Cape Peninsula
University of Technology

IN VITRO PROPAGATION OF *AGATHOSMA BETULINA* AN INDIGENOUS PLANT OF ECONOMIC IMPORTANCE

by

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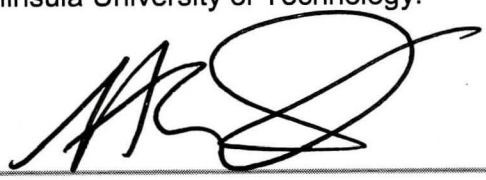
December 2013

DECLARATION

I, Hildegard Witbooi, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



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2 April 2014

Date

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ABSTRACT

Agathosma betulina (Berg.) Pillans, previously known as *Barosma betulina*, is a member of the Rutaceae family, and indigenous to the fynbos botanical biome of the Western Cape of South Africa. It is commonly known as buchu. Extracts as well as powdered leaves have traditionally been used for the treatment of various ailments. The increase in the international demand for *A. betulina* for health as well as food and beverage benefits, have raised concerns over exploitation of wild populations and the lack of horticultural information necessitates this study to evaluate the propagation of this economical important species. The main objective of this study was to establish a simple and highly productive micropropagation protocol for *A. betulina* through experimenting with nodal explants.

Testing of the effect of various treatments (physical scarification, chemical scarification, GA, stratification, smoke and combinations thereof) on the *in vitro* germination of *A. betulina* seeds was done to elucidate the factors which control seed germination. The study revealed that the physical scarification and smoke-induced germination had a significant effect on germination percentages. In terms of germination rate, the radical generally started to appear after approximately 10 days in the physical scarification with smoke treatment.

Initial decontamination methods with the exposure of various concentrations of NaOCl gave fatal results, however 1.5% NaOCl had more phenolic reactions rather than fungal or bacterial contamination. Interestingly, contamination rates of explants were influenced by the stage of maturity of the explant material. This plant material was used to test different strengths of regeneration media, to ensure that the explants receive ample nutrients. Results made exhibited that ½ MS was the best strength for growing *A. betulina* nodal explants. Compared comparison between *in vitro* derived explants and *ex vitro* collected explants showed that the *ex vitro* derived explants had significant results, but the explants lost vigour soon after the initial exponential growth leading to the explants dying off. Furthermore, *ex vitro* decontaminated plant material was not economically viable to continue with.

Seedlings derived from germinated seeds appeared to be the preferred method of propagation as this spent the least time in culture and produced a stable plant with

an established root system, which is essential during the hardening off process after *in vitro* growth. When exposing nodal explants to phytohormone 2,4-D it responds best to dosages 0.5mg L^{-1} and 1mg L^{-1}. Phytohormone BA was very effective in producing soft friable callus. The best results were shown when 0.5mg L^{-1} BA was applied to ½ MS media. For both shoot length and multiple shoot production, a combination of phytohormones BA-NAA (1: 0.5mgL^{-1}) had the most significant results. Interestingly, a higher phytohormone concentration of NAA is necessary to develop multiple adventitious roots. The effect of 3mg L^{-1} was significant in that it resulted in multiple adventitious roots, but fewer calli was observed in this treatment.

Micropropagation becomes valuable as little attention between subcultures is needed; making it less labour intensive compared to conventional nursery propagation systems where weeding watering and spraying of plants are labour intensive.

In the traditional world of medicine, more so in Southern Africa, extracts are prepared by adding boiling water to the plant material; however commercial ethanol is used as an extractant. Establishment of the essential oil quality of the *in vitro* cultures post exposure to various treatments was done. Analysis of essential oils from *A. betulina* resulted in the identification of twenty one compounds. The results showed qualitative as well as quantitative differences amongst the samples used in the study. The highest relative concentration of limonene was observed in the callus of nodal explants after it was exposed to 0.5mg l^{-1} NAA. No pulegone was found in this treatment making it ideal for limonene production. This suggests that liquid culture with the same treatment may produce more calli making it ideal for the production of limonene.

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Abbreviations

ARC	Agricultural Research Council
CFR	Cape Floristic Region
Cm	Centimeter (10^{-2} m)
dH_2O	Distilled water
°C	Degrees Celsius
'Ex vitro'	Cultivated in natural conditions
EtOH	Ethanol
g	Gram
GA_3	Gibberilic acid
HCL	Hydrochloric acid
In vitro	"In glass"
L	Liter
M	Molar
Min	Minute
Mm	Millimeter (10^{-3}m)
MS	Murashige and Skoog (1962) medium
NaOCL	Sodium hypochlorite
WPM	Woody Plant Medium
PGR	Plant growth regulator
IAA	indole-3-acetic acid
BA	N_6-benzylaminopurine
NAA	1-Naphthaleneacetic acid
2, 4-D	Dichlorophenoxy acetic acid
v/v	Volume per volume
WHO	World Health Organisation

CHAPTER ONE

GENERAL INTRODUCTION

Several indigenous South African cultures rely on informal markets to obtain traditional medicine as their primary health care, as this is affordable and easily harvested from the wild. It is estimated that 80% of the world's population, mostly those native of developing countries depend on traditional medicine (Mander *et al*., 2006). Informal markets have proved to provide an income to families as there is a demand for traditional medicine. Very few, about 38 of an estimated 3000- 4000 of medicinal plants in use of Southern Africa have been commercialised for local and international use (Van Wyk, 2008), as there are limited knowledge about the cultivation of these indigenous plants. However, an estimated 25% of all prescribed medicines contain some ingredient(s) derived from plants (Mander *et al.,* 2006).

Unfortunately, the over-exploitation of wild populations of medicinal plants such as *Agathosma betulina* (Berg.) Pillans. (Rutaceae) in the Western Cape presents a biodiversity threat and have a current national status and criteria of declining under the Threatened Species Programme of SANBI and listed in the book, Red list of South African plants. It is cultivated on a small but slowly increasing scale as the availability of seed, the germination thereof and the rooting of cuttings as well as general cultivation practices have been poor and thus commercialisation is difficult. *A. betulina* is often seen in the informal market systems in an intact but dried form and often cut unsustainably in the wild. The leaves are often powdered and available as multi-species blends. It is often not available in any plant nursery and this is a clear indication of the unavailability of cultivation practices as well as the difficulty of growing this plant in areas not identical to that of its natural habitat.

A. betulina, known as buchu is said to have had attractive financial returns in the past few decades. Whether low or high cultivation input is used, the harvesting window is large, stretching from December- February each year. The value of buchu lies in its oil, which has several uses. The oil is distilled from this highly aromatic plant and used in the cosmetic, pharmaceutical, food and beverage industries for its distinctive aroma, taste and medicinal properties. The high demand for such uses presents an opportunity for scientists to investigate novel micropropagation methods to increase cultivation and production practices.

Tissue culture, due to its ability to produce clonal material in mass, was chosen as an *in vitro* conservation strategy. This propagation method would assist with supplying large volumes to meet commercial demands for *A. betulina* and other related taxa. Germination as well as the rooting of cuttings has not been successful and with the application of tissue culture techniques, this can be overcome. To our knowledge, no viable micropropagation methods currently exist to assist with both conservation and commercialisation. The focus of this study was to develop a germination and mass production protocol through the investigation of plant growth regulator requirements for induction of *in vitro* cultures of *Agathosma betulina* using foliage material. Furthermore, the induction of plant material obtained in the field as well as the rooting of plantlets and continued culture to become established plants that can reproduce. This will act as a baseline study to assist in further research on this important medicinal plant of Southern Africa.

CHAPTER TWO

LITERATURE REVIEW

2.1 AGATHOSMA BETULINA, A MEDICINAL PLANT OF SOUTH AFRICA

2.1.1 Classification of *Agathosma* lineage

The genus *Agathosma* consists of approximately 150 indigenous flowering plant species endemic to South Africa. The genus belongs to the family Rutaceae. The Cape region of South Africa has veldt- types with the richest composition of aromatic indigenous plants in the whole of South Africa (Moolla and Viljoen, 2008).

2.1.2 Geographical distribution of *A. betulina* and vernacular names

Buchu species are endemic to the high-altitude regions of the western part of the Cape Floristic Region (CFR) (Goldblatt and Manning, 2000). Mediterranean climate generally prevail in this setting. It roughly extends from North of Clanwilliam to Tulbagh and southward to Paarl and Riversdale (Blommaert and Bartel, 1976). *Agathosma betulina* is commonly known in English as round-leaf buchu and short buchu and in Afrikaans it is referred to as boegoe or bergboegoe, other names include and are not limited to bucco, bookoo and Diosma. This plant is probably one of the best known South African herbs both internationally and locally for its medicinal purposes (Moolla and Viljoen, 2008).

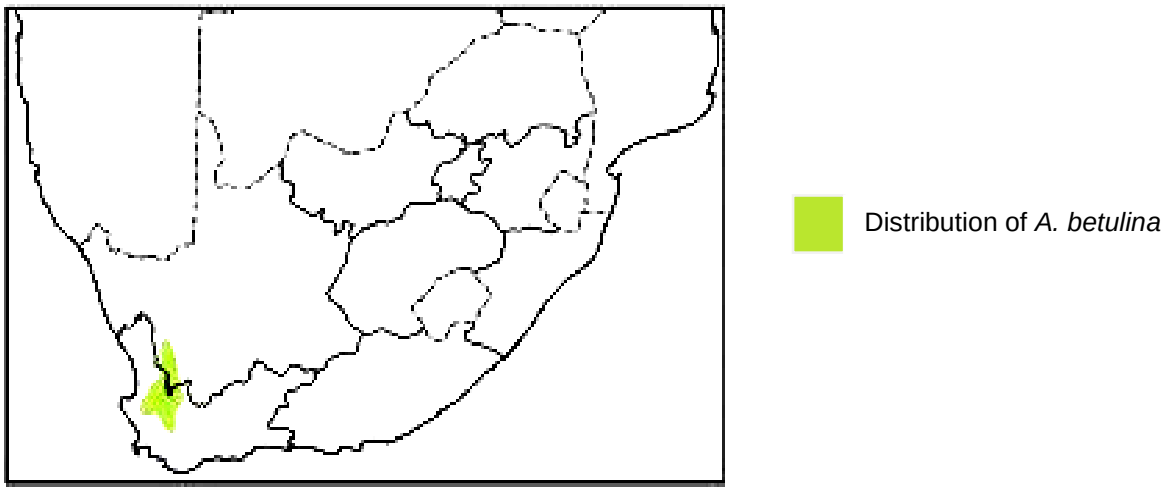

Figure 1: Geographical distribution of *A. betulina*

2.1.3 General botany of *A. betulina*

Buchu is an evergreen, resprouting and broad-leafed shrub (Fig 2A) grows over 1 metre in height (Pillans, 1950, Spreeth, 1976). The pellucid oil glanded leaves (Fig 2C) are oval shaped, concave, minutely toothed and are mostly 10-20 mm long (Fig 2C). The flowers are white to pink and are present in the leaf axils (Fig 2B) with petals 8-10 mm long, lance- shaped staminodes and ovary and fruit are 5- segmented.

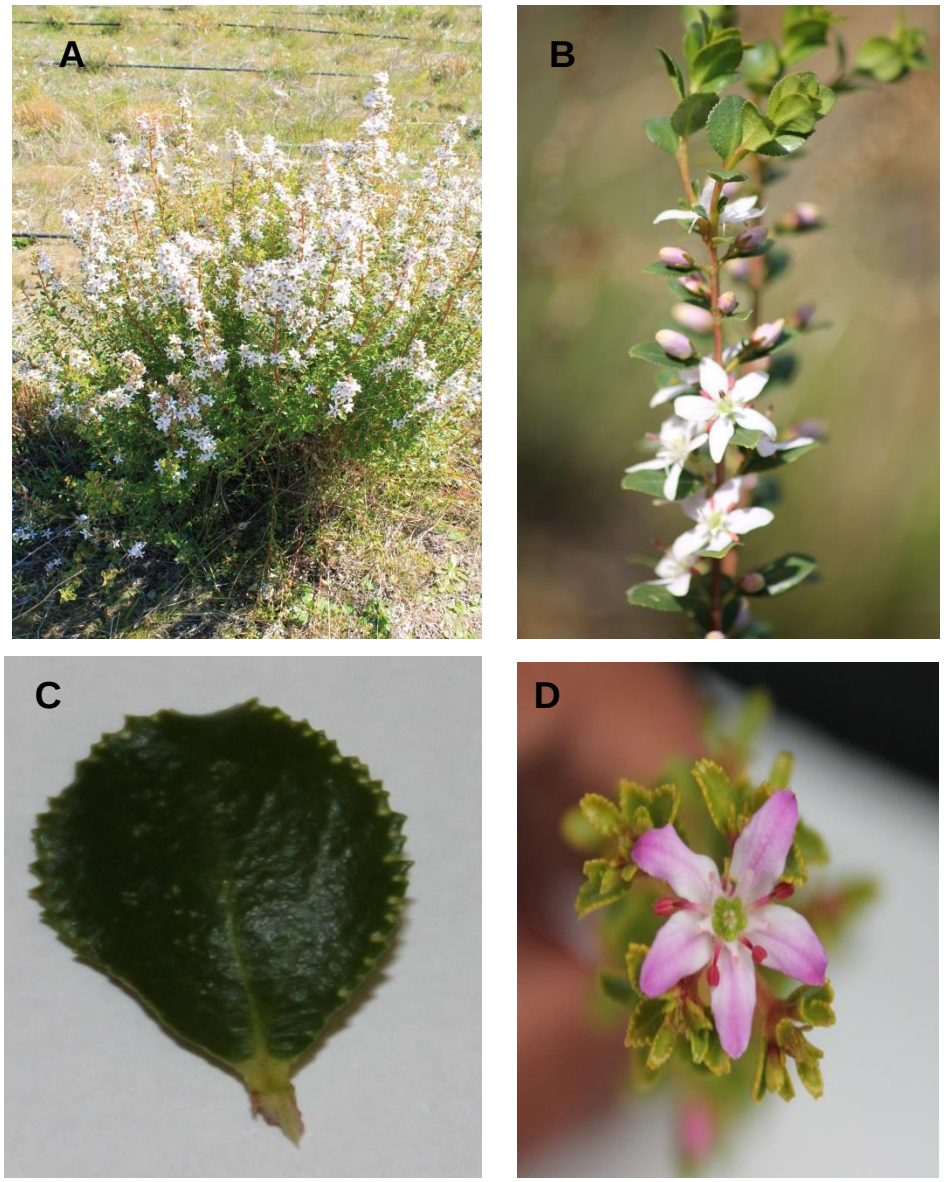

Figure 2: (A) *Agathosma betulina* broad leaved shrub in full flower (B) Flowers and flower buds in leaf axils (C) Concave leaf of *A. betulina* (D) Star shaped white to pink-purplish flowers

Buchu was first introduced by the Khoi-San in the 1650's as a medicinal plant to the European settlers in the Cape (Moolla and Viljoen, 2008). It is therefore endemic to South Africa and grows wild in the mountains of the Western Cape (Von Wielligh, 1913). It is culturally important to the Khoi-San people, whom dried and powdered the leaves mixed with sheep fat to anoint their bodies (Spreeth, 1976). Buchu brandy was prepared by distillation of the plants leaves by the Khoi-San people, which served as a remedy for infections of the stomach, bladder and bowels (Pillans, 1910; Watt and Breyer- Brandwijk, 1962; Roberts, 1990; Bruneton, 1995). Some of the traditional uses of *Agathosma* include: as an antipyretic, a liniment, an antispasmodic, a cough remedy, as well as the treatment of colds and flu; for the treatment of cholera and other stomach ailments; relief of rheumatism, bruises and gout; a diuretic; for the treatment of kidney and urinary tract infections; for the relief of calculus as well as for antiseptic purposes (Watt and Breyer- Brandwijk, 1962; Simpson, 1998). The use of this medicinal plant spread to America and Europe where it was extensively used as medicine (Moolla and Viljoen, 2008). *A. betulina* is now mainly enjoyed as a herbal tea by many health conscious individuals and is available as a single herb or as a blend of other herbal teas. Medicinal plants in Southern Africa have been recognised to be an important provider of primary health care benefits, livelihood opportunities and as being culturally significant (Diederichs, 2006). It is now under the spotlight of environmentalists, economists, social scientists, policy-makers and trade organisations throughout the world (Diederichs, 2006).

2.1.5 Economical importance of *A. betulina*

The value of buchu lies in the high content of its oil, which is used in medicine, cosmetics and food products (van Wyk *et al*. 1997; ICS 2006). Approximately 300 tons of buchu is harvested annually and over 250 tons of that is being distilled into oil and is destined for export (ICS, 2006). South Africa is a key supplier of buchu oil to other countries (Coetzee, 1999).

2.1.6 Growth and soil requirements

Buchu is a member of fynbos vegetation. The fire-prone life span of between 10 to 15 years adheres to the buchu- group, becoming woody as they age. Kirstenbosch National Botanical Garden experimented with Buchu already in the

1920s to determine whether it can be cultivated commercially (Ntwana, 2007). Positive results were achieved and farmers started to cultivate Buchu commercially since then (Werner, 1949). Information on the growth requirements of *Agathosma betulina* is still limited, even after conducting many experiments. Buchu is restricted to specific areas in which it grows naturally and that is at an altitude ranging between 1 737 to 2 028 m above sea level on the south-west facing slopes (Blommaert, 1972a). Low levels of nutrients are characteristics of soils of Mediterranean type climates, yet even within these soils the levels of nutrients vary considerably (Grooves *et al*., 1980). Fynbos plants survive in soils which are notoriously deficient in all the nutrients essential for optimal plant growth (Cowling and Richardson, 1995). Fire is a mineralizing agent in fynbos, due to its returns of mineral elements held in the above ground biomass and litter to the soil (Brown and Mitchell, 1986). Frequent fires in Mediterranean-type climate are a key environmental factor and are coupled with nutrient paucity (Stock and Allsopp, 1992). The smoke treatment technique has been pioneered in South Africa in 1990 to break dormancy of seeds. High tannin concentration as well as essential oils in the sclerophyllous leaves of fynbos plants increases the flammability of the community during periods of water stress (Cloete, 2005). After fire, nutrient flushes become a characteristic feature of fynbos systems and the availability of nitrogen and phosphorus are increased (Musil and Midgley, 1990). Buchu grows well in Mediterranean type of climate, where summers are dry, but with rain during the winter months of May to September (Simpson, 1998). Buchu requires moderate to high water supply (Handforth, 1998).

Buchu grows in soils similar to other fynbos species. Generally the soils are coarse graded, nutrient poor, infertile and acidic (Maitre, Midley, 1992; Arkcoll, 1997). Infertility of fynbos soil is mainly due to its derivatives of rocks which are poor in nutrients (Goldblatt and Manning, 2000). The ecology of the vegetation of the infertile sandy soil is fragile and species are endangered through disturbances and due to their limited ranges (Bond and Goldblatt, 1984). Buchu naturally grows on mountains and the soils of these are whitish, sandy, shallow, rocky and low in pH (Cowling and Richardson, 1995).

2.1.7 Plant propagation

2.1.7.1 Seed germination

Buchu seed is both difficult to obtain and to germinate as well as the rooting of cuttings have not been very successful (Blomerus, 2002). Establishing plants due to poor germination and the survival of seedlings following transplanting in the field are the major problems (Blommaert, 1972b). Buchu plants produce their seeds in a capsule from which its ripening takes place (Werner, 1949). Seed that are collected from fully ripe capsules show a higher germination percentage (Blommaert, 1972a).

2.1.7.2 Vegetative propagation

Buchu may be propagated from cuttings (Karsen, 2003). Heel cuttings are preferred and in practice, when propagating Rutaceae. Cuttings should preferably be 2-4 cm in length. The heel of the cutting may be dipped in a rooting hormone to promote root formation and place in a well drained disinfected medium and placed on a hot bed. Rooting time for semi-hardwood cuttings is 6-10 weeks.

2.2 BIOTECHNOLOGICAL APPROACHES

Plant tissue cultures, are found to have potential as a supplement to traditional agriculture in industrial production of bioactive plant metabolites (Ramachandra Rao, Ravishankar, 2002). Shoot culture remains the most used form of clonal tissue culture and may provide a ready source of contaminant and disease-free plant material (George, 1993). Utilizing whole seeds or seed embryos, are other forms of tissue culture or a variety of non-shoot based explants disinfestations protocols for plant tissue culture have been reported (Bunn and Tan 2002; Pence, 2005) for material which have been sourced from wild populations. Initiation of shoots into culture, involves manipulation and optimization of the growing medium components, mainly plant growth regulators, to achieve shoot multiplication (stage II) and root generation (stage III) prior to adaptation and transferring rooted shoots into an *extra vitrum* environment (stage IV). Degrees of difficulty can be encountered within the stages and optimization may occur. Auxins, abscisic acid, ethylene and gibberellins are commonly recognized as the main groups of natural occurring plant hormones.

2.3 Aims and objectives

A. betulina is an economically important medicinal plant of South Africa and could be utilised more efficiently if the extent of its *in vitro* propagation and the acclimatisation for its cultivation *ex vitro* is fully investigated and understood. Previous studies have focused on some of the pharmacological activities, the effect of pH and some specific nutrients as well as the greenhouse rooting of cuttings. Buchu seed is both difficult to obtain and to germinate as well as the rooting of cuttings have not been very successful (Blomerus, 2002). Due to increased exploitation species survival is jeopardised. Micropropagation circumvent the use of wild collected plants and can provide commercial producers of *A. betulina* with the necessary information for rapid propagation and effortless access to high quality plants. Therefore, this study was undertaken with the following aims: (1) to establish a highly regenerative propagation protocol for *A. betulina*; (2) to determine if tissue cultured and *ex vitro* grown plants differ in essential oil quality. These aims were a first-time attempt and were met by following key objectives including:

(1) establishing a simple and highly productive micropropagation protocol for *A. betulina* through experimenting of seed germination, different explants types; the use of different combinations of auxins and cytokinins *in vitro*; production of roots *in vitro*, followed by mass transfer of *in vitro* propagated plants to the glasshouse for slow acclimatisation;

(2) analysing plant extracts to determine their differences at chemical level using gas chromatographic metabolite tools.

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CHAPTER THREE
IN VITRO SEED GERMINATION OF *AGATHOSMA BETULINA*

3.1 INTRODUCTION

3.1.1 Advantages of *in vitro* seed germination

Conventional propagation methods for *Agathosma spp*. utilise seeds as well as cuttings for plantlet cultivation and can root upon application of auxins. However, the rooting of *A. betulina* has been reported not to be very successful. Germinating seeds *in vitro* often produce a whole plant with established roots and shoots which can easily be acclimatized to be grown outside, either in a glasshouse or in the field. *In vitro* seedling generation produce plants at the same speed repetitively thereby allowing mass production of whole plants in a shorter timeframe. The advantage of using *in vitro* treatments to induce germination is that it increases the likelihood that all seeds will germinate at the same time, which is essential for further experiments.

3.1.2 Aims of the chapter

The aims of this chapter were to determine the factors which are limiting seed germination and to establish a viable protocol for the *in vitro* germination of *A. betulina* seeds. No previous information on *in vitro* seed germination and seedling development thereof has been published. Germinated seeds were then used as the starting point for the *in vitro* multiplication of *A. betulina*, which developed stock culture for further experiments. *In vitro* shoot and root generation is not discussed in this chapter, as the seed developed into seedlings and later established plants. A few rooted plants were acclimatized to grow in the glasshouse as a preliminary attempt. This chapter therefore reports and discusses a germination culture system which was found to be optimal for seedling generation and resulted in established and vigorous *in vitro* plantlets.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

3.2.1.1 Seeds

The fruits of *A. betulina* used for this experiment were harvested from Waboomsrust Farm, a privately owned site in Du Toitskloof, Western Cape Province, South Africa (latitudes 26°32'53, 22" South and longitudes 28°37'05, 22" East). This plant flowers from June up to November and the five segmented fruit pods (Fig 3.1A) were harvested throughout the month of December 2007 and kept in a cool dry box where triggers release seeds (Fig 3.1B) in a shooting action, as a method of harvest. Harvesting of seeds used in this experiment was done five years ago (2007) before a fire outbreak on the farm and the seeds were stored in hessian in a cool dry place.

Figure 3.1: (A) Five chambered fruit pods prior to harvesting of seeds (B) *A. betulina* seeds

3.2.2 Pre-germination treatments and decontamination of *A. betulina* seeds

Several treatments were applied to seeds prior to incubation in the light growth chamber: a) seeds were physically scarified by gently removing the hard seed coat completely; b) seeds were gently rubbed with sand paper to break the seed coat and then soaked in smoke-water overnight; c) the third treatment involved the soaking of seeds overnight in a cold room at 4°C in combination with smoke and sterile distilled water; d) seed coats were physically removed and left overnight in smoked distilled water in a cold room. Untreated seeds were used as control for this experiment.

Following the pre- treatment, the seeds were decontaminated by a 1 min immersion in absolute ethanol, followed by a 10 min wash in commercial bleach solution [3% (v/v) NAOCL] prior to three wash steps for 5 min each in sterile distilled water (dH_2O). Following the wash steps, the seeds were blotted dry on sterile filter paper (Whatman® No.10) and later transferred to the growth medium. Five seeds were placed on each Petri dish and these were incubated in the light growth chamber and the germination was monitored for 35 days.

3.2.3 Media preparation procedures

Full, half and quarter strength of the macro- and micro nutrients described by Murashige and Skoog (1962) were tested in an initial experiment. All experiments which followed were done with ½ strength MS media. The medium was solidified with 1% (w/v) Agar (Biolab, Gauteng, South Africa). All media used were autoclaved at 121°C and 103 kPa (1.1 kg cm^{-2}) for 20 minutes after the pH had been adjusted to 5.7 with 1M KOH or 1M NaOH. All experiments were conducted under laminar flow conditions. Cooled medium was then poured into 10 cm Petri dishes (100 mm x 20 mm, Corning®, USA). For the smoke treatment, concentrated Kirstenbosch liquid smoke [active ingredients: butenolide (3-methyl 2H-furo (2,3-c) pyran-2-one)] ; "Flematti *et al*., 2004; van Staden *et al*., 2004") was added to a concentration at 10% prior to autoclaving. Each treatment had five Petri dishes, each containing five seeds and were sealed with 2cm strip of Parafilm 'M'® (American National Can™, USA). Petri dishes were then placed in the light growth chamber under an illumination of 50 µmol m^{-1} s^{-2} photosynthetic photon flux density (PPFD) and a 16 hour light and 8 hour dark cycle. The light was provided by 'cool-white' fluorescent tubes (L75W/20XOsram, USA, code number F96712). The temperature was maintained at 24°C ± 2°C for all seed lots. The number of seeds that germinated daily per Petri dish was scored for 35 days.

3.2.4 Continuous subculture, rooting and commercial potential for the micropropagation protocol

Microplants were routinely subcultured every four to six weeks and this involved placing whole plantlets on fresh RM to facilitate new and continuous growth. Further investigations were done on the effect of various strengths (1/4, ½ and full strength

MS) of basal RM on the *in vitro* derived nodal explants discussed in Chapter four (Table 4). Therefore, the most suitable medium for plantlet regeneration was ½ MS and all other medium strengths were disregarded.

Seedlings with the primary root attached elongated best over a period of 28 days when transferred to a PGR free medium. It was evident that the rooting of *A. betulina* was not spontaneous, as there was no callus or roots observed when explants were exposed to media without PGR. Further investigations are therefore necessary to determine the factors limiting the rooting of this plant.

In vitro plantlets of *A. betulina* were subcultured to determine the potential amount of plantlets which would arise from the original explants subsection. This involved placing the explants (1.5 cm) sections of the plantlet in glass vessels (90 × 50 mm) containing 25 ml PGR-free medium. The physiological parameters (length of each plantlet, the number of shoots, as well as the amount of internodes) were noted after 28 days in culture.

3.2.5 Acclimatisation of *A. betulina in vitro* plantlets to glasshouse conditions

In vitro cultures with a well developed root system were extracted from tissue culture vessels and gently washed with distilled water. The plants were planted out in pots (13 x 10 cm) containing sand and peat (2:1; v/v) which was autoclaved at 122 kPa and 121 °C for 20 min prior to use. The plants were covered with a plastic bag to assist in slow acclimatisation to glasshouse conditions (a temperature controlled environment: minimum of 15 °C and maximum of 22 °C).

3.2.6 Data collection and statistical analysis

Data were collected on twenty five seeds (five per Petri dish) per treatment. The number of germinated seeds was noted every three days for 35 days. The percentage data for the germinated seeds for each treatment were analyzed using Statistica Release 11 (Statsoft Inc. 2011) using one-way analysis of variance (ANOVA) and the Fischer LSD test. The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant.

