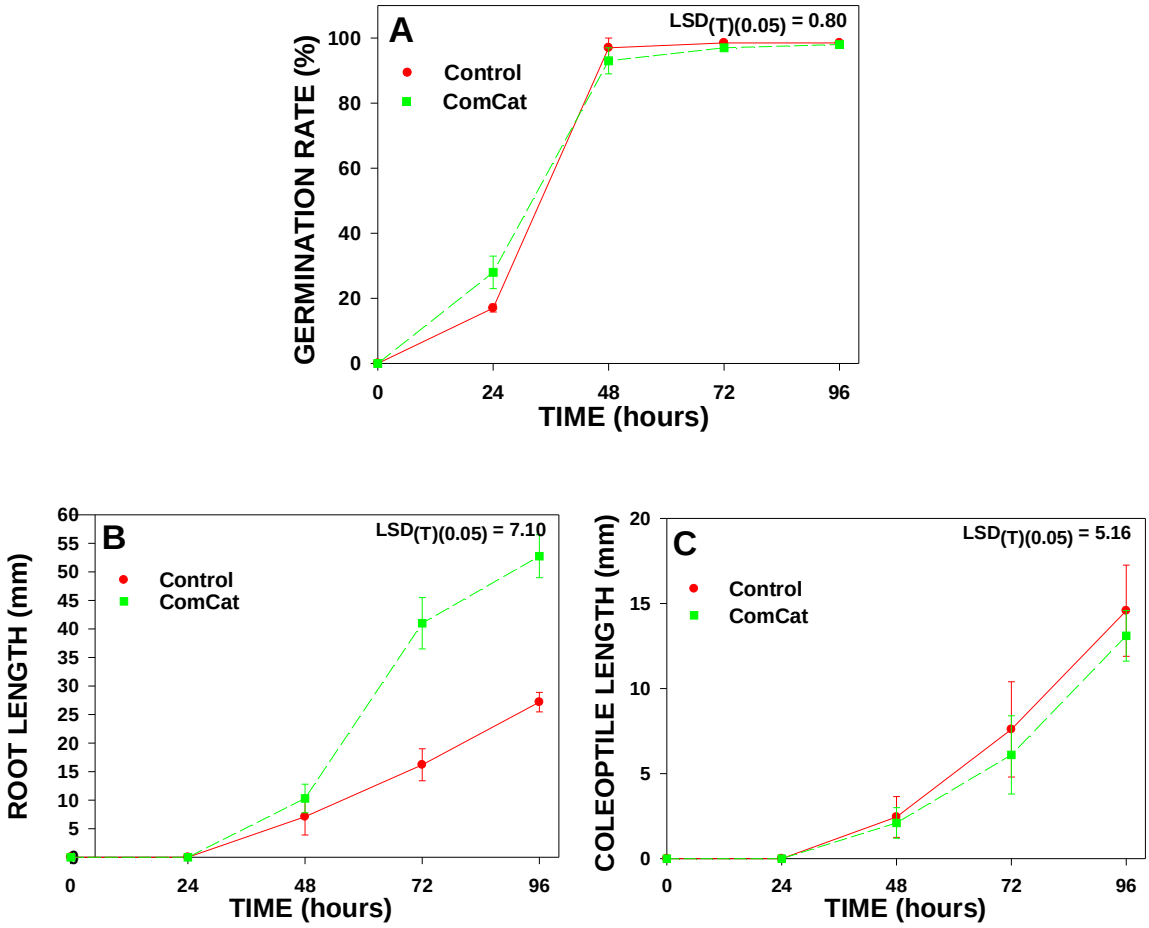

Figure 3.4: The effect *ComCat*® at a concentration of 0.5 mg ℓ^{-1} on A) the germination rate of cabbage seed as well as subsequent B) root and C) coleoptile growth of seedlings at 24 hour intervals over a 96 hour incubation period. $LSD_{(T)(0.05)}$ values at 96 min. are supplied in graphs. Error bars = Standard deviation.

As shown in Figure 3.4 A, at a concentration of 0.5 mg ℓ^{-1} *ComCat*® contributed to a slight increase in the germination rate of cabbage seed at 24 h of incubation,

compared to the water control. However, no significant differences between the control and the *ComCat*® treatment were observed at any of the other time intervals.

Cabbage seedlings responded to the *ComCat*® treatment in a highly significant fashion (Figure 3.4 B). Already at 72 h and also at 96 h of incubation *ComCat*® stimulated root growth significantly ($P<0.05$) compared to the water control. Statistically no significant difference in coleoptile growth between treated and non-treated cabbage seedlings was observed (Figure 3.4 C). However, *ComCat*® tended to inhibit coleoptile growth slightly.

3.3.4.2 Pea (*Pisum sativum*)

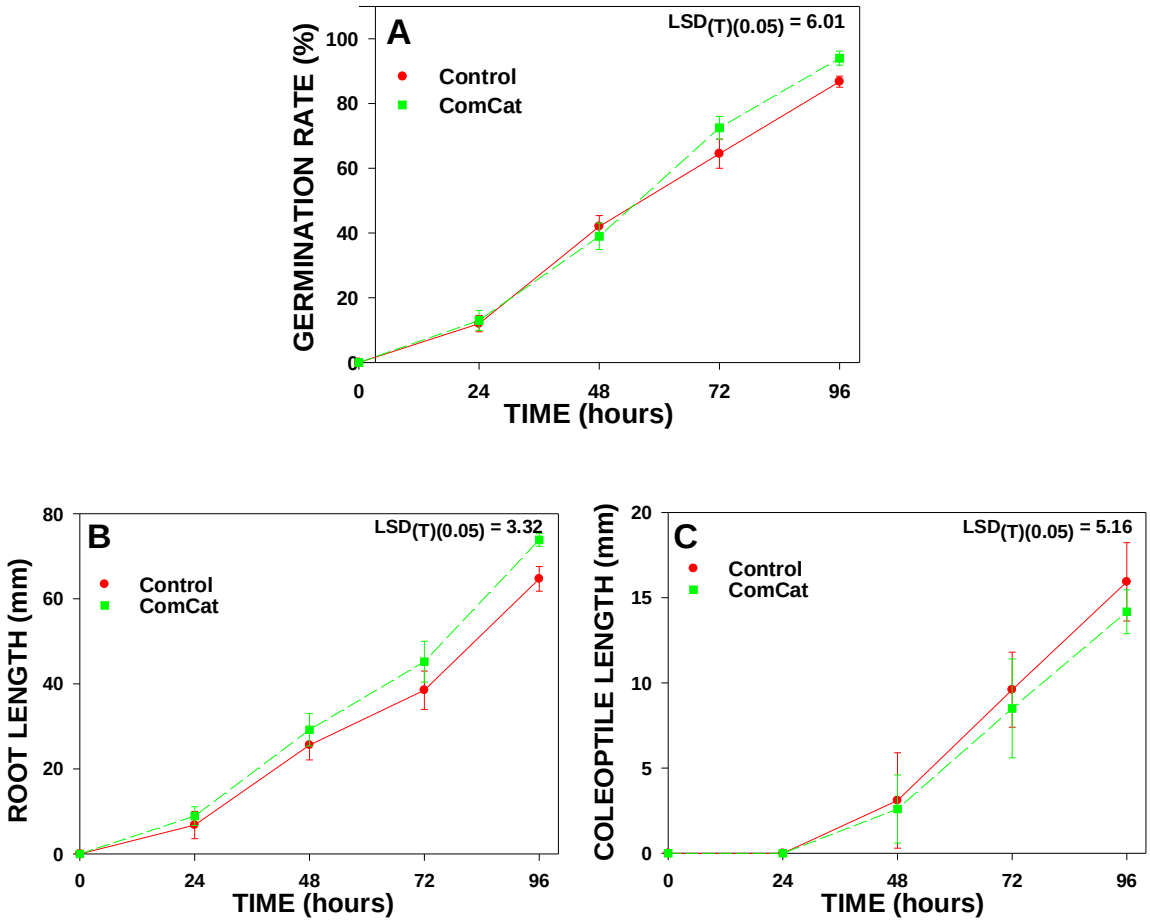

Figure 3.5: The effect *ComCat*® at a concentration of 0.5 mg ℓ^{-1} on A) the germination rate of pea seed as well as subsequent B) root and C) coleoptile growth of seedlings at 24 hour intervals over a 96 hour incubation period. $LSD_{(T)(0.05)}$ values at 96 min. are supplied in graphs. Error bars = Standard deviation.

After 72 h and 96 h of incubation the germination rate of *ComCat*® treated pea seed was markedly higher than that of the water control seed (Figure 3.5A). Similarly, at 72 h and 96 h of incubation *ComCat*® applied at 0.5 mg ℓ^{-1} increased the root length growth of the young pea seedlings significantly ($P<0.05$; Figure 3.5B). However, in the case of coleoptiles the *ComCat*® treatment inhibited growth significantly at 72 h and 96 h of incubation (Figure 3.5C).

3.3.4.3 **Wheat** (*Triticum aestivum)*

No statistically significant differences ($P<0.05$) in either the germination rate (Figure 3.6A) of wheat seeds or both root (Figure 3.6B) and coleoptile (Figure 3.6C) growth of wheat seedlings between *ComCat*® treated and the water control were observed over a 96 h incubation period. (Figure 3.6A, B, C)

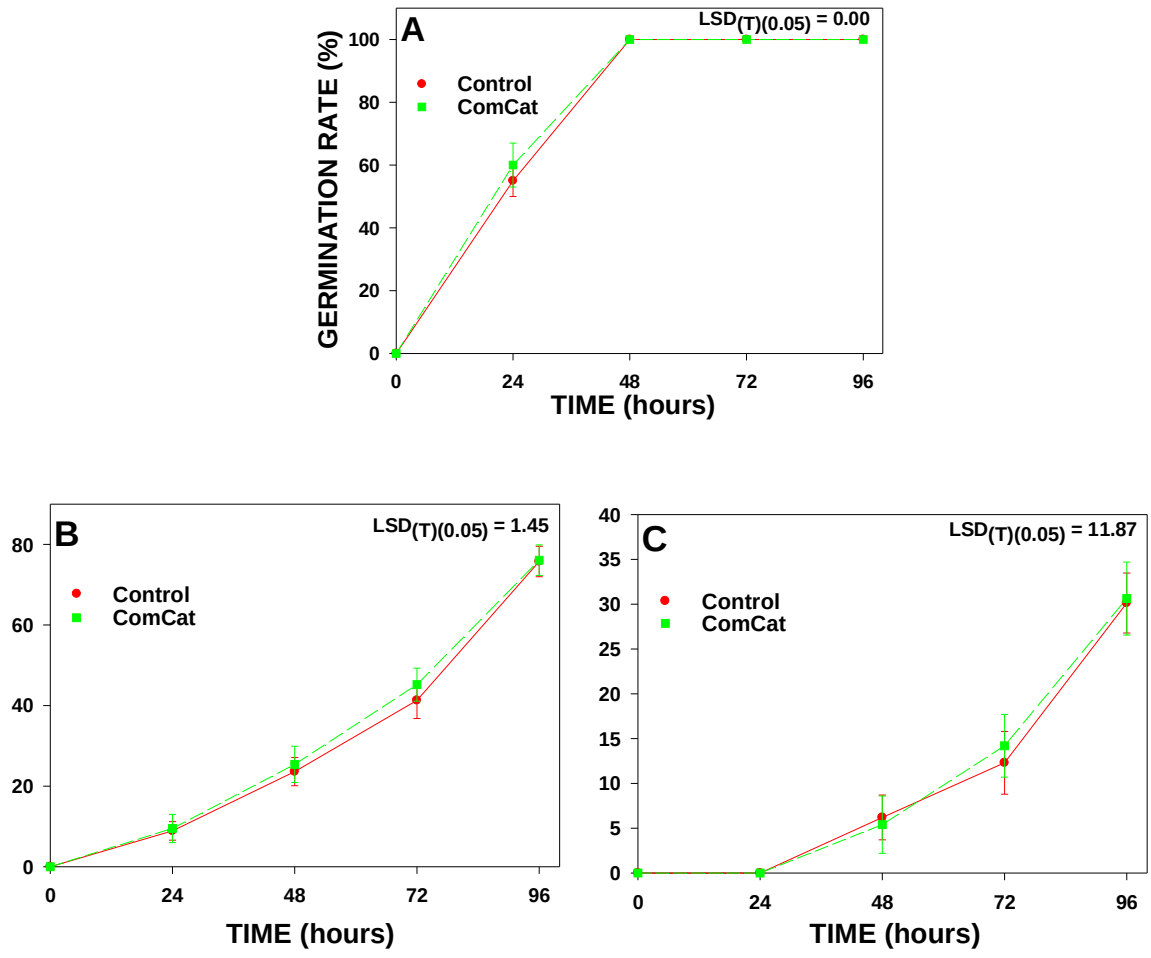

Figure 3.6: The effect *ComCat*® at a concentration of 0.5 mg ℓ^{-1} on A) the germination rate of wheat seed as well as subsequent B) root and C) coleoptile growth of seedlings at 24 hour intervals over a 96 hour incubation period. $LSD_{(T)(0.05)}$ values at 96 min. are supplied in graphs. Error bars = Standard deviation.

3.3.5 Biotest 5: Wheat seedling growth response to treatment with different *ComCat*® concentrations after four weeks under glasshouse conditions

Wheat seedlings responded differently to treatment with *ComCat*® at 0.5 mg ℓ^{-1} (optimum according to biotest 1) than did the other test crops (radish, pea and cabbage) in the sense that no effect was observed. Subsequently, a concentration range was tested. Interestingly, wheat seedlings grown from seeds treated with *ComCat*® in seed trays at the 3-4 leaf growth stage over four weeks under controlled glasshouse conditions (Figure 3.7) again responded differently than those over a shorter (96 h) incubation period (Figure 3.6) under laboratory conditions.

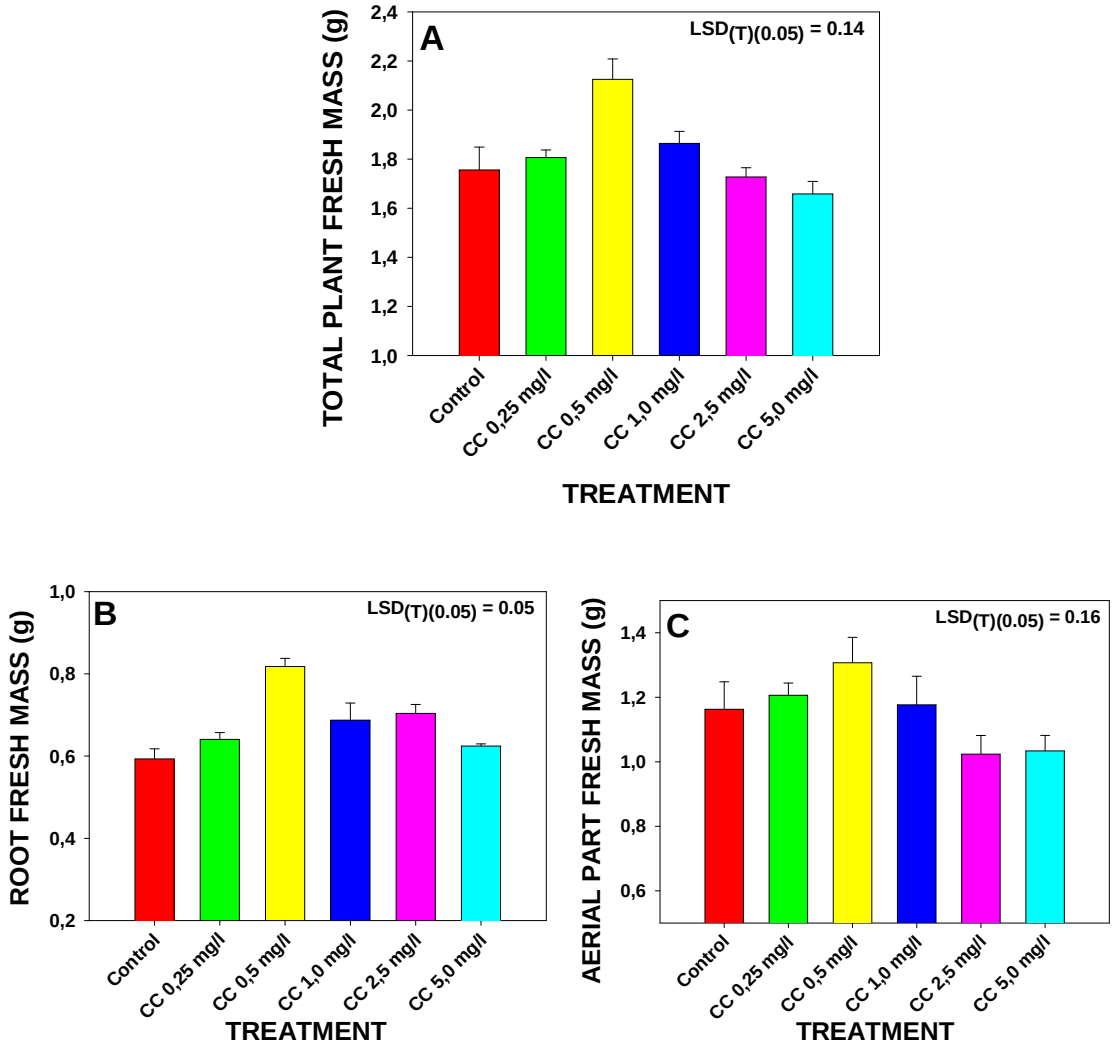

Figure 3.7: The growth response of wheat seedlings cultivated in seed trays four weeks after planting while different *ComCat*® (CC) concentrations were foliar applied at the 3-4 leaf growth stage, under controlled glasshouse conditions. A) = total plant fresh mass, B) = root fresh mass and C) = aerial part fresh mass. $LSD_{(T)\ (0.05)}$ values are supplied in graphs. Error bars = Standard deviation.

The total plant fresh mass (FM) of wheat seedlings, measured four weeks after planting, was significantly (P<0.05) enhanced by treatment with *ComCat*® at the 3-4 leaf growth stage and at 0.5 and 1.0 mg ℓ^{-1}, compared to the other concentrations and the water control (Figure 3.7 A). The higher *ComCat*® concentrations (2.5 and 5.0 mg ℓ^{-1}) decreased the total plant fresh mass, compared to the control, but this was only significant in the case of the highest concentration.

Interestingly, exactly the same tendency as for total plant FM was observed for root FM except that the higher *ComCat*® concentrations all contributed to increased root growth that was only non-significant in the case of the highest concentration (Figure 3.7B). Once again the most significant enhancement of root growth under the influence of *ComCat*® was observed with the 0.5 mg ℓ^{-1} concentration, compared to all of the other treatments and the control.

Almost a mirror image of the results obtained with total plant FM was observed for coleoptile FM (Figure 3.7C) under the influence of *ComCat*®. However, although the lower range *ComCat*® concentrations (0.25. 0.5 and 1.0 mg ℓ^{-1}) tended to increase coleoptile FM, this was not statistically significant. Conversely, both of the higher *ComCat*® concentrations in the range (2.5 and 5.0 mg ℓ^{-1}) significantly reduced coleoptile FM compared to the other concentrations, but not the water control.

3.3.6 Biotest 6: Growth response of lettuce, beetroot and wheat seedlings at four weeks following different *ComCat*® treatments at different growth stages under glasshouse conditions

3.3.6.1 Lettuce

Of the four drench treatments with 0.5 mg ℓ^{-1} *ComCat*®, three (day1, 3 and 6) contributed to significant (P<0.05) increases in leaf fresh mass (FM), compared to the control, when measured four weeks after planting seeds in trays and keeping it under controlled glasshouse conditions (Figure 3.8). Drench treatment of the growing medium at day 0 (when seeds were planted) significantly inhibited leaf growth. The combined drench and foliar treatment at day 8 as well as the two foliar treatments at days 10 and 13 also contributed to significant increases in leaf FM. In terms of aerial part vegetative growth, the drench treatment at day 3 and the foliar spray treatment at day 10 increased leaf FM the most and in a highly significant fashion.

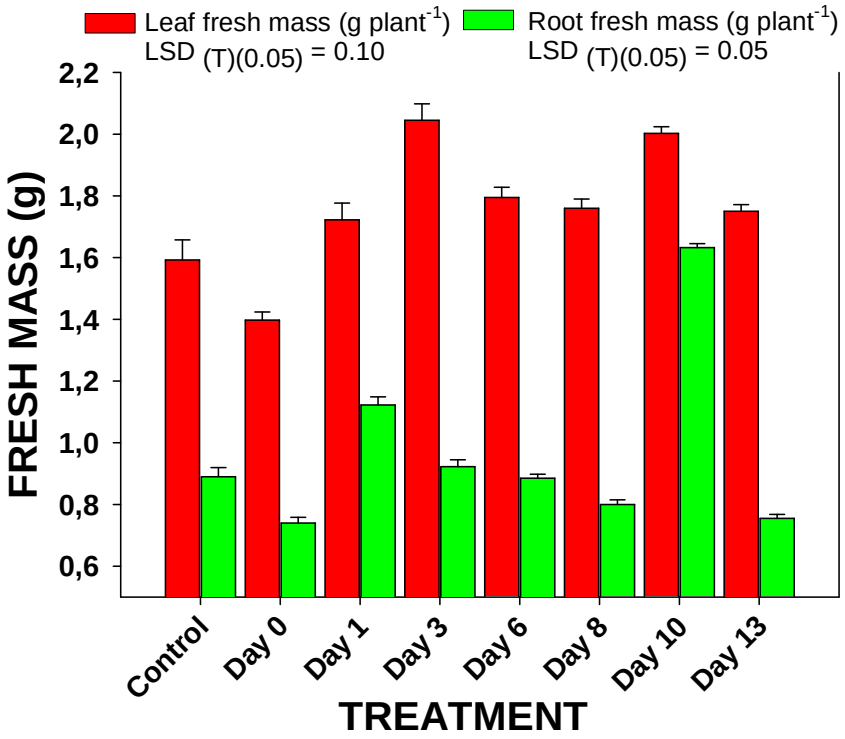

Figure 3.8: The effect of *ComCat*® applied at different growth stages at a concentration of 0.5 mg ℓ^{-1} on leaf and root fresh mass of lettuce grown in seed trays under glasshouse conditions. Fresh mass was measured after four weeks. Day 0, 1, 3 and 6 = drench treatment of growing medium; Day 8 = drench plus foliar spray treatment; Day 10 and 13 = foliar spray treatment only. $LSD_{(T)(0.05)}$ values for leaf and root FM are supplied separately in the graph. Error bars = Standard deviation.

As was the case with leaves, foliar application of *ComCat*® at day 10 resulted in highly significant enhancement of root growth (Figure 3.8) in lettuce seedlings, compared to all other treatments as well as the control. Furthermore, the drench treatment at day 1 enhanced the root growth significantly compared to the control and all other treatments. The drench treatment at day 0 and combined drench and foliar treatment at day 8 as well as foliar treatment at day 13 significantly inhibited root growth while the drench treatments at day 3 and 6 had no effect.

3.3.6.2 Beetroot

As was the case with lettuce, foliar treatment of beetroot with *ComCat*® at day 10 had the most pronounced and highly significant ($P<0.05$) enhancing effect on leaf growth (FM; Figure 3.9) compared to the control. However, drench treatment of the growing medium at days 0, 1 and 3 also significantly enhanced leaf FM while the drench

treatment at day 6, the combined drench and foliar spray treatment at day 8 as well as the foliar treatment at 13 had no significant effect on leaf growth of beetroot seedlings.

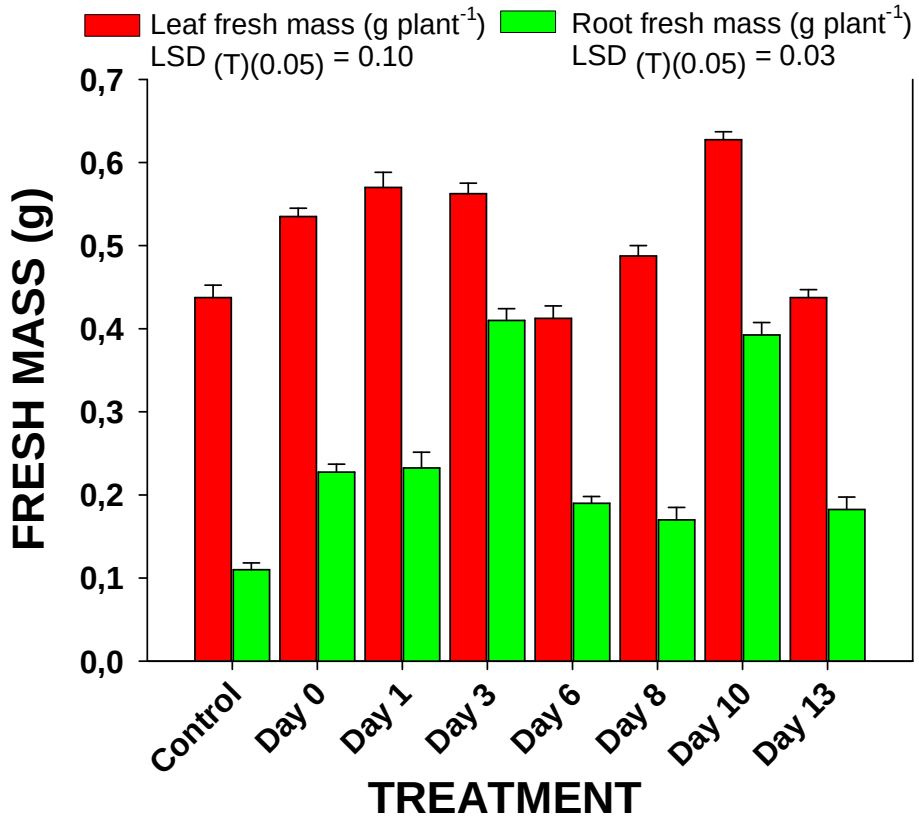

Figure 3.9: The effect of *ComCat*® applied at different growth stages at a concentration of 0.5 mg ℓ^{-1} on leaf and root fresh mass of beetroot grown in seed trays under glasshouse conditions. Fresh mass was measured after four weeks. Day 0, 1, 3 and 6 = drench treatment of growing medium; Day 8 = drench plus foliar spray treatment; Day 10 and 13 = foliar spray treatment only. $LSD_{(T)(0.05)}$ values for leaf and root FM are supplied separately in graph. Error bars = Standard deviation.

All of the different *ComCat*® treatments increased root growth of beetroot significantly (Figure 3.9). Once again the drench treatment at day 3 and the foliar treatment at day 10 induced root growth in the most pronounced and highly significant fashion, as was the case with leaves, but in this case compared to not only the control but also to all other treatments. The increase in root growth under the influence of these two *ComCat*® treatments was 272.7% and 256.8% respectively.

3.3.6.3 Wheat

Wheat leaf growth four weeks after planting was significantly ($P<0.05$) enhanced by drench treatment with *ComCat*® at day 1 and 6 after planting as well as the foliar treatment at day 10, compared to the water control (Figure 3.10). Drench treatment of the growing medium at day 0 and 3 significantly reduced leaf growth. The combined drench and foliar treatment at day 8 as well as foliar treatment at day 13 had no significant effect on leaf growth of wheat.

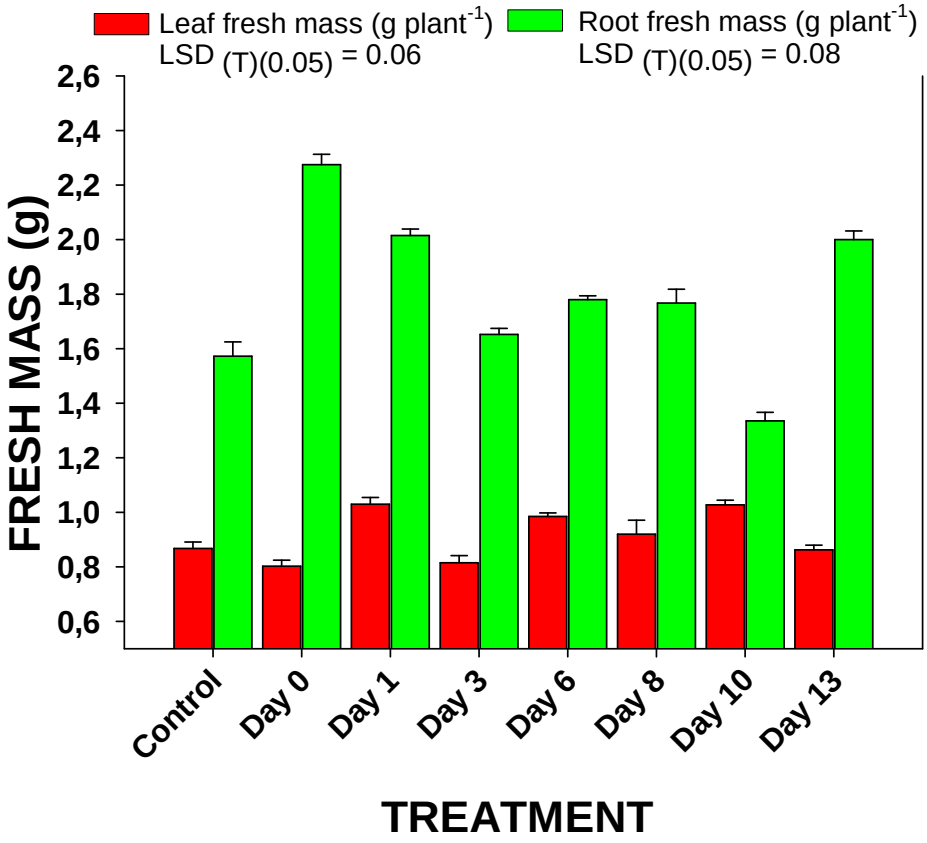

Figure 3.10: The effect of *ComCat*® applied at different growth stages at a concentration of 0.5 mg ℓ^{-1} on aerial part and root fresh mass of wheat grown in seed trays under glasshouse conditions. Fresh mass was measured after four weeks. Day 0, 1, 3 and 6 = drench treatment of growing medium; Day 8 = drench plus foliar spray treatment; Day 10 and 13 = foliar spray treatment only. $LSD_{(T)(0.05)}$ values for leaf and root FM are supplied separately in the graph. Error bars = Standard deviation.