3.3 RESULTS AND DISCUSSION

3.3.1 Germination of seeds

A seed was considered to be germinated when the radicle (1 mm) protruded from the seed capsule. No previous results have been reported on the *in vitro* seed germination of *A. betulina*. The seeds were handled as fynbos seeds. The initial understanding of the buchu seeds were that they would not need to be scarified, as I could easily break the seeds coat mechanically. The first experiment therefore tested the effect of different strengths on MS media (1/4 and ½ strength) with and without smoke solution (results are not shown). Periodic fires are a natural phenomenon in the fynbos vegetation. Fire-stimulated seed germination for various fynbos types has been reported. It was observed in this study that after day 3, the decontamination method was 100% successful (Fig 3.2). However, there were visible signs of germination after 7days in culture. The general trend evident in this study shows that signs of germination occur after 7days in culture especially with the application of smoke solution (Fig 3.3). It was obvious at day 14 that the seeds of *A. betulina* are dependent on scarification for germination, as this had the most significant results (36.00±16.00) Table 3.1.

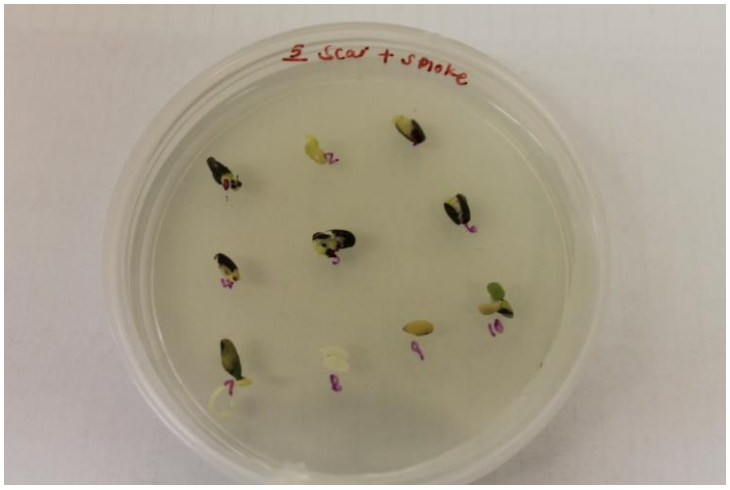

Figure 3.2: 100% decontamination after 7days in culture

To establish synchronized germination, *A. betulina* seeds were exposed to smoke solution, scarified physically and/ stratified; and as anticipated, the germination of *A. betulina* seeds followed a sigmoid pattern over 35 days (Table 3.1). All seed treatments were exposed to light conditions. The dormancy-breaking mechanism of light is linked to the phytochrome function. This then in return activate the biochemical and genetic changes which leads to increased GA3 inside the seed. According to Taiz and Zeiger (2002), the GA3 is important in the activating of α-amylases and other hydrolytic enzymes in seeds resulting in the degradation of stored resources as well as the endosperm.

As a general trend, the first signs of germination were noted in a few seeds pre-treated with scarification and smoke medium under light conditions approximately 7 days after culturing. This was an indication that germination was reliant on the removal of the seed coat (facilitated by physical scarification) and/ or in combination with smoke treatment. Also effective, but to a lesser degree was treatment stratification/scarification and smoke producing only 16% of germinated seeds. Stratification which mimics chilling and moist conditions is a standard method utilized to enhance and speed up dormancy (Rehman and Park 2000). The latter was thought to be a dependant during germination of *A. betulina* as some farmers germinate their seeds ex vitro during the autumn months of the year to allow the plants to grow vegetative during the cooler winter months.

Exogenously applied GAs as a substitute for stratification successfully aid in alleviating dormancy in seed. Germination remained constant in treatment scarification and smoke between days 18 and 28 and picked up again thereafter (Table 3.1). At day 35 however, germination rate dropped drastically therefore, the experiment was terminated at day 40. The seeds which experienced no treatment (control) showed signs of germination at day 64, with only two seeds responding (results not shown).

The final mean percentage germination of *A. betulina* indicated that there were significant differences in the number of germinated seeds among treatments. Physical scarification and smoke proved to be the optimal treatment required for obtaining the highest amount of seed germination (Table 3.1). This germination treatment which involves the removal of the hard testa of seeds resulted in 82% seed germination. This is probably because the scarification process enabled higher

concentrations of smoke compounds to reach the embryo, thus, enhancing germination. The influence of smoke on seed germination is well documented and the trigger of this influence can be assigned to butenolide (3-methyl 2H-furo (2, 3-c) pyran-2-one) as observed by Flematti *et al*., 2004; van Staden et al 2004. This compound promotes germination rate as well as percentage germination (Jain *et al*. 2008). No germination effects in GA and smoke as well as cold and smoke treated seeds can be ascribed to the detrimental effects of the cool climate on the seeds of *A. betulina.*

Table 3.1 Mean percentage germination frequency of *A. betulina* seeds under different pre-germination treatments

	Germination (%)						
Effect	**7days**	**14days**	**18days**	**25days**	**28days**	**33days**	**35days**
Control	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00c	0.00±0.00c	0.00±0.00c
Scarification and smoke	16.00±6.78**b**	36.00±16.00**a**	6.00±1.52**c**	6.00±1.00**c**	6.00±1.00c	7.20±0.80c	8.20±0.92bc
GA and smoke	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00c	0.00±0.00c	0.00±0.00c
Cold and smoke	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00**c**	0.00±0.00c	0.00±0.00c	0.00±0.00c
Cold , scarification and smoke	0.00±0.00**c**	2.00±2.00**c**	2.40±1.94**c**	0.80±0.49**c**	1.40±0.40c	1.80±0.66c	1.60±0.40c

Values presented are means± SE. Different letters in the same column indicate values that are statistically different at a 95% confidence level.

Table 3.2 A summary of the effect of various pre-germination treatments on *A. betulina* seeds after 35days in culture

Effect	Degrees of freedom	F	p
Germination mean	34	4.99911	0.000000*

Includes no treatment as control. *Denotes the treatments (interactions) that had a significant impact on germination at the 95% confidence limit.

The general deduction from the study is that seed coat removal significantly increased germination. Removing a seed coat for successful germination is a sure way of overcoming coat-imposed dormancy (Taiz and Zeiger, 2002). This is because the seed coat is made up of several palisade layers containing lignified cells which are packed closely, containing water repelling compounds (Baskin, 2003). These layers act as a physical barrier to gaseous exchange and water assimilation. Water imbibition is essential for enzymatic function in a seed and gaseous exchange is essential for respiration.

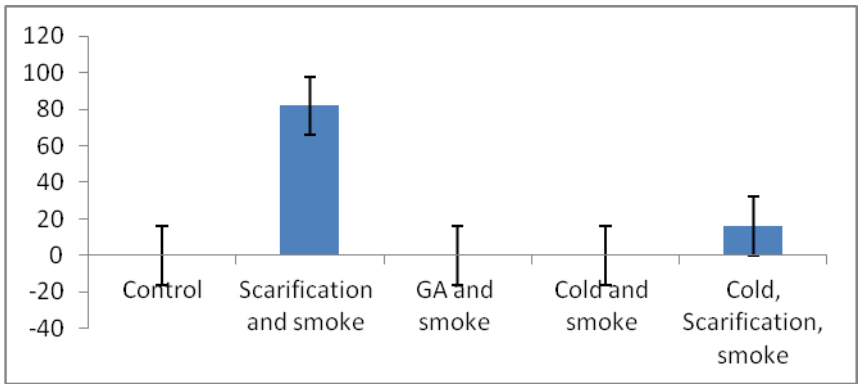

Figure 3.3: *In vitro* germination results of *A. betulina* seeds after 35days in culture

The results presented in Table 3.2 strongly supported the stimulatory role of smoke in breaking coat imposed dormancy and thus, enhancing germination in *A. betulina* seeds. Moreover, chemical scarification of seeds with sulphuric acid resulted in no germination response. Possible explanation for this may be due to the sensitivity of the seeds to the acid concentration as well as time of exposure. The repeated exposure to concentrated sulphuric acid could have damaged the embryos and resulted in no germination as indicated in Table 3.2.

Table 3.3 Pre-germination treatment scarification of *A. betulina* seeds with and without smoke-induced germination after 7days in light conditions

Treatment	Percentage germination (%)
Physical scarification (control)	2.0[b]
Physical scarification and smoke	18.0[a]
Chemical scarification`	0.0[b]
Chemical scarification and smoke	0.0[b]

Different letters indicate statistical significance at the 95% confidence level.

Table 3.4 A summary of the effect of pre-germination treatment scarification of *A. betulina* seeds with and without smoke-induced germination after 7days in culture

Effect	Degrees of freedom	F	p
Germination mean	3	6.333333	0.004903*

Includes scarification without smoke as control. *Denotes the treatments (interactions) that had a significant impact on germination at the 95% confidence limit.

Although physical scarification was most effective for germination, an immense number of seedlings died post germination due to an internal fungal contamination which coincided with seedling emergence (Fig. 3.4A). Interestingly however, a brown-colored substance was released into the cultivation medium surrounding the seeds (Fig. 3.4B). This may be an indication of the allelopathic properties of *Agathosma* seeds. Both findings were similar to those of Colling, 2009 during the germination of *Sutherlandia frutescens*.

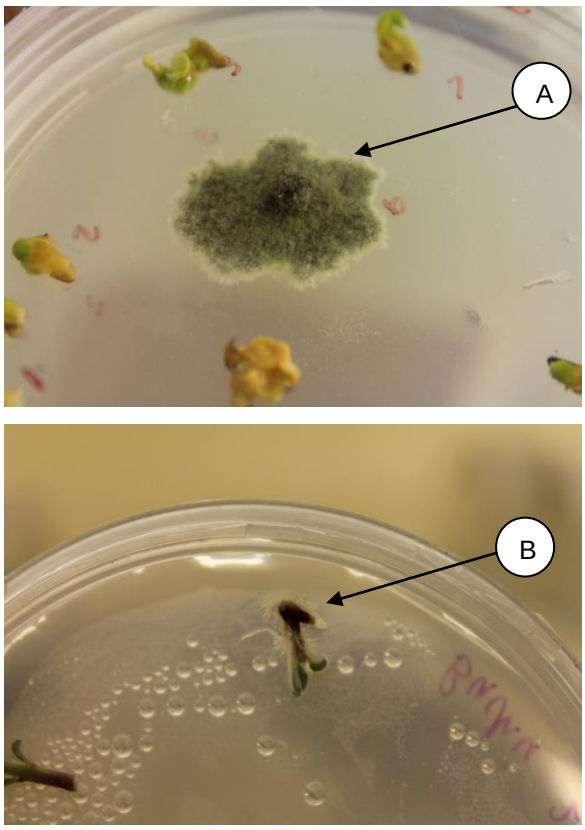

Figure 3.4: *Agathosma betulina* **(A)** internal fungal contamination **(B)** allelopathic response

3.3.2 Continuous subculture, rooting and commercial potential for the micropropagation protocol

Blomerus (2002), reported that the rooting of buchu cuttings have not been very successful, therefore whole plants were initially kept intact with its roots and nodal explants were taken from these. Plantlets with more internodes could be subsectioned for the proliferation of more plantlets in subsequent culture cycles. Each nodal derived plantlet consisted of two shoots and no root formation. This implies that ideally hundreds of new plantlets can be produced from one nodal explant after 28 days in culture.

Table 3.5 Effects of ½ MS medium on the growth response of *A. betulina* nodal explants after 28days in culture

Treatment	No. shoots	Shoot length	No. internodes	No. leaves
½ MS	1.266±0.228	17.933±2.020	4.333±0.557	9.266±1.313

Values presented are means± SE

3.3.3 Acclimatisation of *A. betulina in vitro* plantlets to glasshouse conditions

When the seed germination protocol was followed as described in Fig 3.6, the well established plants were identified to be acclimatized in greenhouse conditions. *In vitro* plantlets were able to adjust to out of culture conditions for up to two weeks plastic bags were cut open, as the plants were ready for further acclimatization. After 17days adjusting to out-of-culture conditions fungus set in and it was too late to save plants.

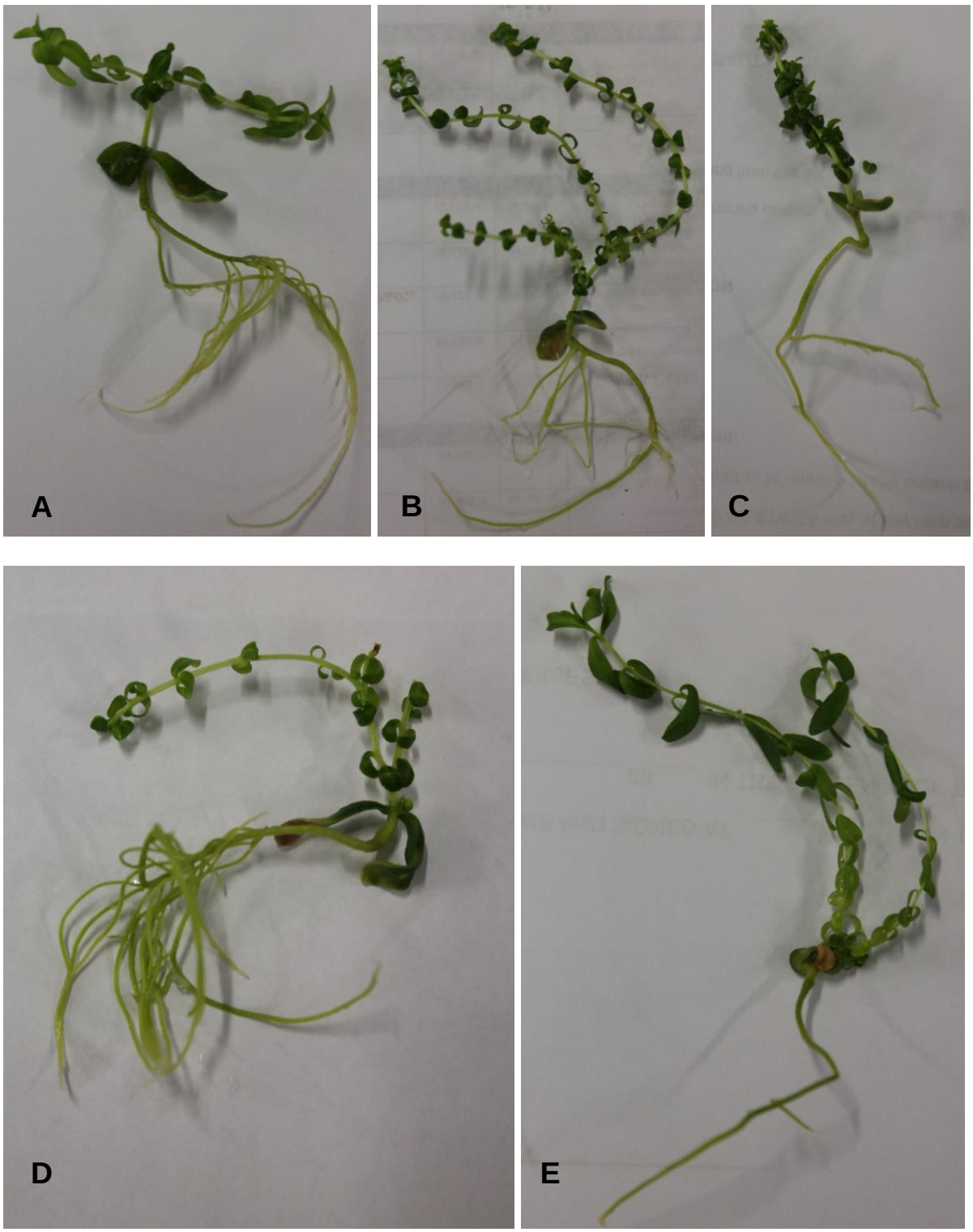

Figure 3.5: Prior to glasshouse acclimatization of established *A. betulina in vitro* plants

3.4 Conclusion

The current lack of a seed germination protocol for *A. betulina* may be a hindrance to the conservation of this species, thereby making the plant species vulnerable to collection for pharmaceutical use. As a result of this, the possibility of the extinction of this plant is more apparent.

In order to circumvent this problem, a novel seed germination protocol was determined for *A. betulina* which can be adopted for commercial production. The seed germination treatment, physical scarification and smoke were the optimal treatment for the germination and further growing of the plant. This treatment gave high seedling count and could promote further vegetative growth as well as provide explants for further investigations. However, the duration of exposure to an acid is important and this treatment should be optimized for each new species. Furthermore, this was the recommended seed germination treatment throughout the study as the seedling grew to develop a strong root system, consisting of a tap root and adventitious roots as well as multi-branched shoots. Thus, potentially developing into a plant that can be acclimatized to be grown in the field. Seedlings could easily be maintained *in vitro* and was bulked up through subculturing in a four to six week cycle. This protocol could play a key role in the large scale mass propagation of *A. betulina* (Berg.) Pillans.

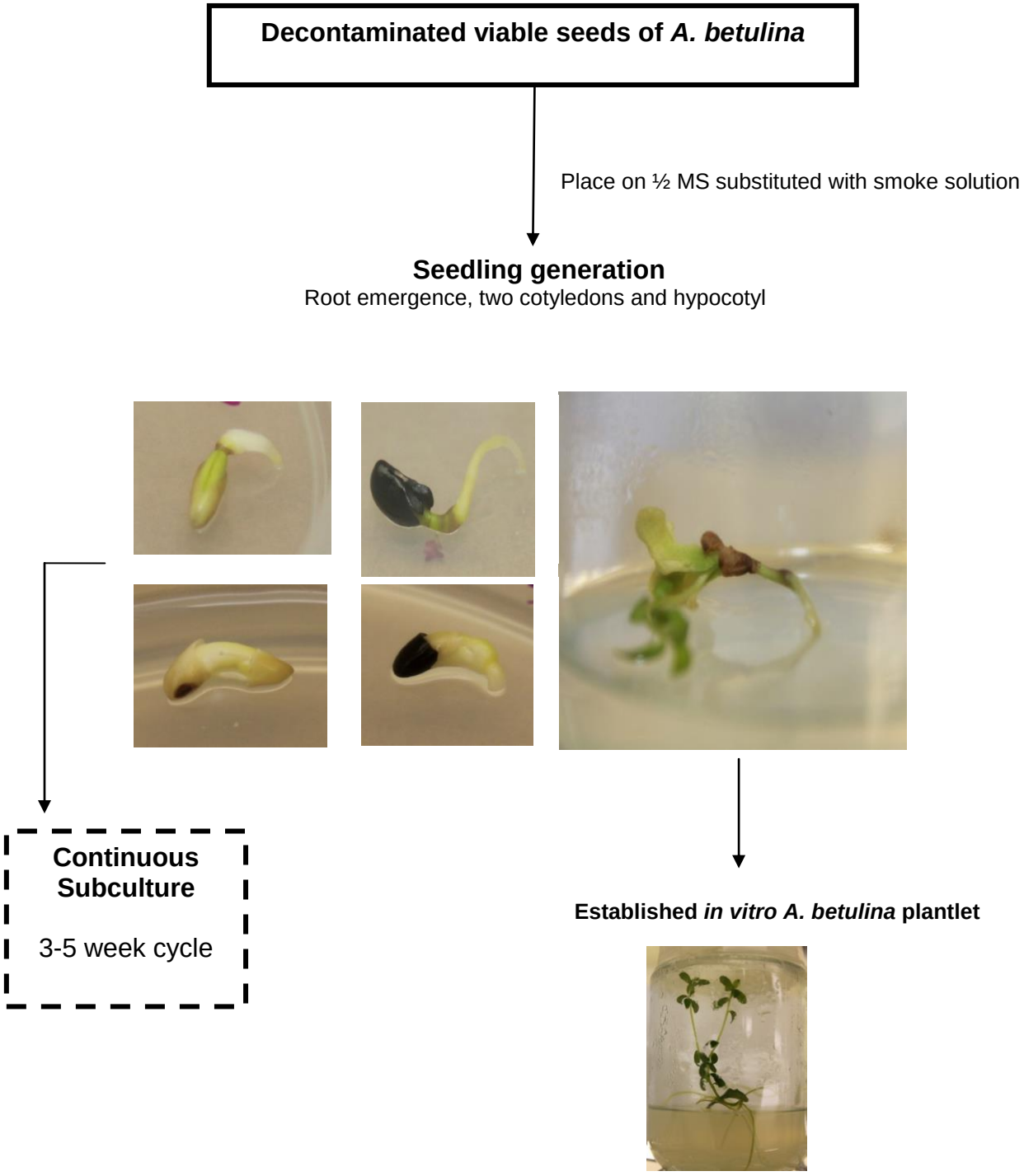

Figure 3.6: Protocol for the seed germination of *A. betulina*

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CHAPTER FOUR
IN VITRO REGENERATION OF SHOOTS AND ROOTS OF *AGATHOSMA BETULINA*

4.1 INTRODUCTION

There are several advantages associated with the *in vitro* propagation or even other biotechnological approaches to aromatic and medicinal plants. *In vitro* regeneration of plants is a biotechnological tool that offers a possible solution to the problem of medicinal plants decimation in South Africa. The World Health Organization (WHO) estimates that up to 80 percent of people still rely on herbal remedies for their health care (Farnsworth *et al*., 1985).

Numerous micropropagation methods such as organogenesis, somatic embryogenesis and axillary shoot proliferation are all techniques that have been tested on woody plants. Jain and Häggman, 2007 have described axillary shoot proliferation as the most widely used method. The adventitious root formation is crucial for the successful vegetative propagation of many woody plants. Conventional propagation methods for *Agathosma spp*. utilise seed as well as cuttings for plantlet cultivation and can root upon application of auxins. However, the rooting of *A. betulina* has been reported not to be very successful. In some cases, micro cuttings root better in *in vitro* environments. According to Lineberger (1983), *in vitro* rooting was superior to *ex vitro* rooting for *Prunus* x ‘Hally Jolivette’. For centuries, this plant has been known as a slow grower and efforts have been made to understand the plant better.

Although the germination of *A. betulina* seeds was 100% successful, this chapter aims to determine the factors that have an influence on multiplication and rooting of *A. betulina* as well as to establish a viable protocol for its continuous *in vitro* propagation. The resulting material was then used for further experimental work (Chapter 5) to provide a stock culture.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

4.2.1.1 *In vitro* derived material

Mature offspring of *in vitro* derived decontaminated and germinated *A. betulina* discussed in Chapter 3 was used as the starting material for this chapter along with the material discussed in (4.3.1.2). Materials derived from germinated seeds usually have more genetic variation and it is therefore important to select the material to be used for multiplication. Decontaminated material from field derived plants was used for comparative study.

4.2.1.2 Field derived material

The youngest tissues of the current year's (2012) growth were harvested in July 2012 from Waboomsrust Farm, a privately owned site in Du Toitskloof, Western Cape Province, South Africa (latitudes 26°32'53, 22" South and longitudes 28°37'05, 22" East). Cuttings taken from the field were kept cool in Zip Seal® bags (Glad®, South Africa) containing water to avoid desiccation during transportation. The plant material was kept in a refrigerator at -4 °C until it was used. Nodal explants of these cuttings were used as starter material and also to test the effect of various basal regeneration media.

4.2.2 Decontamination and culture initiation of field derived foliage plant material

All foliage plant material was decontaminated prior to culture induction. Various decontamination methods were tested. The youngest healthy current year's shoots were selected and: **(A)** rinsed in tap water to wash off all foreign debris prior to testing four solution strengths of a commercial bleach-sodium hypochlorite (NaOCL) at (1) 0.1% NaOCl; (2) 0.5% NaOCl; (3) 1% NaOCl and (4) 1.5% NaOCl which was followed by a wash with sterile distilled water for five min at a time (3×5min) before culture induction.

(B) Rinsed in tap water to wash off all foreign debris prior to incubation in 70% (v/v) ethanol for 2 min. Plant tissues were then immersed in a fungicidal solution of 2% (w/v) Dithane WG-45 solution (active ingredient: mancozeb 750 g kg^{-1}; Efekto, South Africa) and Virikop (active ingredient: copper oxychloride 500 g kg^{-1}) for 20-30 min.

Tissues were transferred and immersed in commercial bleach supplemented with Tween20 for 5 min with the final concentration of the active ingredient, sodium hypochlorite being 3% (w/v). **(C)** Heat treatment was applied by placing the material in sterile distilled water on a hot plate until it came to a boil. At boiling point, the plant material was removed from the hot surface, hot water decanted from them, followed by an immersion in a fungicidal solution of 2% (w/v) Dithane WG-45 solution (active ingredient: mancozeb 750 g kg^{-1}; Efekto, South Africa) and Virikop (active ingredient: copper oxychloride 500 g kg^{-1}) for sixty min. The decontaminated plant material was then washed with sterile distilled water (dH_2O) for five min at a time before an immersion in 3% sodium hypochlorite (NaOCL) for five min. This was followed by a wash three times with sterile distilled water for five min at a time (3×5min) before culture induction.

4.2.3 Media preparation and culture induction (shoots and roots) of *A. betulina*

A. betulina seedlings (90-day-old) were mainly used for culture induction in September 2012. Nodal explants (1.5cm) containing a pair of axillary buds were placed on solid half complement Murashige and Skoog (1962) salts and vitamins (4.4 g l^{-1}; Highveld biological, South Africa) with 0.1g l^{-1} myo-inositol, 30g l^{-1} sucrose and 8g l^{-1} agar (w/v) (5.7-5.8, adjusted with 1 M HCL or 1 M NaOH).

Nodal explants were exposed to various hormone ratios to test the effect thereof. Explants were subcultured onto an appropriate multiplication medium. The experiment was composed of hormonal grid treatments. A system was achieved by experimenting with N_6-benzylaminopurine (BA), 1-Naphthaleneacetic acid (NAA), Dichlorophenoxy acetic acid (2, 4-D) and combinations thereof. The correct auxin: cytokinin ratio is essential as their combination may interact and promote or inhibit the development of shoots and roots. Plants were placed in a growing room with 24-h light illumination to induce multiplication, light was provided by “cool-white” fluorescent tubes and data were collected every 28 days.

After auxin and cytokinin combinations were used to amend the regeneration media, the media was autoclaved at 122 kPa and 121 °C for 20 min. The medium was solidified with 1% Agar-Agar (Biolab, Gauteng, South Africa). Prior to this, the pH had been adjusted to 5.7 with 1M KOH or 1M NaOH. All experiments were conducted

under laminar flow conditions. Details regarding preparation of other different media are provided in Appendix A.

4.2.4 Acclimatisation of *in vitro* plantlets of *A. betulina* to glasshouse conditions

In vitro cultures with a well developed root system were extracted from tissue culture vessels and gently washed with distilled water. The plants were planted out in pots (13 x 10 cm) containing sand and peat (2:1; v/v) which was autoclaved at 122 kPa and 121 °C for 20 min prior to use. The plants were covered with a transparent plastic bag to assist in slow acclimatisation to glasshouse conditions (a temperature controlled environment: minimum of 15 °C and maximum of 25 °C). The survival rate was recorded after 28 days.

4.2.5 Continuous subculture and commercial potential for the micropropagation protocol

Microplants were routinely subcultured every four to six weeks and this involved placing sections of the plantlet on fresh RM to facilitate new growth. *In vitro* plantlets of *A. betulina* were subcultured to determine the potential amount of plantlets which would arise from the original explants subsection. The length of each plantlet, number of shoots and leaves, as well as the amount of internodes was noted.

4.2.6 Statistical analysis

Data were collected on fifteen nodal explants per treatment. The response was scored after 28 days in culture. The data for the growth parameters in response to the various treatments were analyzed with Statistica Release 11 (Statsoft Inc. 2011) using one-way analysis of variance (ANOVA) and the Fischer LSD test. The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Decontamination methods of *ex vitro* derived explants

The initial decontamination method with the various concentrations of NaOCl gave fatal results by, however 1.5% NaOCl had more phenolic reactions rather than fungal

or bacterial contamination. Further methods resulted in decontaminated explants while the use of a bleach/virikop/dithane mix, reduced fungal and bacterial contamination during these first two critical weeks of *in vitro* culture. When using older wood, survival appeared to be more successful than young shoots. This can be ascribed to the higher pulegone levels in younger plants. According to Endenburg (1972), harvesting at different times of the year resulted in differences in pulegone levels which are hepatotoxic and it is a monoterpene that protects source plants against predators (Thomas *et al*., 1990). Due to the former result, pulegone is believed to be higher in younger plants (Esterhuizen, 2013. Per. Comm, Puris Natural Aroma Chemicals (Pty) Ltd.) Further studies may be conducted to determine the best season for micropropagating *A. betulina.* This would require establishing the period in which this plant has lower pulegone levels. After the application of the two decontamination methods it is still unclear whether it is necessary to have the fungicide and bactericide as an extra washing step.

In an effort to improve the survival of the explants placed in culture, another decontamination method was tested using heat. The heat treatment appeared to be lethal as no survival upon the application was recorded. The decontamination of field derived plant material appeared to be unreliable. Interestingly, the method that was optimal did not always give the same results, which is thought to be linked to the genetic make-up of certain individuals (mother stock), harvesting time and physiological state of the plants.

Decontamination posed not to be economically viable. After various methods, resources were exhausted as well as time. It is evident in Fig 4.1 that decontamination methods were successful. Buds in the axils developed to 1.5 cm (Fig 4.1 A) at 14 days in culture. Interestingly, contamination rates of explants were influenced by the stage of maturity of the explant material.

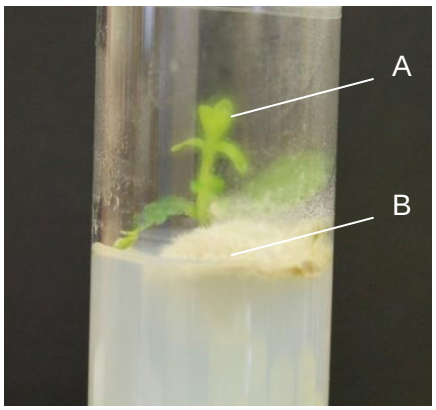


Figure 4.1: Successful decontamination is evident in the new growth **(A)** and fungal contamination **(B)** post decontamination found 14 days after culture

4.3.2 Effect of ¼, ½ and full strength MS on the growth response of nodal explants

The nutritional requirements vary from specie to specie, therefore the general growth responses of the nodal explants cultured on different strengths of MS (1/4, ½, full) were studied and recorded after 28 days in culture (Table 4.1). The analysis indicated that there was significant difference in the number of shoots (Fig 4.3 A) produced by ¼ MS media (1.600±0.190a) and also effective but to a lesser degree was ½ MS media (1.266±0.228ab). Media with the full complement of macro and micro nutrients was the least significant (0.857±0.265b). According to Cowling and Richardson (1995), fynbos naturally occur in nutrient deficient soil. Yet, in order to encourage growth well balanced fertilization is required (Hansforth, 1998). Shoot length clearly showed a significant difference (Fig 4.1 B) in the ½ MS media (17.933±2.02a) compared to ¼ MS (9.733±1.981ab) and least significant in full MS media treatment (3.857±2.655b). The number of internodes corresponded to the shoot length that was recorded at day 28. The treatment ½ MS had a significant amount of internodes (4.333±0.557a), followed with ¼ MS (2.933±0.529ab) and full MS (1.142±0.658b) was least significant in the number of internodes in this treatment. The treatment ½ MS produced the highest number of leaves (9.266±1.313a) when subjected to 28 days in culture. This is an indication that the latter treatment would be most effective when production of leaves is favoured. The ¼ MS treatment was also effective but to a lesser degree (6.866±1.256ab) while full MS produced the least number of leaves. Table 4.1 is a clear representation that the optimal media to be used for the micropropagation of *A. betulina* is a ½ MS; compliment of macro and micro nutrients, as the shoot length, number of internodes

as well as the number of leaves were significantly higher compared to other MS used. The number of shoots was also significantly higher than that of full MS but not significantly different from ¼ MS.

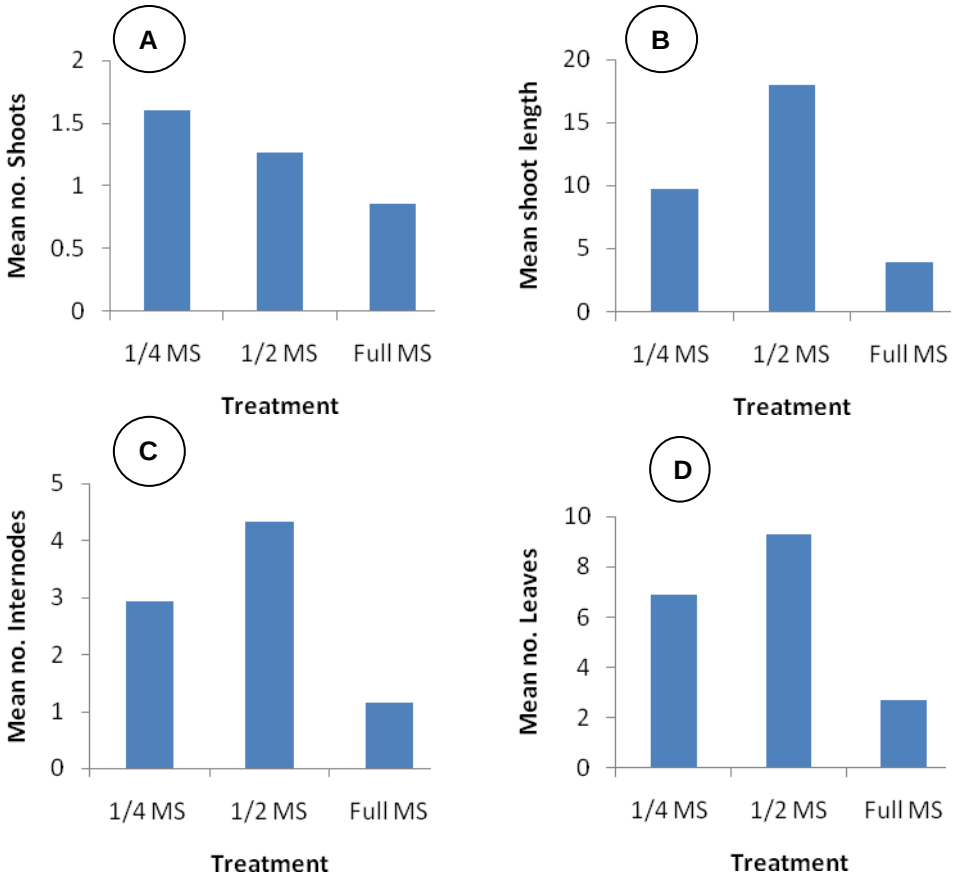


Figure 4.2: Effects of various strengths of MS medium on the growth response of field derived *A. betulina* after 28 days in culture

Table 4.1 Effects of various strengths of MS medium on the growth response of field derived *A. betulina* after 28 days in culture

Treatment	No. shoots	Shoot length	No. internodes	No. leaves
¼ MS	1.600±0.190[a]	9.733±1.981[ab]	2.933±0.529[ab]	6.866±1.256[ab]
½ MS	1.266±0.228[ab]	17.933±2.020[a]	4.333±0.557[a]	9.266±1.313[a]
Full MS	0.857±0.265[b]	3.857±2.655[b]	1.142±0.658[b]	2.714±1.643[b]

*Values presented are means± SE

*Treatment means obtained from 15 replications, means with different letters indicates significant difference at p<0.05 according to Fischer least significance difference

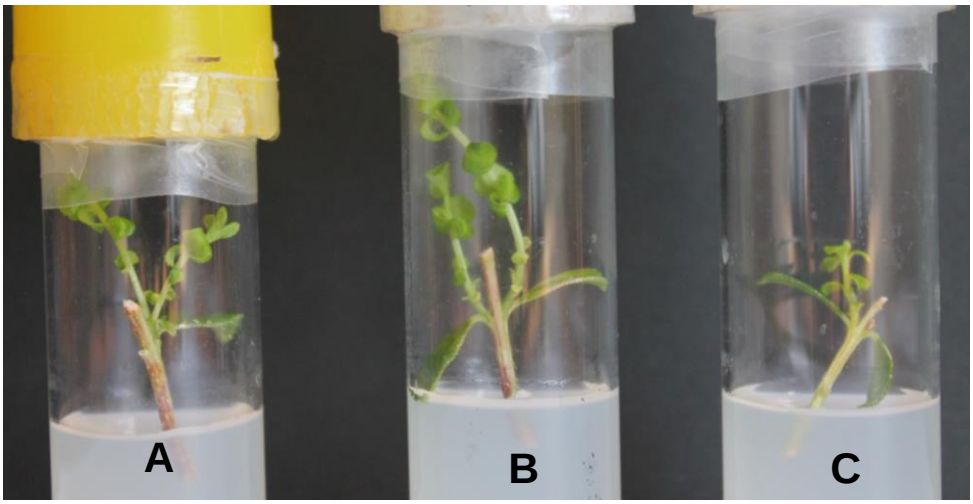


Figure 4.3: Effect of **A**. ¼, **B**. ½ and **C**. full complement MS medium on the growth response of field derived explants and *in vitro* derived explants of *A. betulina* after 28 days in culture

It was worth noting that when young seedlings were grown in a full complement of MS the root had a different growing action. Generally, the roots of plants are found growing downwards and forming a circling action when older inside the glass pot. *A. betulina* plants growing in a full complement of MS roots was found outside the media (Fig 4.4 A and B) with only a minute apart in the agar media. When *A. betulina* is grown in a half complement MS medium the reaction is the opposite of latter (Fig 4.4 C and D).

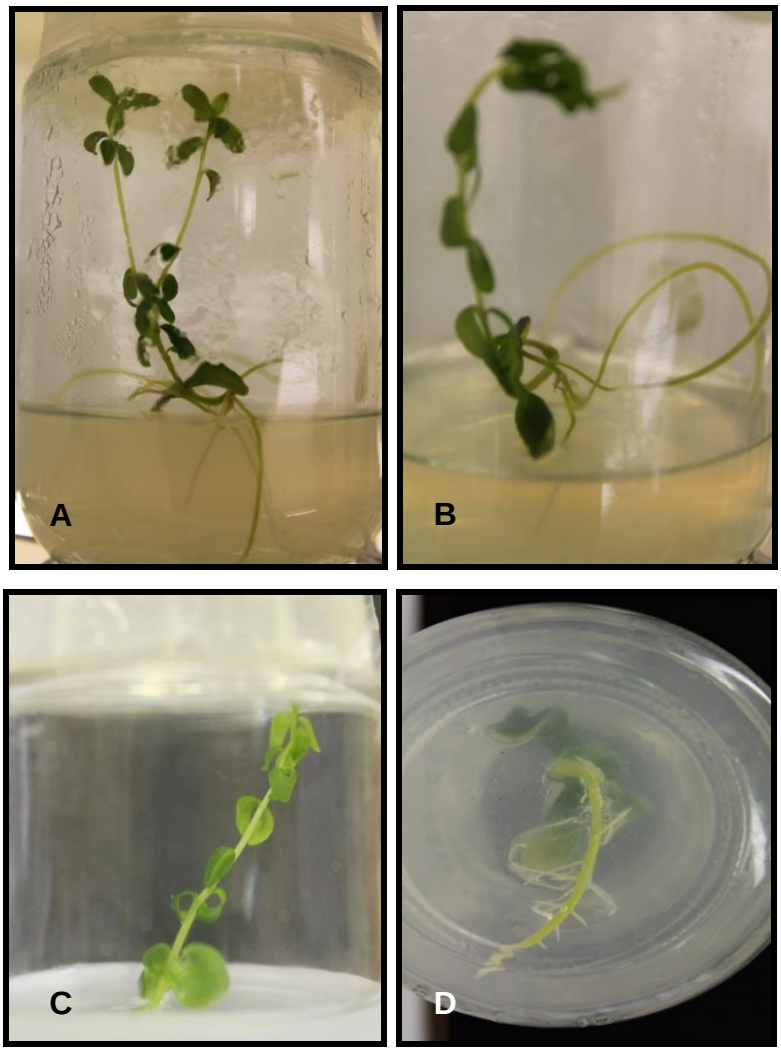


Figure 4.4: Effect of ½ and full complement MS medium on the root growth response *in vitro* derived explants of *A. betulina* after 28days in culture

Table 4.2 Effects of nodal explants on the various strengths of MS medium on the growth response of field derived *A. betulina* after 28 days in culture

Effect	Degree of freedom	F	p
One-way ANOVA			
No. shoots	2	2.54434	0.090863
Shoot length	2	4.94778	0.011889
No. internodes	2	4.02962	0.025252
No. leaves	2	3.52506	0.038666*

Includes ¼, ½, and a full complement MS media. *Denotes the treatments (interactions) that had a significant impact on the establishment of various growth parameters at the 95% confidence limit.

4.3.3 Comparison between field-derived explants and *in vitro* derived explants on ½ MS media

There was no significant difference in the mean number of shoots presented for the two treatments. This can be assigned to the explant type used in this study. Nodal explants tend to produce one or two shoots when initiated in MS basal medium without the addition of plant hormones. However, a significant difference was observed in the shoot length of the explant types being discussed. Shoot length was more than two-fold dominant in the field derived explants (17.933±4.567a) compared to the *in vitro* derived nodal explants (8.000±1.134b). This can be ascribed to the woody shoots which were already established in transporting water and nutrients to the growth tips. The amount of internodes a plant produces generally indicates the amount of cuttings that can be obtained for further propagation. The field derived explants produced higher amount of internodes, therefore this would be a more economical method for mass production of *A. betulina*.

The explants of the field derived material failed to produce callus on ½ MS medium. In contrast, callus was initiated in a few (0.133±0.091a) of the *in vitro* derived explants. The callus appeared as light-yellow in colour with friable texture. The ½ MS basal medium alone did not induce any further morphogenic response from the yellow friable calli and also did not promote further growth. Field derived explants were significantly taller than *in vitro* derived nodal explants throughout the 28-day growth cycle when shoot length was compared (Fig 4.3). Plant height or shoot length is a measure of plant vigour, indicating that field derived plants established more quickly and grew vigorously.

Table 4.3 shows field derived material as favourable explants to be used during the micropropagation of *A. betulina*, however the vigorous growth was limited to the crucial initial period of eight weeks and the explants died off thereafter.

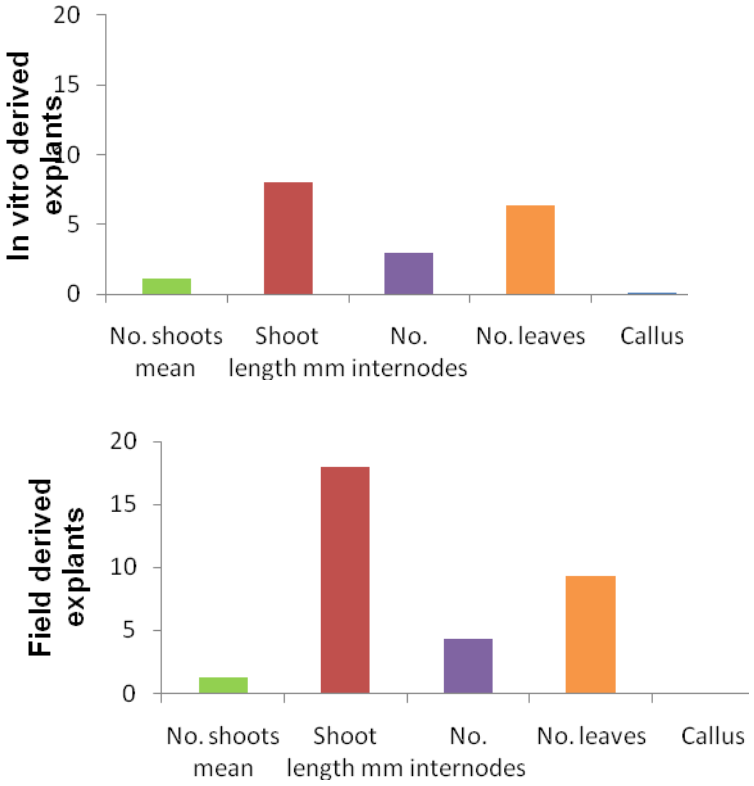


Figure 4.5: Effect of ½ MS medium on the growth response of field derived explants and *in vitro* derived explants of *A. betulina* after 28 days in culture

Table 4.3 Effect of ½ MS medium on shoot differentiation from field derived explants and *in vitro* derived explants of *A. betulina* after 28 days in culture

Explant	No. shoots	Shoot length	No. internodes	No. leaves	Callus
Field derived explants (n=15)	1.266± 0.228[a]	17.933± 4.567[a]	4.333± 1.107[a]	9.266± 2.296[a]	0.000± 0.0[a]
In vitro derived explants (n=15)	1.133± 0.091[a]	8.000± 1.134[b]	2.933± 0.384[a]	6.400± 0.980[a]	0.133± 0.091[a]

*Values presented are means±SE

*Means followed by dissimilar letter(s) in a column are significantly different from each other at P=0.5 according to Fischer least significance difference.

Table 4.4 Effects of nodal explants on the *in vitro* and field derived plant material of MS medium on the growth response of field derived *A. betulina* after 28 days in culture

Effect	Degree of freedom	F	p
One-way ANOVA			
No. shoots	1	0.29474	0.591497
Shoot length	1	4.45522	0.043855
No. internodes	1	1.42784	0.242140
No. leaves	1	1.31883	0.260519
Callus	1	2.153846	0.153357

Includes ¼, ½, and a Full complement MS media. *Denotes the treatments (interactions) that had a significant impact on the establishment of various growth parameters at the 95% confidence limit.

4.3.4 Effect of 2, 4-D PGR on the growth response of *in vitro* derived plant material

The mean number of shoots was significantly higher (2.000±0.00) when 0.5mg l^{-1} 2, 4-D was tested on nodal explants. Also effective but to a lesser degree was 1 mg l^{-1} 2, 4-D (1.300±0.213) but this was not significantly different from the control (no PGR) which had a mean number of shoots of 1.200±0.133. Treatments 3 mg l^{-1} and 5 mg l^{-1} had no significant effect on the mean number of shoots compared to the control (Table 4.2). The mean shoot length was significant in both 0.5mg l^{-1} (14.900±3.407) and 1mg l^{-1} (16.500±3.515) with a slight difference in mean shoot length (Fig 4.3). The internodal elongation appear to be dependent on cytokinin as the length was more than two fold higher on latter treatments compared to the control 7.900±1.615 which is also clear in Fig 4.3. Treatments 3mg l^{-1} and 5mg l^{-1} had no significant effect on the mean shoot length in this experiment. There were significant differences in the mean number of internodes produced with various treatments. Similar to former growth parameter, the mean number of internodes in both 0.5mg l^{-1} (7.400±3.407) and 1mg l^{-1} (6.600±3.515) were significantly higher with a slight difference in mean number of internodes (Fig 4.4). Treatments 3mg l^{-1} and 5mg l^{-1} had no significant effect in the mean number of internodes generated compared to the control (no PGR). When propagating for leaves concentrations 0.5mg l^{-1} (15.600±2.339) and 1mg l^{-1} (13.700±2.828) are the best treatments.

Overall treatment 0.5mg l^{-1} 2, 4-D gave the best results (Fig 4.4). As a result, the former PGR was routinely utilized for continuous multiplication of *A. betulina* nodal explants. The shoot length becomes essential when nodal explants are being utilized for continuous culture as the tallest number of shoots has the higher number of axillary buds for axillary shoot formation (Arikat *et al*., 2004).

Table 4.6 Elongation effects of different concentrations of 2, 4-D plant growth regulator on ½ MS medium after 28 days in culture

2,4-D L^{-1}	Shoot regeneration	Shoot length	Internodes regenerated	Leaf regeneration	Callus formation
No PGR	1.20±0.133^{d}	7.90±1.615^{b}	2.90±0.566**b**	6.60±1.462**b**	0.10±0.1**d**
0.5mg L^{-1}	2.00±0.00^{a}	14.90±3.407^{a}	7.40±1.231**a**	15.60±2.339**a**	1.00±0.00^{a}
1mg L^{-1}	1.30±0.213^{d}	16.50±3.515^{a}	6.60±1.318**a**	13.70±2.828**a**	0.70±0.152^{ba}
3mg L^{-1}	0.70±0.213^{b}	3.60±1.266^{cb}	1.50±0.521^{cb}	3.10±1.1**cb**	0.60±0.163^{cb}
5mg L^{-1}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00**c**	0.30±0.152^{dc}

*PGR denotes plant growth regulator

Values are means ± SE

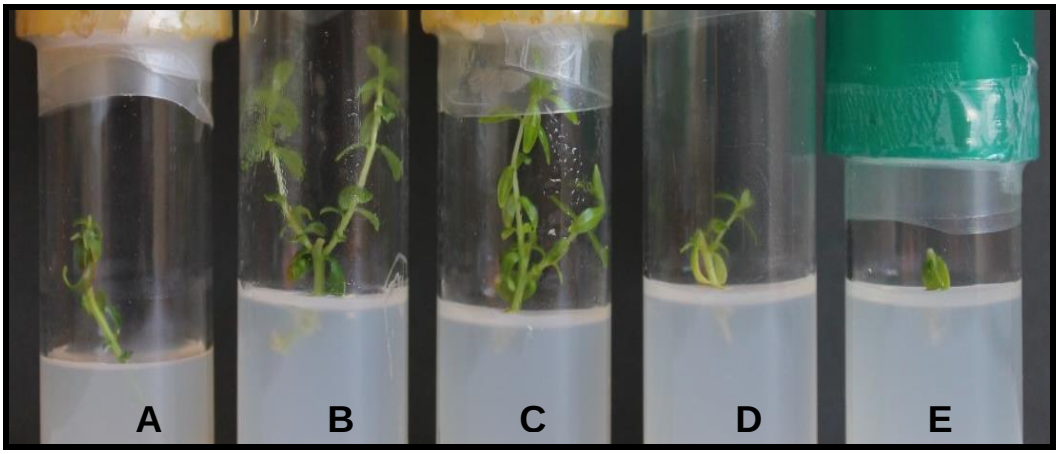


Figure 4.6: Shoot proliferation of *A. betulina* on ½ MS medium containing 2, 4-D phytohormone. Prolific shoot cultures were established from nodal explants on RM with 0.5mgL^{-1} and 1mg L^{-1} respectively **A**. No PGR, **B**. 0.5mgL^{-1} **C**. 1mg L^{-1} **D**. 3mg L^{-1} and **E**. 5mg L^{-1} on the growth response of field derived explants and *in vitro* derived explants of *A. betulina* after 28 days in culture

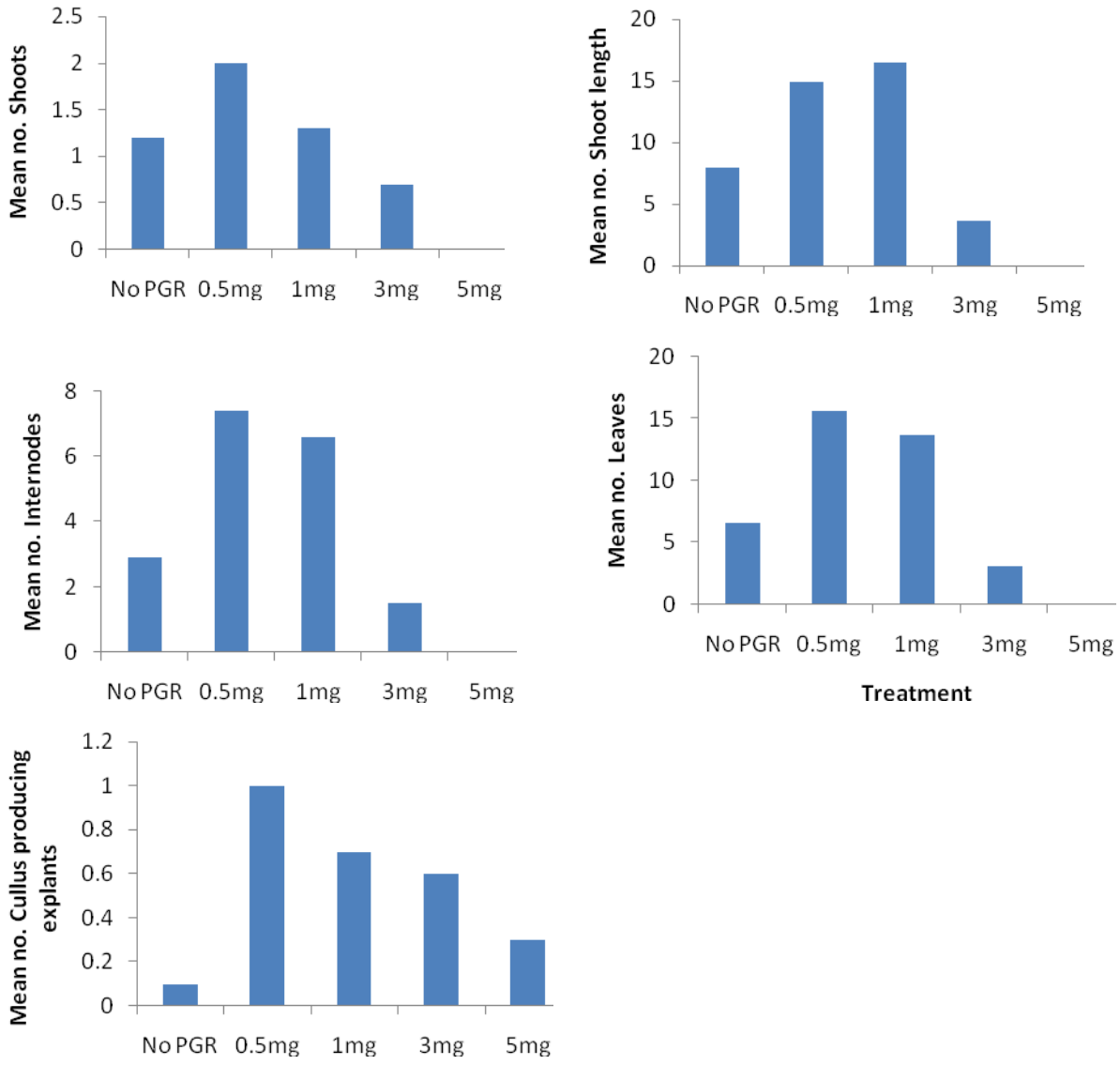


Figure 4.7: Effect of 2, 4-D phytohormone on the growth response of *A. betulina* nodal explants after 28 days in culture.

Table 4.7 Effects of nodal explants on the various strengths of 2, 4-D on ½ MS medium on the growth response of field derived *A. betulina* after 28 days in culture

Effect	Degree of freedom	F	P
One-way ANOVA			
No. shoots	4	25.3929	0.000000*
Shoot length	4	8.93844	0.000021
No. internodes	4	13.41031	0.000000*
No. leaves	4	13.37842	0.000000*
Callus	4	7.38000	0.000117

Includes ½, complement MS media. *Denotes the treatments (interactions) that had a significant impact on the establishment of various growth parameters at the 95% confidence limit.

4.3.5 Effects of BA PGR on the growth response of *in vitro* derived plant material

A. betulina nodal explants responded better to ½ MS containing 0.5mg L^{-1} BA phytohormone as evident in Table 4.3, the mean number of shoots (7.400±0.921), shoot length (16.600±1.528) and callus (1.000±0.00). The former growth responses were all significant ($p<0.001$, Table 4.3) when exposed to treatment 0.5mg L^{-1} BA, although the callus formation had the same response (1.000±0.00) when 1mg L^{-1} BA was noted.

Among the different concentrations tested 0.5mg L^{-1} was most effective for adventitious shoot induction and proliferation of nodal explants (Fig. 4.2.1 DEF). Compared to the control there was a threefold increase in the mean number of shoots (7.400±0.921) and double fold increase in shoot length (16.600±1.528) (Table). But increasing the concentration to 1mg L^{-1} and above resulted in a decrease in growth. The callus formation was significantly higher in BA concentrations 0.5mg L^{-1} and 1mg L^{-1} respectively (1.000±0.00).