Root growth in wheat seedlings treated with *ComCat*® at different stages of development followed a totally different pattern as was observed for the other two

crops (Figure 3.10). Foliar treatment with *ComCat*® at day 10 significantly (P<0.05) inhibited root growth, while the drench treatment at day 3 had no effect. All other treatments significantly enhanced root growth of wheat seedlings while the response to the drench treatment at day 0 and the foliar treatment at day 13 was most pronounced and highly significant.

3.3.7 Biotest 7: The growth response of algae to treatment with *ComCat*®

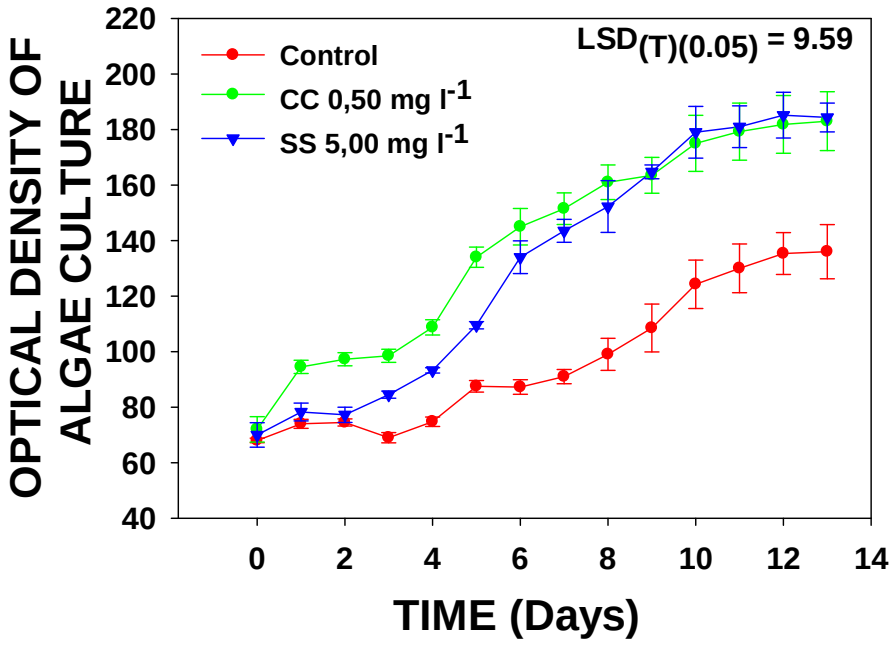

Figure 3.11: The growth response of algae (*Scenedesmus obliquus*) to treatment with *ComCat*® at a concentration of 0.5 mg ℓ^{-1} at one day intervals over a two week incubation period. $LSD_{(T)(0.05)}$.value on day 13 indicated in graph. Error bars = Standard deviation.

The growth rate of *Scenedesmus obliquus* followed a typical sigmoid pattern recognized by an initial lag phase, a linear growth phase and a maximum growth phase, independent of the treatment. Already after one day the growth rate of the algae culture was twofold higher following treatment with *ComCat*® at 0.5 mg ℓ^{-1} than that of the water control and the SS (5.0 mg ℓ^{-1}) treated algae culture. The latter tendency continued over the first three days during the lag phase. The linear growth phase for both the *ComCat*® and SS treated algae cultures followed exactly the same pattern and was not significantly different from each other. However, both of the latter

treatments contributed to a maximum growth rate that was significantly higher than that of the water control at day 13.

3.4 Discussion

Seven biotests were employed to measure the bio-stimulatory activity of *ComCat*®, as claimed by the manufacturers (AgraForUm AG, Germany), under both laboratory and glasshouse conditions. The product was tested at different concentration levels (0.25, 0.5, 1.0, 2.5 and 5.0 mg ℓ^{-1}) in the laboratory (Biotest 1) and in the glasshouse (Biotests 3 and 5), by using selected crops. Additionally, *ComCat*® was applied to seedlings of test crops at different growth stages (Biotest 6) and at a single concentration under glasshouse conditions in order to obtain a complete profile. Further, in some instances (Biotests 2, 4 and 7) only a single *ComCat*® concentration (0.5 mg ℓ^{-1}) was tested as this repeatedly emerged as the optimum concentration. Where uncertainty prevailed (e.g. in the case of wheat), both the multiple and single concentration approaches were followed.

Baker`s yeast, *Saccharomyces cerivisiae*, was used in respiration rate biotest 1 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 in the absence of oxygen (anaerobic) while the carbon dioxide released is stoichiometric with each molecule of glucose metabolized under anaerobic conditions (Amthor, 1989). This supplied a simple screening procedure to ascertain whether the active compounds contained in *ComCat*® possessed the potential to manipulate respiratory metabolism in yeast cells in any way. From the concentration range tested (0.25, 0.5, 1.0, 2.5 and 5.0 mg ℓ^{-1}), 0.5 mg ℓ^{-1} *ComCat*® was clearly the optimal concentration as it significantly increased the respiration rate of monoculture yeast cells compared to all the other concentrations as well as the water control.

Subsequently, in order to ascertain whether this effect on yeast cell respiration was also applicable to plant tissue, the respiratory response of pea seeds to treatment with *ComCat*® at the concentration found optimal for yeast cells (0.5 mg ℓ^{-1}), was tested (Biotest 2). Expressed as CO_2 release over a two hour incubation period, the *ComCat*® treatment increased the respiration rate of pea seeds highly significantly (>200%). However, when the respiration rate was expressed as O_2 consumption, *ComCat*® had no effect. Although both expression forms for the respiration rate are acceptable, they actually supply two perspectives. Carbon dioxide

release collectively indicates either glycolytic activity under anaerobic conditions together with possible CO_2 release via the oxidative pentose phosphate (OPP) pathway or mitochondrial activity under aerobic conditions (Crawford, 1977). On the other hand, O_2 consumption indicates mitochondrial involvement during aerobic respiration only. From the CO_2 release and O_2 consumption data it can be deduced that the pea seeds were in an anaerobic state and mitochondrial activity had not yet commenced, as germination had not been completed and the seed was unable to absorb O_2.

A rationale for this approach is that the role of cell respiration in maintaining sufficient energy levels for germination and subsequent seedling growth is indisputable (Amthor, 1989) and a decrease in growth often observed at later stages of crop development is also due to a decrease in respiration. According to the author, the respiration rate is highest in actively dividing tissue (e.g. axis of a germinating seed or meristematic tissue) where the demand for energy and the biosynthesis of essential intermediates are greatest during seed germination and the first few days of seedling growth. These intermediates are essential and form the building blocks necessary for growth.

However, 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 & Hichman, 2000). This had to be verified experimentally. Subsequently, a third bio-assay involving seed germination and seedling growth was employed. Treatment of radish seeds with *ComCat*® at a concentration range had no effect on germination, but the 0.5 mg ℓ^{-1} concentration had the most pronounced and significant enhancing effect on root growth compared to the other concentrations tested as well as the water control. As was the case with the respiration test. This was in concert with the findings of Channal *et al.* (2002) who reported that fresh leaf extracts from seven tree species had no effect on seed germination, but enhanced sunflower and soybean seedling growth. Although *ComCat*® contributed to a slight increase in coleoptile length growth, this was not significant for any of the concentrations tested. Interestingly, the highest *ComCat*® concentration in the range (5.0 mg ℓ^{-1}) inhibited both root and coleoptile growth as is typical for hormones (Salisbury & Ross, 1992). Cag *et. al.* (2007) also reported that the effect of Epibrassinolid on the growth of red cabbage cotyledons showed differences related to concentration with highest enhancement at 0.001 µM and no

differences at 10 µM. The latter strongly suggested that the initial respiration rate increase observed in yeast cells with the 0.5 mg ℓ^{-1} (optimum) *ComCat*® concentration could be indicative of a positive (stimulatory) rather than a negative (inhibitory) effect on cell metabolism and seedling growth.

In order to verify that not only radish seedlings react to treatment with *ComCat*® but also those of other crops, the seedling growth response of one row crop (wheat) and two vegetable crops (cabbage and pea) were followed after treatment at a concentration of 0.5 mg ℓ^{-1}. From the acquired data it was interesting to note that seedlings from different crops responded differently to treatment with *ComCat*®. In all three cases treatment with *ComCat*® had no effect on seed germination.

Negative effects of plant extracts on seed germination were reported in several publications (Ejaz Ahmad *et al*., 2003; Panahyan-e-Kivi *et al*., 2010; Bhadoria, 2011). Phiri (2010) reported that the application of *Moringa oleifera* leaf extracts to legume seeds had a different effect on different species. It prolonged initial germination of beans, increased the germination percentage of cowpea, but reduced the germination percentage of groundnut seed. In all three crops coleoptile and root growth was suppressed. Phiri & Mbewe (2010) further found that *M. oleifera* leaf extracts reduced the germination percentage of rice by 7%, but did not affect germination of wheat, maize and sorghum. However, treatment of cereal seeds with the *M. oleifera* leaf extracts enhanced root length of maize and hypocotyl length of wheat seedlings. Unfortunately *M. oleifera* leaf extracts showed undesirable effects namely reduction of radical growth in rice as well as hypocotyl growth and seedling survival in sorghum. The results indicate that sorghum is more sensitive to *M. oleifera* leaf extracts compared to other cereals.

But, although the growth of both cabbage and pea seedlings was significantly enhanced in terms of root and coleoptile growth in this study, the *ComCat*® treatment had no effect on wheat seedling growth. In terms of wheat this was in concert with previous findings of Afzal *et al*. (2005) who reported that treatment with both IAA and GA_3, two well known PGRs, failed to improve seedling growth in wheat under normal or saline conditions in the laboratory. Although the authors did not test the response of other crops, it seems that wheat being a C3 plant while belonging to the grass family (Poaceae; previously Gramineae) that mostly represent C4 plants, must be treated with caution or even as an exception at times.

For this reason the response of wheat to treatment with *ComCat*® at a concentration range, but as a foliar application instead of a seed treatment, was repeated under glasshouse conditions (Biotest 4). *ComCat*® was applied foliarly to wheat seedlings at the 3-4 leaf growth stage and growth was measured four weeks after planting. Interestingly, wheat seedling growth, and especially root growth, was increased significantly and again most profoundly by the 0.5 mg ℓ^{-1} concentration. The latter strongly indicates that the growth response of wheat seedlings grown from seeds treated with *ComCat*® differs from the response when the product was applied foliarly after seedling emergence. This is in concert with a report by Harms & Oplinger (1988). According to the authors results obtained under carefully controlled conditions in a laboratory often differ from that obtained under field conditions and are not easy to repeat either in the field or in pots under glasshouse conditions. For example, in field crops (and for that matter in pots) root growth is strongly related to soil nutrient supply and not only to the influence of a specific hormone. They also stated that several PGRs in the auxin family of chemicals stimulate root initiation on plant cuttings, but there is no certainty that it will do the same when seeds are treated.

From this it must be accepted that different crops will respond differently to treatment with PGRs and that the response will also depend on the time of application. Especially regarding soil and foliar application, timing is questioned. At which crop growth stage should *ComCat*® be applied in order to provoke the optimal growth response? For this purpose biotest 5 was employed while wheat and two vegetable crops (lettuce and beetroot) were chosen as test crops. *ComCat*® was applied at 0.5 mg ℓ^{-1} either as a soil drench at different growth stages before the seedlings emerged from the soil or as a foliar spray at different growth stages after seedling emergence. The response of lettuce and beetroot seedling growth was very similar in the sense that, in terms of both root and coleoptile growth, a soil drench with *ComCat*® at day 3 and a foliar application at day 10 (3-4 leaves unfolded) were significant, compared to the control, and optimal in both cases. Again the response of wheat was erratic and totally different from the other two crops.

The optimal root growth response of wheat seedlings were when *ComCat*® was applied either at day 0 or 1 as soil drenches or foliar at day 13 (5-6 leaves unfolded). This was rather unexpected as no response was observed when wheat seeds were treated (biotest 3). However, the response to a foliar treatment at day 13

was more or less in concert with the observation in biotest 4 when *ComCat*® was applied foliarly at the 3-4 leaf growth stage. This confirmed that wheat responded very differently to treatment with *ComCat*® than other crops, that a soil drench was preferred by the crop to seed treatment only and that foliar application at a later stage in seedling development seemed the better option. No information on this unique behaviour of wheat could be traced in the literature. However, from the literature it became clear that different crops respond differently to treatment with natural bio-stimulatory compounds. In this regard Aliotta & Cafiero (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.

From the acquired data it also became clear that a 0.5 mg ℓ^{-1} concentration was optimal for *ComCat*® in most cases, while the higher concentrations tended to have an inhibiting effect. 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. Terefa (2002) also demonstrated that only low concentrations of flower, root and stem extracts of the weed *Parthenium hysterophoru* had a stimulatory effect on seedling growth of tef (*Eragrostis tef*). As previously mentioned, the principle active compounds in *ComCat*® belong to the brassinosteroid (BR) group of hormones (Takeuchi *et al*., 1996; Schnabl *et al*., 2001). It is, therefore understandable that the acquired data on seedling growth is in line with a report by Prakash *et* al. (2001) on the diverse effect of application concentration of brassinosteroids. The authors showed that tomato seed and subsequent seedling behaviour responded differently to different concentrations of BRs in terms of germination and growth, respectively.

Further, the seedling growth response of different crops to treatment with *ComCat*® was more pronounced in terms of root growth stimulation than that of coleoptile growth. This is seen in a positive way by Boiffin *et al*. (2001) who stated that early stimulation of aerial part growth in agricultural crops is not desired because of the negative effect it can have on the final yield. The results obtained with *ComCat*® thus far are also in agreement with the findings of Turk & Tawaha (2003) who reported that water extracts of some plants known for their allelopathic influence

on the environment were more inclined to stimulate root than either coleoptile or shoot growth. Much earlier the work of Chung & Miller (1995) lead the authors to conclude that this might be expected as roots are the first to come into contact with natural allelochemicals following absorption from the soil. Although the latter argument is not applicable to seed treatment, it seems important to test the possibility that translocation of the active substances by different plant tissues might be an issue. For this reason, and since seedlings treated with natural bio-stimulants theoretically have a better chance of either establishing on their own or be transplanted more successfully (Balestri & Bertini, 2003), future research will have to be extended to the response of young seedlings to foliar applications under field conditions. The results obtained in this study supplied the rationale for the latter. A further rationale for extended studies can be found in a statement by Sonnewald & Herbers (2001) namely that a strong root system is directly proportional to optimal vegetative growth and yield of crops.

Finally, biotest 7 was used to follow the growth response of an algal colony of *Scenedesmus obliquus*. *ComCat*® had a highly significant enhancing effect on the growth of this alga. The significance lies in the fact that single cell organisms also seem to respond positively to treatment with the product. The potential is that other single cell organisms, e.g. beneficial soil organisms that play a pivotal role in recycling of nutrients, may react in the same way. This has the potential to open a new avenue for applying the product as a soil treatment. Already with wheat it was observed that seedling growth response was positive when *ComCat*® was applied as a soil drench as opposed to treatment of seeds. A possibility exists that this response was indirectly due to the effect that *ComCat*® might have had on organisms in the growth medium following a drench treatment. In this regard Jolly *et al*. (1999) reported on the significant growth enhancing effect of a PGR (name disclosed) on bamboo (*Dendrocalamus strictus*) when applied as a soil drench. This aspect needs a comprehensive investigation as soil analyses for nutrient content and soil organism activity will have to be followed over time after application.

Finally, due to the rather erratic response of wheat to treatment with *ComCat*®, and due to its importance as a staple food source in the world, a comprehensive study was undertaken with this crop under field conditions in chapter 4. However, maize as well as three vegetable crops were also included in this field study in order to assess also their response under field conditions.

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Chapter 4

YIELD RESPONSE OF SELECTED CROPS TO FOLIAR APPLICATIONS OF *ComCat*® UNDER FIELD CONDITIONS

Abstract

In this study the yield response of five selected crops to treatment with *ComCat*® was followed under field conditions over two seasons. These included two grain crops, maize and wheat, as well as three vegetable crops, cabbage, carrots and onions. In case of the latter two crops, vegetative growth was also quantified due to visible differences observed during the trial period. All test crops were foliarly treated at the 3-4 leaf growth stage, but for maize and wheat this was the only treatment. An additional second foliar spray was applied to all vegetable crops at either 30% head (cabbage), 30% root (carrot) or 30% bulb (onions) development. During the first growing season (2007/08) single cultivars of all crops were treated with the recommended products, *ComCat*® ROW for maize, wheat and onions and *ComCat*® VEG for cabbage and carrots, but at different concentrations in order to determine the optimums. For all test crops the optimum dosages, as recommended by the manufacturers, were confirmed in terms of the potential of *ComCat*® products to increase yields. This was 100 g ha^{-1} for maize, 200 g ha^{-1} for wheat, cabbage and carrots and 400 g ha^{-1} for onions. During the second season (2008/09) only the optimum concentrations identified for each crop during the previous season was applied while two cultivars per crop were compared to each other and to respective untreated controls. For all test crops one of the cultivars used during season two was the same as was tested during the previous season in order to ascertain whether the measured response would repeat over two seasons. In all test crops, and compared to untreated controls, treatment with the different *ComCat*® products contributed to significant yield increases. This tendency repeated over two seasons for one of the cultivars per crop, except in the case of maize and onions where the second season yield increase was non-significant for one of the two test cultivars. For maize this was cv. PAN6043 and for onions cv. Australian Brown. This indicated that maize and onions were cultivar sensitive to treatment with *ComCat*®.

Keywords: Yield, *ComCat*®, grain crops, vegetable crops.

4.1 Introduction

A decade ago it was estimated that between 70 and 80 million people will be added to the world's population every year meaning that the population will increase by almost a third to reach 8 billion in 2020 (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 the 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). Whether the figures presented by the author will be realized is debatable. However, unless the planet is struck by one or other catastrophy, population growth remains the driving force behind food demand increases.

The challenge is to increase both farm productivity and sustainability (Penning de Vries, 2001). 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 (Heidhues, 2001) are probably the most important. 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 & van Keulen, 1995). Other aspects such as the discovery of new soils and the need for comprehensive research to develop proper cultivation practices need to be considered. It therefore seems that, in terms of future food production, improvement will rely on productivity increases on already available arable land (Heidhues, 2001).

The current prognosis is that the production of food on available arable land will simply have to be increased by applying new techniques or cropping systems that will not further deplete this natural resource. Based on a review of more than eighty case studies by Scherr (1999), 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. The search for techniques that could be implemented as alternatives for the restoration of degraded soil as well as to increase crop yield and quality still continues, but this is no small challenge for agricultural research. However, there are reasons to be optimistic based on the

development of new techniques over the past five decades. These include genetic engineering and manipulation of crops via foliar applied agrochemicals.

Genetic engineering and the development of transgenic crops that are more resistant to abiotic and biotic stress factors, have a higher production potential and/or have higher nutrient qualities are regarded as the technological breakthrough of the century by many (Keller & Carabias, 2001). However, according to the authors, there is a general lack of experience with this technology. Moreover, despite the fact that the latter statement was made a decade ago, transgenic crops have not been cultivated long enough to evaluate its short and long-term benefits and risks and this probably still applies today. Subsequently, consumer resistance towards transgenic crops is a reality often heard of in the media. Nevertheless, 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. Collectively, the arguments outlined above are used to promote organic farming as an alternative for future crop production.

According to Kohout *et al.* (1998), natural bio-stimulants extracted from plants as well as their analogues show potential as an additional cultivation practice when applied in crop production systems either as foliar sprays or as a soil drench, 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 is rather well established.

Although natural products are generally considered ideal from an environmental perspective due to their bio-degradability, they may have limitations (Dayan *et al.,* 2009). These may include a) natural compounds with complex chemical structures that are prone to losing 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 and hazardous effects of synthetic chemicals on non-target plants will remain in the minds of consumers. This 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.

The availability of a wide range of natural brassinosteroids (BRs) from wild plants and their low toxicity together with the very low concentrations needed for the manifestation of their effect on plants, make BRs logical candidates for agricultural application (Holá *et al*., 2010). *ComCat*®, the BR-containing natural product under scrutiny, is one of the first that has been commercialized for the agricultural industry. Pure BRs exogenously applied are known to increase yield in some economically important crops that were included in previous studies. Reports on yield increases in BR-treated crops include cotton (Ramraj *et al*., 1997), leguminous crops (Hayat & Ahmad, 2003) and cereals (Ali *et al*., 2008), to name a few.

Despite the confirmed cases where exogenous application of BRs has contributed to yield increases in selected crops, their effect depends on various internal and external factors (Holá *et al*., 2010). These include time of application in relation to the developmental stage of plant, the length of plant exposure to BRs, the frequency of application, the application mode and the concentration at which it is applied (Khripach *et al*., 2000; Amzallag, 2002; Sasse, 2003; Khripach *et al*., 2003; Fariduddin *et al.* 2003, 2008; Holá *et al*., 2010). According to Ali *et al*. (2008) the response of crops to treatment with BRs is also species specific.

Limited reports on the response of crops to treatment with *ComCat*® could be traced in the literature. These included its effect on seedling growth of selected crops (Pretorius *et al*., 2008) and quality and shelf life of carrots (Workneh *et al.*, 2009a, Workneh *et al.*, 2011). Further three publications on tomato included the effects of pre- and post harvest treatments with *ComCat*® on changes in sugar content of tomato as well as its yield, quality and shelf life (Melkamu *et al.* 2008; Melkamu *et al.* 2009, Workneh *et al.*, 2009b). No reports could be traced in literature confirming that *ComCat*® has similar effects on other crops than carrots and tomato. *ComCat*® is a natural bio-stimulant product from seeds of European plants (Agraforum AG, Germany). The BRs contained in the product has been identified as 24-episescasterone and 24-epi-castasterone (Volz, 2000). However, the author indicated that a third brassinosteroid still needed to be identified in *ComCat*®.

Preliminary screening of *ComCat*® for bio-stimulatory activity revealed a statistically significant potential to promote seedling development in terms of coleoptile and root growth (Chapter 3). In this chapter the aim was to ascertain whether foliar applications of *ComCat*® at different concentrations and at the 3-4 leaf growth stage had the potential to enhance the yield of economically important agricultural crops under field conditions. Two grain crops, maize (*Zea mays*) and wheat (*Triticum aestivum*), and three vegetable crops, cabbage (*Brassica oleracea*), carrots (*Daucus carota*) and onions (*Allium cepa*), were selected for this purpose.

Although the recommendations of the manufacturers of *ComCat*® were followed in terms of application time for each crop, different concentrations were tested on one cultivar for each crop during 2007/08 in order to verify the optimum in the light of a report by Khripach *et al*. (2000) that the concentration at which BRs is applied may be critical and vary for different crops. A second cultivar per crop was included during the second season's trials in order to establish 1) whether test crops were cultivar sensitive to treatment with *ComCat*® and 2) whether results obtained with a single cultivar during the first season was repeatable during the second season. A further objective was to assess the product's application potential in the agricultural industry and its possible contribution to economic prosperity for the farmer.

4.2 Materials and Methods

4.2.1 Materials

Maize and wheat seed were purchased locally from SENWES, carrot seed from Stark Ayres and onion seed from Mayford (South Africa). Cabbage seedlings were obtained from PPP Seedlings Pty Ltd (Bloemfontein South Africa). Standard fertilizer products were purchased from Sidi Parani, South Africa and *ComCat*® products (ROW for maize, wheat and onions; VEG for cabbage and carrots) from Agraforum AG, Germany.

4.2.2 Description of trial sites

All grain and vegetable crop trials were conducted over two growing seasons (2007/08 and 2008/09) in the Bainsvlei area (29°01'00"S, 26°08'50"E) of the

Bloemfontein district on the farm Lionsvlei in the Free State Province of South Africa. The site is situated in the summer rainfall area of the Free State province, South Africa, with mean annual precipitation of 344.5 mm. The maximum temperature varies from 19ºC in June to 35ºC in January while the mean minimum varies from 1ºC in June to 18.1ºC in January. The soil is of the Bainsvlei type which is a light red loamy sand soil. Soil samples were taken at a depth of 30 cm and analyzed to determine the soil fertility status (see 4.2.4).

4.2.3 Experimental design and trial layout

In all cases statistical trials were laid out in a complete randomized block design. During the first season (2007/08) a single trial was conducted for each crop in order to test the yield response on treatment with *ComCat*® at different concentrations. In the second season (2008/09) two trials per crop were laid out simultaneously and adjacent to each other, using two different cultivars, while *ComCat*® was applied at only the optimum concentration identified during the previous season. In order to obtain sufficient degrees of freedom for statistical analyses, treatments were replicated six times during season 1 and twelve times during season 2.

Table 4.1: Trial specifics for grain under full irrigation.

Crop	**Maize**	**Wheat**
Cultivar: Season 1 Season 2	 PHI 3394 PHI 3394 PAN6043	 Tugela PAN 3377 Tugela
Replications Concentration trial Yield trials	 6 12	 6 12
Between row spacing	90 cm	50 cm
In row spacing	20 cm	15 cm
Plants/ha	50 500	80 000
Plot size	180 m^2	100 m^2

Plant rows were in an E-W direction. 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 separately for row (Table 4.1) and vegetable crops (Table 4.2).

Table 4.2: Trial specifics for vegetables under semi-irrigation

Vegetable	Cabbage	Carrots	Onions
Cultivar: Season 1 Season 2	Conquistador Conquistador Drumhead	Fancy Fancy Snakpak	Australian Brown Australian Brown & Texas Grano
Replications Concentration trial Yield trials	6 12	6 12	6 12
Between row spacing	65 cm	20 cm	30 cm
In row spacing	50 cm	2.5 cm	10 cm
Plants/ha	30 800	±2 million	333 000
Plot size	58.5 m^2	30 m^2	45 m^2

4.2.4 Planting and fertilizer application

4.2.4.1 Planting

Maize and wheat seeds were planted mechanically with John Deere planters calibrated to deliver the desired seed mass and fertilizer levels per hectare. Wheat and maize were cultivated under full irrigation (pivot system). All vegetable trials were planted and fertilized by hand and cultivated under semi-irrigation conditions using a sprinkler system.

4.2.4.2 Fertilizer application

Fertilizer application (Table 4.5) was based on soil analyses (Table 4.3) and recommended withdrawal norms in terms of the yield potential per crop (Table 4.4). As the withdrawal norms for different cultivars were very similar, N, P and K requirements were met on average. In some cases fertilizer was applied (Table 4.5) slightly above, and in other cases slightly below the withdrawal norms (Table 4.4).

Table 4.3: The average soil fertility status over two seasons (Free State Department of soil analysis, National Department of Agriculture, Glen and SGS Agri-Laboratory Services).

Parameter	Unit	2007/08	2008/09	Norm
		*0-30 cm	*0-30cm	(mg kg^{-1})
Clay & Silt (0-30cm)	%	20	20	
Sand (0-30cm)	%	80	80	
Conductivity	mS m^{-1}	20	21	200-500
pH (KCl)		5.6	5.5	5.5-7.5
Calcium (NH_4OAc)	mg kg^{-1}	441	443	300-2000
Magnesium (NH_4OAc)	mg kg^{-1}	211	192.9	80-300
Potassium (NH4 OAc)	mg kg^{-1}	139	75.5	80-160
Sodium (NH4 OAc)	mg kg^{-1}	5.8	6.2	100-500
Phosphorus (Olsen)	mg kg^{-1}	5.4	7.4	15-30
Ca:Mg		2.09	2.29	1.5-4.5
(Ca+Mg):K		4.69	8.42	10-20
Mg:K		1.51	2.55	3-4
**CEC	$cmol_c kg^{-1}$	3.1	3.86	-

*Average of individual samples taken all over the trial area and pooled together.
**CEC = Cation exchange capacity

The soil analysis during season 1 (2007/08) revealed that the K content was well within the norm and no K was applied. The P-content, however, was well below the norm during both seasons and P was added above the withdrawal norm. The

area used for field trials during season 2 (2008/09) was well below the norm for both P and K and both was added above the withdrawal norm for the expected yield outcome. N was applied according to the withdrawal norms for each crop during both seasons.

Table 4.4: Fertilizer requirements based on withdrawal norms (kg ton^{-1}) and yield potential (ton ha^{-1}) of test crops (FSSA, 2003, 2007).

Crop	Withdrawal Norms (kg ton^{-1}) N	P	K	Cultivar and Yield potential (ton ha^{-1})	Minumum fertilizer need (kg ha^{-1}) N	P	K
Maize	15.0	3.0	3.5	PHI 3394 (10)	150	30	35
				PAN6043 (6)	90	18	21
Wheat	22.0	3.8	4.3	PAN 3377 (8)	176	30	34
				Tugela (6)	132	23	26
Cabbage	3.4	0.6	3.5	Conquistador (70)	238	42	245
				Drumhead (80)	272	48	280
Carrots	4.0	0.75	4.6	Fancy (60)	240	45	276
				Snakpak (60)	240	45	276
Onion	2.85	0.63	3.88	Austr. Brown (60)	171	38	233
				Texas Grano (50)	143	32	194

4.2.5 Treatments under field conditions

4.2.5.1 Foliar application of *ComCat*®

A Solo® 475 knapsack sprayer (Solo Incorporated; Germany) delivering a pressure between 50 and 60 psi was used for foliar application of *ComCat*®, or distilled water in the case of controls, directly over plants in a row. The sprayer was equipped with a flat fan nozzle spraying at a 40º angle (both sides of the perpendicular) covering a width of 1.1 m on the soil surface from a height of 90 cm above the ground. Depending on the plot area per crop and number of rows per plot, the sprayer was calibrated to deliver 300 L ha^{-1} in the case of maize and wheat and 500 L ha^{-1} in the case of vegetable crops.

Table 4.5: Fertilizer applications for two seasons based on soil analysis figures.

Crop	Time	Rate (kg ha^{-1})	Type	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})
			Season 1	**(2007/08)**		
Maize	At plant	350	3:1:0(28)	76	25	0
	Top dressing	200	3:1:0(28)	42	18	0
			TOTAL	126	43	0
Wheat	At plant	500	3:2:0(25)(Zn)	75	50	0
	Top dressing	300	ASN(27)	81	0	0
			TOTAL	156	50	0
Cabbage	At plant	300 / 280	LAN(28) / Super P(10.5)	84	29	0
	Top dressing	400 /100	LAN(28) / Super P(10.5)	112	10	0
			TOTAL	196	39	0
Carrot	At plant	300 / 280	LAN(28) / Super P(10.5)	84	29	0
	Top dressing	400 /100	LAN(28) / Super P(10.5)	112	10	0
			TOTAL	196	39	0
Onion	At plant	300 / 230	LAN(28) / Super P(10.5)	84	24	0
	Top dressing	400 /100	LAN(28) / Super P(10.5)	112	10	0
			TOTAL	196	34	0
			Season 2	**(2008/09)**		
Maize	At plant	400	2:3:2(22)	25	38	25
	Top dressing	400	3:1:0(28)	84	28	0
			TOTAL	109	66	25
Wheat	At plant	600	3:2:1(25)(Zn)	75	50	25
	Top dressing	300	ASN(27)	81	0	0
			TOTAL	156	50	25
Cabbage	At plant	400/200	LAN(28) / 2:3:2(22)	160	19	12.5
	Top dressing	200/200	LAN(28) / 2:3:2(22)	60	20	12.5
			TOTAL	230	39	25
Carrot	At plant	400/200	LAN(28) / 2:3:2(22)	160	19	12.5
	Top dressing	200/200	LAN(28) / 2:3:2(22)	60	20	12.5
			TOTAL	230	39	25
Onion	At plant	400/200	LAN(28) / 2:3:2(22)	160	19	12.5
	Top dressing	200/200	LAN(28) / 2:3:2(22)	60	20	12.5
			TOTAL	230	39	25

ASN = Ammonium sulphate nitrate. LAN = Limestone ammonium nitrate(28). Super P = Super phosphate(10.5).

4.2.5.2 Treatment of grain crops

The recommended foliar application rate for *ComCat*® ROW on wheat is 200 g ha^{-1} and 100 g ha^{-1} for maize (Agraforum AG, Germany). In both cases the product was dissolved in 300 ℓ ha^{-1} of water and applied once at the 3-4 leaf stage (growth stage 13-14; Meier, 1997). However, the recommendation of the manufacturers was tested during season 1 (2007/08) by comparing the product at three different concentrations (100, 200 and 300 g ha^{-1} for wheat and 50, 100 and 200 g ha^{-1} for maize), to an untreated control. Treatments were replicated six times. As the recommended rates of the manufacturers were confirmed in this way (see 4.4), *ComCat*® ROW was applied at only the optimum rates (200 g ha^{-1} for wheat and 100 g ha^{-1} for maize) in the second season (2008/09) and replicated 12 times. The number of replicates was chosen in order to obtain sufficient degrees of freedom for statistical analysis.

4.2.5.3 Treatment of vegetable crops

The recommended application of *ComCat*® VEG on vegetables is 200 g ha^{-1} for cabbage and carrots while *ComCat*® ROW is used for onions at 400 g ha^{-1}. During season 1 (2007/08) different concentrations of the products were once again tested. For cabbage and carrots 100, 200 and 300 g ha^{-1} and for onions 200, 400 and 600 g ha^{-1} were tested. In all cases the products were applied in a volume of 500 ℓ water ha^{-1}.

As recommended by the manufacturers, both *ComCat*® products were applied twice during the growing season on vegetables. For all three vegetables the first application was when the 3rd true leaf had unfolded (growth stage 13; Meier, 1997). The second application was when cabbage reached 20-30% of the expected head size (growth stage 42-43), when 20-30% of the expected root diameter was reached in carrots (growth stage 42-43) and when the bulb reached 30% of the expected diameter in onions (growth stage 43). Concentration trials during season 1 (2007/08) was replicated six times and yield trials at the optimum concentration during season 2 (2008/09) 12 times. The recommended concentrations were applied during both the first and second recommended application times.

4.2.6 Quantification of crop yield

4.2.6.1 Wheat

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}. Calculations were on an area basis (yield per area harvested).

4.2.6.2 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}. Calculations were on an area basis.

4.2.6.3 Cabbage

Twenty cabbage plants per replicate were selected at random and removed from the soil by hand using a pitchfork. Heads were separated from the foliage by means of a sharp knife. The fresh weight of heads was calculated on an area basis and expressed in ton ha^{-1}.

4.2.6.4 Carrots

Fifty carrots per replicate were selected at random 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}. Calculations were on an area basis. Additionally, the lengths of each carrot was measured and noted.

4.2.6.4 Onions

Thirty onions per replicate were selected at random and removed from the soil by hand. Subsequently, the bulb and foliage were separated, the fresh weights determined and the yield expressed in ton ha^{-1}. Calculations were on an area basis. Additionally, the diameter of each onion was measured and noted.

4.3 Statistical analysis

Analysis of variance (ANOVA) was performed on the data, using the NCSS 2000 statistical program, to identify differences between treatments. Tukey's mean significant difference (MSD) procedure for comparison of means (Steele & Torrie, 1980) was applied to separate means on a 5% (P<0.05) probability level.

4.4 RESULTS

4.4.1 The yield response of wheat to foliar spray treatment with *ComCat*® ROW under irrigation conditions

During season 1 (2007/08) *ComCat*® ROW was applied at three different concentrations. Application of the product at all three concentrations contributed to marked increases in dry kernel yield of wheat, cv. Tugela, but compared to the untreated control this was only significant (P<0.05) in the case of the 200 g ha^{-1} treatment (+762 kg ha^{-1}; Figure 4.1). The latter confirmed the application rate recommended by the manufacturers. Treatment at the lower (100 g ha^{-1}) and higher (300 g ha^{-1}) concentrations resulted in almost the same increase in kernel yield of 602 and 594 kg ha^{-1} respectively, but this was not significantly different from the control. No significant differences between treatments at different concentration levels were observed.

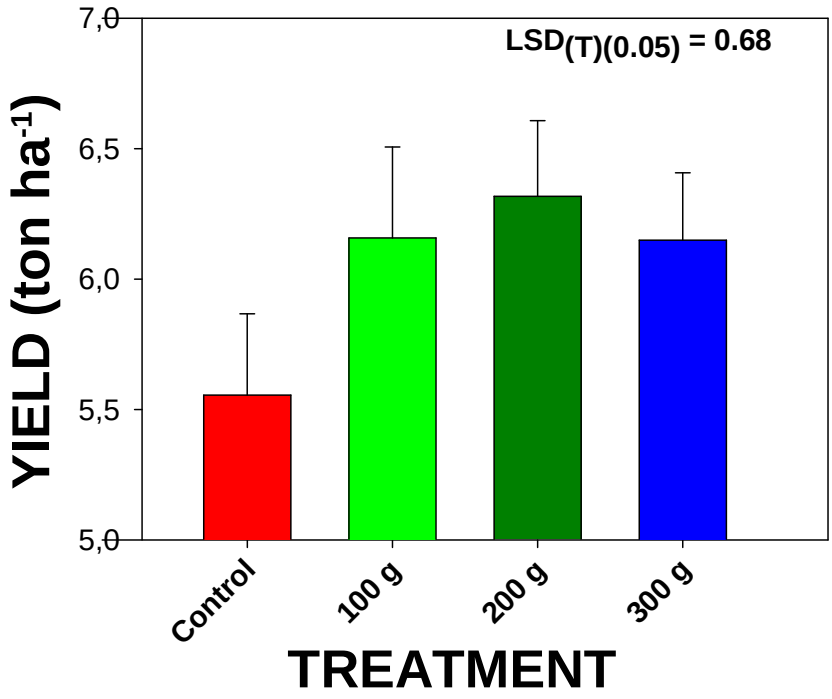

Figure 4.1: The yield response of wheat, cv. Tugela, to treatment with *ComCat*® ROW at different concentrations (100, 200 and 300 g ha^{-1}) when applied only once at the 3-4 leaf growth stage during the 2007/08 growing season. $LSD_{(T)(0.05)}$ value is supplied in the graph. Vertical bars = Standard diviation.

Subsequently, only the recommended 200 g ha^{-1} was applied at the 3-4 leaf growth stage in season 2 (2008/09) using two different wheat cultivars, PAN3377 (Figure 4.2A) and Tugela (Figure 4.2B). Statistical analysis ($P<0.05$) of dry kernel yield data was executed separately for the two cultivars to ascertain the difference from the untreated control. The yield of both cultivars was increased markedly by the treatment, but this was only significant in the case of PAN3377 (Figure 4.2 A). The significant yield increase observed with Tugela during the previous season was not repeated during 2008/09.

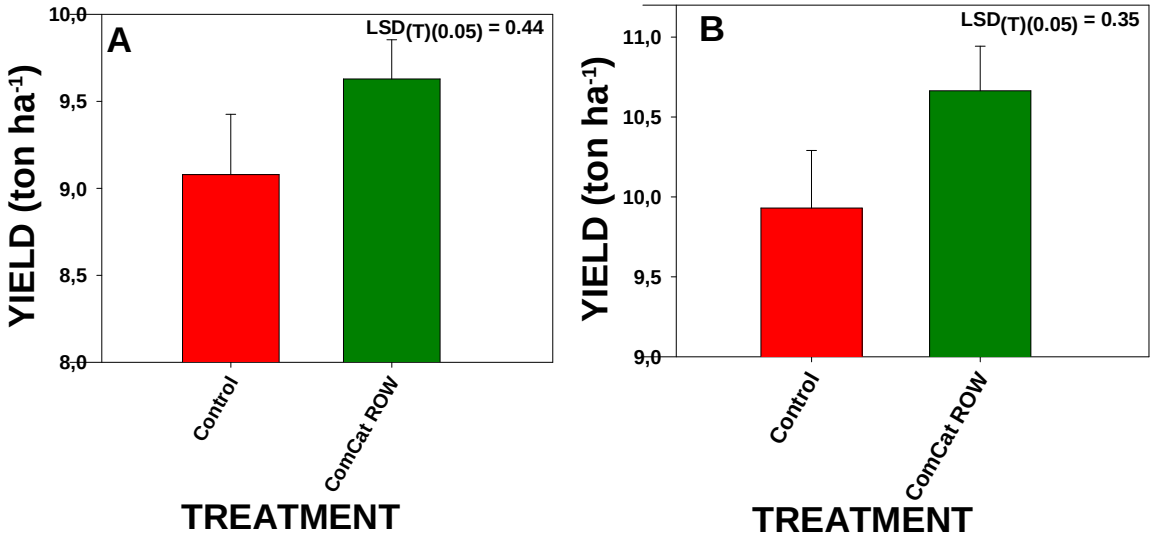

Figure 4.2: The yield response of two different wheat cultivars, A) PAN3377 and B) Tugela, to treatment with *ComCat*® ROW at 200 g ha^{-1} when applied only once at the 3-4 leaf growth during the 2008/09 growing season. $LSD_{(T)(0.05)}$ values are supplied in the graphs. Vertical bars = Standard diviation.

4.4.2 The yield response of maize to foliar spray treatment with ComCat® ROW under irrigation conditions

Foliar spray treatments of maize, cv. PHI3394, with *ComCat*® ROW at the three leaf growth stage and at both 50 g ha^{-1} and 100 g ha^{-1} increased the dry kernel yield markedly by 873 ha^{-1} and 1059 kg ha^{-1} respectively, compared to the untreated control (Figure 4.3). However, this was only significant ($P<0.05$) in case of the 100 g ha^{-1} treatment. Treatment at 200 g ha^{-1} almost had no effect on yield while the slight yield increase observed was significantly lower than that by the other two concentrations tested.

In the following season (2009/09) both maize cultivars, PAN6043 (Figure 4.4A) and PHI3394 (Figure 4,4B) responded positively to treatment with the single 100 g ha^{-1}:

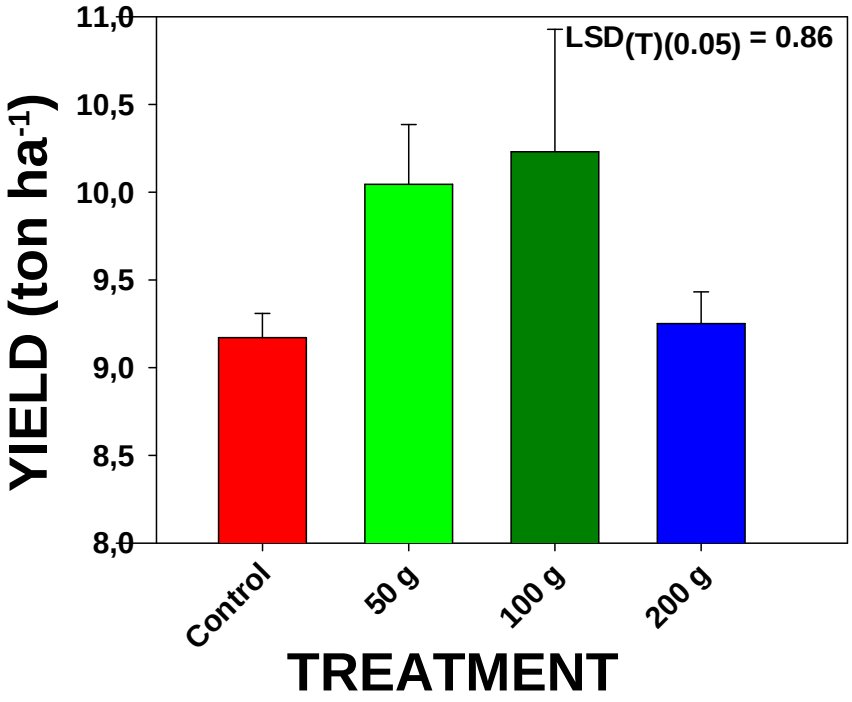

Figure 4.3: The yield response of maize, cv. PHI3394, to treatment with *ComCat*® ROW at different concentrations (50, 100 and 200 g ha^{-1}) when applied only once at the 3-4 leaf growth stage during the 2007/08 growing season. $LSD_{(T)(0.05)}$ value is supplied in the graph. Vertical bars = Standard diviation.

ComCat® ROW application (Figures 4.4A and B), compared to the untreated control. However, the yield increase observed for PAN6043 (587 kg ha^{-1}) was not statistically significant (Figure 4.4A) while the difference in kernel yield (+783 kg ha^{-1}) between treated and non-treated PHI3393 was highly significant (Figure 4.4B). In the case of the latter the same trend was observed over two seasons.

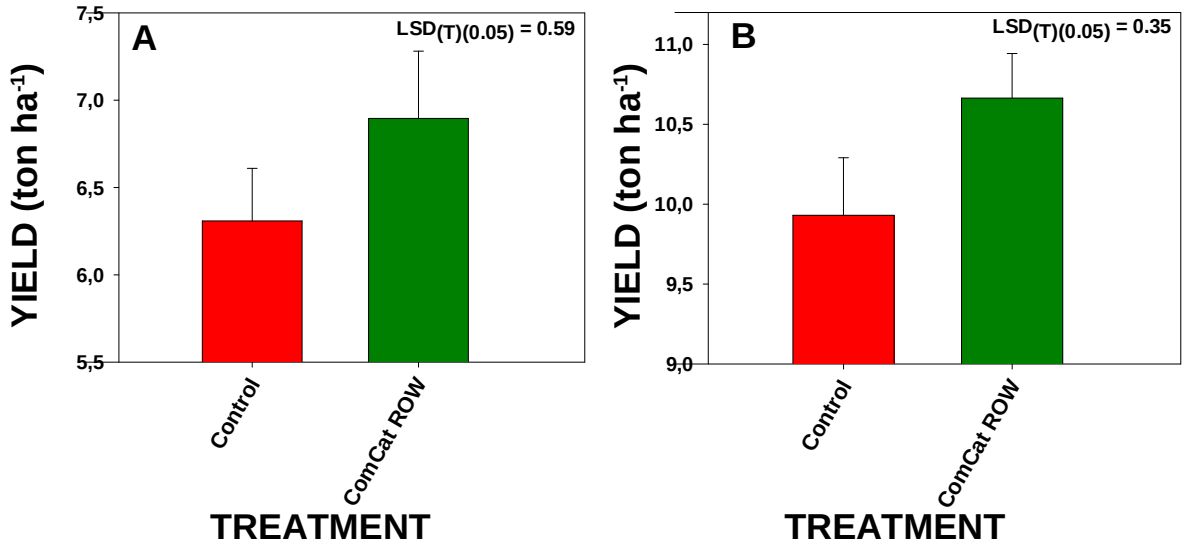

Figure 4.4: The yield response of two different maize cultivars, A) PAN6043 and B) PHI3394, to treatment with *ComCat*® ROW at 100 g ha^{-1} when applied only once at the 3-4 leaf growth during the 2008/09 growing season. $LSD_{(T)(0.05)}$ values are supplied in the graphs. Vertical bars = Standard diviation.

4.4.3 The yield response of cabbage to foliar spray treatment with *ComCat*® VEG under semi-irrigation conditions

Cabbage, cv. Conquistador, also responded differently to the different *ComCat*® VEG concentrations applied at the 3-4 leaf growth stage and at 30% head development (Figure 4.5). Only the recommended dosage of 200 g ha^{-1} contributed to a highly significant increase (+6.86 ton ha^{-1}) in head mass compared to the untreated control as well as the other two concentrations tested. Although the lower (100 g ha^{-1}) and higher (300 g ha^{-1}) concentration rates both tended to increase the head mass slightly, this was not significant.

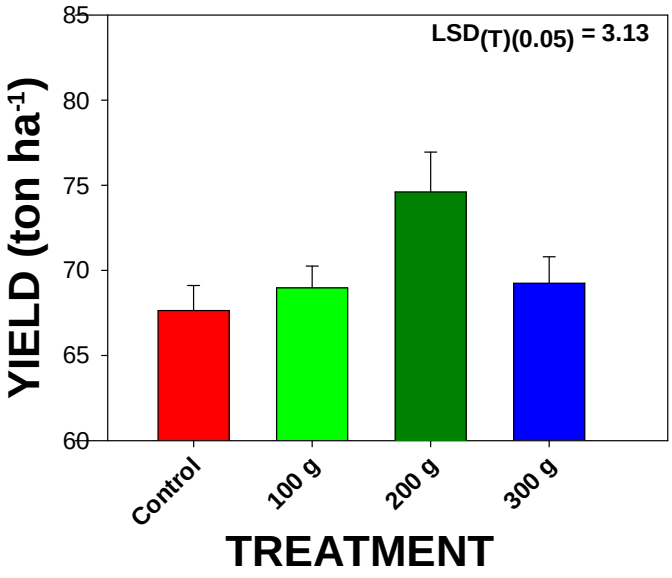

Figure 4.5: The yield response of cabbage, cv. Conquistador, to treatment with *ComCat*® VEG at different concentrations (100, 200 and 300 g ha^{-1}) when applied twice at the 3-4 leaf growth stage and at 30% head development during the 2007/08 growing season. $LSD_{(T)(0.05)}$ value is supplied in the graph. Vertical bars = Standard diviation.

In the following season (Figure 4.6) *ComCat*® VEG was applied twice, at planting and at 30% head development, but only at the recommended dosage of 200 g ha^{-1}. The treatment contributed to significant ($P<0.05$) yield increases in both cultivars, (Conquistador; Figure 4.6A and Drumhead; Figure 4.6B), in terms of head fresh mass.

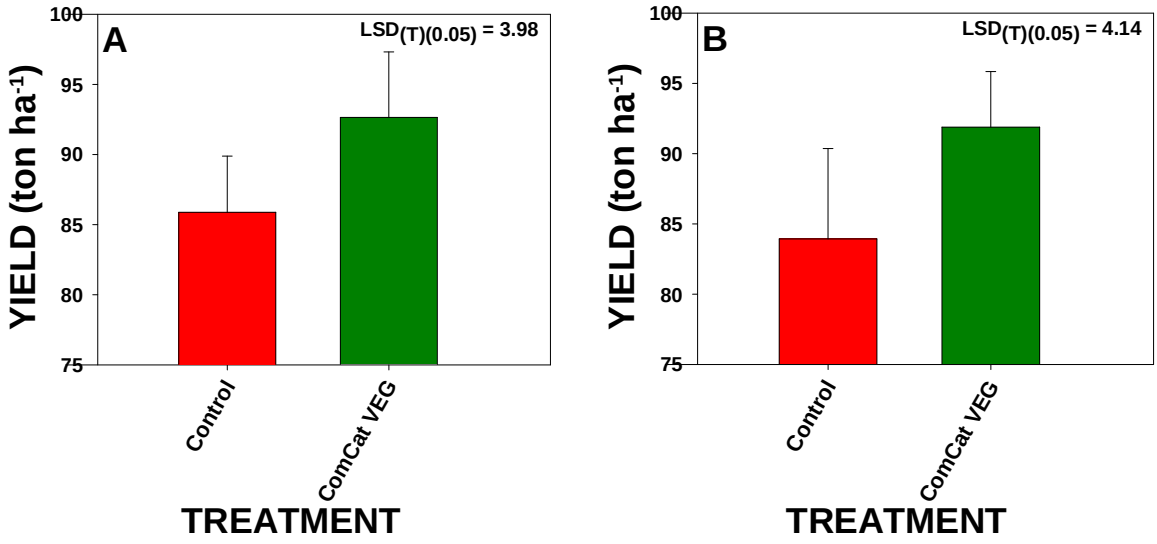

Figure 4.6: The yield response of two different cabbage cultivars, A) Conquistador and B) Drumhead, to treatment with *ComCat*® VEG at 100 g ha^{-1} when applied twice at the 3-4 leaf growth and at 30% head development during the 2008/09 growing season. $LSD_{(T)(0.05)}$ values are supplied in the graphs. Vertical bars = Standard diviation.

4.4.4 The yield response of carrots to foliar spray treatment with *ComCat*® VEG under semi-irrigation conditions

Due to a visual observation of top growth differences, both the growth and yield response of carrots, cv. Snakpak, to treatment with *ComCat*® VEG at different concentrations was determined in 2007/08. All three concentrations of the product increased the leaf fresh mass, but this was only significant in the case of the 100 and 200 g ha^{-1} treatments (Figure 4.7A), compared to the untreated control. Interestingly, the lowest *ComCat*® concentration (100 g ha^{-1}) contributed to the most pronounced increase in leaf growth while the total leaf fresh mass decreased in a linear fashion as the *ComCat*® concentration was increased up to 300 g ha^{-1}. The difference between treatments, in terms of its tendency to decrease leaf growth based on concentration, was only significant in the case of the highest concentration (300 g ha^{-1}) and only when compared to the lowest (100 g ha^{-1}) concentration.

The effect of different *ComCat*® VEG concentrations on root length growth revealed a different pattern than the one that was observed for leaf growth. In contrast to its effect on leaf growth, *ComCat*® applied at 100 g ha^{-1} decreased root length, compared to the control, but this was not significant. However, the decreasing tendency of the low concentration (100 g ha^{-1}) contributed to significant ($P<0.05$)

differences in root length from both the 200 and 300 g ha^{-1} dosages. Applied at both the latter concentrations *ComCat*® increased the carrot root length, but this was only statistically significant in the case of the lower dosage of the two (Figure 4.7B).

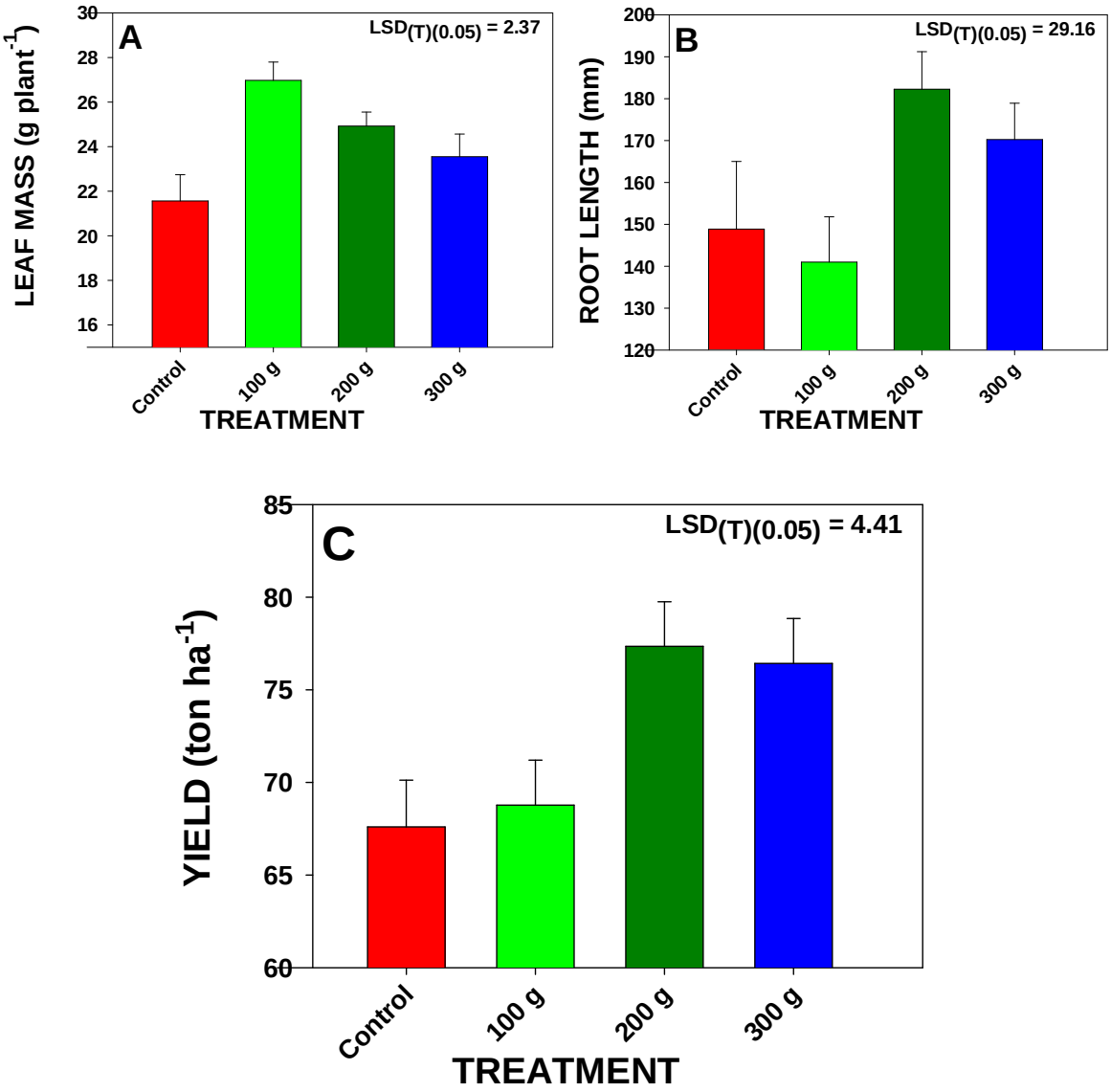

Figure 4.7: The growth and yield response of carrots, cv. Snakpak, to treatment with *ComCat*® VEG at different concentrations (100, 200 and 300 g ha^{-1}) when applied at the 3-4 leaf growth stage and again at 30% root development during the 2007/08 growing season. A = leaf mass, B = root length and C = root yield. $LSD_{(T)(0.05)}$ values are supplied in the graphs. Vertical bars = Standard diviation.

The yield response of carrots to the higher range *ComCat*® VEG concentrations followed the same pattern as did root length growth (Figure 4.7C).

Both the 200 and 300 g ha^{-1} dosages increased the yield significantly by 9.75 and 8.83 ton ha^{-1}, respectively, compared to the untreated control and the 100 g ha^{-1} application. Although the low (100 g ha^{-1}) concentration did not decrease the root yield as was the case with root length, its slightly enhancing effect was non-significant, compared to the control. However, compared to the higher concentration range treatments, the yield obtained with the low concentration was significantly lower.

Subsequently, only the recommended 200 g ha^{-1} was applied at the 3-4 leaf growth stage in season 2 (2008/09) using two different carrot cultivars, Fancy (Figure 4.8A) and Snakpak (Figure 4.8B). Although somewhat lower than the previous season, *ComCat*® VEG applied at the recommended rate increased the yield of both cultivars by 6.5 and 5.88 ton ha^{-1}, respectively. This was statistically significant in both cases.