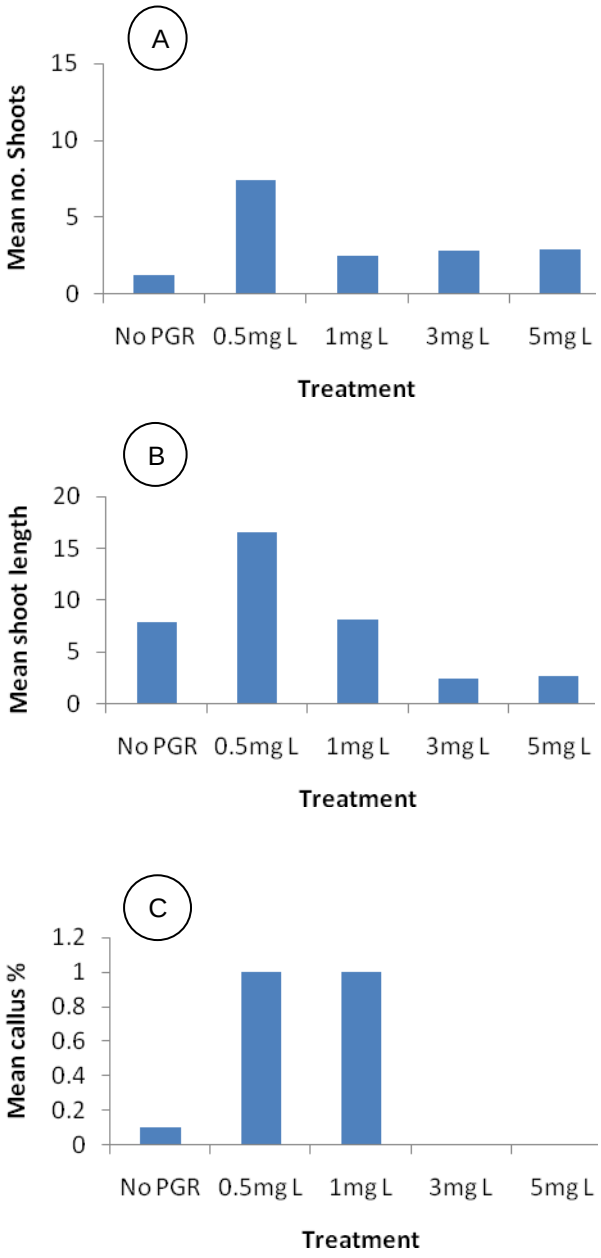


Figure 4.8: Effect of BA plant growth regulator on the growth response of *A. betulina* nodal explants of after 28 days in culture.

Table 4.8 Effects of nodal explants on the various strengths of 2, 4-D on ½ MS medium on the growth response of field derived *A. betulina* after 28 days in culture

BA mg L^{-1}	**Mean no. shoots**	**Shoot length (mm)**	**Callus**
No PGR	1.200±0.133^{b}	7.900±1.615^{b}	0.100±0.1^{b}
0.5mg L^{-1}	7.400±0.921^{a}	16.600±1.528^{a}	1.000±0.00^{a}
1mg L^{-1}	2.500±0.428^{cb}	8.200±1.540^{b}	1.000±0.00^{a}
3mg L^{-1}	2.800±0.290^{c}	2.500±0.223^{c}	0.00±0.00^{b}
5mg L^{-1}	2.900±0.276^{c}	2.700±0.366^{c}	0.00±0.00^{b}

Data indicate mean ± standard error and treatments denoted by the same letter in a column were not significantly different ($P \leq 0.05$) using the LSD test. Ten replicates were used per treatment.

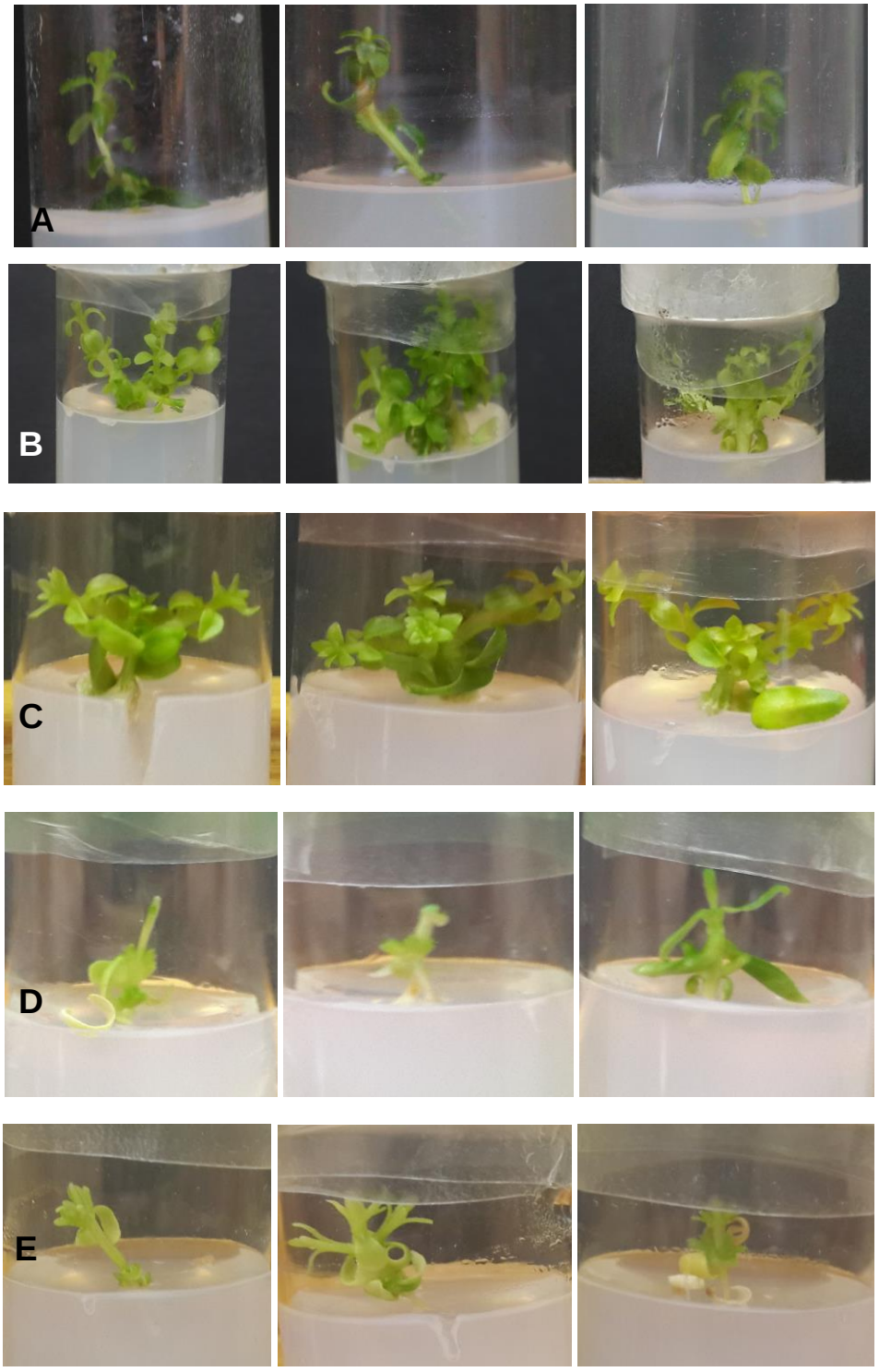


Figure 4.9: Shoot proliferation of *A. betulina* on ½ MS medium containing BA phytohormone. Prolific shoot cultures were established from nodal explants on RM with 0.5mgL^{-1} and 1mg L^{-1} respectively **A**. No PGR, **B**. 0.5mgL^{-1} **C**. 1mg L^{-1} **D**. 3mg L^{-1} and **E**. 5mg L^{-1} on the growth response of field derived explants and *in vitro* derived explants of *A. betulina* after 28 days in culture

Table 4.9 Effects of nodal explants on the phytohormone BA on the growth response of field derived *A. betulina* after 28days in culture

Effect	Degrees of freedom	F	p
Number of shoots	4	22.9665	0.000000*
Shoot length (mm)	4	21.8951	0.000000*
Callus generation	4	141.0000	0.000000*

Includes the basal RM as control. *Denotes the treatments (interactions) that had a significant impact on the establishment of various growth parameters at the 95% confidence limit.

4.3.6 Effects of BA-NAA PGR on the growth response of *in vitro* derived plant material

The effects of BA and NAA on root induction were analyzed by using different concentrations of BA and NAA respectively in half strength media. No rooting response (-) was observed with all the concentrations of BA and NAA combination. Instead of rooting, a result was achieved by the production of adequate callus in half strength media when different strengths of BA and NAA were tested for the induction of roots. Different concentrations of BA and NAA resulted in callogenic and multiple shoot response rather than the induction of roots. When the response of BA was tested excellent multiple shoot results were found. An expected response of NAA was multiple adventitious roots. However the response of the combination of the two phytohormones resulted in compact hard lush green callus. When 0.5mg BA was combined with NAA at 0.5mg shoot proliferation was significantly higher (3.100±0.566) than all the other BA-NAA combinations. According to Tchoundjeu and Leakey (1998), when auxins are too high, they are generally injurious to the cells, this can be seen in treatments 3:0.5mgL^{-1} and 5: 5mgL^{-1} BA-NAA (Fig 4.10). For both shoot length and multiple shoot production, 1: 0.5mgL^{-1} has the most significant results (Fig 4.10 C).

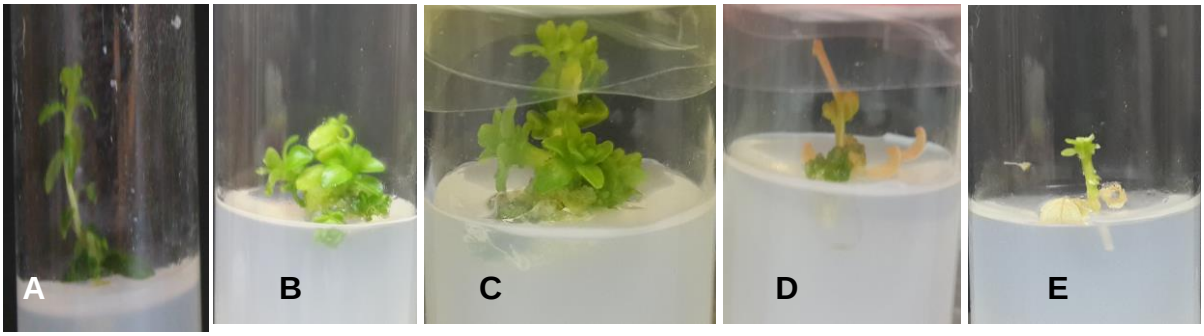
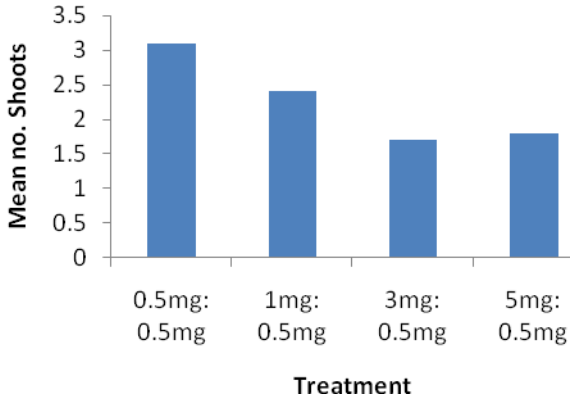


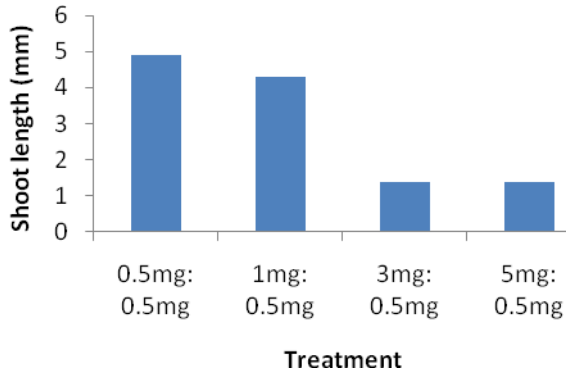
Figure 4.10: Shoot and callus proliferation of *A. betulina* on ½ MS medium containing a combination of BA and NAA phytohormone. Prolific shoot cultures were established from nodal explants on RM with 0.5mgL^{-1} and 1mg L^{-1} respectively **A**. No PGR, **B**. 0.5mgL^{-1} **C**. 1mg L^{-1} **D**. 3mg L^{-1} and **E**. 5mg L^{-1} on the growth response of field derived explants and *in vitro* derived explants of *A. betulina* after 28 days in culture

Table 4.10 Effects of nodal explants on the various combination strengths of BA: NAA on ½ MS medium on the growth response of field derived *A. betulina* after 28 days in culture

BA:NAA (mg L)	Mean no. shoots per plantlet	Mean shoot length (mm)	Callus
No PGR	1.20±1.33^{b}	7.90±1.615^{a}	0.10±0.1^{a}
0.5: 0.5	3.100±0.566^{a}	4.900±1.026^{a}	0.800±0.133^{b}
1: 0.5	2.400±0.733^{a}	4.300±1.044^{a}	1.000±0.00^{a}
3: 0.5	1.700±0.472^{a}	1.400±0.371^{b}	0.00±0.00^{c}
5: 0.5	1.800±0.359^{a}	1.400±0.266^{b}	0.00±0.00^{c}

Data indicate mean ± standard error and treatments denoted by the same letter in a column were not significantly different ($P \leq 0.05$) using the LSD test. Ten replicates were used per treatment.





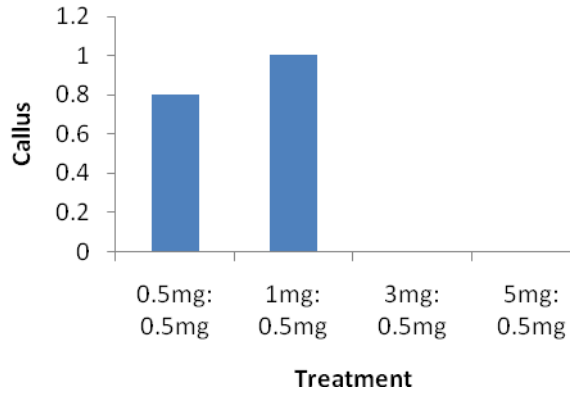


Figure 4.11: Effects of BA: NAA plant growth regulator on the growth response of *A. betulina* nodal explants of after 28 days in culture.

Table 4.11 Effects of nodal explants on the various strengths of BA-NAA on ½ MS medium on the growth response of field derived *A. betulina* after 28 days in culture

Effect	Degrees of freedom	F	p
Number of shoots	3	1.37615	0.265645
Shoot length (mm)	*3*	5.90368	0.002198
Callus	3	62.2500	0.000000

Includes ½, complement MS media. *Denotes the treatments (interactions) that had a significant impact on the establishment of various growth parameters at the 95% confidence limit.

4.3.7 Effect of NAA PGR on the growth response of *in vitro* derived plant material

The highest mean number of shoots was observed in the control (1.133±0.090) and 3mg L^{-1} NAA (1.000±0.00) (Fig 4.13) after 56 days in culture. The effect of 1mg L^{-1} NAA (0.333±0.126) is less effective at 56 days in culture. There was no development observed in former treatments after 28 days in culture. Likewise, no significant difference was observed in explants treated with respectively at 28 days. However, a significantly ($p<0.05$) high mean shoot length was observed in the 0.5 mg L^{-1} NAA (7.200±4.084) at 56 days as well as the controls at 28 days (8.000±1.133) and 56days (7.600±1.218) in culture, respectively. NAA has no significant effect on the mean number of internodes as well as the mean number of leaves per explant. The controls in both these growth parameters are significantly higher than that of the varying levels of NAA. The NAA concentrations 0.5 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1} was stagnant at 28 days in former growth parameters. However, an increase in growth was observed in concentration 1 mg L^{-1} at 56 days for both the mean number of internodes (1.533±0.258) and the mean number of leaves (2.667±0.374). A significant mean number of callus producing nodal explants were observed in 0.5 mg L^{-1} and 1 mg L^{-1} respectively, with no significant difference in the days exposed to treatment. Moreover, the mean number of roots per plant was significantly higher in concentration 3 mg L^{-1} at 28 days and in the same treatment fewer calli was observed (Fig 4.12). Also significant but to a lesser degree was concentration 1 mg L^{-1} at day 56. The results of this experiment showed that phytohormone NAA promoted the rooting of nodal cutting of *A. betulina*. Numerous researchers have

reported promotion of rooting by auxins in other plant species (Cope and Mandel, 2000).

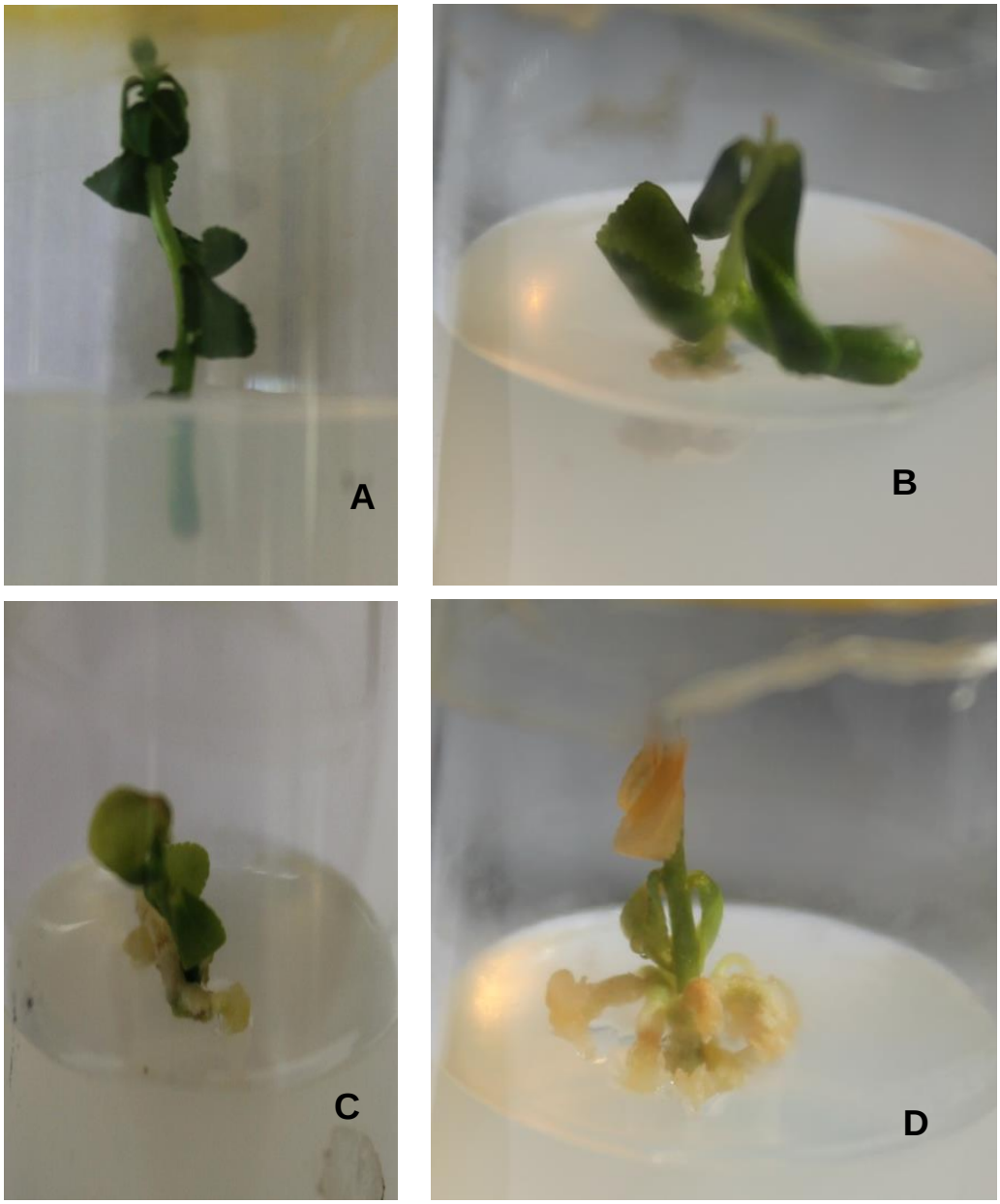


Figure 4.12: Shoot proliferation of *A. betulina* on ½ MS medium containing NAA phytohormone. Prolific root cultures were established from nodal explants on RM with 0.5mgL^{-1} and 1mg L^{-1} respectively **A**. No PGR, **B**. 0.5mgL^{-1} **C**. 1mg L^{-1} and **D**. 3mg L^{-1} on the growth response of field derived explants and *in vitro* derived explants of *A. betulina* after 28 days in culture

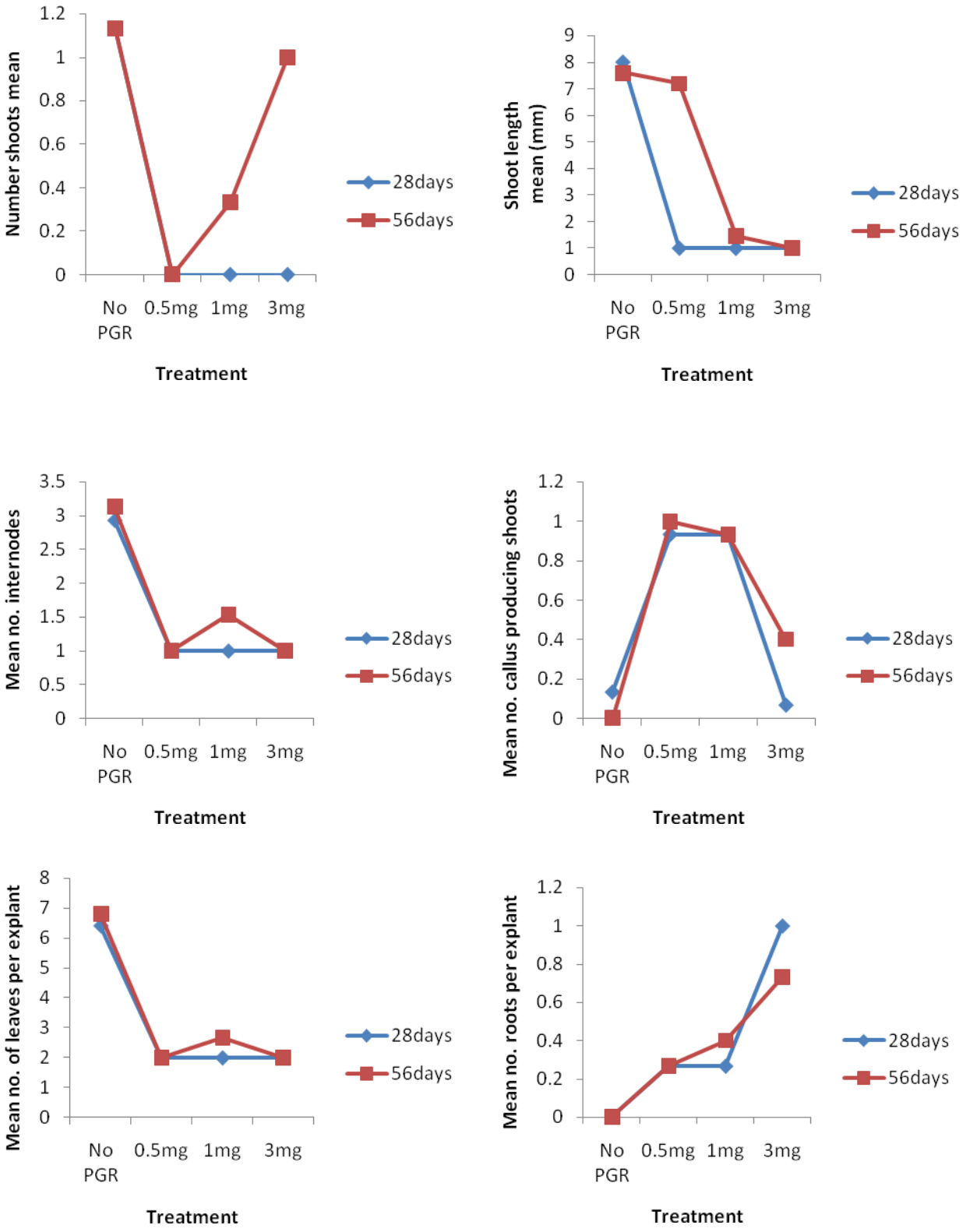


Figure 4.13: Effect of NAA plant growth regulator on the growth response of *A. betulina* nodal explants after 28 days in culture.

Table 4.12 Effects of nodal explants on the various combination strengths of NAA on ½ MS medium on the growth response of field derived *A. betulina* after 28 days in culture

No. days	NAA	No. shoots mean	Shoot length	No. internodes	No. leaves	Callus	No. roots
28 days	No PGR	1.13±0.09^{b}	8.00±1.13^{a}	2.93±0.38^{a}	6.40±0.97^{a}	0.13±0.09^{b}	0.00±0.00^{b}
	0.5mg L^{-1}	0.00±0.00^{c}	1.00±0.00^{b}	1.00±0.00^{b}	2.00±0.00^{b}	0.93±0.06^{c}	0.26±0.26^{ba}
	1mg^{-1}	0.00±0.00^{c}	1.00±0.00^{b}	1.00±0.00^{b}	2.00±0.00^{b}	0.93±0.06^{c}	0.26±0.11^{ba}
	3mg L^{-1}	0.00±0.00^{c}	1.00±0.00^{b}	1.00±0.00^{b}	2.00±0.00^{b}	0.06±0.06^{b}	1.00±0.33^{a}
56 days	No PGR	1.13±0.09^{b}	7.60±1.21^{a}	3.13±0.40^{a}	6.80±0.81^{a}	0.00±0.00^{b}	0.00±0.00^{b}
	0.5mg L^{-1}	0.20±0.10^{c} ^{a}	7.20±4.08^{a}	1.00±0.00^{b}	2.00±0.00^{b}	1.00±0.00^{c}	0.26±0.26^{ba}
	1mg L^{-1}	0.33±0.12a	1.46±0.61^{b}	1.53±0.25^{b}	2.66±0.37^{b}	0.93±0.66^{c}	0.40±0.33^{ba}
	3mg L^{-1}	1.00±0.00b	1.00±0.00^{b}	1.00±0.00^{b}	2.00±0.00^{b}	0.40±0.13^{a}	0.73±0.49^{ba}

*PGR denotes plant growth regulator

Values are means ± SE

Table 4.13 Effects of nodal explants on the MS medium supplemented with NAA on the growth response of field derived *A. betulina* after 28 days in culture

Effect	Degree of freedom	F	p
One-way ANOVA			
No. shoots	7	49.9565	0.000000*
Shoot length	7	4.60407	0.000148
No. internodes	7	18.1480	0.000000*
No. leaves	7	19.9336	0.000000*
Callus	7	36.4118	0.000000*
Roots	7	1.52690	0.165321

Including various strengths of quarter (¼), half (½), and a full complement MS media. *Denotes the treatments (interactions) that had a significant impact on the establishment of various growth parameters at the 95% confidence limit.

4.3.7 Commercial potential of the micropropagation protocol

Variance in microplants is detected in the phenotypic performance of the plants at a later growing stage, more so in the performance of field growing. Genetic changes are the most serious types of aberration because they are permanent and may be difficult to detect during the micropropagation process. The plantlets gave significant results when exposed to the phytohormone 2, 4-D (Table 4.6). Plantlets with more internodes could be devided for the proliferation of more plantlets in subsequent culture cycles. Each plantlet could be devided into a node and internode of about 1cm in length. This implies that ideally hundreds of new plantlets can be manufactured from one *A. betulina* seedling.

4.3.8 Acclimatisation of *A. betulina in vitro* plantlets to glasshouse conditions

The acclimatisation of well rooted cultures (Fig 3.4) was 100% successful after the plants grew independently after only three weeks out of culture. However, unfortunately the plants experienced a fatal fungal attack, which was treated with a fungicide 2% Dithane solution, but appeared to be detrimental to the young acclimatised plants. The experiment could not be repeated due to lack of rooted plant material as well as time constraints.