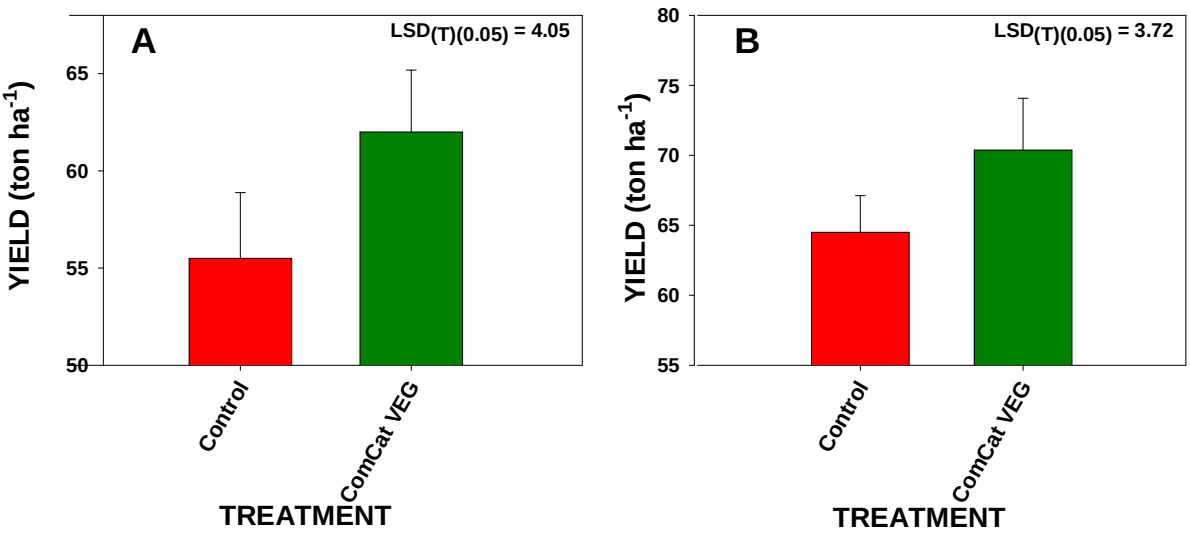

Figure 4.8: The yield response of two different carrot cultivars, A) Fancy and B) Snakpak, to treatment with *ComCat*® VEG at 100 g ha^{-1} when applied at the 3-4 leaf growth and again at 30% root development during the 2008/09 growing season. $LSD_{(T)(0.05)}$ values are supplied in the graphs. Vertical bars = Standard diviation.

4.4.5 The yield response of onions to foliar spray treatment with *ComCat*® ROW under semi-irrigation conditions

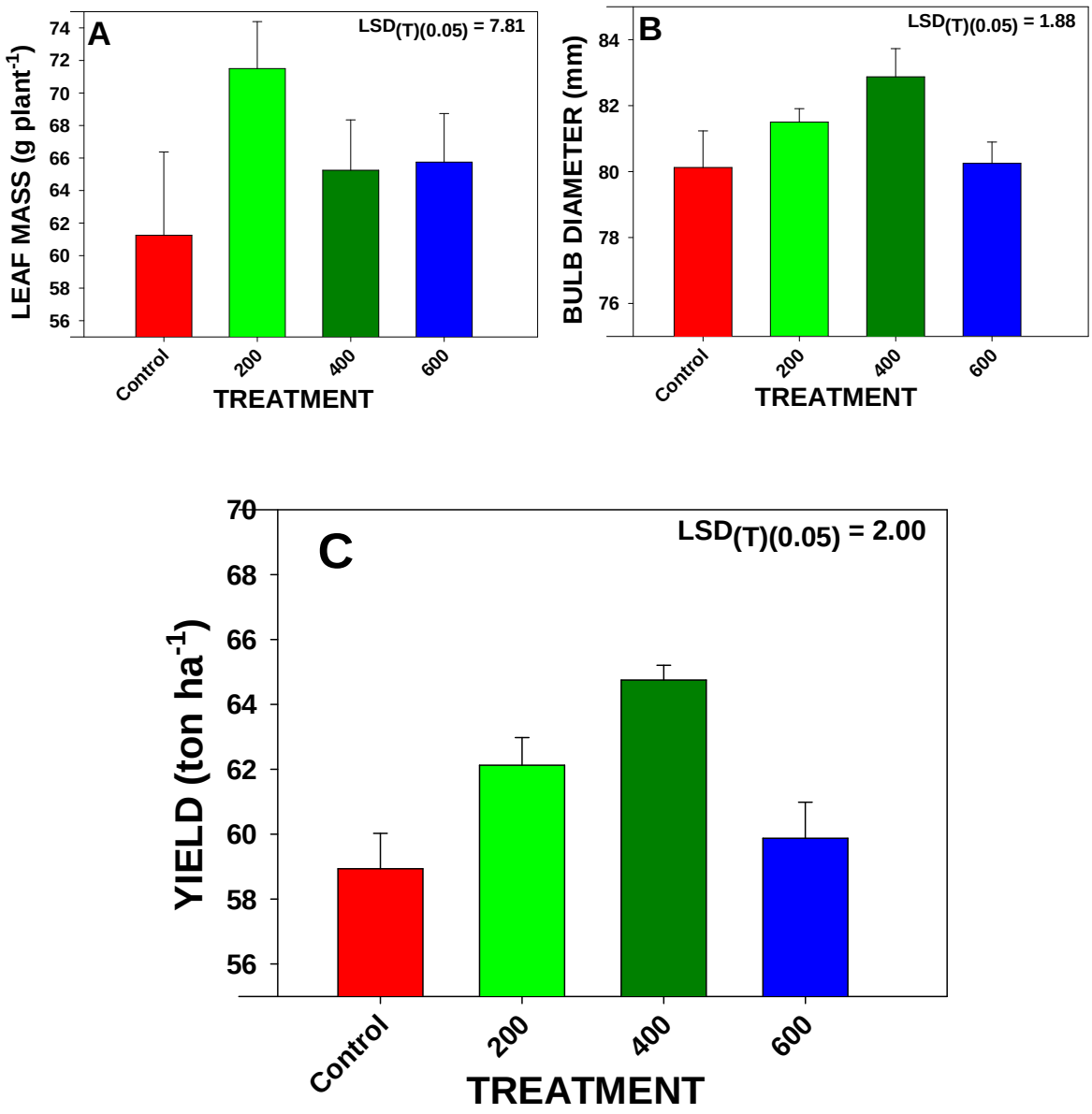

Figure 4.9: The growth and yield response of onions, cv. Australian Brown, to treatment with *ComCat*® VEG at different concentrations (100, 200 and 300 g ha^{-1}) when applied at the 3-4 leaf growth stage and again at 30% root development during the 2007/08 growing season. A = leaf mass, B = bulb diamete and C = bulb yield. $LSD_{(T)(0.05)}$ values are supplied in the graphs. Vertical bars = Standard diviation.

As was the case with carrots, a visual difference between top growth in onions, cv. Australian Brown, after treatment with *ComCat*® ROW at different concentrations led to measurement of both growth and yield during 2007/08. Similar to the response of carrots, the lowest concentration (200 g ha^{-1}) contributed to significant ($P<0.05$) and

the most pronounced (16.7%) enhancement of the onion leaf fresh mass (Figure 4.9A) compared to the untreated control. Although both the 400 and 600 g ha^{-1} concentrations contributed to an increase in leaf fresh mass, this was not statistically significant.

Treatment with *ComCat*® ROW at 200 and 400 g ha^{-1} resulted in a marked increase in onion bulb diameter (Figure 4.9B), compared to the untreated control, but this was only significant in case of the latter. The 600 g ha^{-1} concentration had neither a positive nor a negative effect on bulb diameter, compared to the control, but this was significantly lower compared to the 400 g ha^{-1} treatment.

Exactly the same pattern was observed for final bulb yield (Figure 4.9C) as was observed for bulb diameter, except that both the 200 (+3.29 ton ha^{-1}) and 400 g ha^{-1} (+5.91 ton ha^{-1}) treatments contributed to significant yield increases, compared to the control.

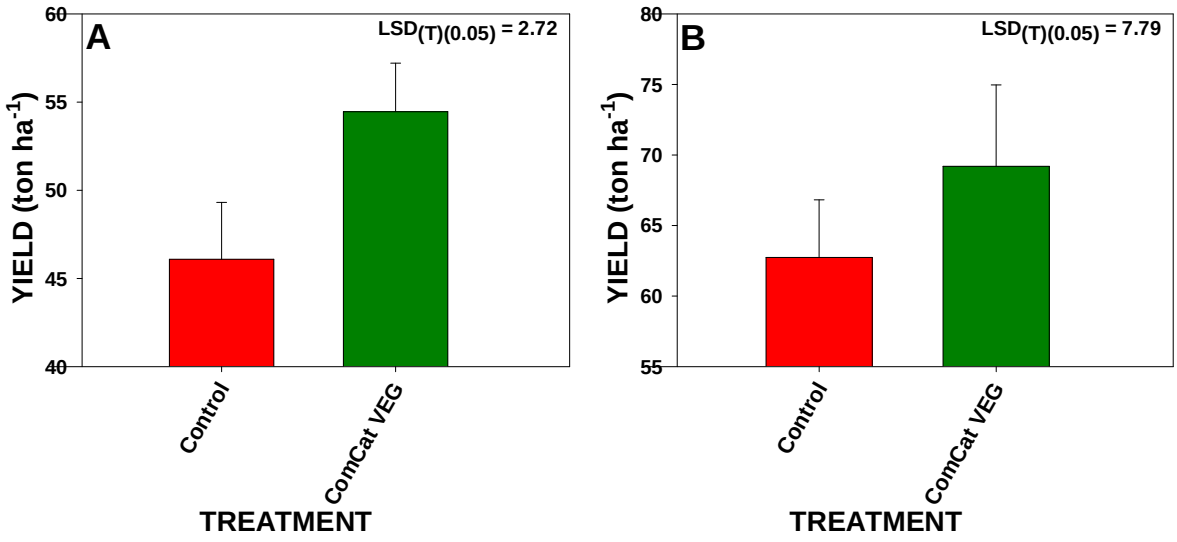

Figure 4.10: The yield response of two different onion cultivars, A) Texas Grano and B) Australian Brown, to treatment with *ComCat*® VEG at 400 g ha^{-1} when applied at the 3-4 leaf growth and again at 30% root development during the 2008/09 growing season. $LSD_{(T)(0.05)}$ values are supplied in the graphs. Vertical bars = Standard diviation.

During the 2008/09 growing season only the 400 g ha^{-1} *ComCat*® ROW treatment, that was found being optimal during the previous season, was tested by using two onion cultivars, Texas Grano (Figure 4.10A) and Australian Brown (Figure 4.10B). The yield of both cultivars was increased markedly by the treatment, but this

was only significant in the case of Texas Grano. The significant yield increase observed with Australian Brown during the previous season was not repeated during 2008/09.

4.5 Discussion

Exogenously applied brassinosteroids (BRs) have long been known to increase growth and yield in many economically useful plant species. However, its effectiveness on the growth and yield of crops depends on the plant species and concentration applied (Amzallag, 2002). For example, exogenous application of 100 µM of 24-epibrassinolide as a foliar spray was optimal for the growth and yield of rice (Krishnan *et al*., 1999), while for tomato it was 3 µM (Vardhini & Rao, 2001). These contrasting reports are probably due to the involvement of BRs in different biochemical reactions or via regulation of the concentrations of other hormones like auxin, gibberellins and cytokinins, which are involved in different growth aspects (Hola *et al*., 2010). According to Mussig *et al*. (2003) exogenously applied BRs had a positive influence on root growth in plants if its concentration is greater than its threshold value and this concentration is genotype specific.

Exogenous application of BRs improves the potential productivity of crops by activating cell elongation and vascular differentiation (Hayat & Ahmad, 2003) and/or the translocation of photosynthate from the source (leaves) to sinks by regulating the proton pump mechanism in membranes (Fujii & Saka 2001). Hayat & Ahmad (2003) reported a significant yield increase in wheat after foliar treatment with epibrassinolide at a rather low concentration. Hola *et al*. (2010) observed a significant yield increase in maize under field conditions when treated with 24-epibrassinolide or synthetic androstane, the analogue of castasterone, when applied either at the 3-4 or the 6-7 leaf stages. However, foliar application of BRs to maize plants at the 3-4 leaf growth stage also influenced vegetative growth by increasing the lengths of the 7^{th} to 10^{th} leaves, whereas application at the 6-7 leaf growth stage had the opposite effect. The authors concluded that the effect of BRs on crops depended on the developmental stage during which the application of BRs occured, the plant genotype, the type of BR and its concentration.

From the literature, and from an agricultural perspective, it is clear that comprehensive studies on each and every commercial crop will have to be

conducted in order to determine the commercial potential of BRs in the industry. Moreover, BRs will have to be tested at different concentrations and application times for each crop making it a vast venture (Khripach *et al*., 2000). However, based on the comprehensive studies that have been undertaken on BRs since its discovery as well as the very low concentrations needed for the manifestation of their effects on plants, BRs are logical candidates for agricultural application (Hola *et al*., 2010). The latter supports the rationale for the current study as stated in chapter 1.

What is important in terms of the current study is that the research done to date, as far as could be established, was on pure BRs either extracted from plants or synthesized. A vast number of publications are available in literature that confirm the positive growth and yield response of plants to treatment with purified BRs. To name a few, these include cereals (Takematsu & Takeuchi 1989; Sairam, 1994; Ramraj *et al*., 1997; Rao *et al*., 2002; Ali *et al*., 2008), leguminous crops (Vardhini & Rao, 1998), mustard and rapeseed (Hayat *et al*., 2000, 2001) and cotton (Ramraj *et al*., 1997).

Though pure BRs generally have a positive influence on growth, physiological and yield parameters of plants, their effect depends on various internal and external factors as mentioned previously (Hola *et al*., 2010). According to the authors, many BRs and BR-analogues that showed high biological activity in bioassays or controlled-environment experiments failed to stimulate plants grown in the field conditions. This is explained on grounds of various reasons including, inter alia, time of application in relation to the developmental stage of the plant (Khripach et al., 2000; Amzallag, 2002; Sasse, 2003), the length of plant exposure to BRs (Fariduddin *et al*., 2003, 2008), the frequency of application (Khripach *et al*., 2003) as well as plant species and genotype (Ali *et al*., 2008).

In this study a recently commercialized natural product that contains two known and one unknown brassinosteroid (Volz, 2000), *ComCat*®, was scrutinized for its potential to be applied in the agricultural industry. The rationale for the study was that *ComCat*® is probably one of the first BR-containing natural products that have been commercialized and, in the light of the statement by Ramraj *et al*. (1997) that chances are slim for BRs to be introduced into the agricultural industry on a commercial level, a comprehensive study on its potential was undertaken. The purpose of this study was to evaluate the efficacy of exogenously applied *ComCat*® to grain and vegetable crops regarding its potential to increase yields under field conditions. As a point of departure the recommendations of the manufacturers were

followed to a large extent in terms of the time of application and concentrations for the chosen test crops, except that different concentrations were tested during the first season (2007/08).

Results confirmed these optimal concentration recommendations for single cultivars of each crop as 100 g ha^{-1} for maize, 200 g ha^{-1} for wheat, cabbage and carrots and 400 g ha^{-1} for onions. As a result, only the optimal concentrations were applied during the second season (2008/09) while the cultivars tested during season one was included in the second season in order to establish the potential of the product to deliver repeatable results. Additionally, a second cultivar per crop was included during the second season in order to ascertain whether the product was cultivar sensitive in terms of its effect on yield.

Both cabbage cultivars (Conquistador and Drumhead) and carrot cultivars (Fancy and Snakpak) responded positively to treatment with *ComCat*® in the sense that the yield increases to which it contributed were statistically significant for all test cultivars, compared to the respective untreated controls. Clearly more cultivars of these crops will have to be tested to verify this assumption.

However, the yield increase induced in the maize cultivar PHI3394 was statistically significant over two seasons, but the tendency to increase the yield of the second cultivar, PAN6043, was not. This was positive for maize in terms of the product's repeatability, but indicated that it was cultivar sensitive. This was in concert with the genotype specificity of maize to treatment with BRs reported by Hola *et al*. (2010). A possible consideration is that maize was the only one belonging to the C4 group, but no indication could be traced in literature that emphasized this possibility. Nevertheless, a wider range of maize cultivars needs to be tested.

The opposite tendency was observed for wheat and onions where treatment with *ComCat*® significantly increased the yield of the wheat cultivar, Tugela, and onion cultivar, Australian Brown, during season one, but not during the second season, although a tendency to increase its yield was observed. Further, the yield of the second wheat cultivar, PAN3377, and onion cultivar, Texas Grano, was also increased significantly by the treatment, compared to the untreated control, indicating that it might be less cultivar sensitive. Again, more information on cultivar response to treatment with *ComCat*® is needed to verify the latter statement.

Importantly, *ComCat*® is not a pure BR-containing product, but also contains auxin, gibberillin, cytokinin, phytosterols, flavonoids and free amino acids in its composition (Agraforum AG, Germany). The latter, therefore, points towards the possibility that synergism between BRs and other natural hormones (Mandava, 1988) is a factor that might be involved in the product's mechanism of action. In this regard Tanaka *et al*. (2003) reported on the synergistic effect between BRs, auxin and gibberellin in terms of vegetative growth in *Arabidopsis thaliana*. They used brassinazole (Brz), a BR biosynthesis inhibitor, to elucidate the significance of endogenous BRs. It inhibited growth of roots, hypocotyls and cotyledonous leaf blades dose-dependently and independent of light conditions. However, cytological observation disclosed that a synergistic relationship of BR with gibberellin A3 (GA3) and indole-3-acetic acid (IAA) to induce elongation growth in light-grown hypocotyls exists. The authors concluded that endogenous BRs play an important role in the early growth of *Arabidopsis* and act on lightgrown hypocotyl elongation independent of, but cooperatively with, gibberellins and auxin.

However, contradictory evidence to the above has also been published. For example, BR-induced cell enlargement in an auxin-starved cultured cell line of carrot (Sala & Sala, 1985) and BR-promoted elongation of auxin-depleted soybean epicotyls (Clouse *et al*., 1992). Although this aspect does not fall within the scope of this study, future work with *ComCat*®, because it contains BRs and other plant hormones, should be aware of possible synergistic or antagonistic effects between BRs and these hormones. Further, no reports on the possible effect of synergism and/or antagonism between BRs, auxin and gibberellin on yield outcome could be traced in literature. This aspect needs to be considered in future research, if only by the manufacturers of the product that might feel the need to improve its efficacy.

The reason for the latter suggestion is that a strong relationship between vegetative growth and yield exists and too much vegetative growth too soon in a crop's life cycle might have a negative effect on its yield outcome (Boiffin *et al*., 2001). This is important when manipulatory chemicals such as fertilizer, or bio-stimulants for that matter, are applied to crops. In this study visual observation of top growth differences between untreated and *ComCat*® treated carrot and onion plants lead to the quantification of leaf growth in these two crops. In both cases the optimum *ComCat*® concentration applied induced aerial part growth markedly, but this was only significant in the case of carrots, compared to the untreated control.

Interestingly, the carrot cultivar where this was observed was Australian Brown that responded to treatment with a significant yield increase during season one and a non-significant tendency to do the same during the second season, despite the significant aerial part growth that was induced by the *ComCat*® treatment. The latter makes it difficult to come to a foregone conclusion in terms of the possible synergistic effect between BRs and other growth hormones contained in *ComCat*® and its collective effect on growth, and eventually yield. Nevertheless, future research on other test crops should consider the fact that *ComCat*® contains all of the naturally occurring phyto hormones in its composition and this might be one of the reasons why some crops are growth stage and concentration sensitive towards its application while others are cultivar sensitive.

In summary, laboratory bio-tests (chapter 3) and the outcome of field trials in this chapter have confirmed that *ComCat*® not only has bio-stimulatory properties, but has the potential to increase yields of the five crops tested and, therefore, possesses the potential to have an economic impact on the agricultural industry. Subsequently, it has been decided to continue with the search for the third unknown BR (Volz, 2000) contained in *ComCat*® by extraction, fractionation and purification (chapter 5).

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CHAPTER 5

ISOLATION, PURIFICATION AND IDENTIFICATION OF AN UNKNOWN BRASSINOSTEROID CONTAINED IN *ComCat*®

Abstract

Two liquid-liquid extraction methods, using organic solvents of different polarity, were employed to identify a new brassinosteroid (BR) in *ComCat*®, a natural product with bio-stimulatory activity recently commercialized for the agricultural industry. Six bio-assays were used during the activity directed fractionation procedure to follow the isolation of the unknown BR. These included *in vitro* bioassays such as the effect of *ComCat*® or fractions thereof on the respiration rate of monoculture yeast cells, seed germination, seedling and algae growth under laboratory conditions as well as *in vivo* bioassays such as crop growth under glasshouse and yield response under field conditions, as indicators of activity. Three out of the nine semi-purified fractions obtained with the two extraction methods showed high bio-stimulatory activity. By means of gas chromatographic analyses, using external BR-standards, the previously unknown compound was identified as 24-*epi*-brassinolide. Most of the latter was contained in the hexane fraction of method 1 and ethyl acetate fraction II of method 2.

Keywords: *ComCat*®, liquid-liquid extraction, semi-purification, active compounds, bio-stimulatory activity, 24-*epi*-brassinolide,

5.1 Introduction

Brassinosteroids (BRs) are found in very small amounts in plants (Zullo & Adam, 2002). It ranges from 10-100 mg kg^{-1} in pollen (Adam & Marquardt, 1986), 1-100 mg kg^{-1} in immature seeds (Mandava, 1988) and as low as 10-100 ng kg^{-1} in shoots and leaves (Takatsuto, 1994). Subsequently, special methods were developed for their detection and identification. Extraction of BRs from plant material can be achieved with standard partitioning and chromatographic processes where methanol or methanol/ethyl acetate followed by partitioning between water/chloroform and methanol/hexane can be used with success (Zullo & Adam, 2002). However, Gamoh *et al*. (1989) developed an extraction and semi-purification routine that has been applied by many researchers in the field. This implies initial extraction from plant

material with 100% methanol followed by extraction with a methanol:ethyl acetate (1:1; v/v) mixture. Subsequent fractionation with ethyl acetate:water (1:1; v/v) yields an ethyl acetate fraction that contains the BRs which are purified with further steps. The method of Gamoh *et al*. (1989), together with a standard extraction procedure using organic solvents with increasing polarity (Pretorius *et al*., 2008) was followed in this study.

After extraction and purification a sensitive bioassay is necessary to identify the BR-containing fractions obtained during the chromatographic steps. This is referred to as activity directed extraction. Initially, biological detection of BRs was performed by the bean second internode bioassay, a test gradually substituted by the rice lamina inclination bioassay (Wada *et al*., 1981, 1984; Takeno & Pharis, 1982; Kim *et al*., 1990) and the wheat leaf unrolling bioassay (Wada *et al*., 1985; Takatsuto, 1994). Although these bioassays are still used today, other bioassays were developed in this study (chapter 3) of which some were also applied in this chapter. These include *in vitro* bioassays such as the effect of *ComCat*® or fractions thereof on the respiration rate of monoculture yeast cells, seed germination and seedling growth under laboratory conditions as well as *in vivo* bioassays such as crop growth under glasshouse and yield response under field conditions.

Following activity directed extraction, fractionation and purification of specific active ingredients in a plant extract, different methods can be employed to identify the compounds. If external standards are available, gas chromatography (GC) and high performance liquid chromatography (HPLC) are popular and fast methods for this purpose (Takatsuto *et al*., 1982). High performance liquid chromatography is the method of choice for final purification in the isolation of natural BRs, but it is unusual for their detection in plant sources (Konstantinova *et al.*, 2001). Reversed phase high performance liquid chromatography is less sensitive than gas chromatography for the detection and quantification of BRs (Zullo & Adam, 2002)

Gas chromatography (GC), is a common type of chromatography used in analytic chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance or separating the different components of a mixture. The relative amounts of such components can also be determined and GC may help in identifying a compound after injection into the system in minute amounts (Pavia *et al*., 2006).

In GC the mobile phase is a carrier gas, usually an inert gas such as helium or a non-reactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing referred to as a column. The gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. As the chemicals exit the end of the column, they are detected and identified electronically while the different stationary phases cause each compound to elute at a different time, known as the retention time of the compound. The comparison of retention times is what gives GC its analytical usefulness (Pavia *et al*., 2006).

However, many natural compounds are difficult to detect if not derivatized. The latter entails the replacement or substitution of hydrogen in the compound with another functional group that is detectable by detectors forming part of GC or HPLC systems. Gas chromatography (GC) is the preferred method to detect BRs in plant material (Takatsuto *et al*., 1990). However, BRs have to be derivatized in order to be detected. For this purpose silylation, the replacement of hydrogen on the compound with an alkylsilyl group, is the most widely used derivatization technique in gas chromatogaphic separation (Sigma-Aldrich, 1997). The introduction of a silyl group serves to enhance mass spectrometric properties of derivatives by producing either more favourable diagnostic fragmentation patterns of use in structure investigations, or characteristic ions of use in trace analyses employing selected ion monitoring and related techniques.

In this study *ComCat*® was analysed gas chromatographically in its crude form as well as after fractionation by means of a number of organic solvents differing in polarity. In order to identify the third unknown BR contained in *ComCat*® (Volz, 2000) different internal standards were used.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 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). A mixture of two algal strains (*Scenedesmus obliquus*; 276-3a and 211-8b) used in one of the

bio-tests, was obtained from the Department of Plant Sciences, University of the Free State, South Africa.

5.2.1.2 Other materials

ComCat® in its crude form (active ingredients only) was purchased from Agraforum AG, Germany. Most of the organic solvents used during the purification process, namely methanol, ethanol, hexane, ethyl acetate, diethyl ether, dichloro methane and sodium bicarbonate, were purchased from Merck (Germany) and were of the purest grade available.

The following reagents were used for sample preparation or as standards for GC analysis: Ethyl acetate (Merck; Cat. No. 1.09623); Chloroform (Merck; Cat. No. 1.02445); Acetone (Merck; Cat. No. 1.00012); N,N Dimethylformamid (DMF; Merck; Cat. No. 1.03053); BIS Trimethylsilyl trifluoracetamide (=BSTFA; Sigma; Cat. No. T6381); 1-Trimethylsilyl imidazol (=TMSI; Aldrich; Cat. No. 15,358-3) and Sodium sulphate (Na_2SO_4 water free; Merck; Cat. No. 1.06649).

Betulin (Cat. No. B9757), used as an internal marker, was purchased from Sigma (Germany). Internal brassinosteroid standards included Epibrassinolide (Sigma; Cat. No. E1641), Brassinolide (CHEMICLONES INC. Cat No. 101), Castasterone (CHEMICLONES INC. Cat No. 102), 28-Homobrassinolide (CHEMICLONES INC. Cat No. 105) and 28-Homocastasterone (CHEMICLONES INC. Cat No. 106). All of these represented known active BRs were found in plants.

5.2.2 Methods

5.2.2.1 Preparation of crude extracts for fractionation

Two kg of crude *ComCat*® powder (one kg per extraction method; see 5.2.2.2), obtained from seeds of wild plants (names disclosed by manufacturer), that was ground to a fine powder using a Jet mill (Type PMT SJ50-ER100; GmbH, Austria) to particle size <100 micron, was used to prepare crude extracts initially. Either 95% or 100% methanol was used to prepare crude extracts depending on further purification steps (see.5.2.2.2), by covering the *ComCat*® powder with methanol at a rate of 4 ml g^{-1} and allowing extraction on a roller system. The 95% methanol extraction was done over 96 h, but the 100% methanol extraction over a period of one week. In both

cases the methanol supernatant was removed every 12 h by means of vacuum filtration through a double layer of Whatman No. 1 filter paper using a Buchner funnel placed on an Erlenmeyr flask fitted with a side arm connected to a suction pump. The removed supernatant was replaced with the same volume of methanol between removals. Pooled methanol extracts were vacuum distilled and concentrated at 35^{o}C using a Buchi rotavapor (Bibby Sterlin LTD, England) equipped with a cooled Liebig condenser, in order to remove the bulk organic solvent and fractionated further.

5.2.2.2 Fractionation of methanolic *ComCat*® crude extracts using two different methods

5.2.2.2.1 Method 1

A liquid-liquid extraction procedure (Pretorius *et al*., 2008) was followed in order to fractionate the aqueous crude extract remaining after vacuum distillation of the 95% methanol extract as outlined in 5.2.2.1. Fractionation was achieved by using organic solvents with increasing polarity at a ratio of 2:1 (v/v; solvent : crude extract). Solvents included hexane (DC= 1.9), diethyl ether (DC= 4.3), ethyl acetate (DC= 6.0) and dichloro methane (DC= 8.9; Figure 5.1). This was repeated 8 times with each solvent over a 96 h period by recovering each fraction between replacements through vacuum filtration. Pooled extracts were vacuum distilled to dryness overnight at 35°C using a Buchi rotavapor and the recovered contents were weighed (Figure 5.1).