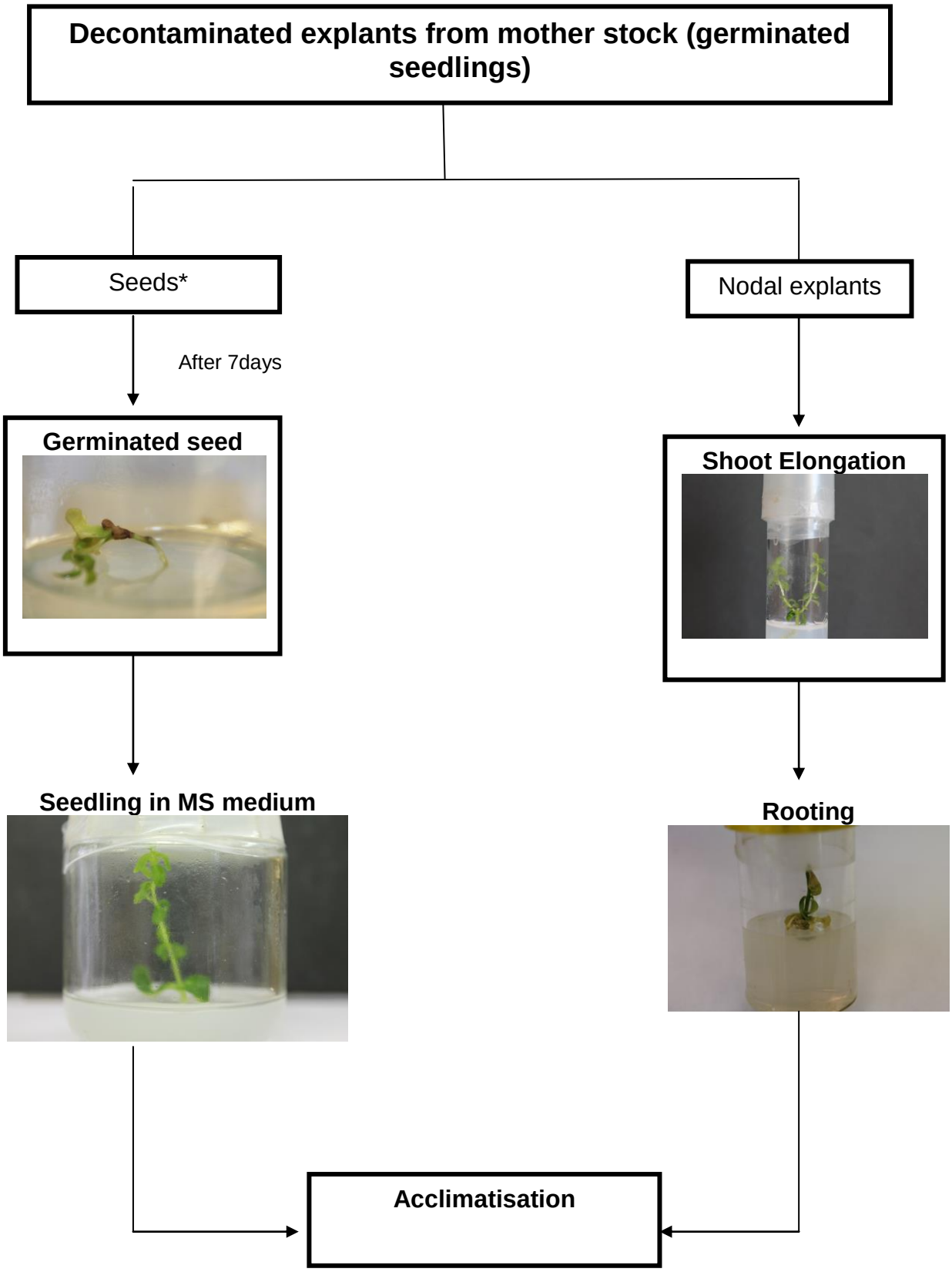


Figure 4.14: Micropropagation of *A. betulina*. * Denotes the preferred method.

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CHAPTER FIVE
ESSENTIAL OIL, AROMA AND FLAVOUR COMPOUND ANALYSIS OF *IN VITRO* PROPAGATED CULTURES OF TREATED AND NON TREATED *AGATHOSMA BETULINA*

5.1 INTRODUCTION

Extracts from medicinal plants are often used for the treatment of various ailments globally. These extracts are prepared through different methods depending on their final usage. In the traditional world of medicine, more so in Southern Africa, these extracts are prepared by adding boiling water to the plant material; however commercial ethanol is used as an extractant. The purpose of this chapter was to establish the essential oil quality of the *in vitro* cultures post exposure to various treatments. To supply market demands and industries requirements it is necessary to maintain a stable essential oil production and quality, mainly in terms of chemical composition (Silva, 2002).

The chapter intended to determine the effect of the various treatments on the chemical constituents of essential oils of *A. betulina*. The various plant organs of the same plant produce different compounds. Each organ type may have a distinctive chemical profile. The extracts of the various organs may contain the same compounds but at variable concentrations, depending on which part of the plant the extract is made from (Zschocke *et al.*, 2000). Individuals of different populations of the same species may also differ in their chemical composition as environmental factors play a role in secondary metabolite production (White *et al*., 2008). The major chemical constituents of *A. betulina* have been recorded and listed as menthone, isomenthone, diosphenol, limonene, pulegone, isopulegone, and both *cis*- and trans-8-mercapto-*p*-menthane-3-ones.

These micropropagated plants often produce similar compound profiles to those found in the native plants and can therefore be used as an alternative source of these compounds (Kolewe *et al*., 2008).

5.2 Materials and methods

In vitro grown *A. betulina* plant material as described in Chapter 4 was used for the purpose of this study. Explants were exposed to various treatments and then grown in half MS basal regeneration medium to ensure continuous growth. All plants were subcultured at a four to six week interval. Observations were made on each glass jar before chemical analysis was done (Table 5.1).

Ex vitro grown seedlings were germinated in river sand after an initial smoke treatment. The seedling germinated after one month and was used on the fifth month after germination.

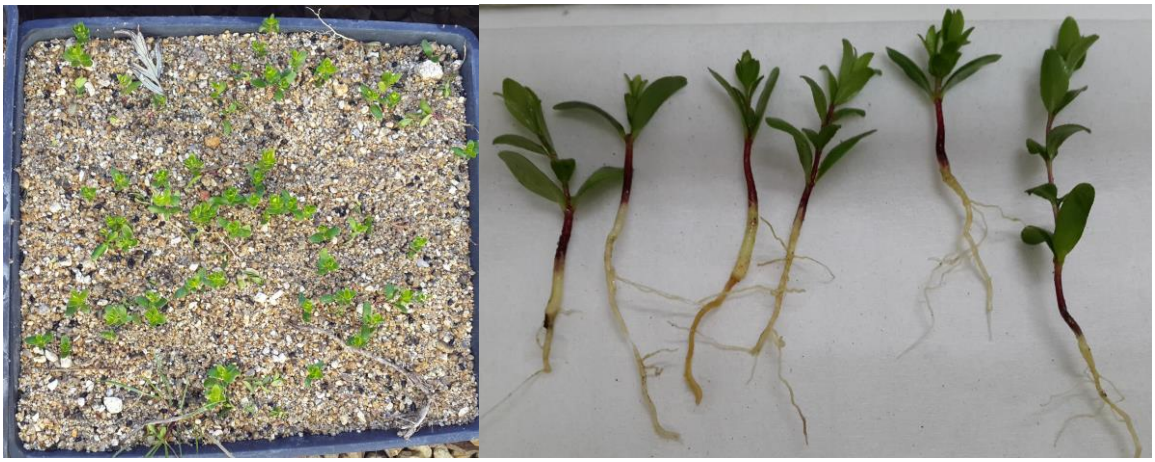

Figure 5.1: *A. betulina* seedlings grown *ex vitro* for gas chromatography mass spectrometry analysis as a control.

5.2.1 Extraction preparation and analysis

Gas chromatography mass spectrometry (GC-MS) aids in structural identification by separating the compounds and providing information about each compound (Sarker and Nahar, 2006). This is particularly useful for volatile compounds.

The *in vitro* plantlets were separated into their organs: leaves, stems and roots. Where no roots occurred, brown calluses were used to analyse chemical composition. Dichloromethane solvent extractions were executed on the various treatments and organs.

Chromatograms were conducted using Thermo Finnigan Focus gas chromatograph equipped with a 30 meter SGE BP-5 glass capillary column and flame ionization

detector. The inlet temperature was 220°C and the detector temperature at 250°C. Nitrogen was used as the carrier gas throughout at 1.1ml/min. Temperature programme used was 70°C (3min), temperature ramp 1.5°C/ min to 130°C (0 min) and temperature ramp 2 was 10°C/min to 200°C (4 min).

5.3 Results and Discussion

Analysis resulted in the identification of 21 compounds. To evaluate the chemical similarities and differences, the percentage of peak area was used to assess the essential oil composition of the samples. The results showed qualitative as well as quantitative differences amongst the samples.

5.3.1 Relative concentration of compounds identified in greenhouse germinated *A. betulina* seedlings

A significant lower concentration of a-Pinene was evident in the leaves (0.5%). The stems showed a concentration of 2.6% and the roots showed significant presence of latter compound in the roots at a concentration of 10.1%. The leaves and stems of the young seedling showed significantly lower sabinene concentrations at 0.3% and 0.9% respectively compared to a significantly higher concentration of 20.2% in the roots. There were no traces of b-Pinene found in the leaves of the young seedling. A less significant concentration was found in the stems (1.0%) compared to the significant concentration of 29.4% present in the roots. Low myrcene compound concentration was found in all plant parts tested. The leaves had a concentration of 2.4% similar to that of the stems at 2.8% of peak area of the compound identified. Interestingly the leaves had a concentration of 25.0% limonene, less significant than that of the stems (40.5%). Even more, the roots have higher limonene concentration (29.0%) than the leaves. Significantly lower than all other compounds identified, cineole compound was present in the leaves and stems at 0.2% and 0.1% respectively. This compound showed no traces in the roots. The leaves and stems showed the same concentration percentage peak area (1.2%) of (E)-b-ocimene and no activity was found in the roots of the plant. The relative concentration of linalool was low in both the leaves and the stems with 0.4% and 0.3% respectively. No activity of the linalool was found in the roots of the plant. Menthone had higher relative concentration and this was evident in the stems (0.7%) compared to the leaves (0.3%), with the roots showing low level of 0.1%. The leaves of the young seedling showed 0.5% relative concentration isomethone and the stems showed

double that of the leaves (1.0%). The roots showed significantly lower level concentration at 0.2%. The leaves had significantly high relative concentration (9.6%) c- and t- Isopulegone. The stems showed 2.4% activity while the roots had no activity. As expected the leaves showed high pulegone concentration levels (47.1%) which are evident in most young seedlings grown in *ex vitro* conditions. Also significant but to a lesser degree was the concentration level of the stems (35.8%). The roots posed very low concentration of pulegone (0.9%).

The leaves of this young seedling had significantly higher relative concentration (1.3%) psi-diosphenol compared to the stem (0.3%). The roots showed no activity of the discussed compound. No 2-Hydroxyiosmenthone level was detected. The seedling had significantly higher diosphenol concentration in the leaves (1.2%) compared to the stems (0.3%) and no traces in the roots of the plant. No 1-Hydroxydiosphenol activity detected. Both the leaves and stems had similar t-8-SH-p-menthanone relative concentration of 0.6% and 0.4% respectively. The roots had significantly higher concentration of 5.9%. The leaves and stems showed similar c-8-SH-p-menthanone significant relative concentrations of 9.4% and 9.7% respectively. The roots resulted in significantly low c-8-SH-p-menthanone (0.8%). No methyl Eugenol, t-8-acetylthiomenthone, c-8-acetylthiomenthone levels was detected.

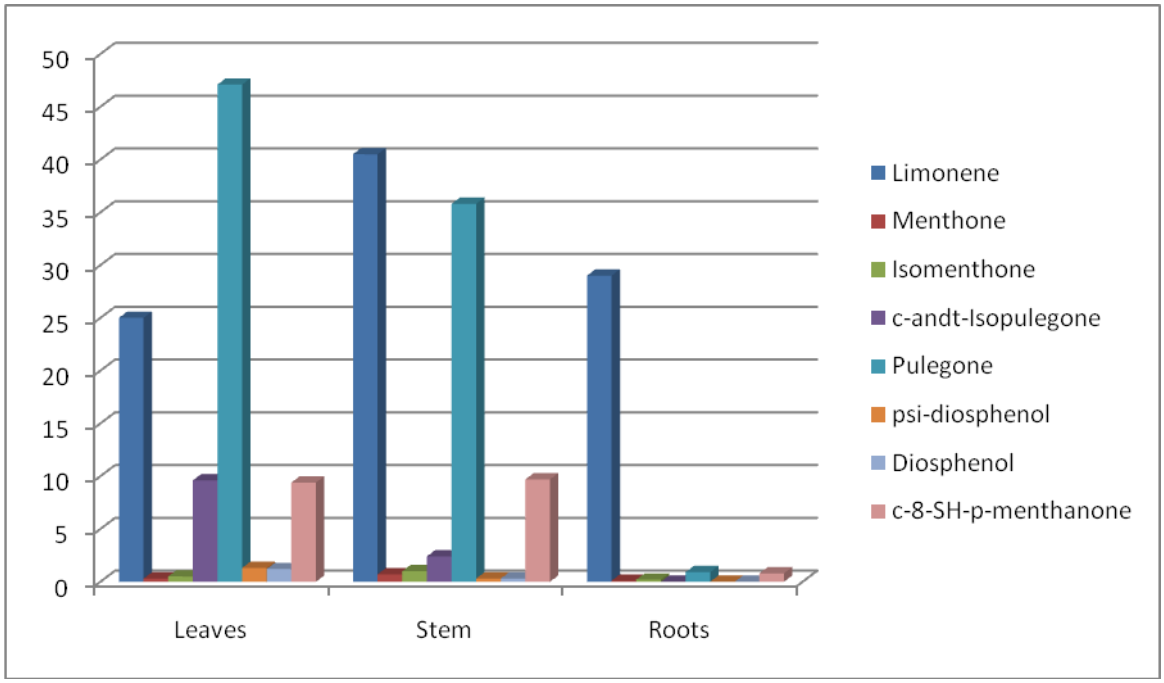

Figure 5.2: Relative percentage of peak area of compounds identified in *ex vitro* grown *A. betulina* seedling

5.3.1 Relative concentration of compounds identified in various *in vitro* phytohormone treated *A. betulina* cultures

5.3.1.1 The effect of phytohormone BA (0.5mg l^{-1}) on the concentration of compounds in *A. betulina* cultures

Interestingly the leaves had a similar limonene (24.8%) concentration of the control 25.0%, more significant than that of the stems (18.2%). Even more, the callus had higher limonene concentration (20.0%) than the stems. With menthone being one of the main constituents in the chemical profile of *A. betulina*, it interestingly showed a significant higher presence in the callus compared to the leaves and the stems, with the leaves having the least relative concentration. Isomethone appeared to be significant in the callus.

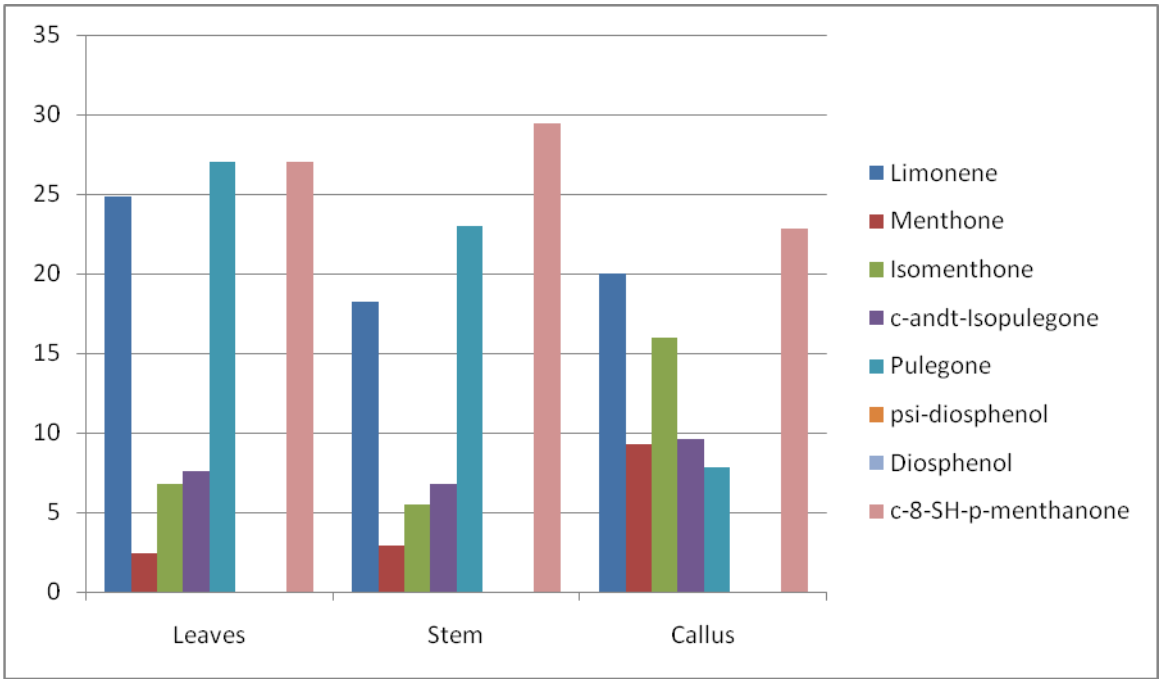

Figure 5.3: Relative percentage of peak area of compounds identified in *ex vitro* grown *A. betulina* seedling

5.3.1.2 The effect of phytohormone NAA (0.5mg l^{-1}) on the concentration of compounds in *A. betulina* cultures

Limonene was present in all plant parts, though in the callus had a significant 67.7% sharing the total peak area with a-Pinene at 32.3%. The leaves and stem enjoyed similar percentage of peak area at 19.6% and 20.7% respectively. In the roots that were present, only 3.4% limonene was found. Menthone, isomenthone, c- and t-Isopulegone and pulegone was only found in the leaves and stems. No diosphenol was present when the plant was subjected to 0.5mg l^{-1} NAA.

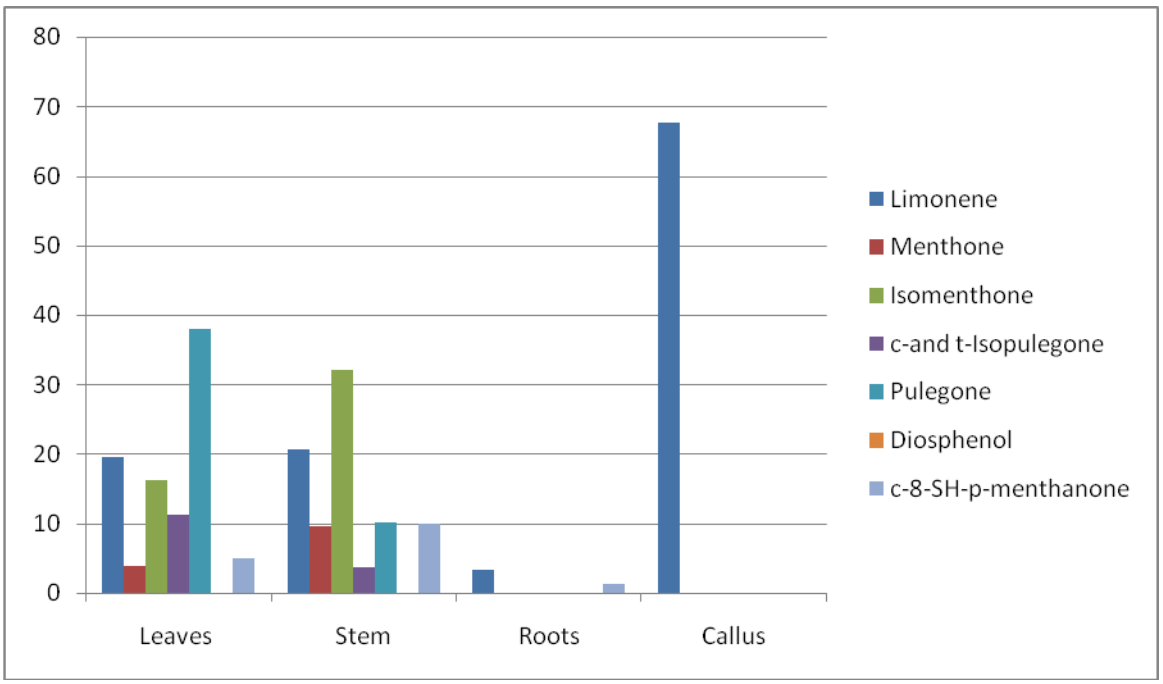

Figure 5.4: Relative percentage of peak area of compounds identified in *A. betulina*

5.3.1.3 Effect of phytohormone 2,4D (1mg l^{-1}) and NAA (0.5mg l^{-1}) on the concentration of compounds in *A. betulina* cultures

When the plants were exposed to 2,4-D first and then NAA the limonene relative concentration was still significant in the callus (36.0%) similar to when it was only exposed to 0.5mg l^{-1} NAA. Significant levels of isomenthone were detected in the callus (20.9%). Pulegone levels were equally significant in both the leaves (39.9%) and stems (41.5%). No diosphenol was detected.

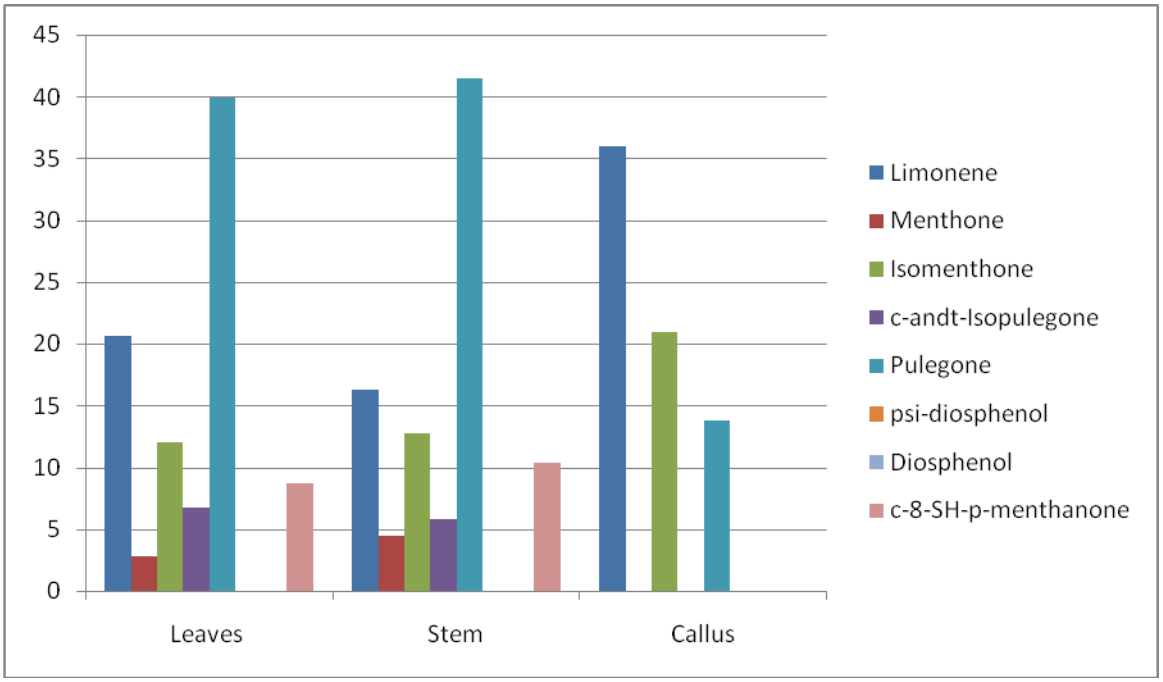

Figure 5.5 Relative percentage of peak area of compounds identified in *A. betulina*

5.3.1.4 Effect of phytohormone BA (0.5mg l^{-1}): NAA (0.5mg l^{-1}) on the concentration of compounds in *A. betulina* cultures

When the plants were exposed to the above treatment, the stems had significant limonene levels (25.3%). Isomenthone was prominent in the callus with a significant 44.4%. The pulegone levels were significant in the leaves (53.5%).

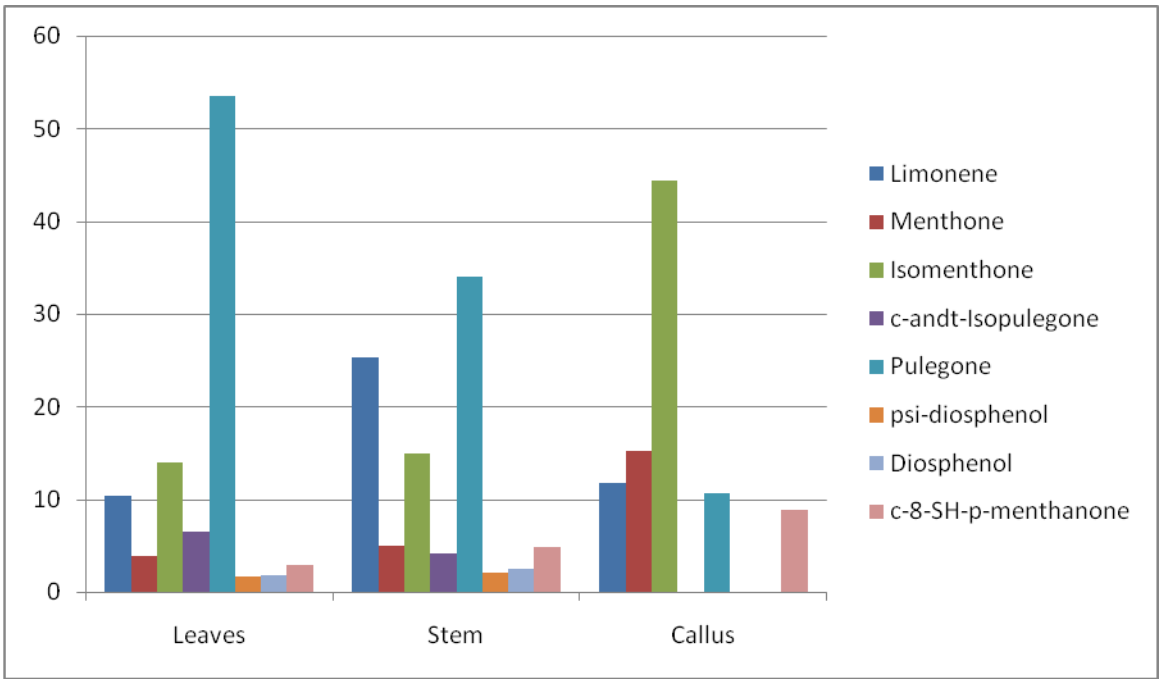

Figure 5.6: Relative percentage of peak area of compounds identified in *A. betulina*

5.3.1.3 The effect of phytohormone 2,4D (0.5mg l^{-1}) on the concentration of compounds in *A. betulina* cultures

A similar trend followed in the discussed treatment with the callus having significant limonene levels (44.9%). All three plant organs had exceptional concentrations of isomenthone with the leaves (33.8%) being significant followed by the callus (27.3%) and then the stems (23.0%). Pulegone levels were significant in the stem (27.0%).

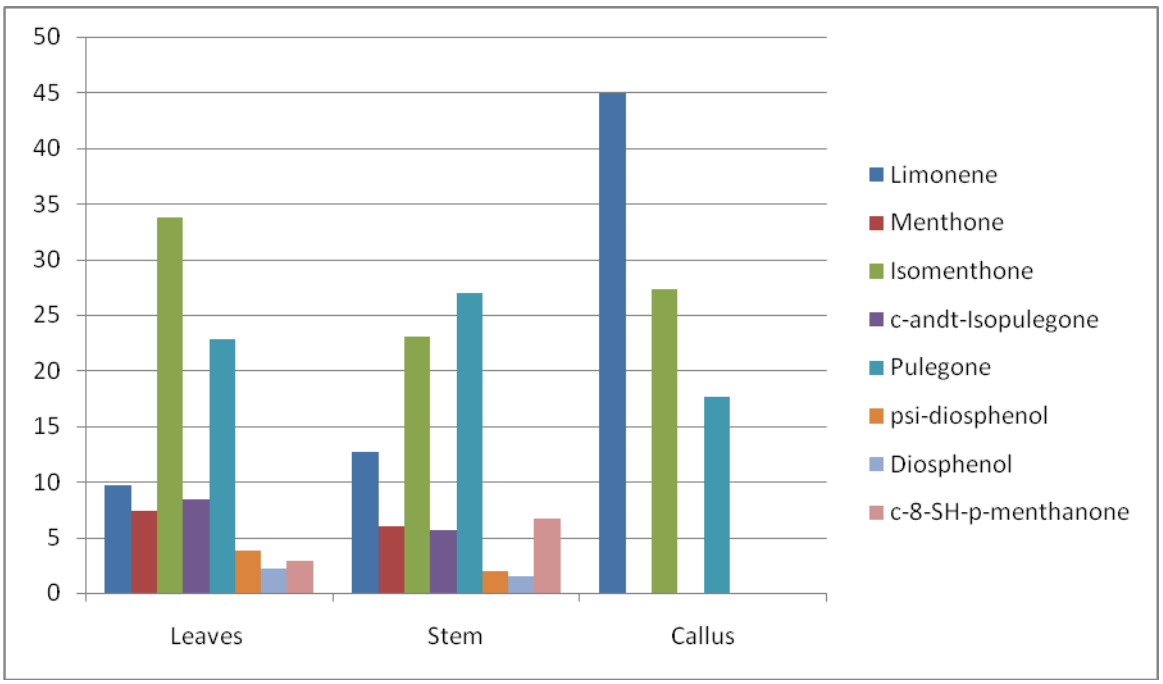

Figure 5.7: Relative percentage of peak area of compounds identified in *A. betulina*

5.3.1.3 The effect of phytohormone BA (1mg l^{-1}) and NAA (0.5mg l^{-1}) on the concentration of compounds in *A. betulina* cultures

When the *in vitro* plantlets were exposed to BA and then subcultured onto NAA, the callus showed significant relative concentration of a-Pinene (32.7%). Limonene levels were significant in the stem (26.7%) when this treatment was applied. Isomethone and pulegone were both compounds that were significant in presence in the leaves.