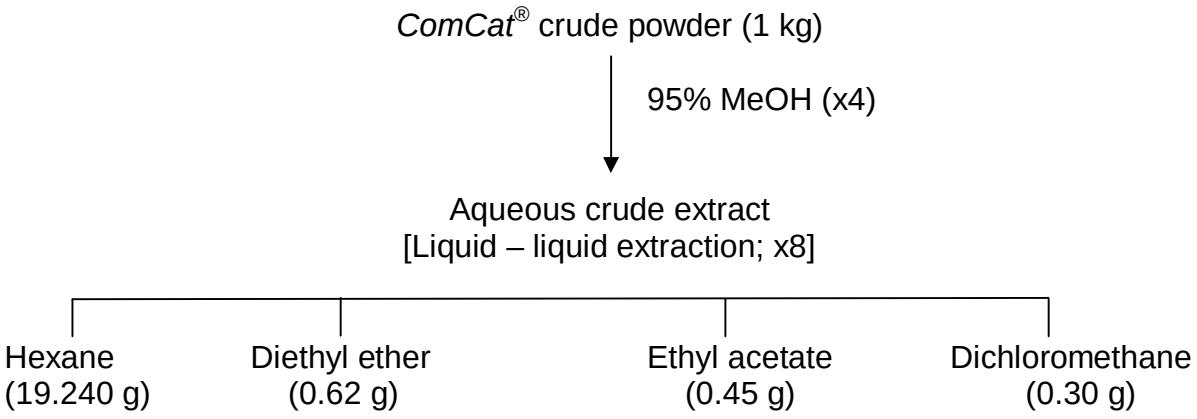


Figure 5.1: Liquid – Liquid extraction of crude *ComCat*® powder using organic solvents with increasing polarity. Masses of compounds recovered in each solvent from the initial kilogram of crude material are indicated in brackets.

5.2.2.2.2 Method 2

A liquid-liquid extraction procedure to specifically fractionate brassinosteroids and phytosterols from a 100% methanolic crude extract (5.2.2.1), as developed by Gamoh *et al.* (1989) and generally applied by Takatsuto *et al.* (1989) and Schmidt *et al.* (1996), was followed as outlined in Figure 5.2. This was done in order to separate any possible brassinosteroid or related compounds from any other possible active substances contained in the crude *ComCat*® extract. The most important fractions are numbered in Figure 5.2. These fractions are underlined and their corresponding numbers indicated in bold in the text.

One kg crude *ComCat*® powder was initially extracted with 100% methanol over one week as outlined in 5.2.2.1. Subsequently, the same plant material was extracted with an ethyl acetate:methanol mixture (1:1; v/v) for another week by following the same procedure as explained above. The methanol and ethyl acetate:methanol extractants were combined, mixed, vacuum filtered and distilled.

The compounds in the combined MeOH:Ethyl acetate fraction were further separated by fractionating three times overnight between ethyl acetate and water (1:1; v/v) and, finally, for 3 x ½ hour. Both the water and ethyl acetate fraction I obtained in this way were concentrated by means of vacuum distillation. Subsequently, compounds contained in both of these fractions were further separated by fractionating between hexane and 90% methanol (1:1; v/v) on a mechanical shaker for 3 x ½ hours. From this four important (according to Gamoh *et al*., 1989) fractions were obtained namely Hexane fraction I **(1)**, Hexane fraction II **(2)**, MeOH:Water fraction I **(3**) and MeOH:Water fraction II **(4)**. The mass of recovered compounds were determined for each of these fractions except **(4)** as this was fractionated further.

MeOH : Water fraction II **(4)** was fractionated further on grounds of a report by Gamoh *et al*. (1989) that BRs are contained in this fraction, together with other compounds. The methodology of the authors was followed in order to separate the BRs from these other compounds. This was achieved by first concentrating MeOH:Water fraction II **(4)** by vacuum distillation and separating the compounds contained in it by fractionating between a saturated $NaHCO_3$ solution (m/v; water) and ethyl acetate (1:1; v/v) on a mechanical shaker for 3 x ½ hour. The two fractions separated naturally in a separating funnel as ethyl acetate fraction II **(5)** and a

$NaHCO_3$ fraction **(6)** due to differences in specific gravity. According to Gamoh *et al*. (1989), the BRs are contained in ethyl acetate fraction II **(5).** Nevertheless, in this study all of the *ComCat*® fractions collected by means of methods 1 and 2 were assumed to be active and were, therefore, tested for bio-stimulatory activity by means of bio-tests (see 5.2.2.3). Only the most active fractions were further tested in glasshouse and field trials.

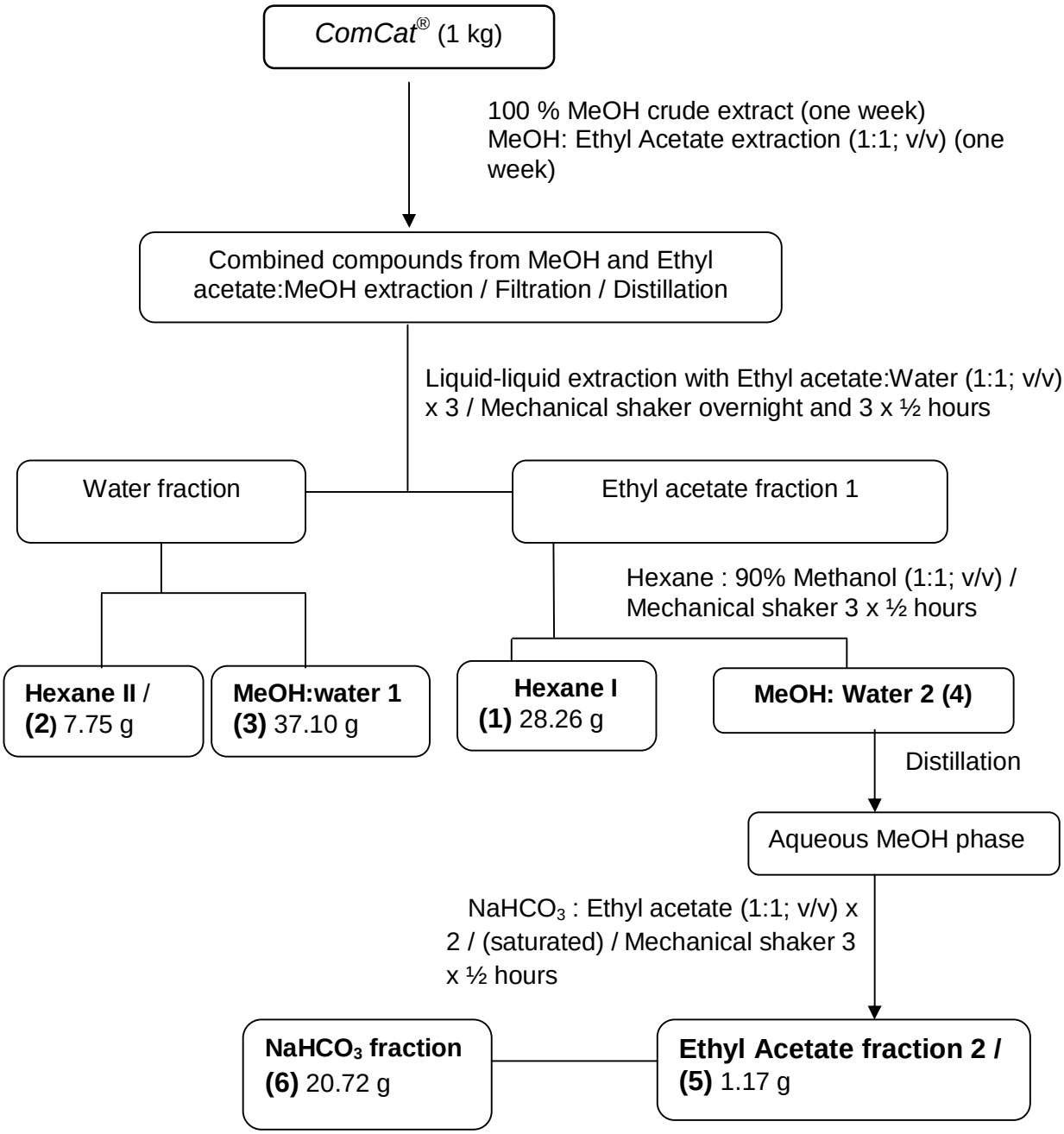


Figure 5.2: Outline of the procedure to specifically extract and fractionate brassinosteroids and phytosterols according to the method developed by Gamoh *et al.*(1989)**.** Masses of compounds recovered in each solvent from the initial kilogram of crude material are indicated in brackets.

5.2.2.3 Biotests

All four *ComCat*® extracts obtained with semi-purification method 1 (hexane, diethyl ether, ethyl acetate and dichloro methane) and five fractions obtained with method 2 (hexane I, hexane II, ethyl acetate II, $NaHCO_3$ and MeOH:Water 2) were subjected to two different biotests in order to assess bio-activity. These included the effect of semi-purified fractions on 1) the respiration rate of monoculture yeast cells and 2) the germination of pea seeds (5.2.2.3.1).

Subsequently, only the most active fractions from both methods were subjected to four additional biotests, namely the hexane fraction from method 1 as well as ethyl acetate II and the MeOH:Water fraction from method 2. The biotests employed in this case were two *in vitro* tests under laboratory conditions and two *in vivo* tests under either glasshouse or field conditions. *In vitro* biotests included the effect of semi-purified fractions on 1) seed germination and seedling growth of selected crops and 2) algae growth while *in vivo* tests included 3) wheat seedling growth over four weeks under glasshouse conditions and 4) the yield response of wheat under field conditions (5.2.2.3.2).

5.2.2.3.1 *In vitro* biotests for all fractions obtained with two extraction methods

The *ComCat*® product was included in all biotests as a positive control at 0.5 mg ℓ^{-1} (optimum concentration obtained previously; see chapter 3) while water served as a negative control. As the semi-purified fractions obtained by means of two extraction methods were concentrated, the following formula was used to calculate the % recovery of compounds per fraction and multiply it with the optimum 0.5 mg ℓ^{-1} concentration used for *ComCat*® in order to adjust fraction concentrations to a comparable level (calculated data is supplied in Table 5.1):

$$\frac{\text{Compounds (mg) recovered from 1kg } \textit{ComCat}^{®}}{\text{1 000 000 mg original } \textit{ComCat}^{®}} \times \frac{100}{1} \times 0.5 \text{ mg } \ell^{-1}$$

$$= \text{mg } \ell^{-1} \text{ of a specific fraction}$$

Table 5.1 Calculated concentrations of semi-purified fractions, obtained by means of two extraction methods, used in biotests.

Method and fractions	Compounds (mg) recovered from 1 kg *ComCat*® **[A]**	% Recovered $\frac{[A]}{1000000} \times 100$ **[B]**	Calculated concentrations of fractions based on 0.5 mg ℓ^{-1} for *ComCat*® **[B]** x 0.5 **(mg ℓ^{-1})**
Method 1			
Hexane	19240	1.920	0.00962
Diethyl ether	620	0.062	0.00031
Ethyl acetate	450	0.045	0.000225
Dichloromethane	30	0.003	0.00015
Method 2			
Hexane I	28260	2.826	0.01413
Hexane II	7750	0.775	0.003875
MeOH:water	37070	3.707	0.018535
Ethyl Acetate fraction 2	1170	0.117	0.00059
$NaHCO_3$ fraction	20720	2.072	0.01036

Biotest 1: Respiration rate of monoculture yeast cells

All semi-purified *ComCat*® fractions obtained by means of semi-purification method 1 and five fractions obtained by means of method 2 were tested as explained in chapter 3 (3.2.2.1).

Biotest 2: Germination rate of pea seeds

All semi-purified *ComCat*® fractions obtained by means of semi-purification method 1 and five fractions obtained by means of method 2 were tested as explained in chapter 3 (3.2.2.3).

5.2.2.3.2 *In vitro* and *in vivo* biotests for three most active fractions obtained with semi-purification methods 1 and 2

5.2.2.3.2.1 *In vitro* biotests

Biotest 3: **Seed germination and subsequent seedling growth of selected crops**

The three most active *ComCat*® fractions identified with biotests 1 and 2, namely the hexane fraction obtained with method 1 as well as ethyl acetate II and the MeOH:Water fractions from method 2, were tested as explained in chapter 3 (3.2.2.4).

Biotest 4: **The growth of algae under controlled conditions**

The three most active *ComCat*® fractions identified with biotests 1 and 2 were tested as explained in chapter 3 (3.2.2.7).

5.2.2.3.2.2 *In vivo* biotests

Biotest 5: Wheat seedling growth under glasshouse conditions

The three most active *ComCat*® fractions identified with biotests 1 and 2 were tested as explained in chapter 3 (3.2.2.6). The wheat cultivar, Tugela, was used. *ComCat*® ROW served as a positive control. Foliar applications were made at the 3-4 leaf growth stage (approximately two weeks after planting) and growth measured in terms of fresh mass two weeks later (four weeks after planting).

Biotest 6: Yield response of wheat under field conditions

The three most active *ComCat*® fractions identified with biotests 1 and 2 were tested under field conditions during the 2008/09 growing season as explained in chapter 4 (4.2.2 to 4.2.5). The wheat cultivar, Tugela, was used. The same trial outlay, planting conditions and fertilizer application that was used for wheat in the 2008/09 season was applied. Fertilizer was applied according to the withdrawal norms for wheat (Chapter 4; Table 4.4).

ComCat® ROW was used as a positive control at a rate of 200 g ha^{-1} while water was used as a negative control. The fractions obtained with semi-purification methods 1 and 2 were applied on a concentration basis (0.5 mg ℓ^{-1}) and not an area basis. The knapsack sprayer was calibrated to deliver a volume of 300 ℓ ha^{-1}.

5.2.2.4 Identification of the third unknown brassinosteroid contained in *ComCat*®

5.2.2.4.1 Preparation of reference solutions

5.2.2.4.1.1 Internal Standard Solution

One hundred mg Betulin was initially dissolved in 50 ml chloroform in a 100 ml measuring cylinder, chloroform added to the 100 ml mark and mixed thoroughly. The internal standard solution was added to all specimens during GC-analysis in order to standardize the volume injected.

5.2.2.4.1.2 Calibration solution

Five mg Betulin and 5 mg of a given brassinosteroid standard were initially dissolved in 4 ml chloroform, diluted to a final volume of 5 ml and mixed thoroughly. The calibration solution was used to determine the GC-profiles and retention time of the different BR-standards separately. This information was used to identify the unknown BR contained in *ComCat*®.

5.2.2.4.2 Sample preparation of a *ComCat*® crude extract and fractions thereof for gas chromatographic analysis using two methods

Method 1: Ethyl acetate used for preparing the *ComCat*® crude extract and fractions thereof

One hundred grams of ground seeds were initially extracted twice with 800 ml of 100% methanol in a steel container on a mechanical shaker for 1 h at room temperature. Each of the eluates was decanted after one hour and filtered separately through Whatman No. 1 filter paper. Both eluates were combined and vacuum distilled to dryness at 35°C in order to remove the methanol completely (see 5.2.2.1). This dry compound mixture is referred to as the crude extract.

Two to three grams of the crude extract were transferred to a 24 ml vial and mixed with 1 ml of an internal standard solution and 15 ml ethyl acetate. The mixture was well agitated on a mechanical shaker, centrifuged, dried over sodium sulphate and filtered into a 50 ml round-bottom flask. Subsequently, the filtrate was distilled under vacuum using a rotary evaporator and the dried extract redissolved in 1 ml N,N dimethylformamid (DMF) and 1 ml chloroform. Two hundred µl of the latter was silylated in a 1 ml vial with 100 µl N,O-Bis(trimethylsilyl)trifluoracetamide (BSTFA) and 100 µl 1-trimethylsilyl imidazol (TMSI) in an oven at 90°C for 12 hours.

Both TMSI and BSTFA are silylation agents. Silylation involves the replacement of hydrogen on the compound with an alkylsilyl group and is the most widely used derivatization technique in gas chromatogaphic separation. Functional groups derivatized by TMSI include hydroxyl, carboxylic acid, amine, thiol and phosphate groups while BSTFA is the preferred reagent for trimethylsilylation of steroids (Sigma-Aldrich, 1997).

Method 2: Acetone used for preparing the *ComCat*® crude extract and fractions thereof

Ten grams of ground seeds were initially extracted twice with 100 ml acetone in a steel container on a mechanical shaker for 1 h at room temperature. Subsequently, the same procedure was followed as described for method 1.

5.2.2.4.3 Gaschromatographic identification of the unknown brassinosteroid (Finzelberg method) in semi-purified *ComCat*® fractions after silylation using internal standards

A Hewlett Packard model HP 6890 gas chromatograph (GC) equipped with a fused-Silica-capillary column (CP SIL 5CB; 25 m in length; iD 0.25 mm; dF 0.4 µm) which provides the best resolution (highest efficiency) for the identification of the third BS containing 100% dimethylpolysiloxan as stationary phase, was used. For screening analyses of semi-purified *ComCat*® fractions a Shimadzu GC- 2010 Plus gas chromatograph (GC) equipped with a fused-Silica-capillary column (ZB-5HT; 15 m in length; iD 0.53 mm; dF 0.15 µm) which provide the greatest sample capacity and a shorter runtime containing 100% dimethylpolysiloxan as stationary phase, was used. The GC was operated via a computer using the GC Solution Software. In both GC-systems the conditions were as outlined in Table 5.2.

Table 5.2: GC conditions.

1. <u>Temperature</u>	
• Column oven	120°C (1 min isotherm) at 14°C min^{-} increased to 300°C (16,2 min isotherm) Run time : 30 min
• Injector	300°C
• Detector	310°C
2. <u>Gas supply</u>	
• Carrier gases	Hydrogen: Flow rate : 0.9 ml min^{-1} Split : 10:1 Septum Purge: 4.6 ml min^{-1}
• FID Detector	Flame gas: Hydrogen: 30 ml min^{-1} Flame air: Synthetic air: 400 ml min^{-1} Make-Up-Gas: Nitrogen: 30 ml min^{-1}
3. <u>Injection volume</u>	1 µl

5.2.2.5 Calculation for quantifying the % of the identified BR contained in the crude *ComCat*® extract and the three most active fractions thereof

$$KE = \frac{CS_E \cdot FS_{ISTD}}{FS_E \cdot CS_{ISTD}} = \text{Correction factor}$$

$$\% = \frac{K_E \cdot FP_E \cdot CP_{ISTD}}{CP \cdot FP_{ISTD}} \times \frac{\cdot 100}{1}$$

Where:

CS_{ISTD} = Concentration Betulin (mg ml^{-1}) in the calibration solution
CS_E = Concentration BR-standard (mg ml^{-1}) in the calibration solution
CP_{ISTD} = Concentration Betulin (mg ml^{-1}) in the sample solution
CP = Concentration of the sample (mg ml^{-1}) in the sample solution
FS_{ISTD} = Area of Betulin calibration solution
FS_E = Area of BR-standard in the calibration solution
FP_{ISTD} = Area of Betulin in the sample solution
FP_E = Area of BR-standard in the sample solution

5.2.5 Statistical analysis

Analysis of variance (ANOVA) was performed on the data, using the NCSS 2000 statistical program, to identify differences between treatments. Tukey's mean significant difference (MSD) procedure for comparison of means (Steele & Torrie, 1980) was applied to separate means on a 5% (P<0.05) probability level. Although the response of seeds, seedlings, algae or yeast cells to treatment with *ComCat*® or fractions thereof was measured at intervals over a specific time period, statistical analysis was only done at the end of the period.

5.3 Results

5.3.1 Biotest 1: *In vitro* effect of semi-purified *ComCat*® fractions on the respiration rate of monoculture yeast cells

Although the respiration rate of monoculture yeast cells treated with different semi-purified *ComCat*® fractions was measured at 15 min. intervals over a two hour incubation period, statistical analysis was only done once after two hours. Already over the first 80 min all of the fractions contributed to a marked increase in the respiration rate of yeast cells, compared to the negative control (Figure 5.3).

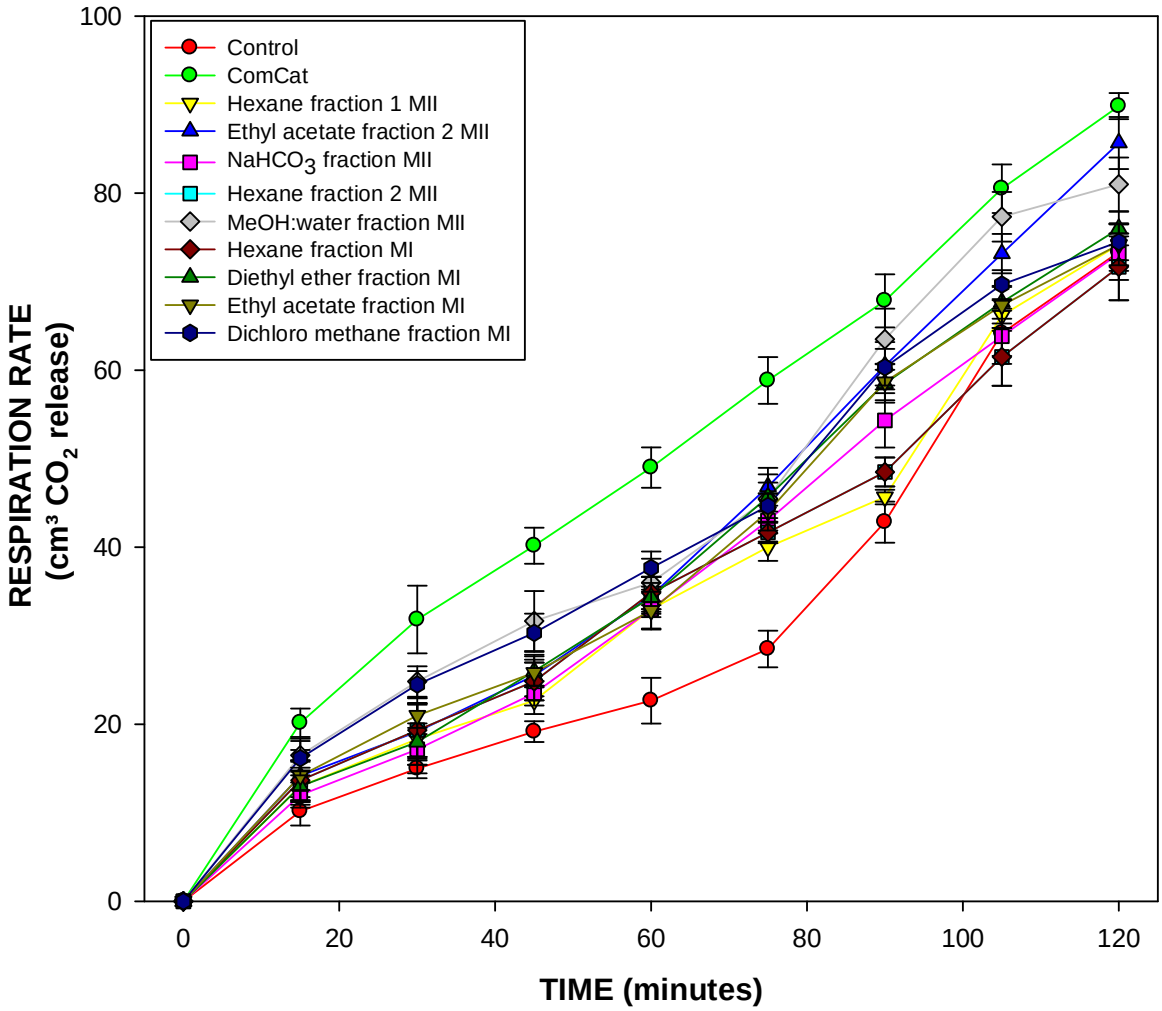


Figure 5.3: The respiratory response of monoculture yeast cells to treatment with 10 different semi-purified *ComCat*® fractions at 15 minute intervals over a 2 hour incubation period. *ComCat*® ROW was used as a positive control while water, containing only glucose as a respiratory substrate, served as a negative control. M I = semi-purification method 1 and M II is method 2 (see Figures 5.1 and 5.2). Vertical bars = Standard diviation.

However, after 120 min the respiration rate of monoculture yeast treated with only the positive *ComCat*® control, ethyl acetate fraction 2 and the MeOH : water fraction obtained with semi-purification method two, differed significantly from the negative control (Table 5.3). None of the fractions obtained with the liquid-liquid extraction method 1 contributed to significant inhibition or stimulation of the respiration rate of monoculture yeast cells.

Table 5.3: Statistical analysis of the respiration rate of monoculture yeast cells 120 minutes after treatment with semi-purified *ComCat*® fractions. The commercial product, *ComCat*® ROW, was used as a positive control while water containing only glucose as respiration substrate served as a negative control

Application	Respiration rate (cm^3 CO_2 release)	
Control (glucose solution)	73.33 ± 3.14	
ComCat® 0.5 mg ℓ^{-1}	89.83 ± 1.47	s
Hexane fraction 1 M II	74.17 ± 2.40	ns
Ethyl acetate fraction 2 M II	85.67 ± 2.94	s
$NaHCO_3$ fraction M II	73.17 ± 1.94	ns
Hexane fraction 2 M II	71.67 ± 3.78	ns
MeOH : water fraction M II	81.00 ± 3.03	s
Hexane fraction M I	71.50 ± 1.52	ns
Diethyl ether fraction M I	76.00 ± 1.90	ns
Ethyl acetate fraction M I	74.17 ± 2.32	ns
Dichloro methane fraction M I	74.50 ± 2.07	ns
$LSD_{(T)(0.05)}$	**5.62**	

M I = semi-purification method one and M II = method two. **s** = significant difference and **ns** = non-significant difference from negative control.

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 of *ComCat*® fractions on seed germination and seedling growth was also tested.

5.3.2 Biotest 2: *In vitro* effect of semi-purified *ComCat*® fractions on the germination rate of pea seeds

Although the germination rate of pea seeds treated with different semi-purified *ComCat*® fractions was measured at 24 h intervals over a 96 h incubation period, statistical analysis was only done once after 96 h. After 24 h it was only the hexane fraction obtained with Method I as well as hexane fraction 1 and the MeOH : water fraction obtained with Method II that accelerated the germination of pea seeds markedly compared to both the positive and negative controls (Figure 5.4). At 48 h exactly the same pattern was observed except that hexane fraction 2 from Method II had a similar enhancing effect on pea seed germination as did the previous three fractions. This tendency prevailed at 72 h of incubation. However, at 96 h the positive control as well as hexane fraction MI, MeOH : Water fraction MII and ethyl acetate fraction 2 MII contributed to significant enhancement of pea seed germination (Figure 5.4; Table 5.4).

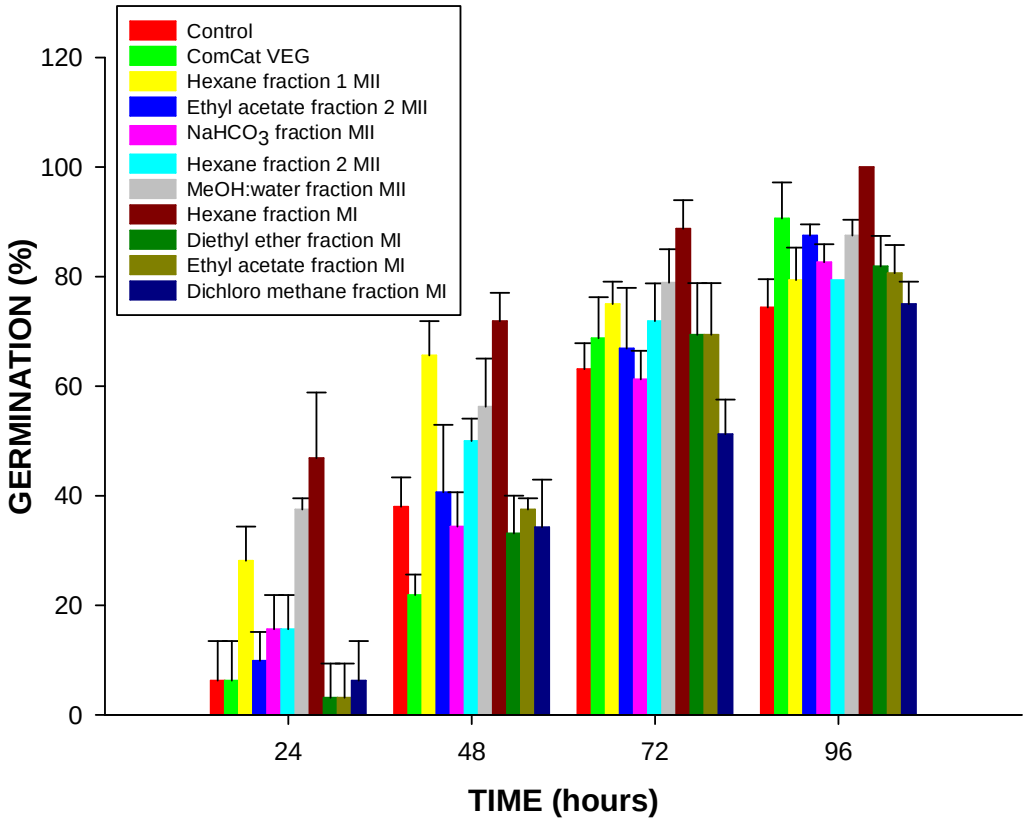


Figure 5.4: The germination response of pea seeds to treatment with 10 different semi-purified *ComCat*® fractions at 24 h intervals over a 96 hour incubation period. *ComCat*® ROW was used as a positive control while water served as a negative control. M I = semi-purification method 1 and M II is method 2 (see Figures 5.1 and 5.2). Vertical bars = Standard diviation.

Table 5.4: Statistical analysis of the germination rate of pea seeds 96 h after treatment with semi-purified *ComCat*® fractions. The commercial product, *ComCat*® ROW, was used as a positive control while water served as a negative control.

Application	Germination rate (%)	
Control (water)	74.38 ± 5.15	
ComCat® 0.5 mg ℓ^{-1}	90.63 ± 6.57	s
Hexane fraction 1 M II	79.38 ± 5.91	ns
Ethyl acetate fraction 2 M II	90.63 ± 2.04	s
$NaHCO_3$ fraction M II	82.63 ± 3.30	ns
Hexane fraction2 M II	85.63 ± 3.75	ns
MeOH : water fraction M II	87.50 ± 2.89	s
Hexane fraction M I	100.0 ± 0	s
Diethyl ether fraction M I	81.86 ± 5.54	ns
Ethyl acetate fraction M I	80.63 ± 5.15	ns
Dichloro methane fraction M I	75.00 ± 4.08	ns
LSD $_{(T)(0.05)}$	**12.39**	

M I = semi-purification method one and M II = method two. **s** = significant difference and **ns** = non-significant difference from negative control.

Based on biotests 1 and 2, only the three most active fractions were tested further. These included the hexane fraction from semi-purification method 1 as well as ethyl acetate fraction 2 and the Methanol : Water fraction obtained with method 2.

5.3.3 Biotest 3: *In vitro* effect of the three most active *ComCat*® fractions on seed germination and seedling growth of selected crops

Although the germination rate for seeds from selected crops, treated with different semi-purified *ComCat*® fractions, as well as subsequent seedling growth were measured at 24 h intervals over a 96 h incubation period, statistical analysis was only done once after 96 h.

5.3.3.1 Cabbage

As shown in Figure 5.5A, *ComCat*® (0.5 mg ℓ^{-1}) that served as a positive control as well as all the three semi-purified fractions tended to enhance the germination of cabbage seeds over the first 72 h of incubation. However, at 96 h none of these differed significantly from the negative water control. The same tendency prevailed for subsequent seedling growth, but only in terms of root growth (Figure 5.5B), where growth induction was significant in all cases compared to the water control. No significant differences in coleoptile growth (Figure 5.5C) between any of the treatments were observed, compared to the negative control.

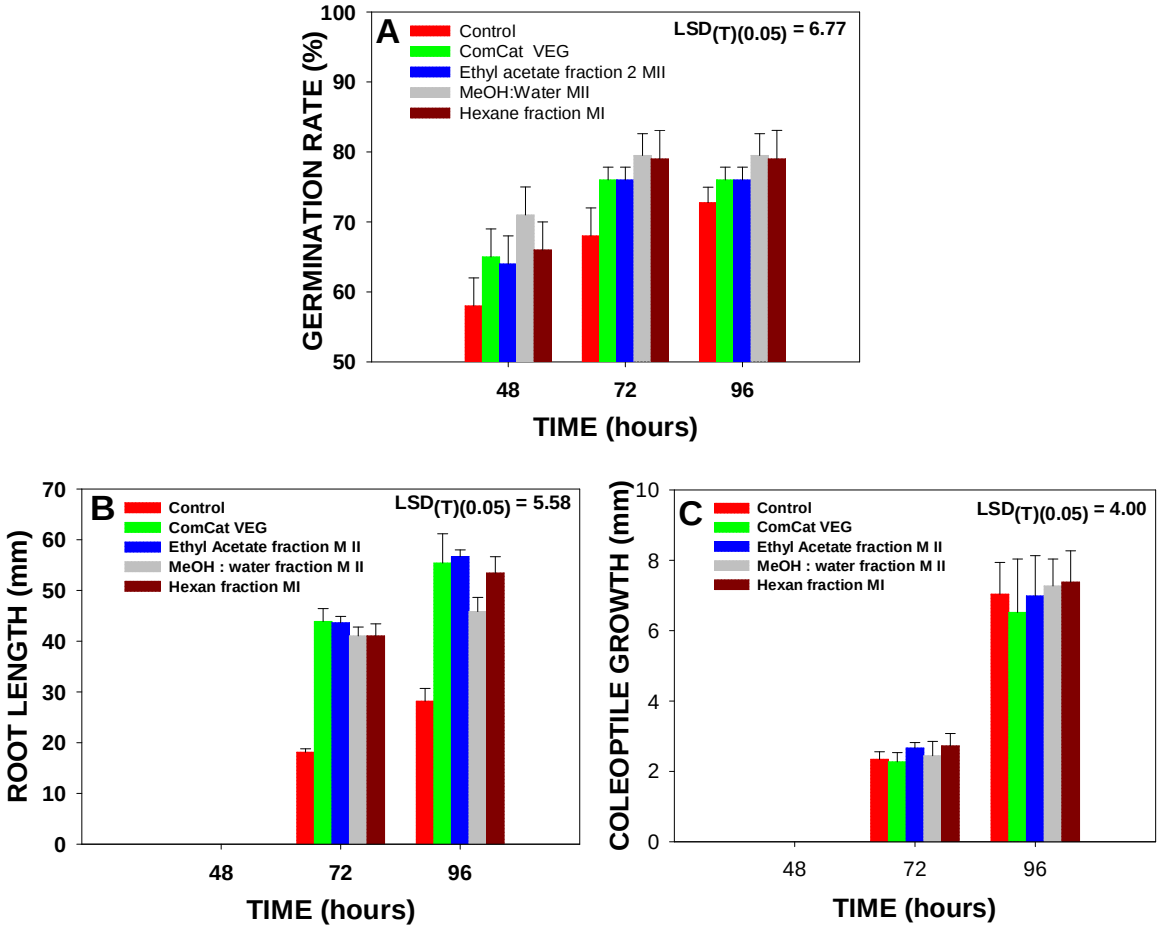


Figure 5.5: The germination and seedling growth response of cabbage to treatment with the three most active semi-purified *ComCat*® fractions at 24 h intervals over a 96 hour incubation period. *ComCat*® VEG was used as a positive control while water served as a negative control. M I = semi-purification method 1 and M II is method 2 (see Figures 5.1 and 5.2). Vertical bars = Standard diviation. LSD $_{(T)(0.05)}$ values are indicated in the graphs.

5.3.3.2 Pea

Over the first 72 h it was only the hexane fraction (method 1) and the MeOH : Water fraction (method 2) that tended to accelerate the germination of pea seeds while ethyl acetate fraction 2 (method 2) had no effect compared to the negative control (Figure 5.6A). Interestingly, during the same time period *ComCat*® VEG, that served as positive control, either delayed germination at 48 h or had no marked effect after 72 h of incubation. However, compared to the negative control, all fractions as well as the positive control contributed to a significant increase in pea seed germination while the hexane fraction (method 1) differed significantly from both controls (Figure 5.6A).

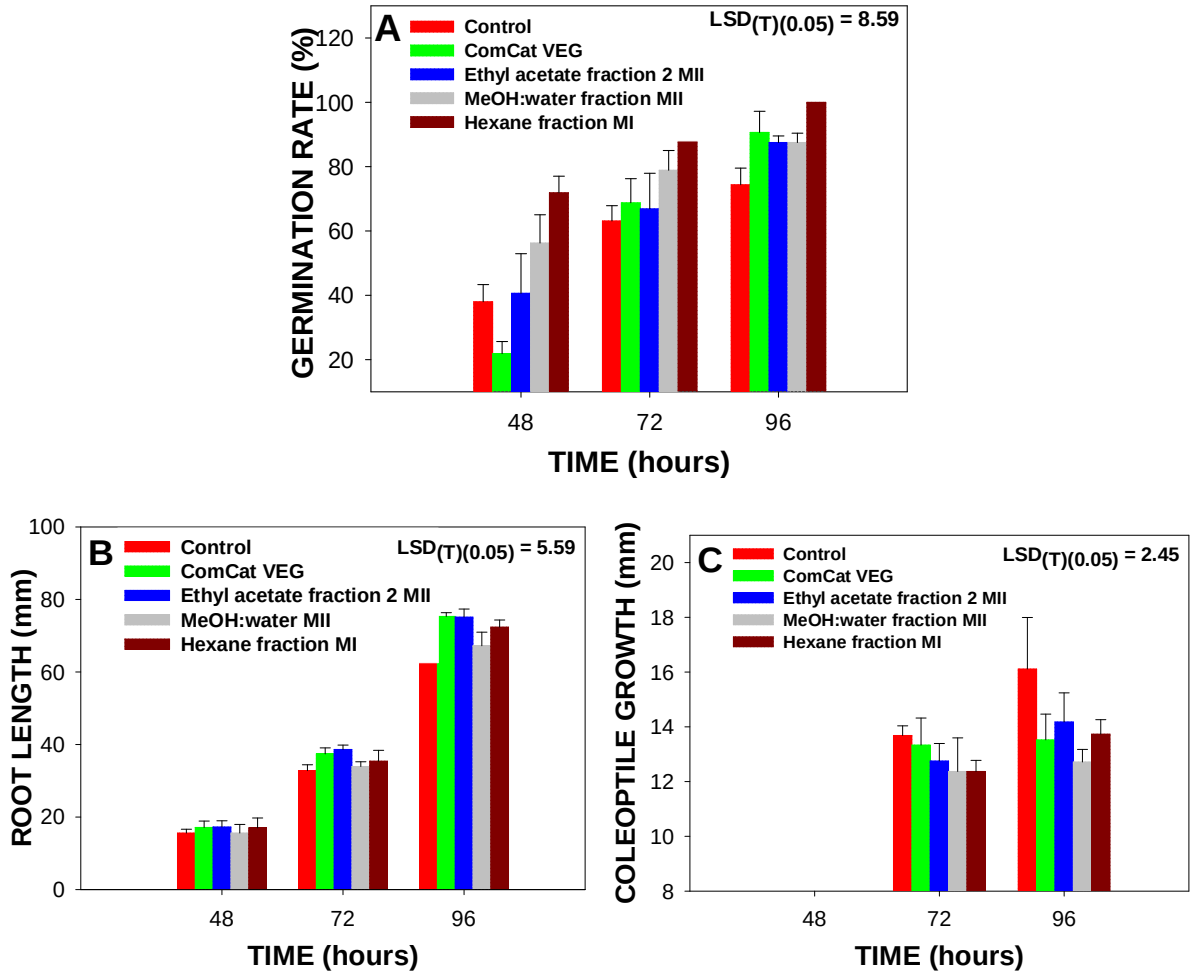


Figure 5.6: The germination and seedling growth response of pea to treatment with the three most active semi-purified *ComCat*® fractions at 24 h intervals over a 96 hour incubation period. *ComCat*® VEG was used as a positive control while water served as a negative control. M I = semi-purification method 1 and M II is method 2 (see Figures 5.1 and 5.2). Vertical bars = Standard diviation. LSD $_{(T)(0.05)}$ values are indicated in the graphs.

After 96 hours of incubation the hexane fraction (method 1) induced root growth of pea seedlings markedly while *ComCat*® and ethyl acetate fraction 2 (method 2) increased root length significantly ($P<0.05$) in pea seedlings, compared to the water control (Figure 5.6B). This tendency was already observed after 72 h of incubation. However, *ComCat*® and all of the fractions thereof significantly inhibited coleoptile growth in pea seedlings (Figure 5.6C).

5.3.3.3 Wheat

Although *ComCat*® accelerated wheat seed germination 48 h after treatment and the ethyl acetate fraction (method 2) tended to have the same effect at 72 h of

incubation, compared to the negative control, none of these differences were statistically significant at 96 h (Figure 5.7A).

Interestingly, and different from the vegetable crops, *ComCat*® ROW that served as a positive control had no effect on either root (Figure 5.7B) or coleoptile (Figure 5.7C) growth of wheat seedlings, compared to the water control. However, at 96 h incubation all the three semi-purified fractions thereof markedly induced both root and coleoptile growth. In terms of root growth (Figure 5.7B) this was only significant ($P<0.05$) in case of the ethyl acetate fraction while growth induction by all three fractions was significant in case of coleoptile growth (Figure 5.7C).

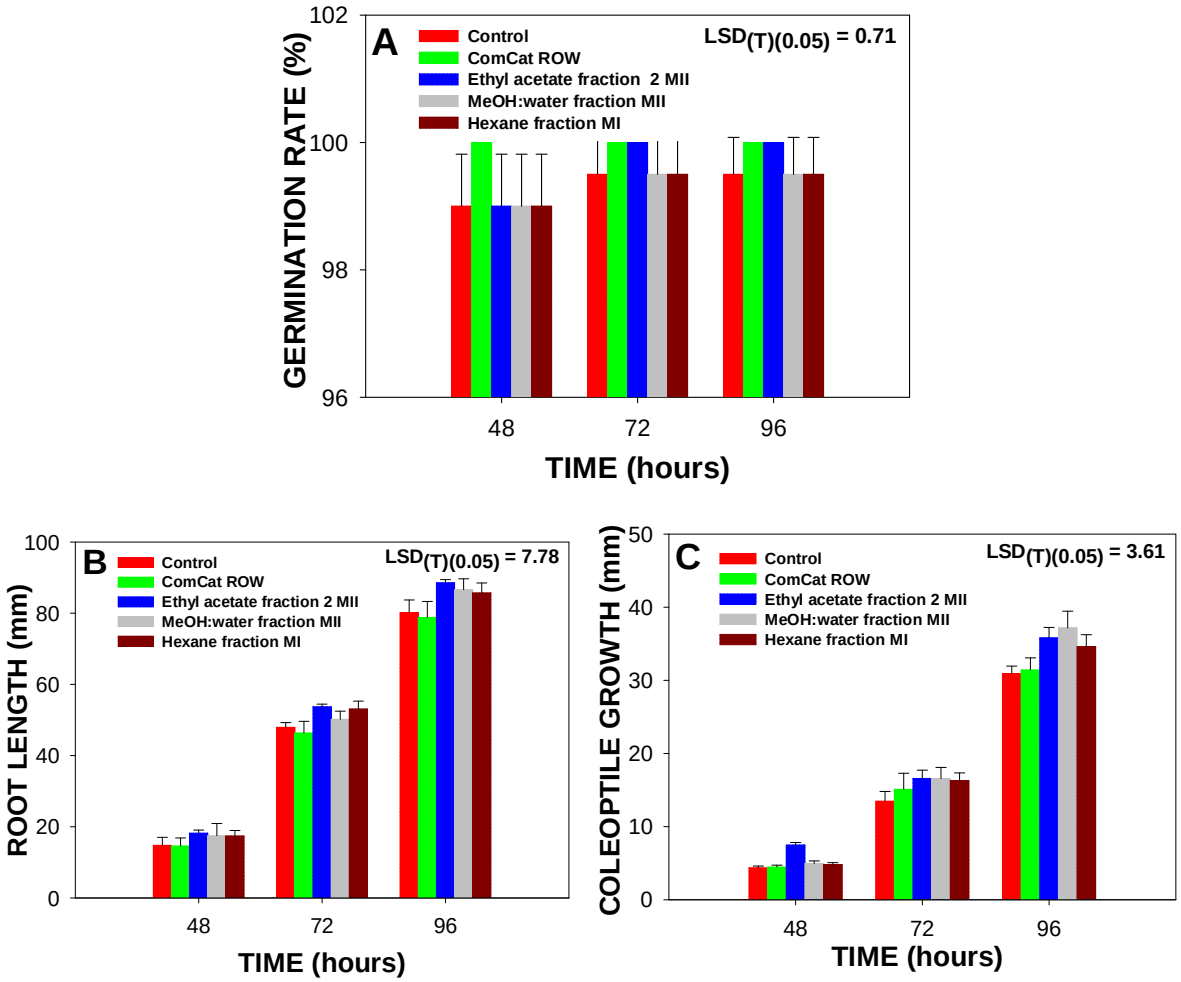


Figure 5.7: The germination and seedling growth response of wheat to treatment with the three most active semi-purified *ComCat*® fractions at 24 h intervals over a 96 hour incubation period. *ComCat*® ROW was used as a positive control while water served as a negative control. M I = semi-purification method 1 and M II is method 2 (see Figures 5.1 and 5.2). Vertical bars = Standard diviation. LSD $_{(T)(0.05)}$ values are indicated in the graphs.

5.3.4 Biotest 4: Effect of the three most active *ComCat*® fractions on the wheat seedling growth after 4 weeks under glasshouse conditions

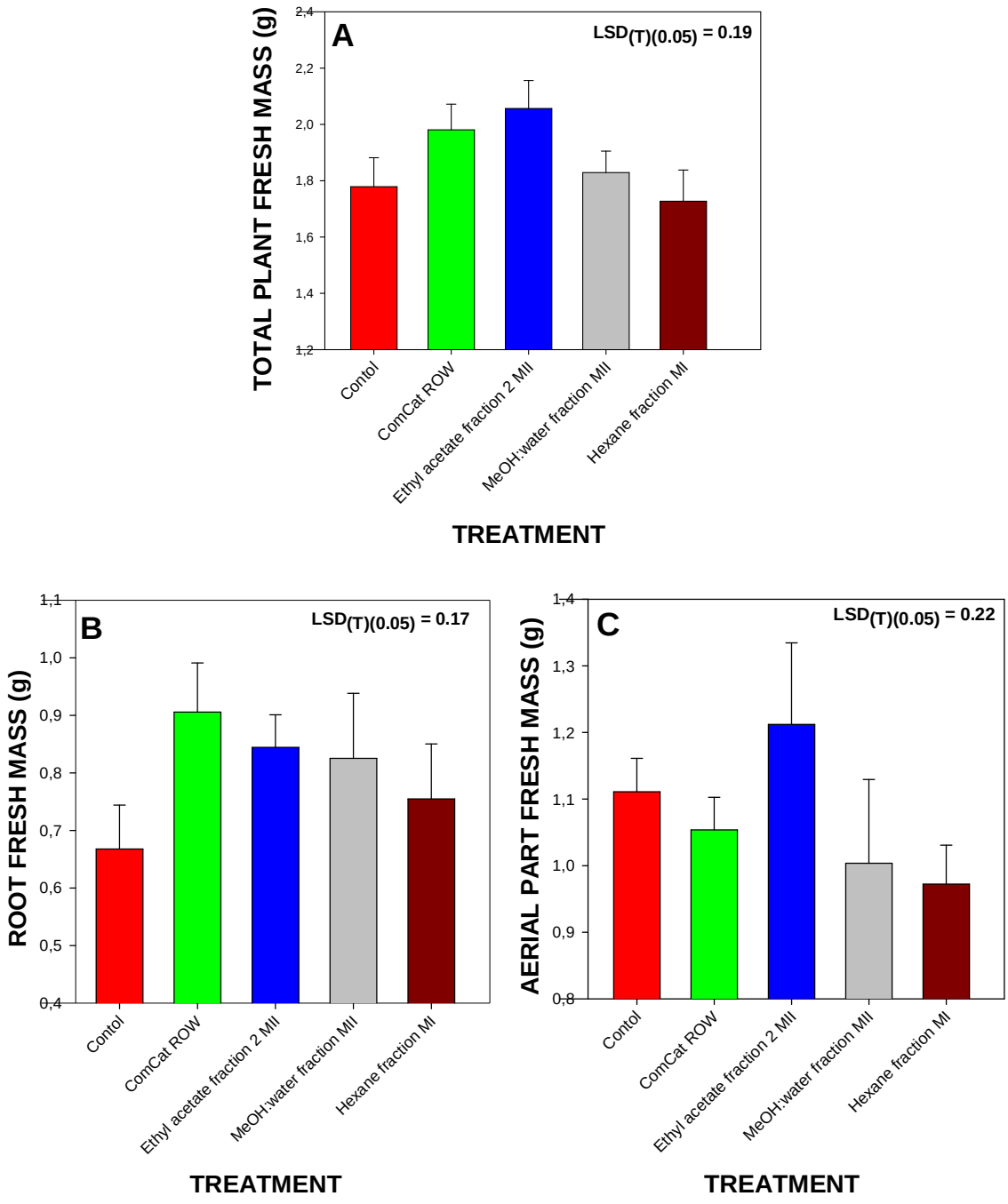


Figure 5.8: Effect of the three most active *ComCat*® fractions, foliarly applied two weeks after planting, on root and aerial part growth of wheat seedlings measured four weeks after planting. *ComCat*® ROW was used as a positive control and water as a negative control. Vertical bars = Standard diviation. LSD $_{(T)(0.05)}$ values are indicated in the graphs.

Compared to the water control, total plant fresh mass of wheat seedlings was enhanced markedly by *ComCat*® and ethyl acetate fraction 2 (method 2) four weeks after planting, but this was only significant ($P<0.05$) in case of the latter (Figure 5.8A). However, all three fractions as well as the positive control contributed to a marked increase in root fresh mass (Figure 5.8B). Significant differences, however, were only observed in case of the enhancing effect by *ComCat*® (+35.61 %) and ethyl acetate fraction 2 (26.44 %; Figure 5.8B).

None of the treatments stimulated aerial part growth (Figure 5.8C) in the four week old wheat seedlings, compared to the water control. Although application of the ethyl acetate fraction tended to do so (9.1%), this was not statistically significant. Compared to the negative control, *ComCat*® and all other fractions thereof contributed to a reduction in aerial part fresh, although not significantly. A significant difference between the slight enhancement of aerial part growth by ethyl acetate fraction 2 and the reduction by the hexane fraction obtained with method 1 was observed. Interestingly, treatment of wheat seeds before planting with this hexane fraction tended to decrease total plant as well as aerial part fresh mass.

5.3.5 Biotest 5: The growth response of algae to treatment with the three most active *ComCat*® fractions

Treatment of an algae culture with *ComCat*® and all three of the fractions thereof increased algae growth markedly after nine days of incubation (Figure 5.9). Compared to the water control, this was statistically significant ($P<0.05$) in all cases except for the hexane fraction. Although statistical analysis was only performed after the ninth day, the tendency to enhance algae growth by all treatments was already observed three days after treatment and the pattern remained up to the end of the incubation period. The most pronounced growth enhancing effect was observed for the ethyl acetate fraction (+67 %) followed by the MeOH : Water fraction (+47.87 %) and *ComCat*® (+37.23 %). After nine days of incubation algae growth induction by the ethyl acetate fraction was also significantly higher than that by the hexane fraction.

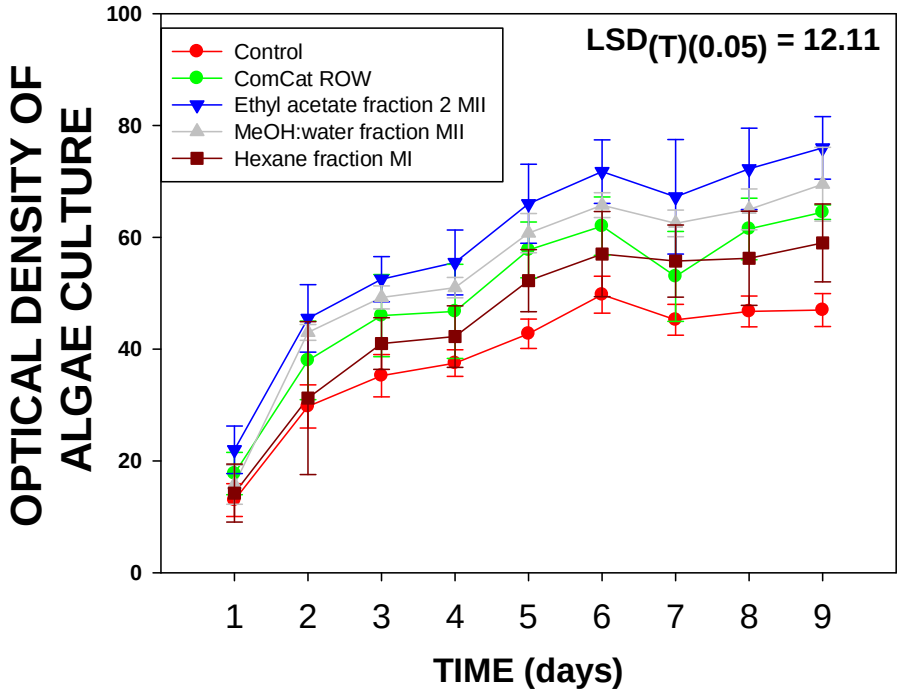


Figure 5.9: The growth response of algae (*Scenedesmus obliquus*) to treatment with the three most active *ComCat*® fractions at one day intervals over nine days. *ComCat*® ROW was used as a positive control and water as a negative control. Vertical bars = Standard diviation. The LSD $_{(T)(0.05)}$ value is indicated in the graph.

5.3.6 Biotest 6: The effect of the three most active *ComCat*® fractions on the final yield of wheat under field conditions

A single foliar spray treatment of wheat, cv. Tugela, at the 3-4 leaf growth stage (growth stage 13-14; Meier, 1997) with *ComCat*® (+0.43 ton ha^{-1}) and the ethyl acetate fraction (+0.31 ton ha^{-1}) increased the kernel yield significantly ($P<0.05$) compared to the untreated control (Figure 5.10). Although treatment with the MeOH : Water as well as the hexane fraction enhanced the kernel yield by 0.24 and 0.16 ton ha^{-1} respectively, this was not statistically significant. The yield increase obtained with *ComCat*® (positive control) was also significantly higher than that obtained with the hexane fraction. The effect of the latter was not significantly different from the untreated control.

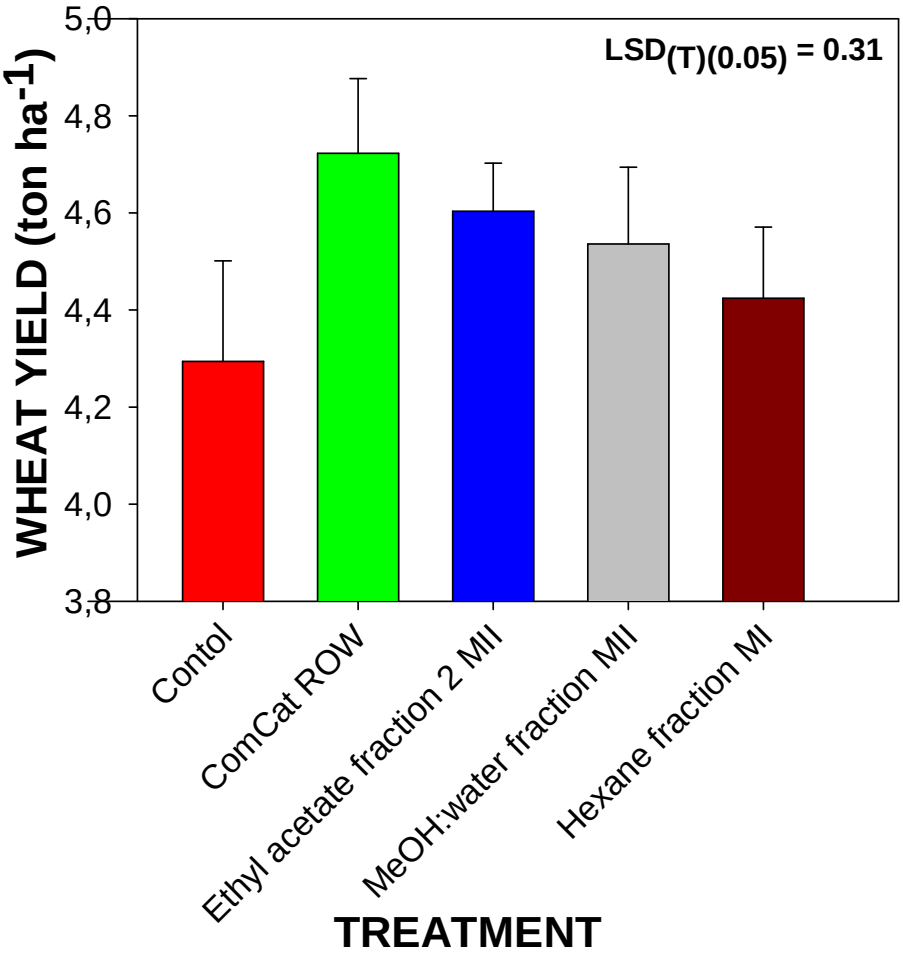


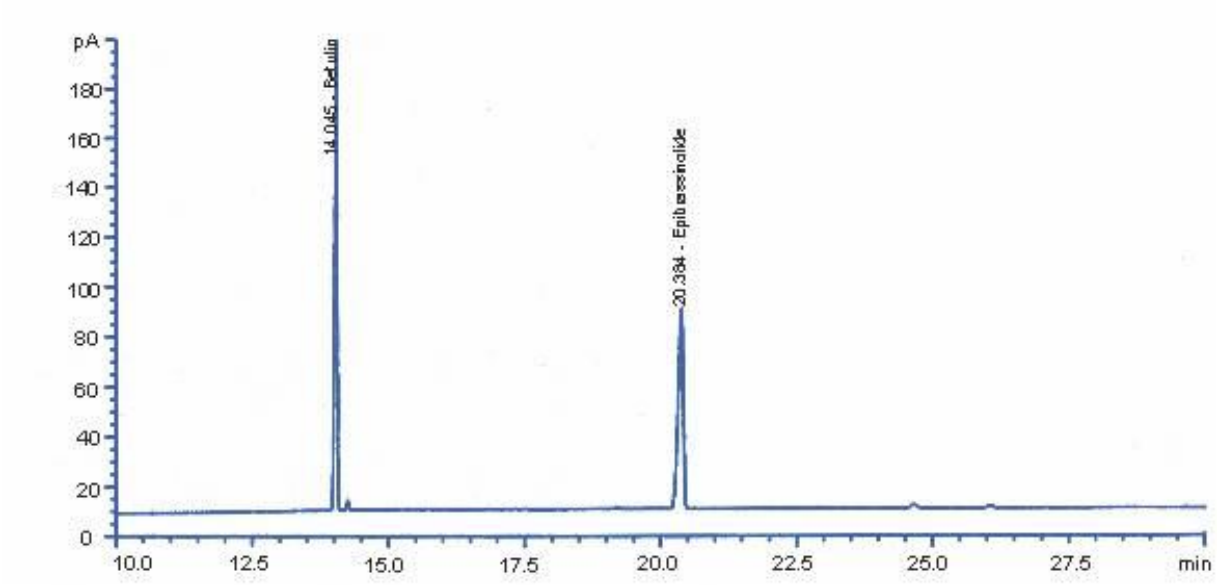
Figure 5.10: The effect of the three most active ComCat® fractions on the yield of wheat, cv. Tugela. *ComCat®* ROW was used as a positive control and water as a negative control. Vertical bars = Standard diviation. The LSD $_{(T)(0.05)}$ value is indicated in the graph**.**

5.4 Identification of the third unknown BR contained in *ComCat®* by means of gas chromatography (GC)

With the aim of identifying the unknown BR (Volz, 2000) contained in *ComCat®* gas chromatographically, a series of internal BR-standards was used. These included Brassinolide, Castasterone, 28-Homobrassinolide, 28-Homocastasterone and Epibrassinolide. Initially calibration with each BR-standard was performed separately in order to obtain GC-profiles as well as repeatable retention times for each. Betulin was included as standard in all GC-analyses to ensure that the injection volume maintained constant for all GC-runs. Additionally, a full gas chromatographic profile of the *ComCat®* extract was obtained. Due to the voluminous extent of data presentation for each BR-standard, GC-profiles of only the eventually identified BR, namely Epibrassinolide, are presented.