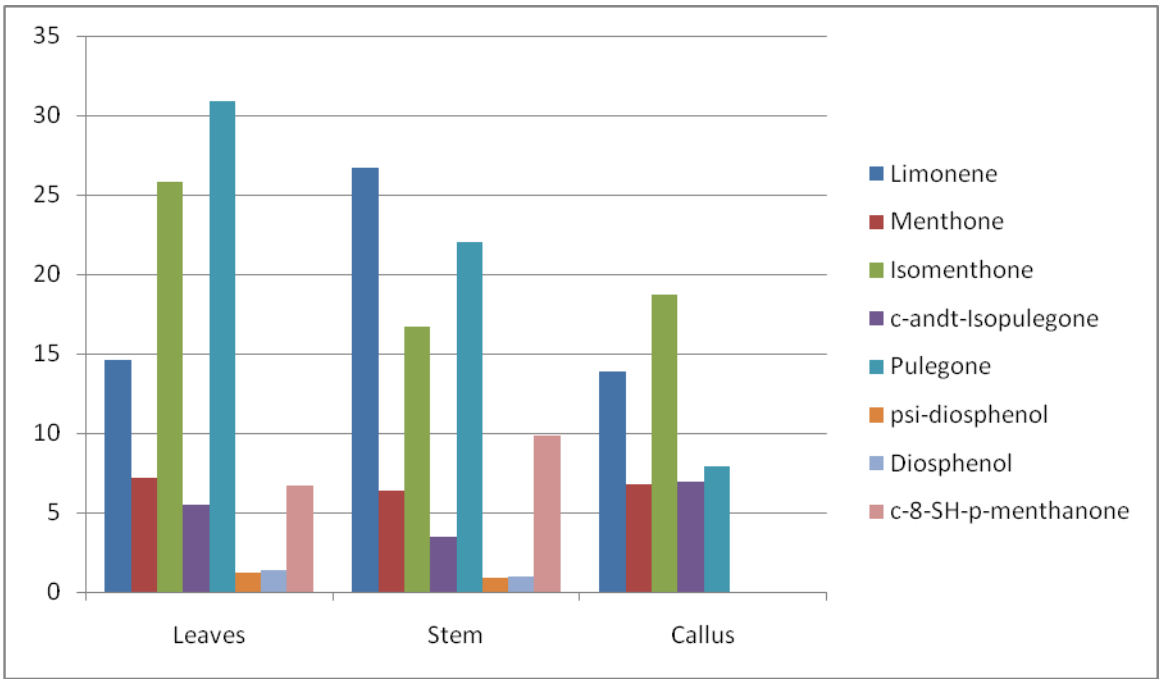

Figure 5.8: Relative percentage of peak area of compounds identified in *A. betulina*

5.3.1.3 The effect of phytohormone NAA (3mg l^{-1}) on the concentration of compounds in *A. betulina* cultures

The production of flavour compounds in response to 3mg l^{-1} NAA is shown in Fig 5.8. The increase in phytohormone concentrations in the culture medium increased the type and quantity of volatile and flavour compounds compared to the control, but not at 0.5mg l^{-1} NAA. Significant quantities of a-Pinene (37.9%), sabinene (16.2%), b-Pinene (24.1%) was prominent, all of which are not the main compounds used commercially. The highest level of limonene was found in the leaves (27.0%). Charlwood *et al*., (1989) similarly reported that the oil accumulation increased significantly after the callus had been transferred onto the medium containing 0.5 mg/L (w/v) BAP plus 0.5 mg/L (w/v) NAA. But, higher concentrations of NAA e.g. 3mg l^{-1} (w/v), decreased the level of flavour compounds in callus.

According to Backer and Saverwein (1990), an increase in the auxin level causes cell division and finally diminishes the production of plant secondary metabolites. An interesting study on the effect of NAA carried out by Banthrope (1996) showed that shoot tips culture of *Mentha spicata* grown on the basal Gamborg B5 medium supplemented with NAA, produced high yields of carvone and limonene.

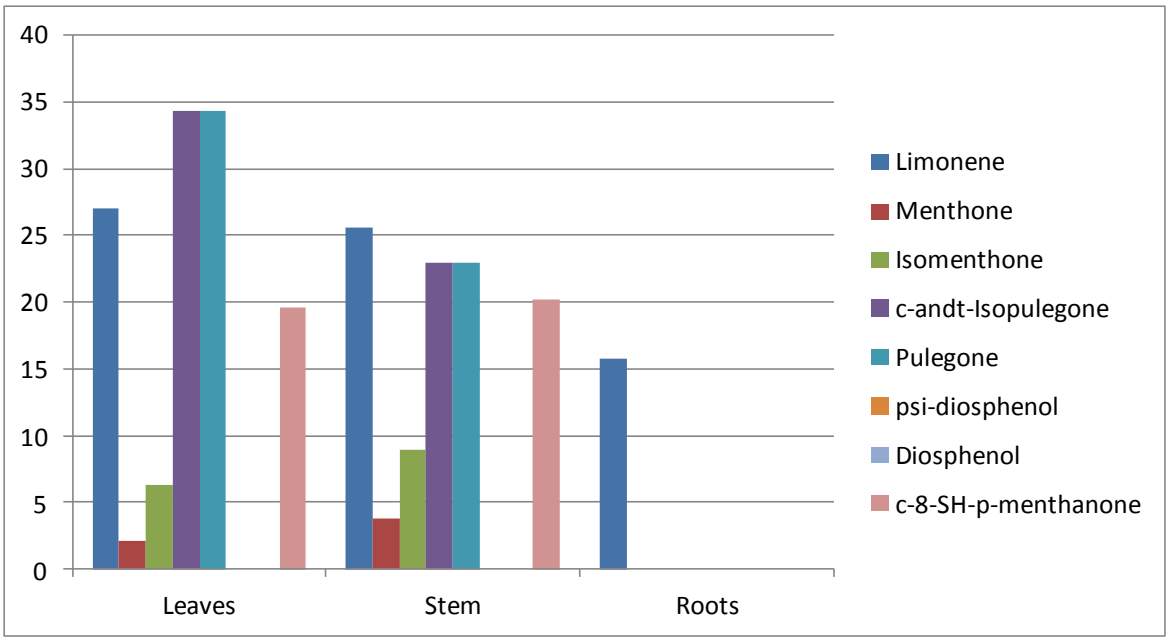

Figure 5.9: Relative percentage of peak area of compounds identified in *A. betulina*

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CHAPTER SIX
GENERAL DISCUSSION AND CONCLUSIONS

As the interest in natural products and their commercialization have increased over the decades, many phytochemical studies have been conducted. Knowledge exists on the chemistry of many plants as well as the conservation status of many of these medicinal plants exists in South Africa. Accumulation of this knowledge is key as South Africa has been relying on the oral histories of the indigenous and rural communities to pass on this knowledge. This would build up to a formal pharmacopeia of the medicinal plants of South Africa. Due to the increase in the interest, resources are decreasing at an alarming rate as a direct result of over-exploitation. After all, this study focused solely on the establishment of a micropropagation method via the initiation of seed and using the germinated seedlings as starter material for further propagation and testing. *Ex vitro* plant material was also tested to view the possibilities of this avenue.

This study confirmed that the *in vitro* germination of *A. betulina* is possible within only seven days in culture. Physical scarification and smoke was the optimal treatment for the germination and further growing of *A. betulina* (Berg.) Pillans. The general deduction from the study is that seed coat removal significantly increased germination. The experiment demonstrated that peak of the germination takes place after 14 days and then slows down after 35 days with 82% germination rate. The advantage of using *in vitro* treatments to induce germination is that it increases the likelihood that all seeds will germinate at the same time, which is essential for further experiments as well as to determine if growing a specific crop is viable. This treatment obtained high seedling count and could grow further vegetatively and provide explants for further investigations. Furthermore, this was the recommended seed germination treatment throughout the study as the seedling grew to develop a strong root system, consisting of a tap root and adventitious roots as well as multi-branched shoots, thus potentially developing into a plant that can be acclimatized to be grown in the field. This protocol could play a key role in the large scale mass propagation of *A. betulina* (Berg.) Pillans.

The initiation of *A. betulina* from the field experienced numerous tests to decontaminate the plant material to be grown in sterile *in vitro* conditions. Decontamination was not economically viable and after various methods, resources were exhausted as well as time. Contamination rates of explants were influenced by the stage of maturity of the explant material, making results unstable. This plant material was used to test different strengths of regeneration media, to ensure that the explants receive ample nutrients. Results made it clear that ½ MS was the best strength for growing *A. betulina* nodal explants. Further experiments compared *in vitro* derived explants versus *ex vitro* collected explants. It was clear that the *ex vitro* derived explants had significant results, but the explants lost vigour soon after the initial exponential growth leading to the explants dying off. Furthermore, *ex vitro* decontaminated plant material was not economically viable to continue with.

Growing of the germinated seedlings were limited to nodal cuttings, though other plant organs may be researched for the initiation of *in vitro* cultures. Seedlings derived from germinated seedlings appeared to be the preferred method of propagation as this spent the least time in culture and produced a stable plant with an established root system, which is essential during the hardening off process after *in vitro* growth. When exposing nodal explants to phytohormone 2,4-D it responds best to dosages 0.5mg L^{-1} and 1mg L^{-1}. Phytohormone BA was very effective in producing soft friable callus. This suggests that this hormone may produce good liquid cultures for further and continuous research on *A. betulina*. The best results were shown when 0.5mg L^{-1} BA was applied to ½ MS media. For both shoot length and multiple shoot production, a combination of phytohormones BA-NAA (1: 0.5mgL^{-1}) had the most significant results. Interestingly, a higher phytohormone concentration of NAA is necessary to develop multiple adventitious roots. The effect of 3mg L^{-1} was significant in that it resulted in multiple adventitious roots, but fewer calli was observed in this treatment. Micropropagation becomes valuable as little attention between subcultures is needed; making it less labour intensive compared to conventional nursery propagation systems where weeding watering and spraying of plants are labour intensive. In addition to this, a viable propagation system of *A. betulina,* may be mass produced for pharmaceutical companies and it may also be provided to traditional medical practitioners and small

scale farmers to grow their own crop, indirectly providing them a secure source of income.

Traditionally medicines derived from plants, more so in Southern Africa, are prepared by adding boiling water to extract the active medicinal compounds; however commercial ethanol is now used as an extractant. Establishment of the essential oil quality of the *in vitro* cultures post exposure to various treatments was done. After the analysis of the *A. betulina* treated cultures, the dichloromenthane solvent extracts through GC (gas chromatograph) resulted in the identification of twenty one compounds. All percentages refer to the percentage of total integrated peak area calculated directly from the total ion chromatogram as recorded on the GC. The results showed qualitative as well as quantitative differences amongst the samples used in the study. The highest relative concentration of limonene was observed in the callus of nodal explants after it was exposed to 0.5mg l^{-1} NAA. No pulegone was found in this treatment making it ideal for limonene production. This suggests that liquid culture with the same treatment may produce more calluses making it ideal for the production of limonene.

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This study thus contributed towards the understanding of an important indigenous South African plant and serves as a basis for the development of further studies that will in future benefit the medicinal as well as the horticultural industries.

Appendix A
Media preparation

The components of the media used in this study are listed in Table A. The medium was adjusted for the lower strength medium experiments.

Table A1: Media composition of Murashige and Skoog (basic RM)

Components	Amount (mg L^{-1})
Macronutrients	
MgSO4.7H2O	**370**
KH2PO4	**170**
KNO3	**1900**
NH4NO3	**1650**
CaCl2.2H2O	**440**
Micronutrients	
H3BO3	**6.2**
MnSO4.4H2O	**22.3**
ZnSO4.7H2O	**8.6**
Na2MoO4.2H2O	**0.25**
CuSO4.5H2O	**0.025**
CoCl2.6H2O	**0.025**
KI	**0.83**
FeSO4.7H2O	**27.8**
Na2EDTA.2H2O	**37.3**
Organic supplements	
Thiamine-HCl	**0.5**
Myo-inositol	**100**
Sucrose	**30000**

Dissolve in distilled water and amend the pH to 5.7-5.8 with 1 M NaOH and 1 M HCI.

To amend the medium with phytohormones, add NAA/BA/IAA/kinetin prior to changing the pH.

Appendix B

Table B1: Relative concentration of *A. betulina ex vitro* grown seedling

Chemical Components	Relative concentration (percentage of peak area) of compounds identified in the *A. betulina* seedling sample		
Plant organ	Leave	Stem	Root
Puris Lot #	HB2308N1	HB2308N1	HB2308N1
a-Pinene	0.	2.	10.
Sabinene	0.	0.	20.
b-Pinene	-	1.	29.
Myrcene	2.	2.	3.
Limonene	25.	40.	29.
Cineole	0.	0.	-
(E)-b-ocimene	1.	1.	-
Linalool	0.	0.	-
Menthone	0.	0.	0.
Isomenthone	0.	1.	0.
c- and t- Isopulegone	9.	2.	-
Pulegone	47.	35.	0.
psi-diosphenol	1.	0.	-
2-Hydroxyiosmenthone	-	-	-
Diosphenol	1.	0.	-
t-8-SH-p-menthanone	0.	0.	5.
c-8-SH-p-menthanone	9.	9.	0.
Total:	100.	100.	100.

Table B2: Chemical composition of phytohormone treated *in vitro* grown plants of *A. betulina*

Chemical Components	Relative concentration (percentage of peak area) of compounds identified in the Buchu samples									
	0,5mg l^{-1} BA			0,5mg l^{-1} NAA				1mg l^{-1} 2,4-D: 0,5mg l^{-1}NAA		
Plant organ	Leaves	Stems	Callus	Leaves	Stems	Roots	Callus*	Leaves	Stems	Callus*
Puris Lot #	HB1408N1L	HB1408N1S	HB1408N1R	HB1408N2L	HB1408N2S	HB1408N2R	HB1408N2C	HB1408N3L	HB1408N3S	HB1408N3R
a-Pinene	-	8.5	12.4	0.6	7.6	32.4	32.3	0.7	1.5	29.3
Sabinene	-	0.4	-	0.9	1.3	22.8	-	1.1	1.1	-
b-Pinene	-	0.3	-	-	1.2	34.7	-	0.4	0.3	-
Myrcene	2.3	2.4	2.1	2.1	3.7	3.0	-	1.8	2.2	-
Limonene	24.8	18.2	20.0	19.6	20.7	3.4	67.7	20.6	16.3	36.0
Cineole	-	-	-	1.1		-	-	3.4	1.7	-
(E)-b-ocimene	-	0.6	-	1.0		-	-	0.8	0.7	-
Linalool	-	-	-	0.4		-	-	0.6	0.4	-
Menthone	2.4	2.9	9.3	3.8	9.6	-	-	2.8	4.5	-
Isomenthone	6.8	5.5	16.0	16.3	32.0	-	-	12.0	12.8	20.9
c- and t- Isopulegone	7.6	6.8	9.6	11.2	3.7	-	-	6.7	5.8	-
Pulegone	27.0	23.0	7.8	38.0	10.2	-	-	39.9	41.5	13.8
psi-diosphenol	-	-	-	-		-	-	-	-	-
2-Hydroxyiosmenthone	-	-	-	-		-	-	-	-	-
Diosphenol	-	-	-	-		-	-	-	-	-
1-Hydroxydiosphenol	-	-	-	-		-	-	-	-	-
t-8-SH-p-menthanone	2.1	2.0	-	-		2.4	-	0.5	0.8	-
c-8-SH-p-menthanone	27.0	29.4	22.8	5.0	10.0	1.3	-	8.7	10.4	-
Methyl Eugenol	-	-	-	-		-	-	-	-	-
t-8-acetylthiomenthone	-	-	-	-		-	-	-	-	-
c-8-acetylthiomenthone	-	-	-	-		-	-	-	-	-
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

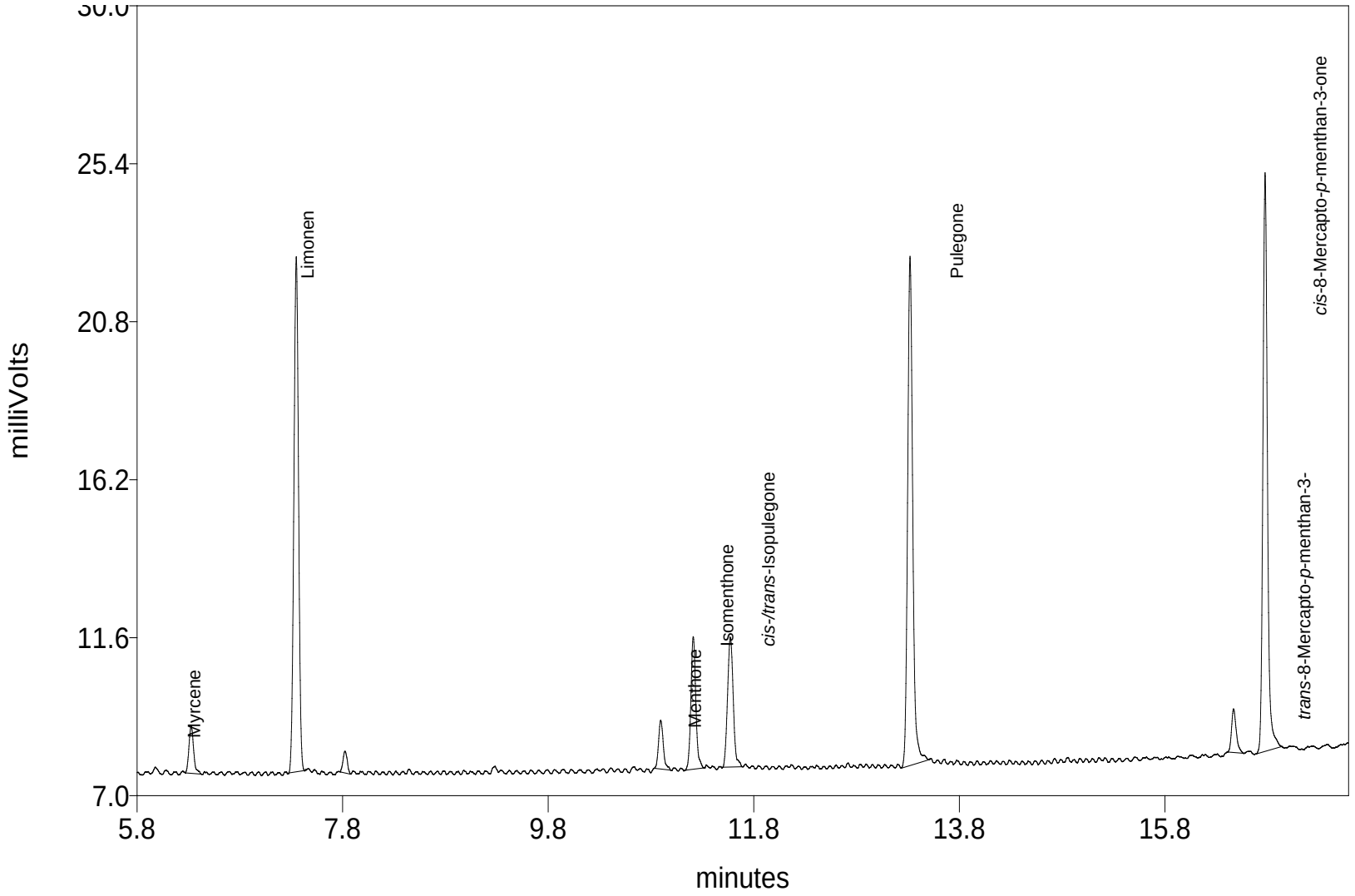
Table B3: Chemical composition of phytohormone treated *in vitro* grown plants of *A. betulina*

Chemical	Relative concentration (percentage of peak area) of compounds identified in the Buchu samples								
	0.5mg l^{-1} BA: 0.5mg l^{-1} NAA			0.5mg l^{-1} 2,4-D			1mg l^{-1} BA: 0.5mg l^{-1} NAA		
Plant organ	Leaves	Stems	Callus*	Leaves	Stems	Callus*	Leaves	Stems	Callus*
Puris Lot #	HB1408N4L	HB1408N4S	HB1408N4R	HB1408N6L	HB1408N6S	HB1408N6R	HB1408N7L	HB1408N7S	HB1408N7R
a-Pinene	0.3	1.3	9.2	0.4	6.6	10.2	0.5	4.1	32.7
Sabinene	0.4	0.7	-	0.6	1.0	-	0.8	1.5	4.0
b-Pinene	0.1	0.2	-	-	1.0	-	0.2	0.9	5.7
Myrcene	1.3	2.3	-	1.7	3.0	-	1.4	2.8	3.4
Limonene	10.4	25.3	11.8	9.7	12.7	44.9	14.6	26.7	13.9
Cineole	1.6	1.1	-	1.3	0.1	-	1.9	1.4	-
(E)-b-	0.6	0.9	-	0.7	1.1	-	0.6	0.9	-
Linalool	0.5	0.4	-	0.7	0.4	-	0.3	0.3	-
Menthone	3.9	5.0	15.2	7.4	6.0	-	7.2	6.4	6.8
Isomenthone	14.0	14.9	44.4	33.8	23.0	27.3	25.8	16.7	18.7
c- and t-	6.5	4.1	-	8.4	5.6	-	5.5	3.5	6.9
Pulegone	53.5	34.0	10.6	22.8	27.0	17.6	30.9	22.0	7.9
psi-	1.6	2.1	-	3.8	2.0	-	1.2	0.9	-
Diosphenol	1.8	2.5	-	2.2	1.5	-	1.4	1.0	-
t-8-SH-p-	0.6	0.5	-	3.0	2.3	-	1.0	1.1	-
c-8-SH-p-	2.9	4.8	8.8	2.9	6.7	-	6.7	9.8	-
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table B4: Chemical composition of phytohormone treated *in vitro* grown

Chemical compounds	Relative concentration (percentage of peak area) of compounds identified in the Buchu samples					
	3mg l^{-1} NAA			1mg l^{-1} BA: 0.5mg l^{-1} NAA		
Part of plant	Leaves	Stems	Roots*	Leaves	Stems	Callus
Puris Lot #	HB1408N8L	HB1408N8S	HB1408N8R	HB1408N9L	HB1408N9S	HB1408N9R
a-Pinene	0.6	5.9	37.9	0.4	13.5	8.4
Sabinene	0.2	0.9	16.2	0.5	2.3	5.0
b-Pinene	-	1.1	24.1	0.1	2.6	3.4
Myrcene	2.7	3.0	4.1	1.4	2.9	3.3
Limonene	27.0	25.6	15.7	9.4	18.4	23.7
Cineole	0.1	-	-	2.2	1.1	-
(E)-b-ocimene	0.9	0.7	-	0.5	0.6	-
Linalool	0.8	0.6	-	0.4	0.3	-
Menthone	2.1	3.8	-	8.7	6.1	15.8
Isomenthone	6.3	8.9	-	22.9	14.2	32.7
c- and t- Isopulegone	3.7	4.2	-	3.9	4.5	1.1
Pulegone	34.3	22.9	-	38.4	21.1	4.1
psi-diosphenol	-	-	-	-	-	-
2-Hydroxyiosmenthone	-	-	-	-	-	-
Diosphenol	-	-	-	-	-	-
1-Hydroxydiosphenol	-	-	-	-	-	-
t-8-SH-p-menthanone	1.7	2.2	2.0	1.6	1.4	-
c-8-SH-p-menthanone	19.6	20.2	-	9.6	11.0	2.5
Methyl Eugenol	-	-	-	-	-	-
t-8-acetylthiomenthone	-	-	-	-	-	-
c-8-acetylthiomenthone	-	-	-	-	-	-
Total:	100.0	100.0	100.0	100.0	100.0	100.0

Gas chromatogram of Buchu oil Lot# HB1408N1L



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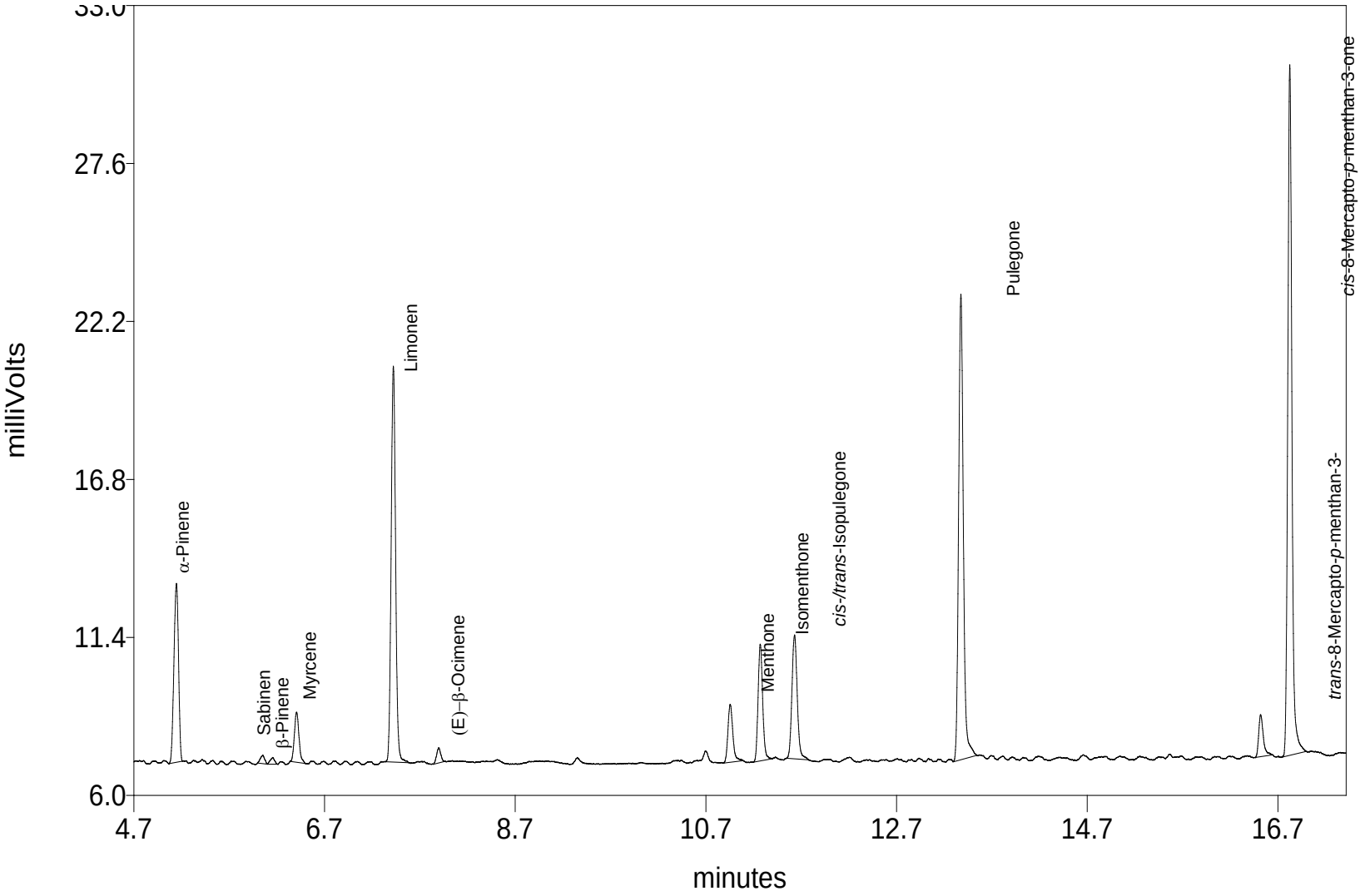