5.4.1 GC-profile of the Epibrassinolide standard

The GC-profile of Epibrassinolide, including the Betulin standard, is shown in Figure 5.11.



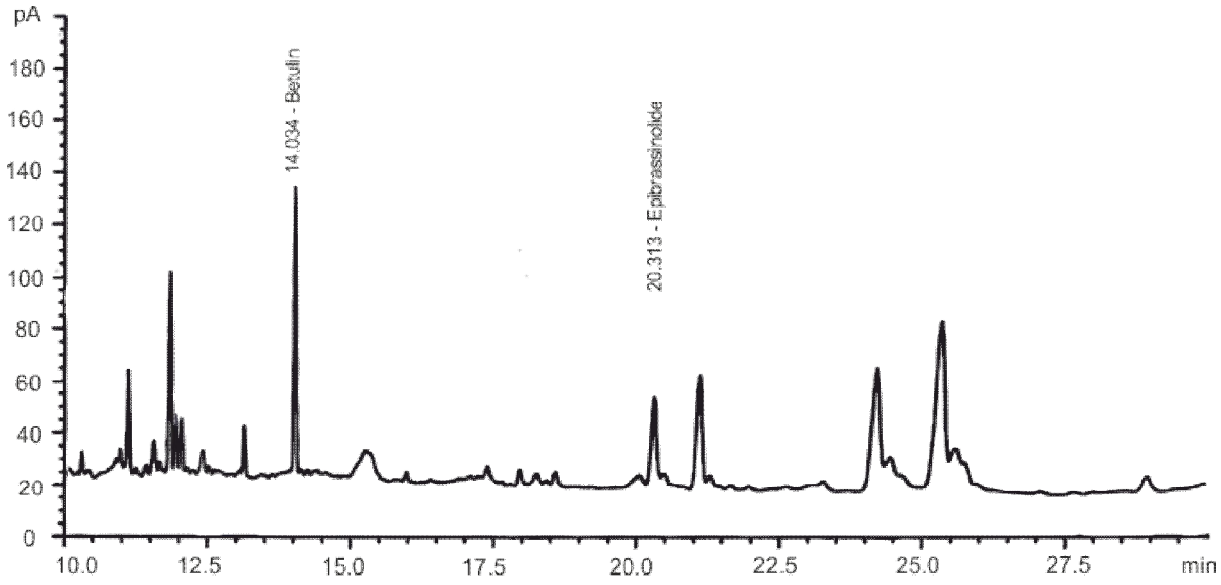
ISTD Percent Report

Retention time (min)	Area (pA*s)	Name of standard
14.028	632.54999	Betulin
20.384	520.30579	Epibrassinolide

Figure5.11 Gas chromatographic profile for the calibration solution containing Epibrassinolid and Betulin as internal standards.

5.4.2 GC-profile of crude *ComCat*® extract with ethyl acetate (method 1) containing the Betulin standard but no BR-standard

The GC-profile of the crude *ComCat*® extract, extracted with ethyl acetate according to method 1(5.2.2.4.2) and containing only the Betulin standard, confirmed that the unknown BR in *ComCat*® was Epibrassinolide (Figure 5.12). The volume of Betulin injected was half that of the calibration run (Figure 5.11) and contained 1.0 x 10^{-4} mg of crude *ComCat*®.



ISTD Percent Report

Retention time (min)	Area (pA*s)	Name of standard
14.034	310.75000	Betulin
20.313	205.60272	Epibrassinolide

Figure 5.12: Gas chromatographic profile of crude *ComCat*® extracted by means of method 1 (5.2.2.4.2).

5.4.3 Overlay GC-profile of the crude *ComCat*® extract and the calibration solution containing Betulin and BR-standards

In order to assess whether retention times of pure standards in GC profiles (Figure 5.11) coincided with that of the crude *ComCat*® extract (ethyl acetate extracted; Figure 5.12) an overlay profile was prepared (Figure 5.13). The main reason was to ascertain whether compounds other than BRs contained in *ComCat*® had an effect on retention times observed when GC profiles of pure standards were obtained separately. From this it could be observed that the peaks of standards corresponded with the peaks of the crude extract and were a perfect match.

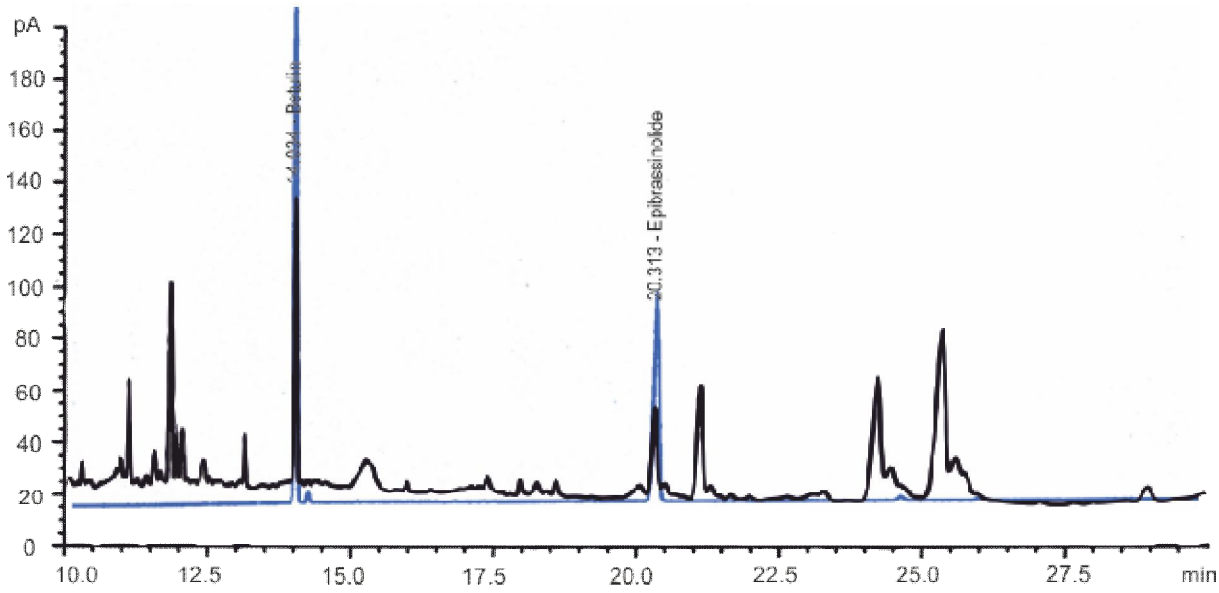
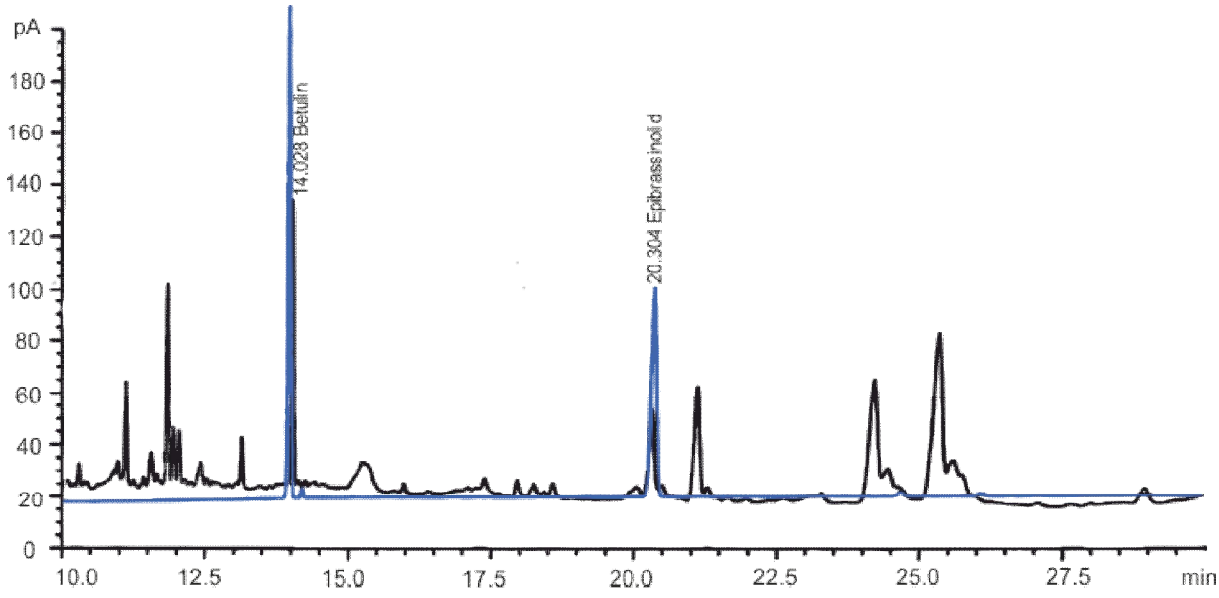


Figure 5.13: Overlay gas chromatographic profile of internal standards (blue line) and the *ComCat*® crude extract (black line).

5.4.4 Overlay GC-profile of crude *ComCat*® extract with acetone (method 2) containing the Betulin standard but no BR-standard



ISTD Percent Report

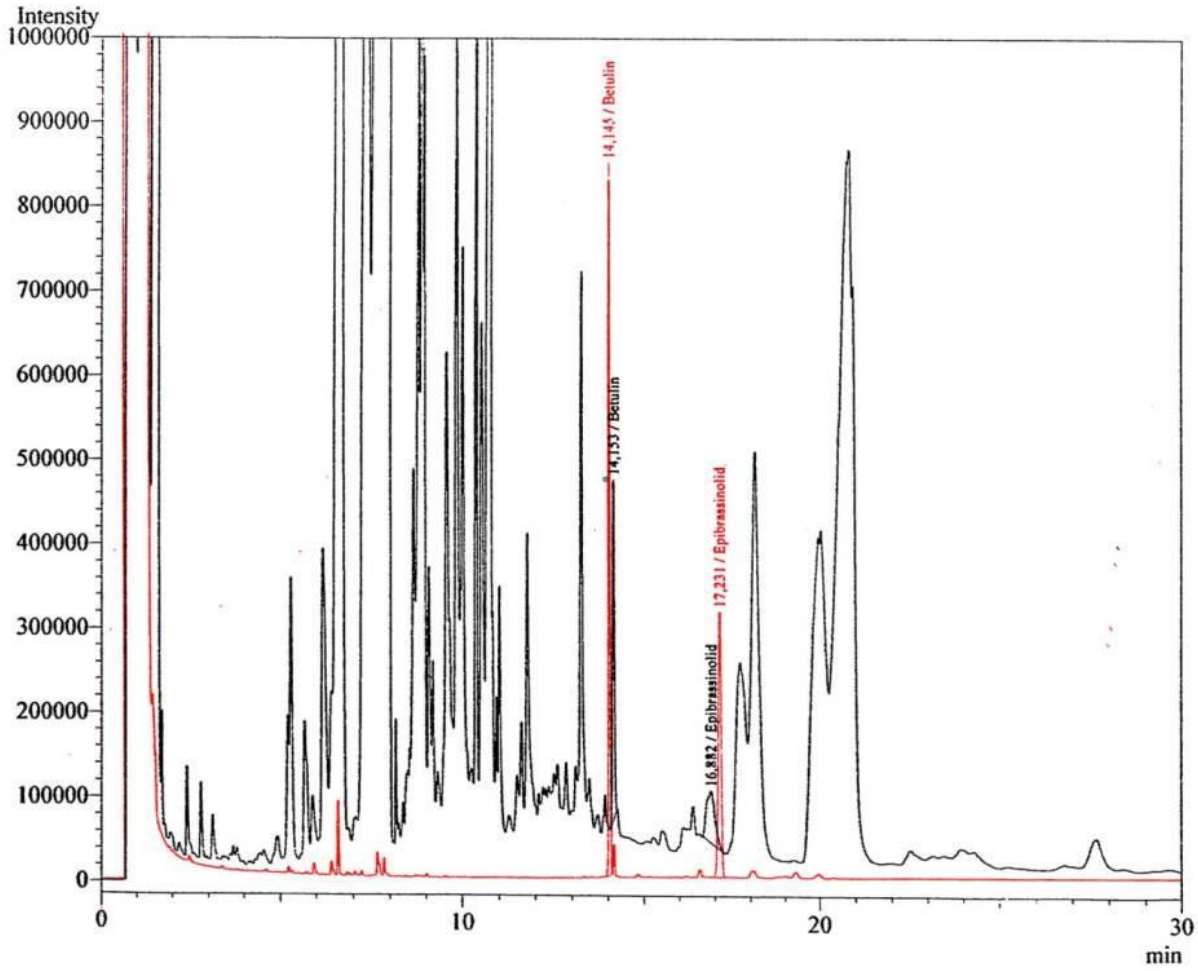
Retention time (min)	Area (pA*s)	Name of standard
14.028	290.02115	Betulin
20.304	139.24292	Epibrassinolide

Figure 5.14: Overlay gas chromatographic profile of internal standards (blue line) and crude *ComCat*® (black line) extracted by means of method 2 (5.2.2.4.2).

In order to confirm these results a second sample preparation with acetone as solvent was done (Figure 5.14). Here also Epibrassinolide could be identified.

5.5 Gas chromatographic profiles of the *ComCat*® crude extract as well as hexane, ethyl acetate and MeOH : water fractions thereof

5.5.1 Overlay GC-profiles of the crude *ComCat*® extract and the calibration solution containing Betulin and BR-standards



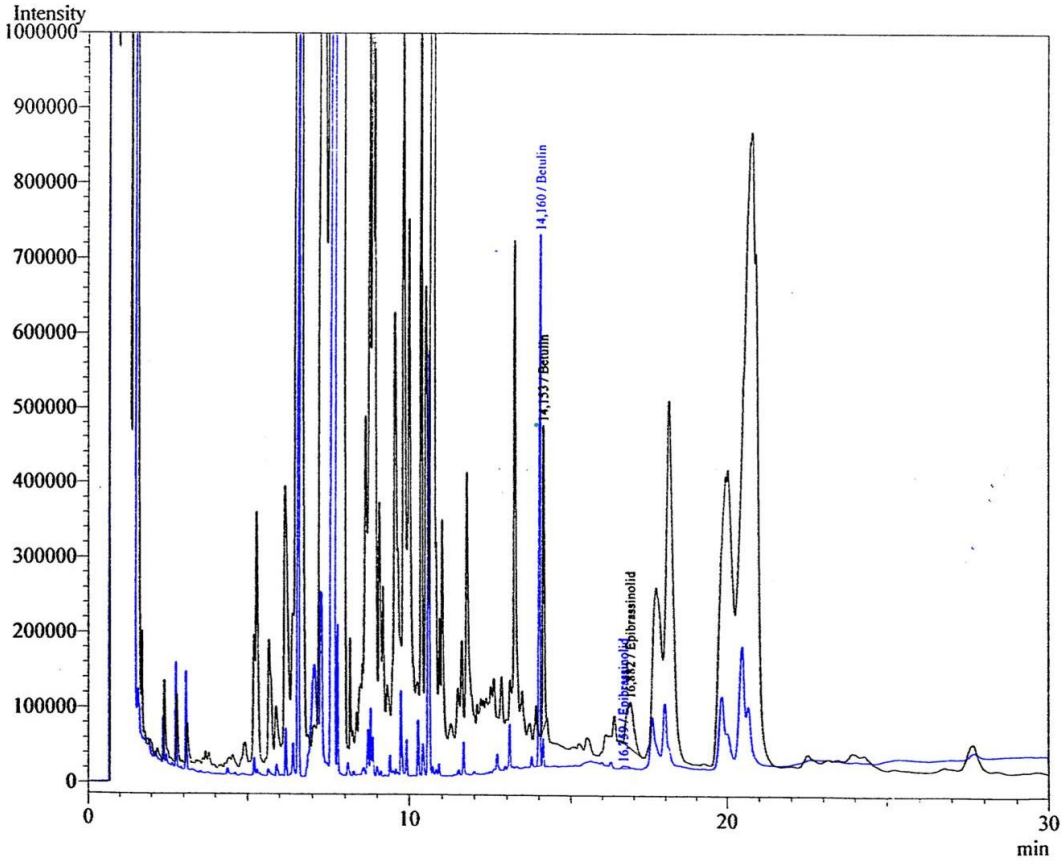
ID#	Name	Ret. Time	Area	Conc.	Units
1	Betulin	14.145	2308061	20.000	µg ml^{-1}
2	Epibrassinolide	17.231	1830774	20.000	µg ml^{-1}
Peak#	**Name**	**Ret. Time**	**Area**	**Conc.**	**Units**
1	Betulin	14.153	1521342	13.372	µg ml^{-1}
2	Epibrassinolide	16.882	1005198	10.981	µg ml^{-1}

Figure 5.15: Overlay gas chromatographic profiles of internal standards (red line) and the *ComCat*® crude extract (black line), using a different GC-system.

For comparing the gas chromatographic profiles of *ComCat*® crude extract with hexane, ethyl acetate and MeOH : water fractions thereof, a different Gas

chromatograph with a shorter column was used. To obtain a profile of the full range of compounds in the product and fractions thereof, gas chromatographic detection was started at 0 min up to 30 min to compare the three fraction profiles with that of the crude extract (Figure 5.15). Here Epibrassinolide was detected at an earlier retention time due to the shorter column. The peaks of standards corresponded with the peaks of the crude extract.

5.5.2 Overlay gas chromatographic profile of the *ComCat*® crude extract and a hexane fraction thereof



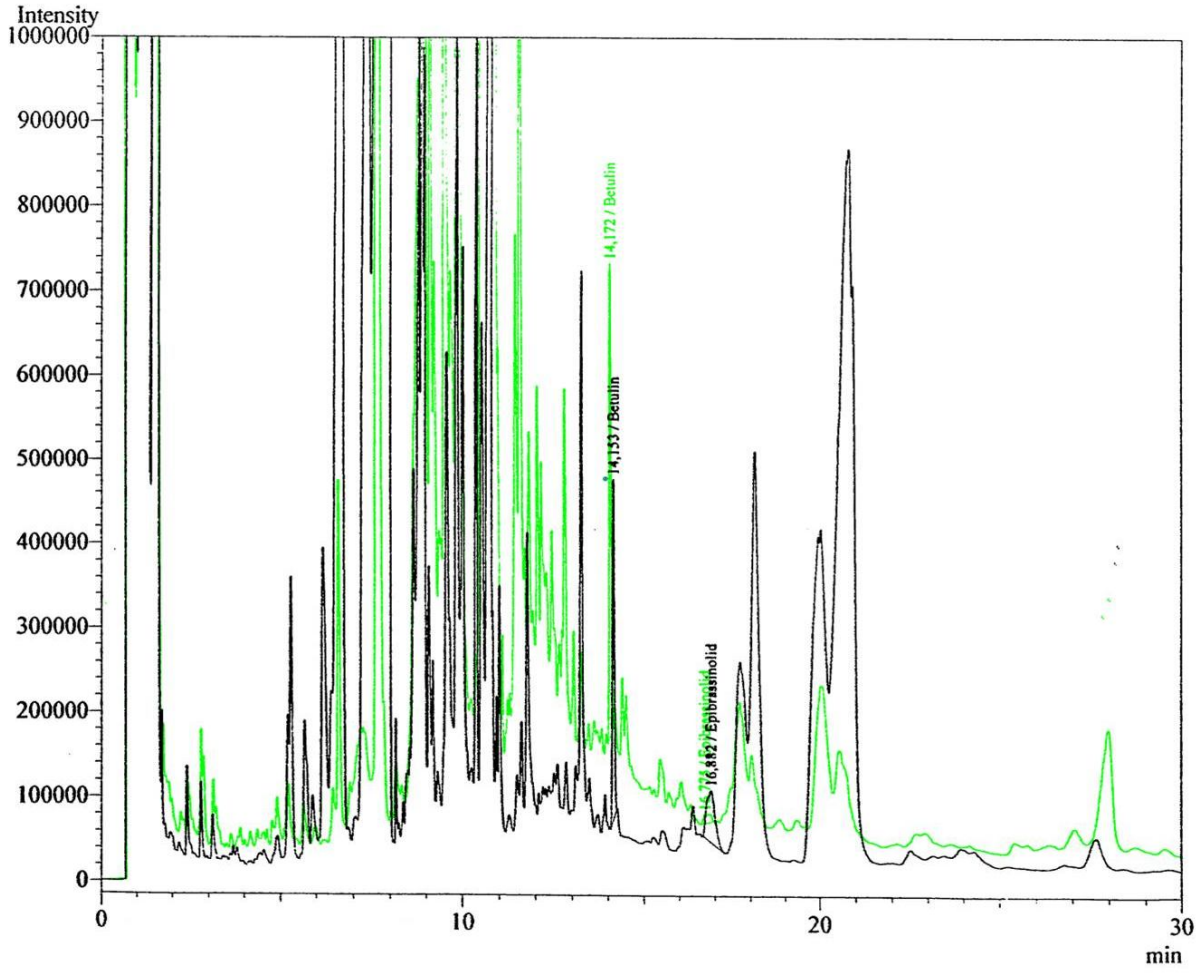
ID#	Name	Ret. Time	Area	Conc.	Units
1	Betulin	14.160	1989524	17.487	µg ml^{-1}
2	Epibrassinolide	16.759	38186	0.417	µg ml^{-1}

Figure 5.16: Overlay gas chromatographic profiles of the *ComCat*® crude extract (black line) and the hexane fraction (MI; blue line).

Much less and/or much lower concentrations of total compounds contained in the *ComCat*® crude extract were observed in the GC-profile of the hexane fraction (Figure 5.16) indicating selective extraction of more non-polar substances by the

hexane. Importantly, the hexane fraction contained a much lower concentration of Epibrassinolide compared to that found in the crude extract (3.8%).

5.5.3 Overlay gas chromatographic profile of the *ComCat*® crude extract and an ethyl acetate 2 (MII) fraction thereof



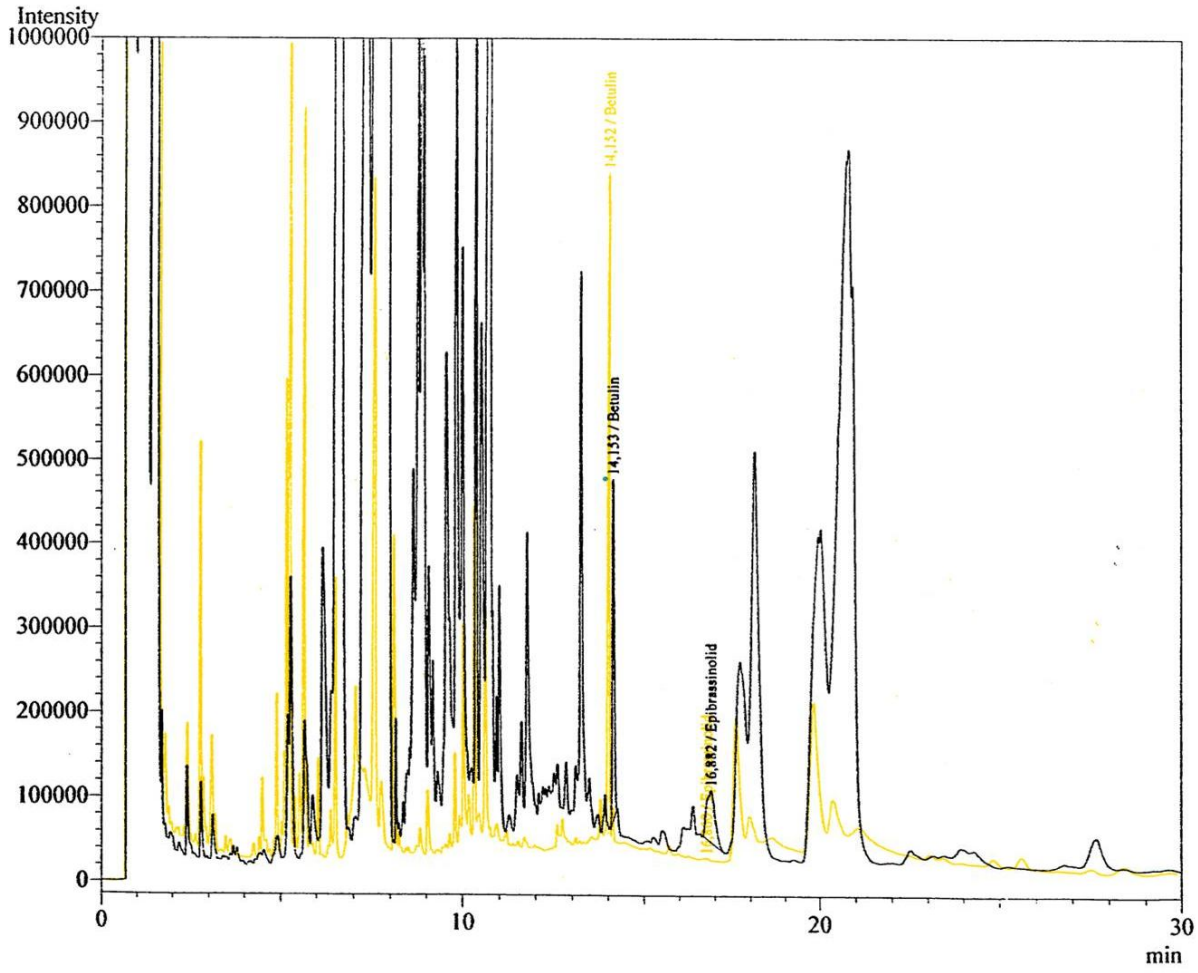
ID#	Name	Ret. Time	Area	Conc.	Units
1	Betulin	14.172	1968762	17.305	µg ml^{-1}
2	Epibrassinolide	16.775	145388	1.588	µg ml^{-1}

Figure 5.17: Overlay gas chromatographic profiles of the *ComCat*® crude extract (black line) and ethyl acetate fraction 2 (MII; green line).

Compared to the hexane fraction profile (Figure 5.16; blue line) much more compounds were detected in the slightly more polar ethyl acetate fraction (Figure 5.17; green line) between 10 and 15 minutes retention time. The Epibrassinolide

concentration in the ethyl acetate fraction was only 14.5% of that contained in the crude extract, but almost four fold higher than that in the hexane fraction.

5.5.4 Overlay gas chromatographic profile of the *ComCat*® crude extract and an MeOH : Water (MII) fraction thereof



ID#	Name	Ret. Time	Area	Conc.	Units
1	Betulin	14.152	2259557	19.861	µg ml^{-1}
2	Epibrassinolide	16.860	3249	0.035	µg ml^{-1}

Figure 5.18: Overlay gas chromatographic profiles of the *ComCat*® crude extract (black line) and Me : OH :water fraction (MII; yellow line).

Compared to the ethyl acetate fraction profile (Figure 5.17; green line) much less compounds were detected in the more polar MeOH: water fraction (Figure 5.18; yellow line) between 7 and 15 minutes retention time. Whereas between 4.5 and 6 minutes retention times a high concentration of compounds were detected. The

Epibrassinolide content in the Me OH : water fraction was very low and represented only 0.32 % of that contained in the crude extract.

5.6 Comparison of the Epibrassinolide content contained in a crude *ComCat*® extract and fractions thereof

Table 5.5: Epibrassinolide content of the *ComCat*® crude extract and fractions thereof.

Pos.	Parameter	Fraction	Analysis results mg kg^{-1}
01	Epibrassinolide	*ComCat* Plant extract powder	248.00
02	Epibrassinolide	Hexane Method I	230.00
03	Epibrassinolide	Ethyl Acetate Method II	190.00
04	Epibrassinolide	MeOH : Water fraction Method II	1.85

The figures in Table 5.5 indicate that 92.7% of the Epibrassinolide contained in the *ComCat*® crude extract was extracted in the hexane fraction of semi-purification method 1 (Pretorius *et al*., 2008) while method 2 (Gamoh *et al*., 1989) yielded only 76.6%.