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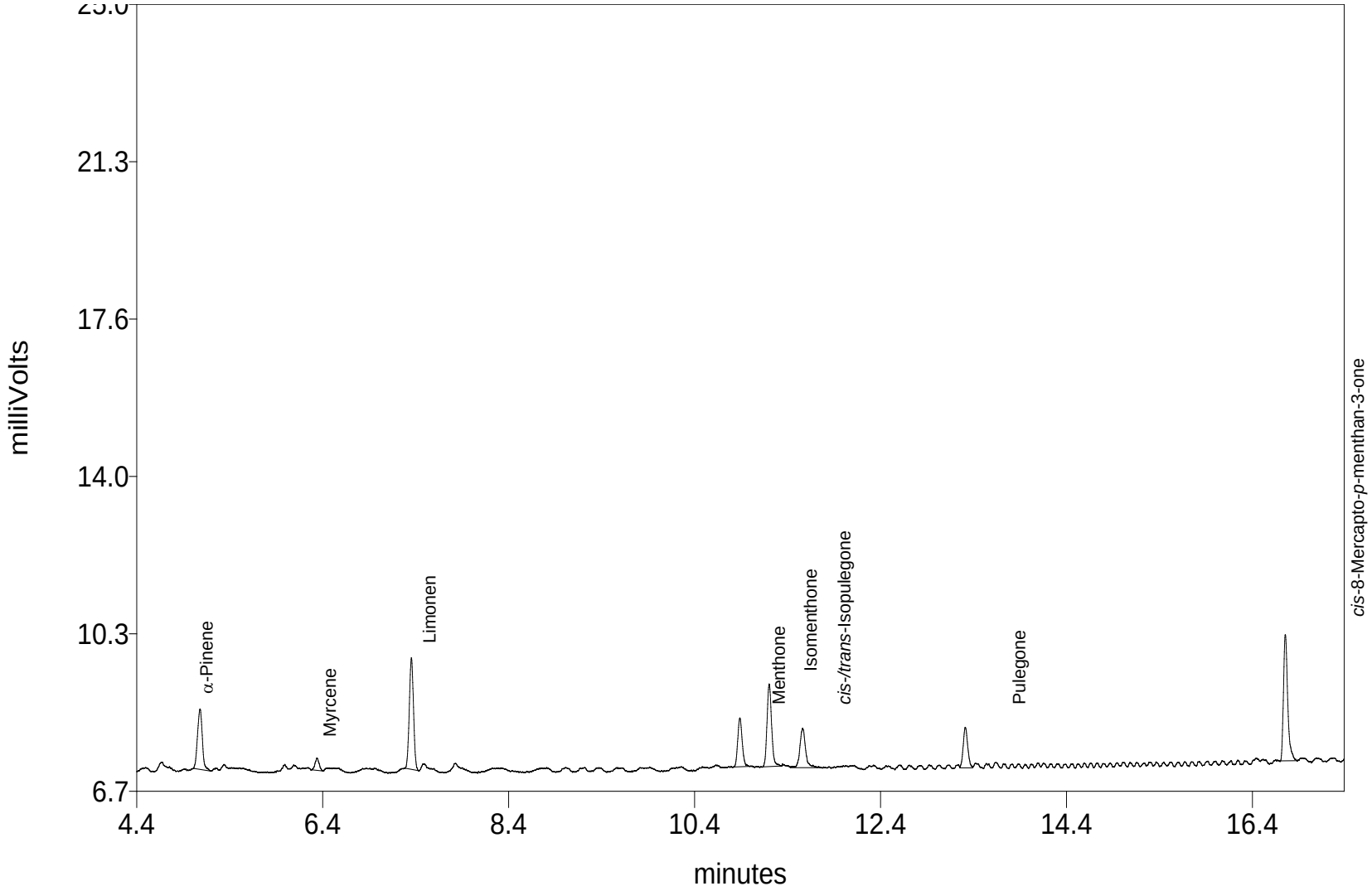

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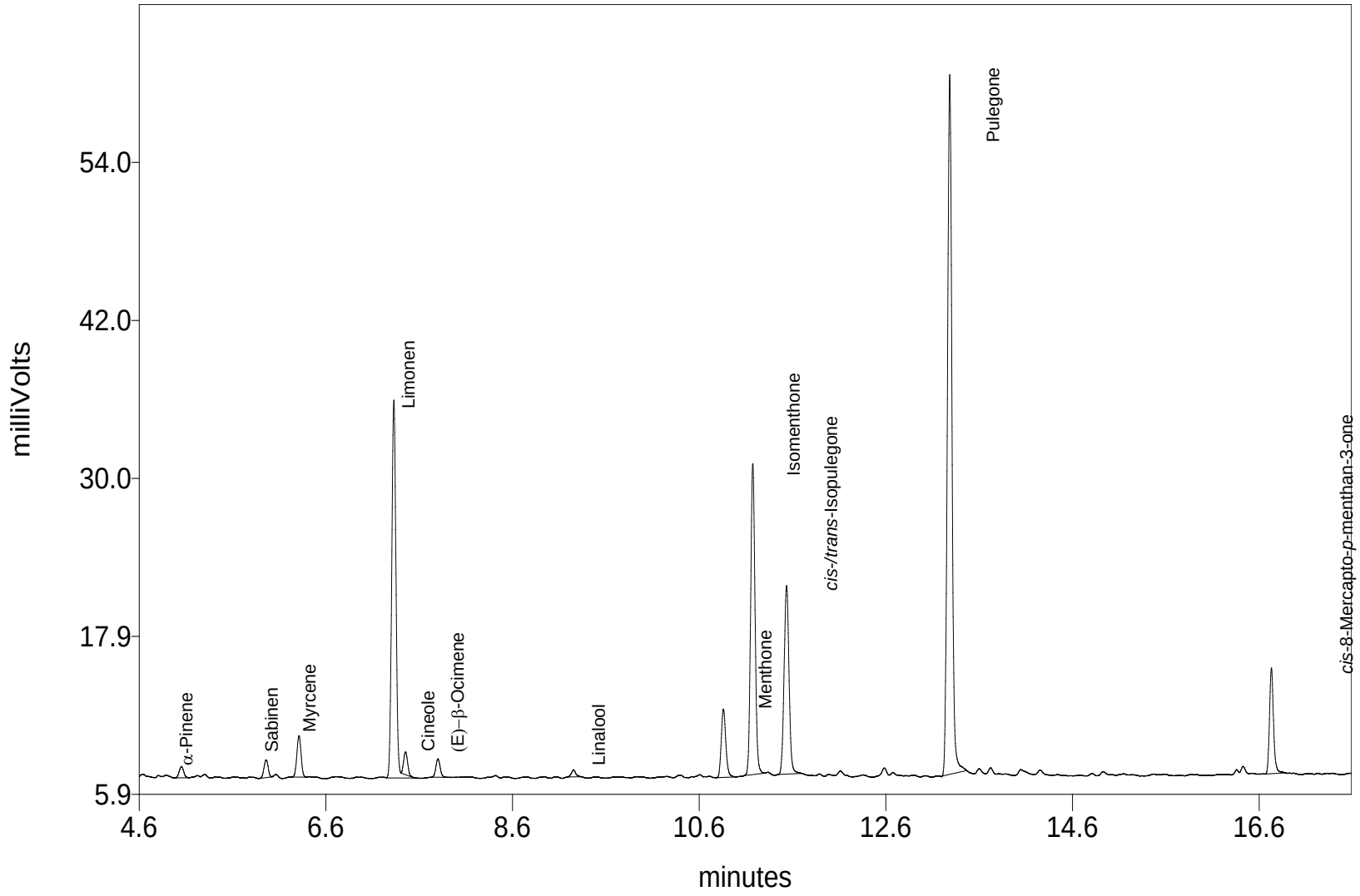

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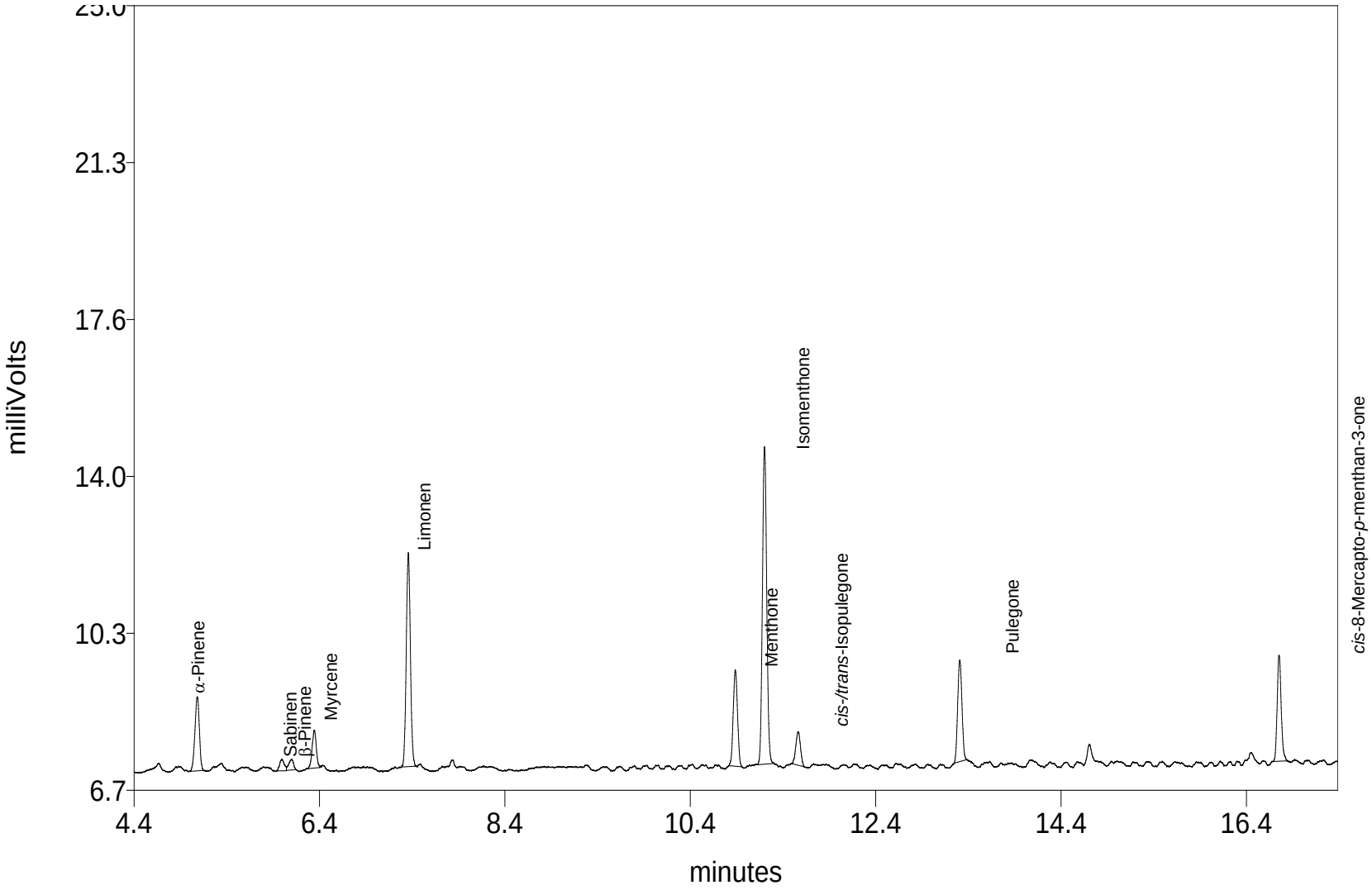

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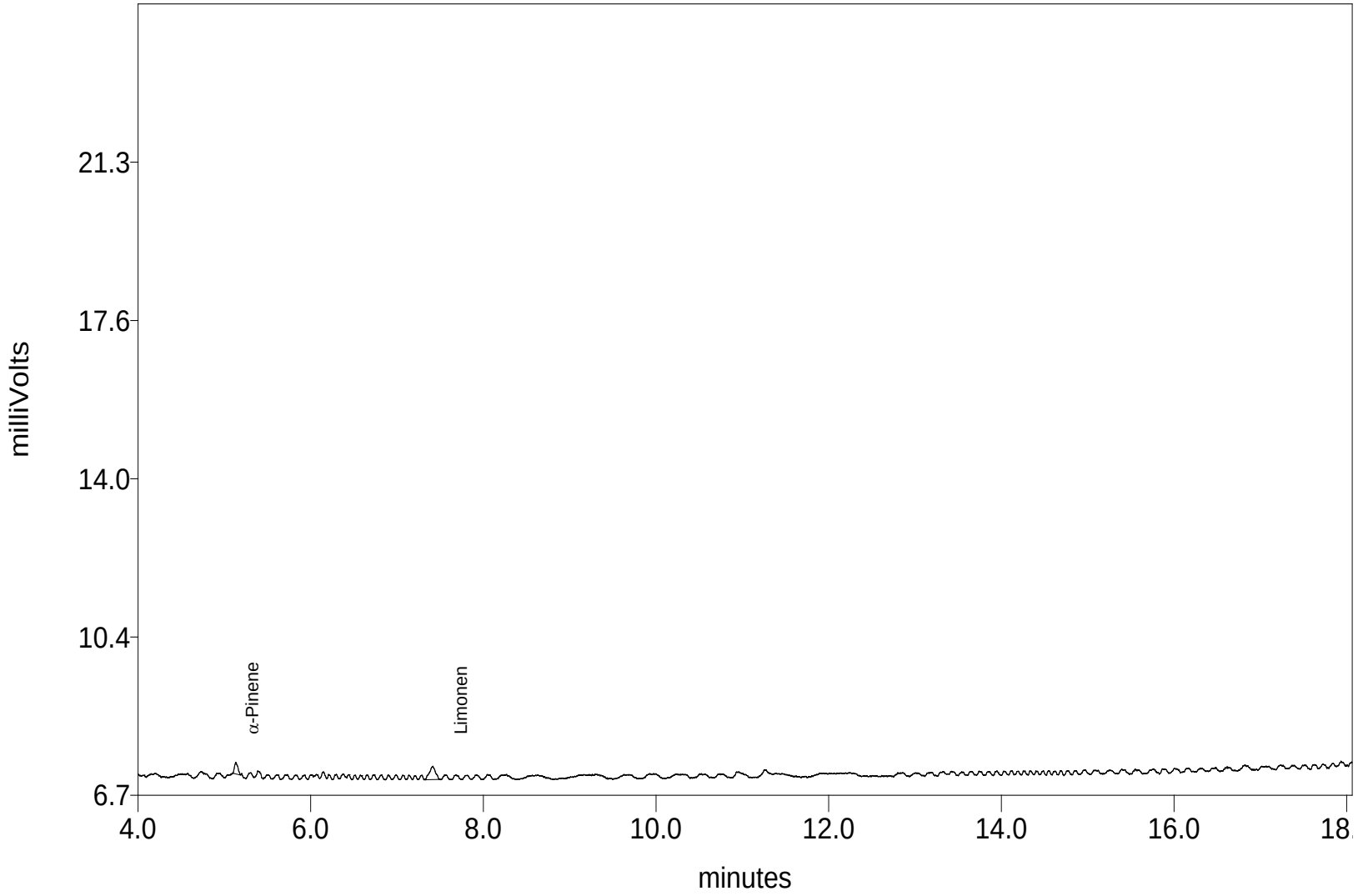

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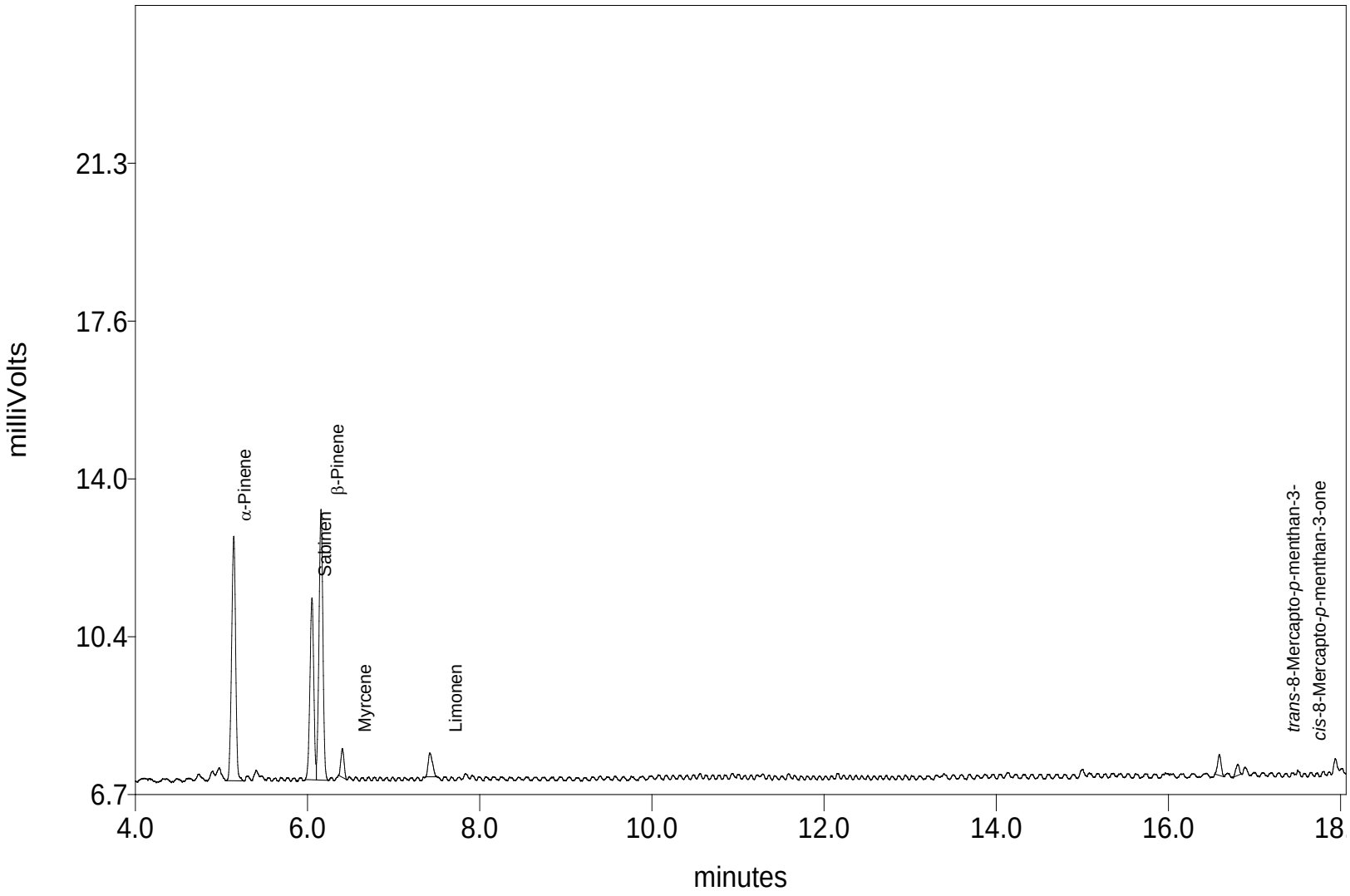
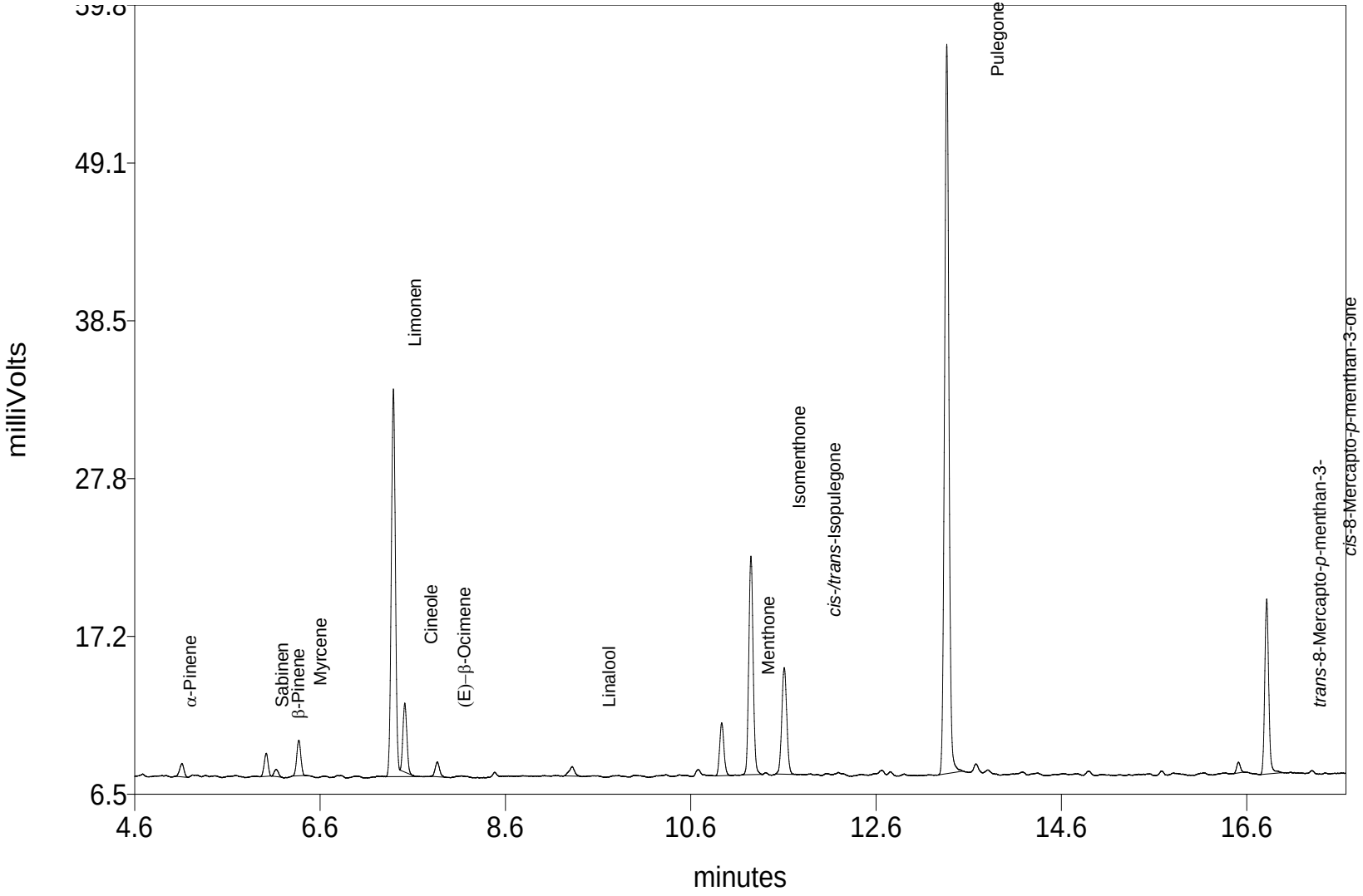
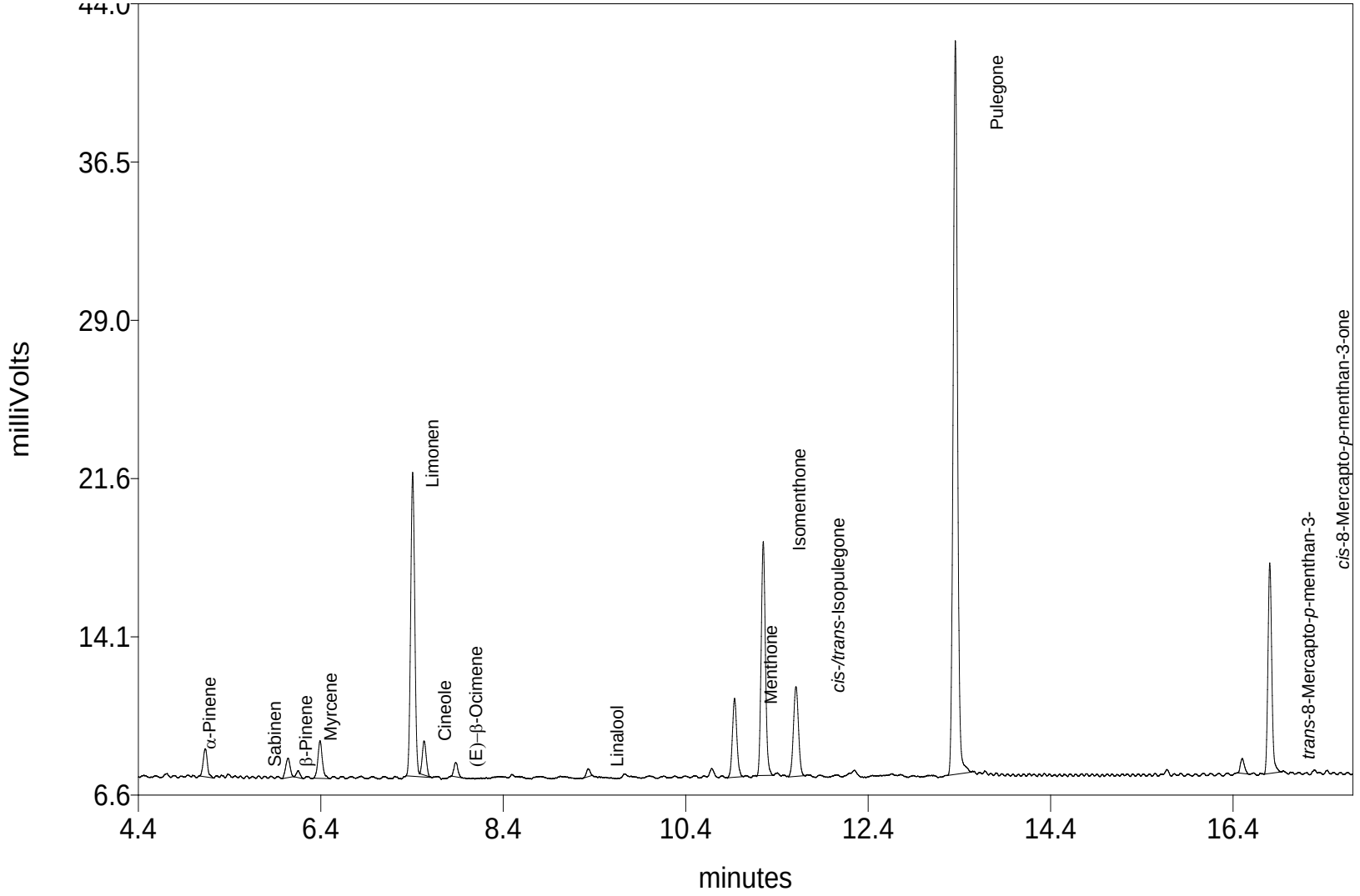


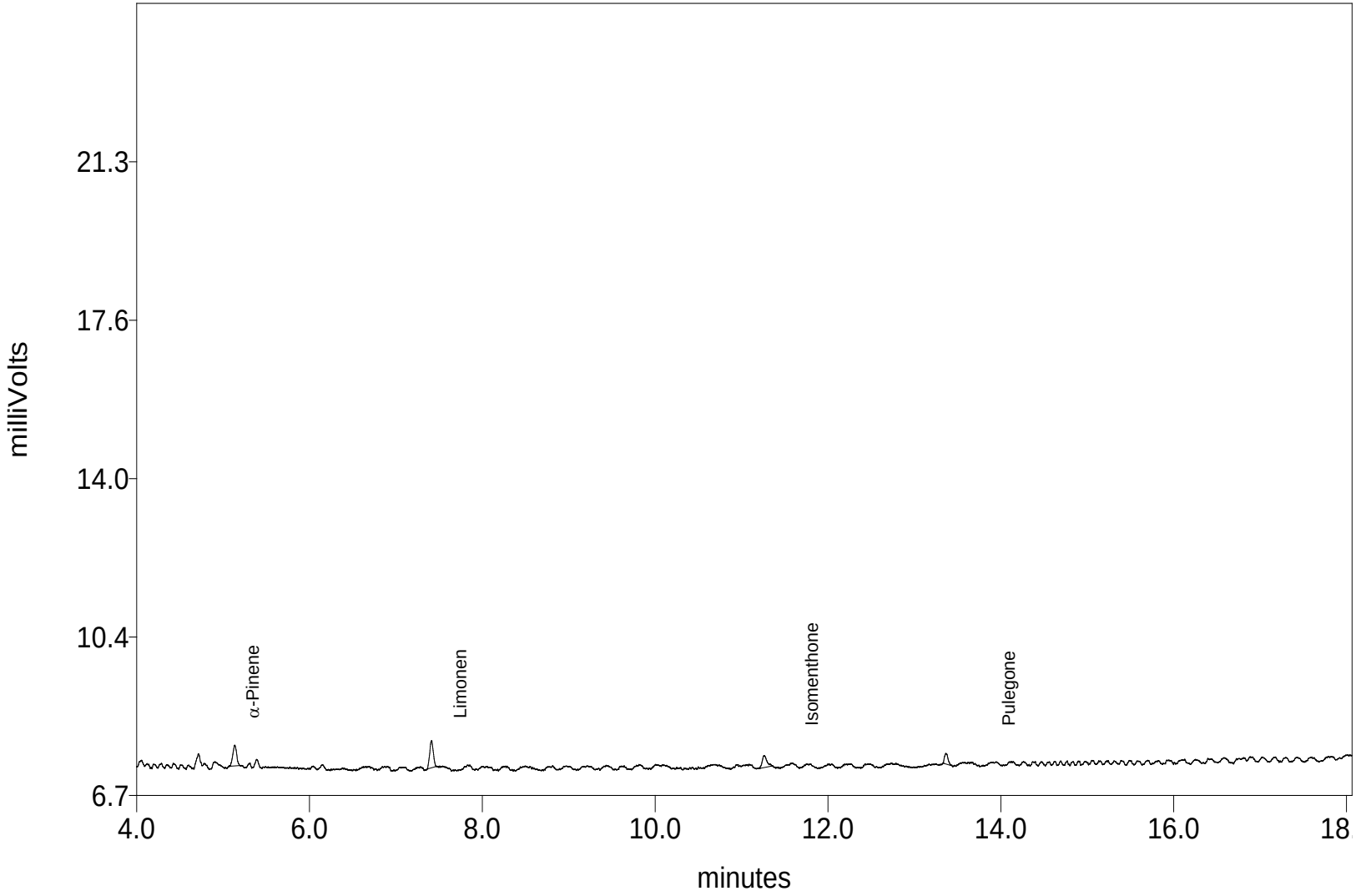
Gas chromatogram of Buchu oil Lot# HB1408N3L