5.7 Discussion

The activity directed semi-purification of bio-stimulatory compounds contained in *ComCat*® was based on two different liquid-liquid extraction methods. Bio-stimulatory activity in liquid-liquid extraction fractions was followed using six bio-assays developed in this study (chapter 3 and 4) as indicators of activity. These included *in vitro* bioassays such as the effect of *ComCat*® or fractions thereof on the respiration rate of monoculture yeast cells, seed germination, seedling growth and algae growth under laboratory conditions as well as *in vivo* bioassays such as crop growth under glasshouse and yield response under field conditions. Two controls were included in all bioassays namely water as a negative and *ComCat*® as a positive.

Based on biotests 1 and 2, only the three most active fractions were tested further. From the liquid-liquid extraction procedure of Pretorius *et al.* (2008) the hexane fraction (biotest 2) as well as of the liquid-liquid extraction procedure of Gamoh *et al*. (1989) for specifically extracting brassinosteroids (BRs) from plant material, two fractions were collected namely ethyl acetate fraction 2 and the

Methanol : Water fraction (biotest 1+2). According to Gamoh *et al*. (1989) ethyl acetate fraction 2 contains the brassinosteroids. In this fraction as well as in the hexane fraction of semi-purification method 1 (Pretorius *et al*., 2008) the previously unknown brassinosteroid (BR; Volz, 2000) contained in *ComCat*® was identified gas chromatographically as 24-*epi*-brassinolide by using internal BR-standards (Figure 5.19).

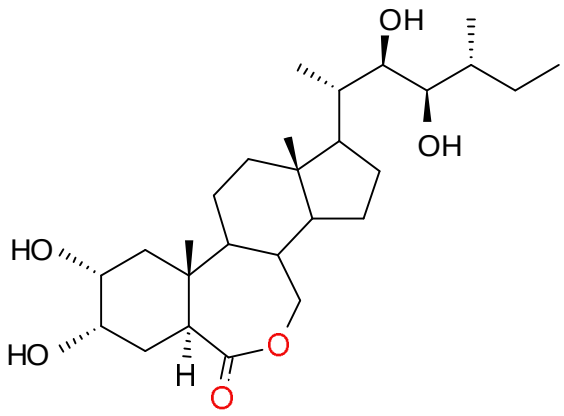


Figure 5.19: Structure of 24-*epi*-brassinolide.

Based on the comparative data obtained in terms of the amount of Epibrassinolide contained in the *ComCat*® crude extract and in semi-purified fractions thereof, the hexane fraction from extraction method 1 (Pretorius *et al*., 2008) yielded 92.7% Epibrassinolide while only 76.6% was recovered in the ethyl acetate fraction 2 of method II (Gamoh *et al*., 1989). The significance of this finding is that, firstly, with method I only one hexane step repeated over 96 h is needed to extract BRs compared to method II that proceeds over a period of two weeks. Secondly, only one solvent is used in method I compared to four in method II making the former not only a faster, but also a cheaper method to follow.

According to Zullo & Adam (2002), Adam & Marquardt (1986) and Mandava (1988) normally between 10-100 mg kg^{-1} of BR in pollen or seeds can be detected. *ComCat*®, which is an extract of *inter alia Lychnis viscaria* seed, contains 248 mg kg^{-1} of Epibrassinolide alone. This is more than twofold higher than the average calculated by these authors.

In this study it was confirmed that these two BR-containing fractions exogenously applied in nearly all bio tests tested positively for bio-stimulatory activity

similar to that of the *ComCat*® product itself. Interestingly, the third semi purified fraction (MeOH : Water fraction obtained with extraction method II), contained only 1.85% Epibrassinolide compared to the crude *ComCat*® extract. However, this fraction was also highly active in the case of all biotests employed except for seedling aerial part growth. The small amount of Epibrassinolide that ended up in this fraction could be due to streaking or imperfect extraction during the previous fractionation steps. What is significant about this state of affairs is that the MeOH : Water fraction could contain compounds, other than BRs, with bio-stimulatory characteristics. This aspect needs to be addressed in a future study as, currently, not much is known about the bio-activities of unidentified compounds in this fraction.

5.8 References

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CHAPTER 6

GENERAL DISCUSSION

From a technical point of view it is probably safe to say that the development of farming practices, in terms of plant production, has come a long way and are largely established. These include techniques such as planting, fertilization, irrigation and harvesting as well as pest and disease control. Moreover, from a scientific perspective it is also safe to say that discoveries over many years have contributed to improved crop production through a better understanding of plants on morphological, anatomical, physiological and genetic levels. However, none of these developments nor discoveries have in all probability reached their ultimate levels in the sense that complacency towards further development can be allowed to set in. Solely for this reason research is an ongoing process. Further, a changing environment and ever increasing world population are compelling reasons for mankind to continue with his endeavours to approach food security issues with vigilance while being conscious of specific problem areas in terms of food production that still exist. *Inter alia* these include below average crop yields in poverty stricken areas of the world. Even if only for this reason, new technology to improve the livelihood of people is welcomed. The latter was addressed in this study.

After obtaining permission from the manufacturers, a recently commercialized natural product, *ComCat*®, that contains three active brassinosteroids (BRs) of which two were identified by Volz (2000), was scrutinized in this study for its potential to be applied in the agricultural industry. Firstly, justification for the investigation is to be found in the fact that *ComCat*® is one of the first brassinosteroid (BR) containing natural products that have been commercialized since the discovery of BRs (Grove *et al*., 1979). Elucidation of BR's role as a plant hormone over a period of three decades established anticipated credence in its application potential in agriculture (Zullo and Adam, 2002). Secondly, from a farming perspective, the potential new technology introduced by the product and claimed by the manufacturers falls within the need for increased food production expressed higher up. However, scepticism towards the application potential of BRs on an economic level expressed by Ramraj *et al*. (1997) supplied a further rationale for scrutinizing the characteristics of *ComCat*® under

laboratory and glasshouse conditions and its application potential on a practical level under field conditions.

Many of the advantages of treating crops with pure BRs that were either isolated from wild plants or synthesized, and published over the past three decades, coincide with claims made by the manufacturers of *ComCat*® (AgraForUm AG). These, *inter alia*, include *ComCat's* enhancing effect on plant growth (Mandava, 1979; 1988; Wang *et al*., 2006), its contribution towards increasing the yield of various crops (Fariduddin *et al*., 2008; Hasan *et al*., 2008) and its ability to increase resistance towards abiotic and biotic stress factors (Fariduddin *et al*., 2009). The latter fell outside the scope of this study.

A first report on *ComCat*®, developed from a BR-containing extract of *Lychnis viscaria,* came from Friebe *et al*. (1999). In 2003 the product was listed in Germany as a plant strengthening agent under the trade name *ComCat*® and commercialized by a German company, AgraForUm AG. The three main objectives of this study were (1) to quantify its bio-stimulatory activity by means of different bio-tests, (2) to measure the yield response of two grain (maize and wheat) and three vegetable (cabbage, carrots and onions) crops to treatment with *ComCat*® under field conditions and (3) to isolate, purify and identify the third unknown BR reported by Volz (2000). It was anticipated that the outcome of these objectives would collectively serve as a means for assessing whether the properties of BRs can be exploited in organised agriculture, whether a BR-containing natural product such as *ComCat*® can repeatedly be applied in agriculture in a sustainable fashion over seasons and whether application of the product is acceptable from an economic perspective. A recent statement by Janeczko *et al*. (2010), supporting the scepticism towards the application potential of BRs in agriculture expressed earlier by Ramraj *et al*. (1997), confirmed the need to assess this BR-containing commercialized product concerning the potential contribution it could make in the crop production industry.

Concerning objective (1), seven biotests were employed to measure the bio-stimulatory activity of *ComCat*®, as claimed by the manufacturers (AgraForUm AG, Germany), under laboratory and glasshouse conditions. The product was tested at different concentration levels (0.25, 0.5, 1.0, 2.5 and 5.0 mg ℓ^{-1}). Although the biotest was structured to measure the effect of *ComCat*® on either the respiration rate of monoculture yeast cells and pea seeds or algae growth or seedling growth of different test crops in the laboratory or glasshouse, the 0.5 mg ℓ^{-1} application repeatedly

emerged as the optimum concentration that contributed to an increase in these parameters. This was in agreement with a report by Neelam *et al.* (2002) who showed that the lower concentration range of hormone products often contribute to growth stimulation while concentrations in the higher range inhibit plant growth. This was also confirmed by Prakash *et al*. (2001) for the effect of BRs on tomato seedling growth.

While treatment with *ComCat*® had little or no effect on seed germination, especially root growth of seedlings from different crops was significantly and consistently induced. Although *ComCat*® contributed to slight increases in coleoptile length growth of seedlings from some of the test crops, this was not significant for any of the concentrations tested. It was, however, interesting to note that seedlings from different crops responded differently to treatment with *ComCat*®. On the one hand seedling growth of both cabbage and peas was significantly enhanced in terms of root and coleoptile growth upon seed treatment while, on the other hand, seed treatment with *ComCat*® had no effect on wheat seedling growth.

The growth response of wheat to foliar treatment with *ComCat*® at the 3-4 leaf stage was, subsequently, followed under glasshouse conditions. Different from the response to seed treatment, wheat seedling growth and especially root growth was increased significantly by the 0.5 mg ℓ^{-1} concentration when measured four weeks after foliar treatment. The latter strongly indicated that not only the type of crop, but also the time of application were factors to consider when manipulation of crops by means of hormone related products was intended. Further, more than two decades ago Harms and Oplinger (1988) reported that growth data obtained under controlled conditions in a laboratory often differ and are not easy to repeat either in the field or in pots under glasshouse conditions.

Arabidopsis thaliana has often been used in the past to unravel the mechanism by which BRs influence plant growth. Especially BR-deficient or BR-insensitive *Arabidopsis* mutants confirmed the essential role of BRs for cell elongation (Altmann, 1998; Clouse and Sasse, 1998). Hu *et al*. (2000) reported that the *CycD3* gene, involved in promoting cell division in *Arabidopsis*, was upregulated by treatment with Epibrassinolide. As the authors used cell cultures it was not possible to predict whether this mechanism applied to the promotion of aerial part or root growth or both in *Arabidopsis*.

However, Müssig *et al*. (2003) reported that low concentrations of BRs such as 24-epicatasterone and 24-epibrassinolide specifically promote root elongation in *Arabidopsis* wild type plants and BR-deficient mutants (e.g. *dwf1-6*). They also showed that the root growth stimulating effect of BRs appears to be largely independent of auxin and gibberellin action. Most importantly, in their study the authors confirmed that shoot and root growth stimulation by BRs is highly concentration dependent. Shoot growth is promoted at the higher concentration levels and root growth at the lower concentration levels tested, but BRs act inhibitory if a threshold level is exceeded. Also, other than the findings of Hu *et al*. (2000), Müssig *et al*. (2003) observed a positive interaction of BR and auxin in promoting root growth in *Arabidopsis* and concluded that this could provide the basis for impaired root growth of BR-deficient mutants.

Recently Cheon *et al*. (2010) stated that, despite clear evidence that BRs regulate cell elongation; their roles in cell division have remained elusive. This supplied the rationale for the authors to compare callus growth from a BR-deficient *Arabidopsis* mutant, *dwarf7-1*, to that of the wild type. They showed that the mutant exhibited a noticeably slower callus growth rate and shoot induction relative to the wild type control. The authors were able to identify the genes involved and concluded that BRs play important roles in both cell division and cell differentiation in *Arabidopsis*.

Brassinosteroid involvement in root and shoot growth in plants has been well documented over the past decade, although *Arabidopsis* was used as test organism in most of the studies. In this study, laboratory screening procedures confirmed that *ComCat*®, similar to the findings of Turk and Tawaha (2003) with pure BR's, possessed the inherent characteristic of inducing seedling growth, albeit mostly root growth, in most of the five test crops. This is perceived in a rather positive way as induction of aerial part growth too soon in a crop's life cycle might have negative effects on yield as was shown for early treatment of crops with excessive nitrogen (Boiffin *et al*., 2001). For this reason, and in the light of the growth enhancing effect observed in this study for all test crops treated with *ComCat*®, it was essential to follow the yield response of different crops to foliar treatment with *ComCat*® under field conditions (objective 2).

Concerning objective (2), and due to the rather erratic response of wheat to treatment with *ComCat*®, a comprehensive study was undertaken with this crop under field conditions. However, a second grain crop, maize, as well as three

vegetable crops were also included in this field study in order to assess their yield response to foliar treatment with *ComCat*®. The reason for including a variety of crops is found in published data of Krishnan *et al*. (1999), Vardhini & Rao (2001) and Amzallag (2002), who all reported that the effectiveness of BR application on the growth and yield of crops depends on the plant species and concentration applied.

During the 2007/08 growing season, one cultivar for each test crops was treated with different *ComCat*® concentrations and at different growth stages. For maize and wheat a single application at the 3-4 leaf growth stage and for vegetable crops, cabbage, carrots and onions, a double application applied. In the case of vegetables the second application was at either 30% head development (cabbage), root development (carrots) or bulb development (onions). Results obtained during the first season revealed that the optimal concentrations were 100 g ha^{-1} for maize, 200 g ha^{-1} for wheat, cabbage and carrots and 400 g ha^{-1} for onions confirming the concentration sensitivity of different crops to treatment with *ComCat*®. During the second season (2008/09), only these optimal concentrations were foliarly applied to test crops. However, additional to the single cultivar tested during the first season a second cultivar for each crop was included during the second season. In this way the repeatability of results was tested over two seasons for one of the cultivars while possible cultivar sensitivity was also followed in terms of its yield response to treatment with *ComCat*®.

In the case of both cabbage (Conquistador and Drumhead) and carrot (Fancy and Snakpak) cultivars, treatment with *ComCat*® contributed to significant increases in yield that repeated over two seasons for at least one of the cultivars from each vegetable crop. Compared to maize, but admittedly based on limited data, it seems that the vegetable crops used in this study are less cultivar sensitive. The yield increase induced by treatment with *ComCat*® in the maize cultivar PHI3394 was statistically significant over two seasons, but the tendency to increase the yield of the second cultivar, PAN6043, was not. This indicated that maize was more cultivar sensitive to treatment with *ComCat*® than the vegetable crops tested and this was in concert with the genotype specificity of maize to treatment with BRs reported by Hola *et al*. (2010). Clearly more cultivars of these crops will have to be tested to verify this assumption.

The opposite tendency was observed for wheat and onions where treatment with *ComCat*® significantly increased the yield of the wheat cultivar, Tugela, and

onion cultivar, Australian Brown, during season one, but not during the second season, questioning the product's repeatability for these two crops. Further, the yield of the second wheat cultivar included in this study, PAN3377, and onion cultivar, Texas Grano, was also increased significantly by the treatment, compared to the untreated control, indicating that it might be less cultivar sensitive. Again, more information on cultivar response to treatment with *ComCat*® is needed to verify the latter statement. However, overall the significant yield increases measured in one or both cultivars of the five different test crops and in one or both seasons confirmed the potential of *ComCat*® to be applied in agriculture and to contribute to economic prosperity for a farmer.

The mechanism by which BRs contribute to increased yields in crop plants was questioned by Wu *et al*. (2008), using rice as test crop. The authors proceeded from the postulate that BRs control seed size or weight or both. Although the mechanism for this is unclear, it has been shown in the past that BRs can regulate the initial carboxylation activity of ribulose-1,5-bisphosphate carboxylase (RUBISCO) and thereby influence photosynthetic CO_2 assimilation (Yu *et al*., 2004). However, what is important is seed filling or the eventual translocation of photosynthate to the harvestable parts of crops, an aspect that was investigated by Wu *et al*. (2008) in rice.

Seed filling depends on the flow of sucrose from the leaves to the embryo and seed endosperm. This flow is determined by the photosynthate production rate and the loading of the phloem with sucrose. Wu *et al*. (2008) showed that the increase in seed weight, after foliar treatment of rice plants with epibrassinolide, resulted from increased quantum yield of electron transport in photosystem II and enhanced filling of the seed with sugars transported to the kernels from the leaves.

Concerning objective (3), gas chromatographic analyses of a crude *ComCat*® extract as well as liquid fractions thereof by using external BR standards confirmed the unidentified BR (Volz, 2000) to be 24-*epi*-brassinolide. The latter as well as the previously identified 24-*epi*-castasterone and 24-*epi*-secasterone (Volz, 2000) are known to be active BRs of the brassinosteroid group (Swaczynová *et al*., 2007). The similarities between the growth and yield response of crop plants to treatment with pure BRs as reported in the literature and *ComCat*® as outlined in this study, strongly indicate that the three active BRs in *ComCat*® are most probably accountable for the typical responses observed throughout the investigation.

However, *ComCat*® does not only contain BRs but also phytohormones such as auxin, gibberillin and cytokinin as well as other natural compounds such as phytosterols, flavonoids and free amino acids in its composition (AgraForUm AG, Germany). Although scrutiny of the actual mechanism of action of BRs fell outside the scope of this study, it seemed appropriate to speculate on this action mechanism in the light of the other natural compounds contained in *ComCat*®. In other words, to postulate or propose a possible action mechanism that differs from that of pure BRs due to possible interaction between some or all of the compounds contained in the product. However, at the onset it must be noted that, despite significant progress in understanding the molecular and cellular effects of BRs in plants, key issues about their biological activity and mode of action remain unknown (Fellner, 2003). Further, although that which follows is rather speculative, consideration of possible interaction between BRs and other compounds contained in *ComCat*®, as part of an action mechanism, is regarded important in order to envisage response differences of crops treated with either the product or pure BRs.

As mentioned in a previous chapter Tanaka *et al*. (2003) reported on the synergistic effect between BRs, auxin and gibberellin in terms of vegetative growth in *Arabidopsis thaliana*. By using a BR biosynthesis inhibitor, brassinazole (Brz), the authors showed that root and hypocotyl growth was inhibited in *Arabidopsis* indicating that cooperative action between BRs and known growth hormones is essential for growth regulation. The fact that *ComCat*® contains all of these hormones and that synergism between them seems likely, the product might have an edge on pure BRs in terms of evoking a response from crops when applied foliar. The latter might explain why field results obtained with *ComCat*® in this study were rather consistent over two seasons compared to the inconsistent results obtained with pure BRs at times as mentioned by Ramraj *et al*. (1997). However, the latter cannot explain cultivar differences observed in some of the test crops except if intrinsic concentration level differences between cultivars might be a factor. This aspect needs to be investigated on its own. A rationale for such a study is to be found in the rather contradictory results published by Sala & Sala (1985) who reported BR-induced cell enlargement in an auxin-starved cultured cell line of carrots. Clouse *et al*. (1992) reported similar results demonstrating BR-promoted elongation of auxin-depleted soybean epicotyls. In both these instances low inherent auxin content favoured BR-activity.

Although no interaction between BRs and flavonoids could be traced in literature, a rather comprehensive publication on the interaction between BRs and essential amino acids recently came from Morera-Boado *et al*. (2010). The authors reported that the interaction of BRs with amino acids is essential to understand how the binding of these phytohormones with the BRI1 receptor in plants occur. According to the authors, the BRI1 receptor (brassinosteroid receptor insensitive 1), which is a leucine rich repeat (LRR) receptor kinase, is localized in the plasma membrane and is a critical component of a receptor complex for BR. In simple terms this means that interaction between BRs and specific amino acids might be the key to BR-binding and, subsequently, BR-activity in plants.

To explain this Morera-Boado *et al*. (2010) compared the typical structure of the BR molecule to that of a typical amino acid and showed specific zones of hydrophyllic interaction that exists between the two (Figure 6.1). The authors proposed a model for brassinolide (Figure 6.1A), the most active BR found in plants. It has three regions of interaction [hydroxyl groups in the A-ring (1), a diol group with R configuration at C22/C23 (2) and a lactone group in C6/C7 (3)] that are implicated in its state of activity.

On the other hand, a model proposed by Morera-Boado *et al*. (2010) for the interactivity of amino acids (Figure 6.1B) entails peptide bonds that are simulated with methylamine ($NHCH_3$) and acetyl ($COCH_3$) groups. Three areas of possible interaction are shown in Figure 6.1B. In order to explore the conformational space in the interaction of BR with the twenty naturally occurring amino acids, the MMH (multiple minima hypersurfaces) procedure was followed by the authors. This is a highly specialized field that includes quantum mechanics. Without going in too much further detail, it is concluded that the authors contributed greatly to understanding the interaction between BRs and amino acids, based on the molecular configuration in their chemical structures, albeit in the form of a theoretical model.

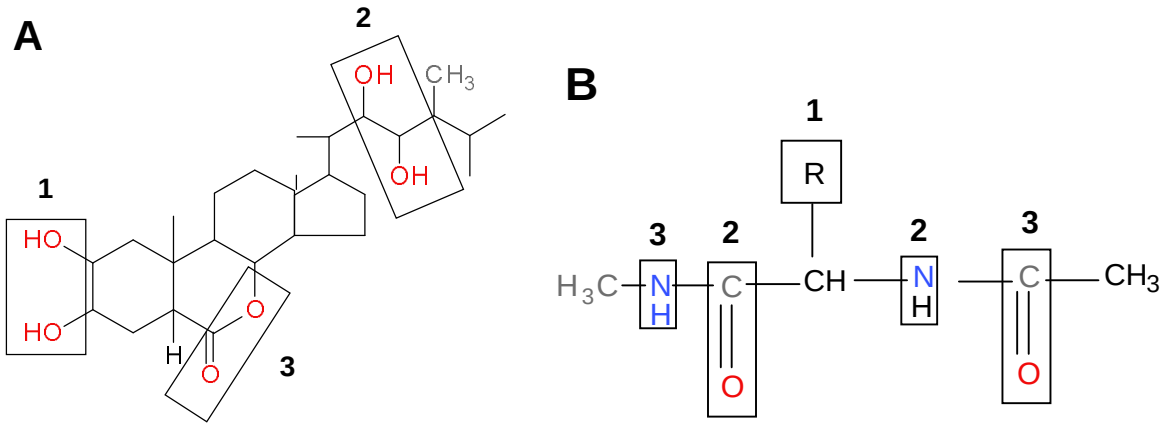

Figure 6.1: Structure of A) brassinolide and B) a blocked amino acid indicating three specific hydrophyllic zones and, therefore, three possible areas of interaction in each case (After Morera-Boado *et al*., 2010).

The underlying question that now comes to mind is what effect do free amino acids contained in *ComCat*® have on either the activity or stability of the three BRs in the product? When speculating on this matter it must be kept in mind that the work of Morera-Boado *et al*. (2010) showed interaction between BRs and amino acids contained in cell membranes and not free amino acids. Theoretically there is a possibility that free amino acids can act as a link between BRs and amino acid side chains of peptides that form part of the membrane structure. This might mean that the free amino acids contained in *ComCat*® may interact with the three BRs via hydrophyllic association that in turn promotes interaction with membranes and, subsequently, BR-activity. The latter might explain why *ComCat*®, consisting of a mixture of natural compounds, has a better chance of inducing a stable response from crops under field conditions than does any active pure BR. However, following the approach of Morera-Boado *et al*. (2010), future research is needed to verify this postulate.

In terms of brassinosteroid action mechanism a recent review by Yang *et al*. (2011) needs to be mentioned. Without going into any detail, this is regarded as an excellent review on the progress that has been made to date over a period of three decades in terms of understanding the action mechanism by which BRs exert their influence in plant growth and development. It is actually amazing to see how important BRs are for normal plant development and the pivotal role it plays in this regard. The mechanism of action is not one sided, but entails an array of activities

that are regulated by a series of genes depending on the activity that is under scrutiny. Although the perception of the BR action mechanism is still in the form of a theoretical model, Yang *et al*. (2011) succeeded in integrating the work of many scientists and supplied a working model that can be refined in future as more information is accumulated.

In summary, the bio-stimulatory characteristic of *ComCat*® was confirmed both *in vitro* and *in vivo* under laboratory, glasshouse and field conditions in this study. Importantly, it was also shown that the significant growth response of different crops to treatment with *ComCat*® did not have a negative effect on final yield. To the contrary, significant yield increases in all of the test crops over two seasons in some instances, confirmed the potential of *ComCat*® to be applied sustainably in the agricultural practice. On average yield increases obtained in this study over two seasons with two cultivars for each crop were 8.7% for wheat, 8.9% for maize, 9.7% for cabbage, 11.6% for carrots and 13.2% for onions. Compared to current annual bank interest rates and considering that the growing cycle of all test crops do not exceed six months, all yield percentage increases fall within the economic viable range. Further, the measured yield increases for the different crops were statistically significant in most, but not all cases. As statistical insignificance is often related to large standard deviations between replicates, it is suggested that the number of replicas should be increased in future field trials. Finally, based on the results obtained in this study, three possible application methods of *ComCat*® are suggested. These include 1) seed treatment, 2) foliar application or 3) soil application.

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SUMMARY

A recently commercialized natural bio-stimulant, *ComCat*®, that contains three active brassinosteroids (BRs) of which two were identified previously, was scrutinized in this study for its potential to be applied in the agricultural industry. Concerning objective (1), seven biotests were employed to quantify the bio-stimulatory activity of *ComCat*® under laboratory and glasshouse conditions. The product was tested at different concentration levels and the 0.5 mg ℓ^{-1} application repeatedly emerged as the optimum concentration in terms of contributing towards increases in all of the parameters measured. Treatment with *ComCat*® had little or no effect on seed germination, but especially root growth of seedlings from different crops was significantly and consistently induced.

Concerning objective (2), two cultivars for each of five test crops were treated with *ComCat*® over two growing seasons under field conditions in order to ascertain their yield response. Some of the test crops, but not all, showed cultivar sensitivity to treatment with *ComCat*®. However, overall the significant yield increases measured in one or both cultivars of the five different test crops and in one or both seasons confirmed the potential of *ComCat*® to be applied in agriculture and to contribute to economic prosperity for a farmer.

Concerning objective (3), gas chromatographic analyses of a crude extract of the product as well as semi-purified fractions thereof by, using internal BR standards, were employed to identify an unknown BR contained in *ComCat*®. This was confirmed to be 24-*epi*-brassinolide. The latter as well as the previously identified 24-*epi*-castasterone and 24-*epi*-secasterone are known to be active BRs of the brassinosteroid group.

In conclusion, the bio-stimulatory characteristic of *ComCat*® was confirmed both *in vitro* and *in vivo* under laboratory, glasshouse and field conditions in this study. Significant yield increases in all of the test crops over two seasons in some instances, confirmed the potential of *ComCat*® to be applied sustainably in the agricultural practice. Based on the results obtained in this study, three possible application methods of *ComCat*® are suggested. These include 1) seed treatment, 2) foliar application or 3) soil application.

OPSOMMING

‘n Onlangs gekommersialiseerde natuurlike biostimulant, *ComCat*®, wat drie aktiewe brassinosteroïede (BRs) waarvan twee voorheen geïdentifiseer is bevat, is in hierdie studie t.o.v. die potensiaal om in die landboupraktyk toegepas te word ondersoek. M.b.t. doelwit (1) is sewe biotoetse ingespan om die biostimulerende aktiwiteit van *ComCat*® onder laboratorium- en glashuistoestande te kwantifiseer. Die produk is teen verskillende konsentrasies getoets en 0.5 mg ℓ^{-1} is herhalend as die optimum konsentrasie, in terme van bydra tot verhoging in al die gemete parameters, geïdentifiseer. Behandeling met *ComCat*® het weinig of geen effek op saadkieming gehad nie, maar veral wortelgroei in saailinge van verskillende gewasse is betekenisvol en konsekwent geïnduseer.

Ten opsigte van doelwit (2) is twee cultivars vir elk van vyf toetsgewasse oor twee seisoene met *ComCat*® behandel en die oesopbrengs onder veldtoestande gemeet. Sommige van die toetsgewasse, maar nie almal nie, was cultivar sensitief t.o.v. behandeling met *ComCat*®. Maar, oorkoepelend het die betekenisvolle oesopbrengsverhogings wat in een of beide cultivars van die vyf verskillende toetsgewasse en oor een of beide groeiseisoene gemeet is, die potensiaal vir *ComCat*® om in die landbou toegepas te word en tot ekonomiese welvaart vir die boer by te dra, bevestig.

Met betrekking tot doelwit (3) is ‘n ru-ekstrak van die produk asook semi-gesuiwerde fraksies daarvan gaschromatografies geanaliseer, deur van interne BR-standaarde gebruik te maak, ten einde die onbekende BR in *ComCat*® te identifiseer. Laasgenoemde is bevestig om 24-*epi*-brassinolied te wees wat, tesame met die voorheen geïdentifiseerde 24-*epi*-castasteroon en 24-*epi*-secasteroon, bekend is as aktiewe BRs van die brassinosteroïed groep.

Ten slotte is beide die *in vitro* en *in vivo* bio-stimulerende eienskappe van *ComCat*® onder laboratorium-, glashuis- en veldtoestande in hierdie studie bevestig. Betekenisvolle oesopbrengsverhoging wat in al die toetsgewasse, in sommige gevalle oor twee seisoene verkry is, het die potensiaal vir *ComCat*® om volhoubaar in die landboupraktyk toegepas te word, bevestig. Gebaseer op die resultate wat in hierdie studie bekom is, word drie moontlike aanwensdingsmetodes vir *ComCat*® voorgestel. Dit sluit 1) ‘n saadbehandeling, 2) ‘n blaarbespuiting of 3) ‘n grondbehandeling in.