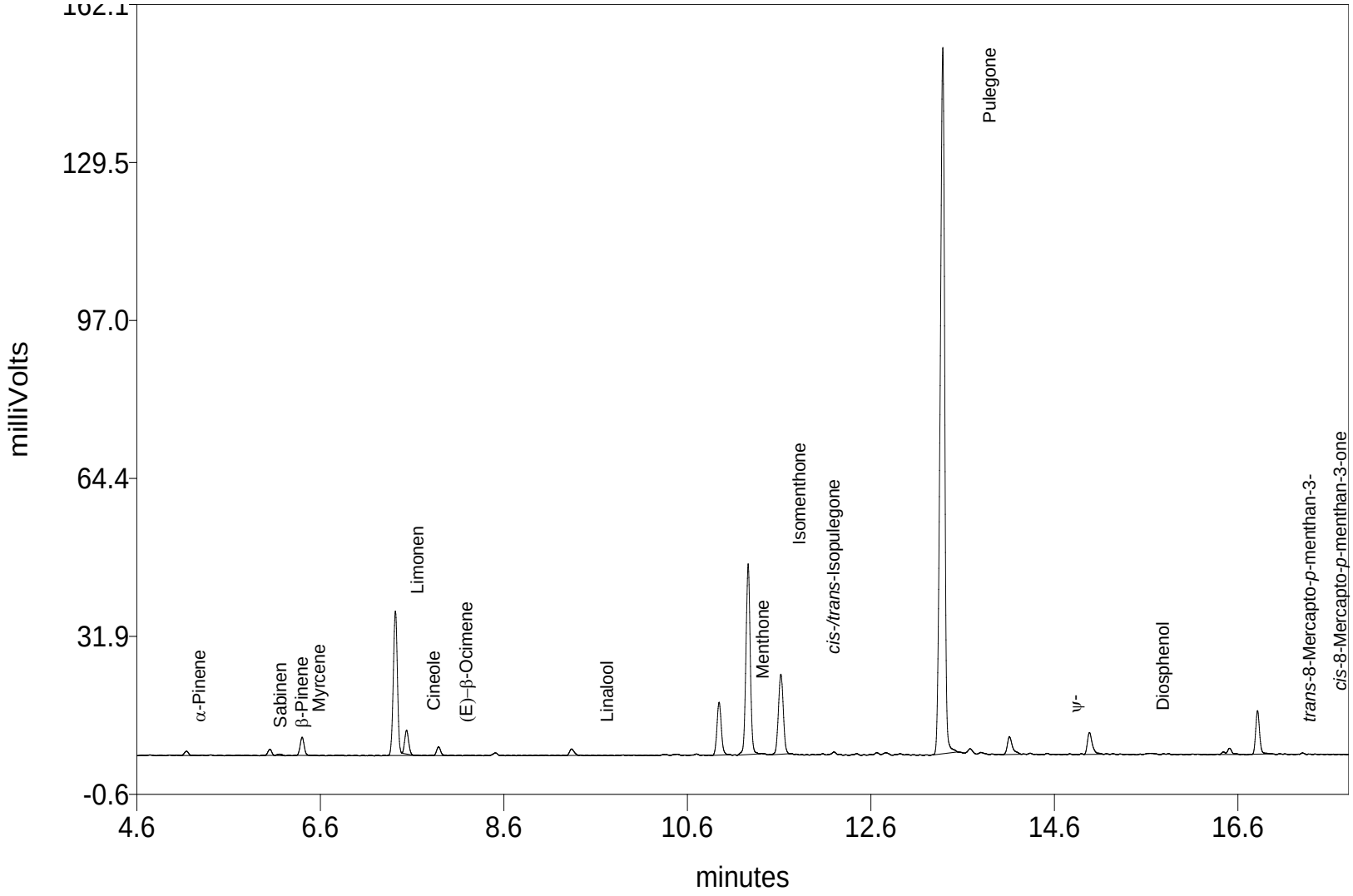
Gas chromatogram of Buchu oil Lot# HB1408N3S



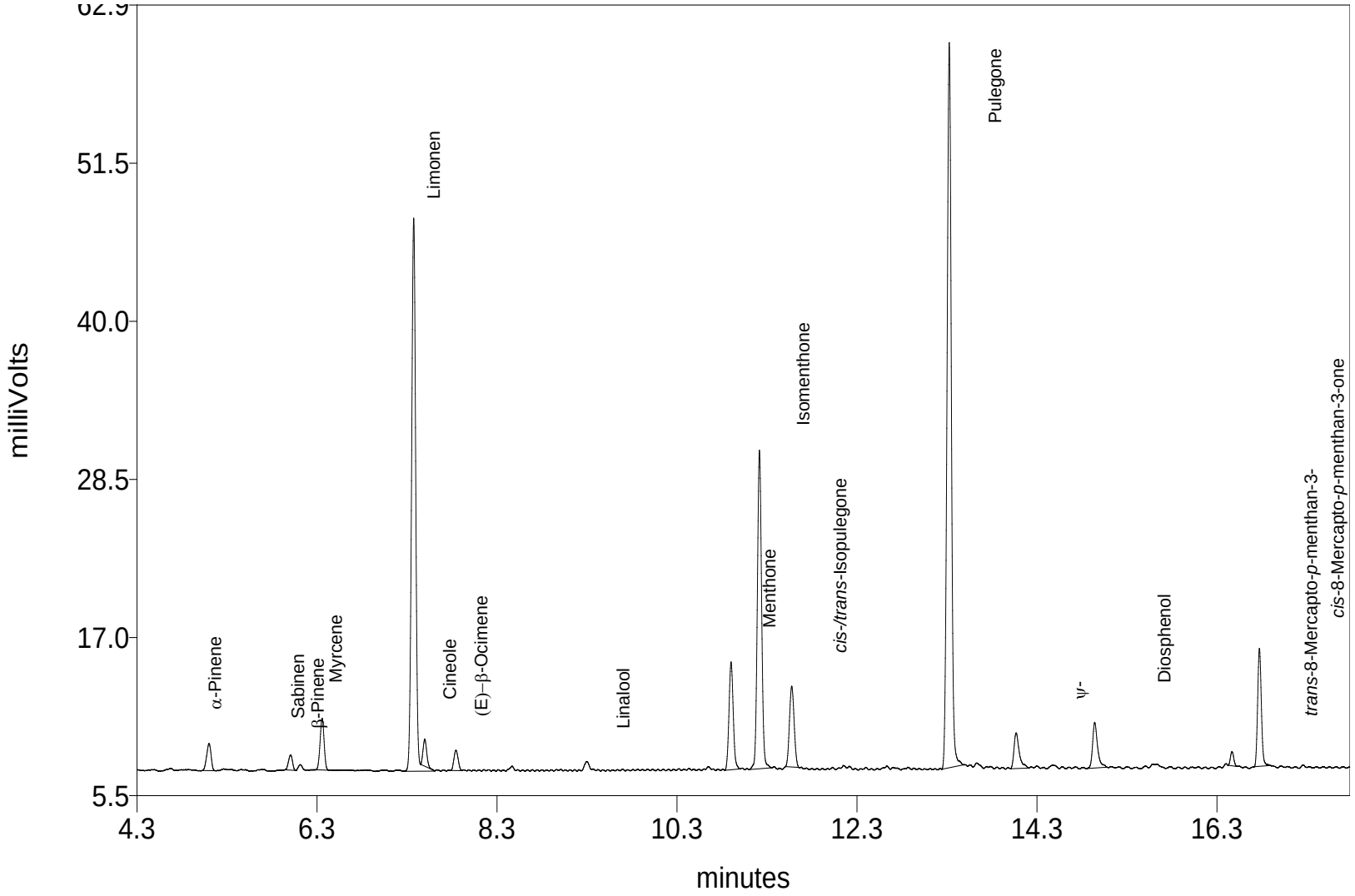
Gas chromatogram of Buchu oil Lot# HB1408N3R



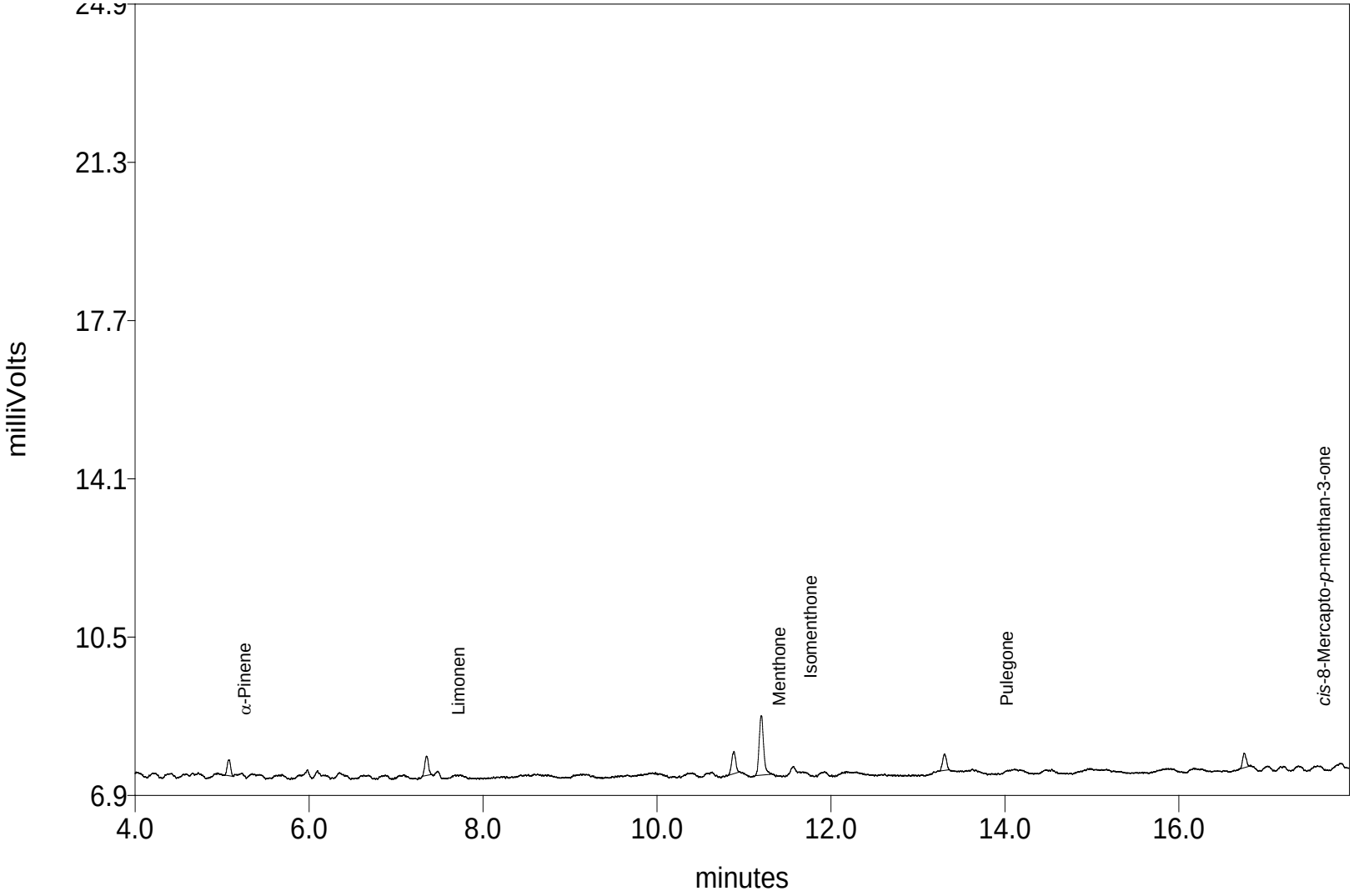
Gas chromatogram of Buchu oil Lot# HB1408N4L



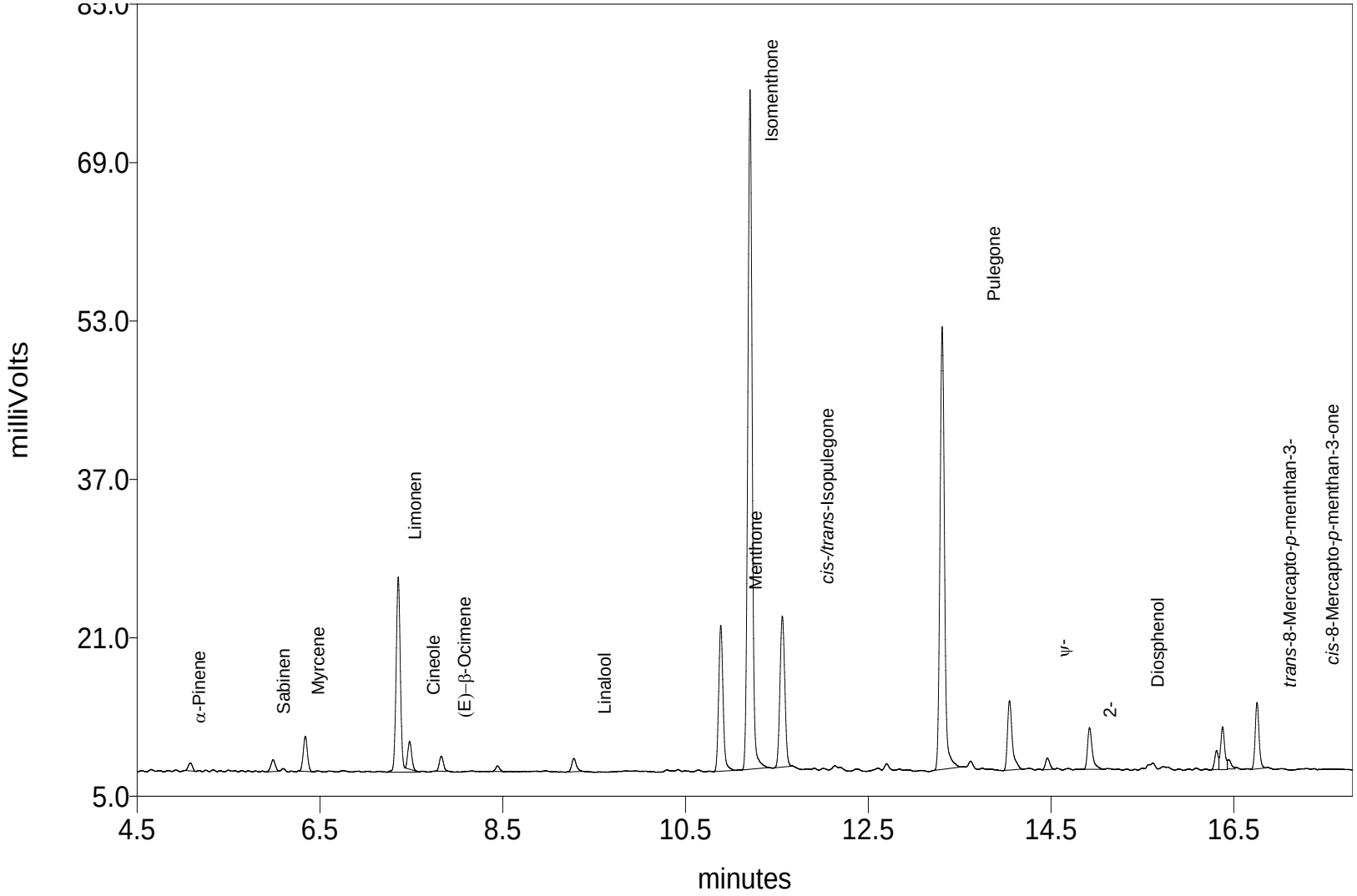
Gas chromatogram of Buchu oil Lot# HB1408N4S



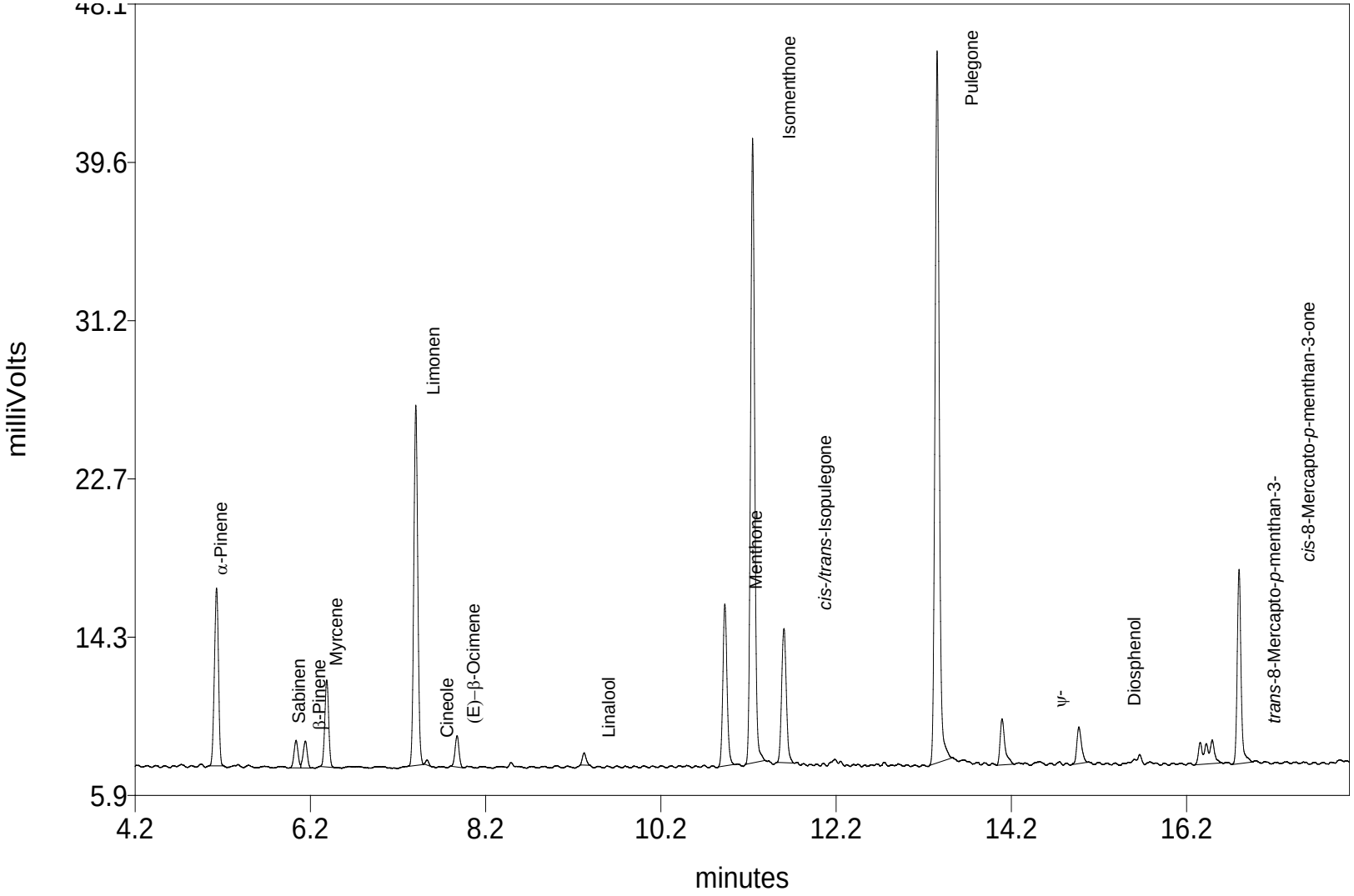
Gas chromatogram of Buchu oil Lot# HB1408N4R



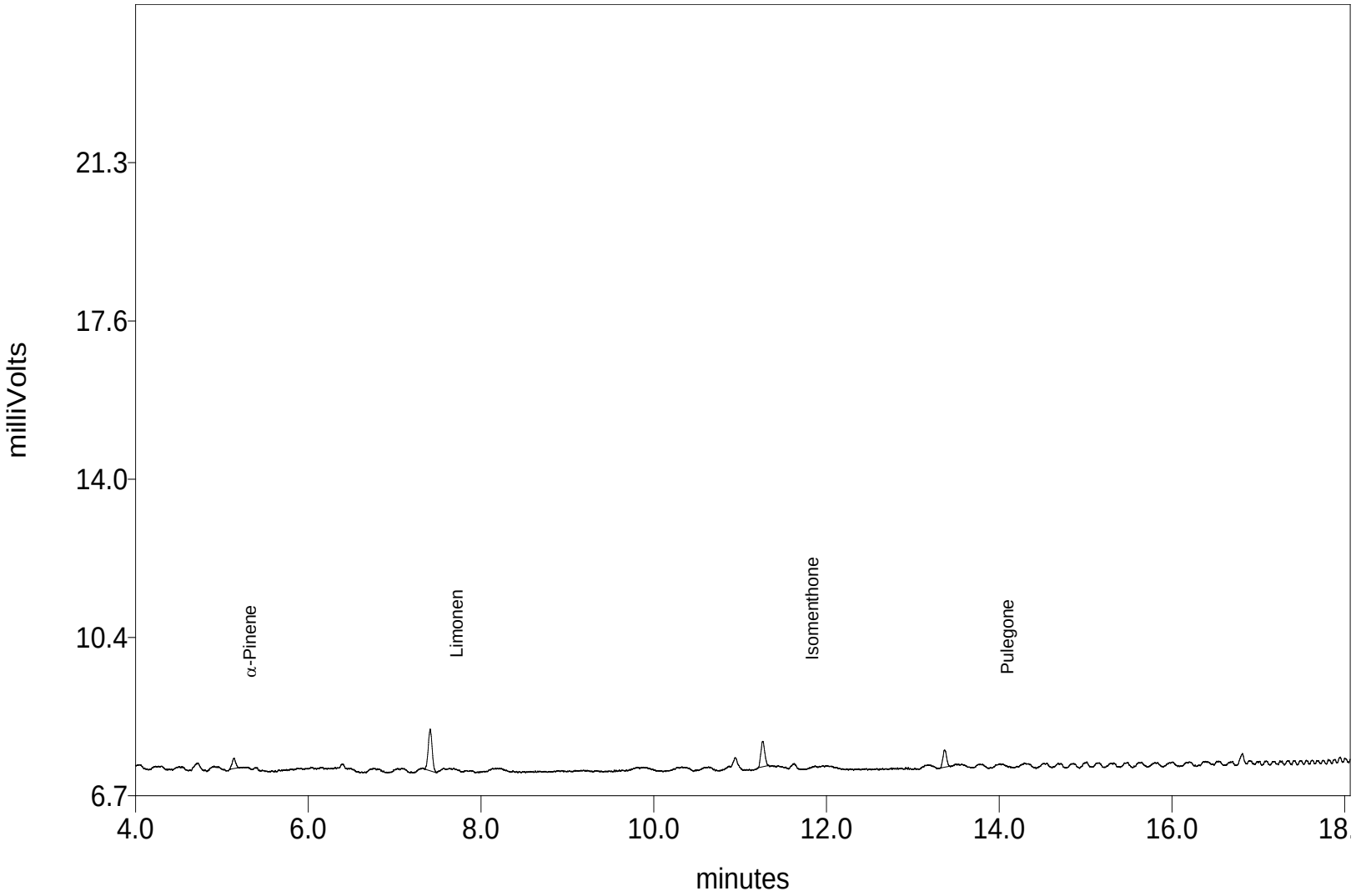
Gas chromatogram of Buchu oil Lot# HB1408N6L



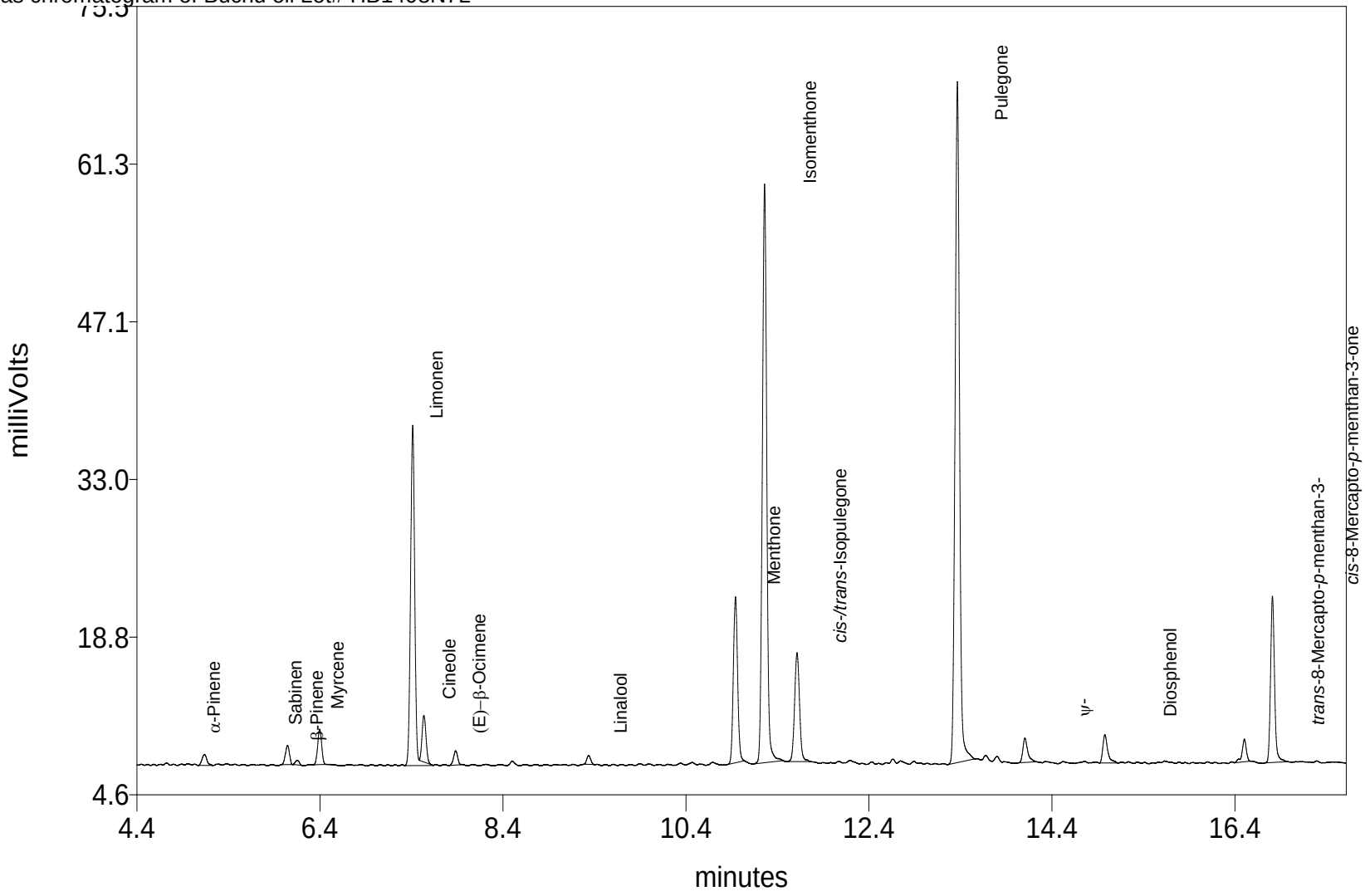
Gas chromatogram of Buchu oil Lot# HB1408N6S



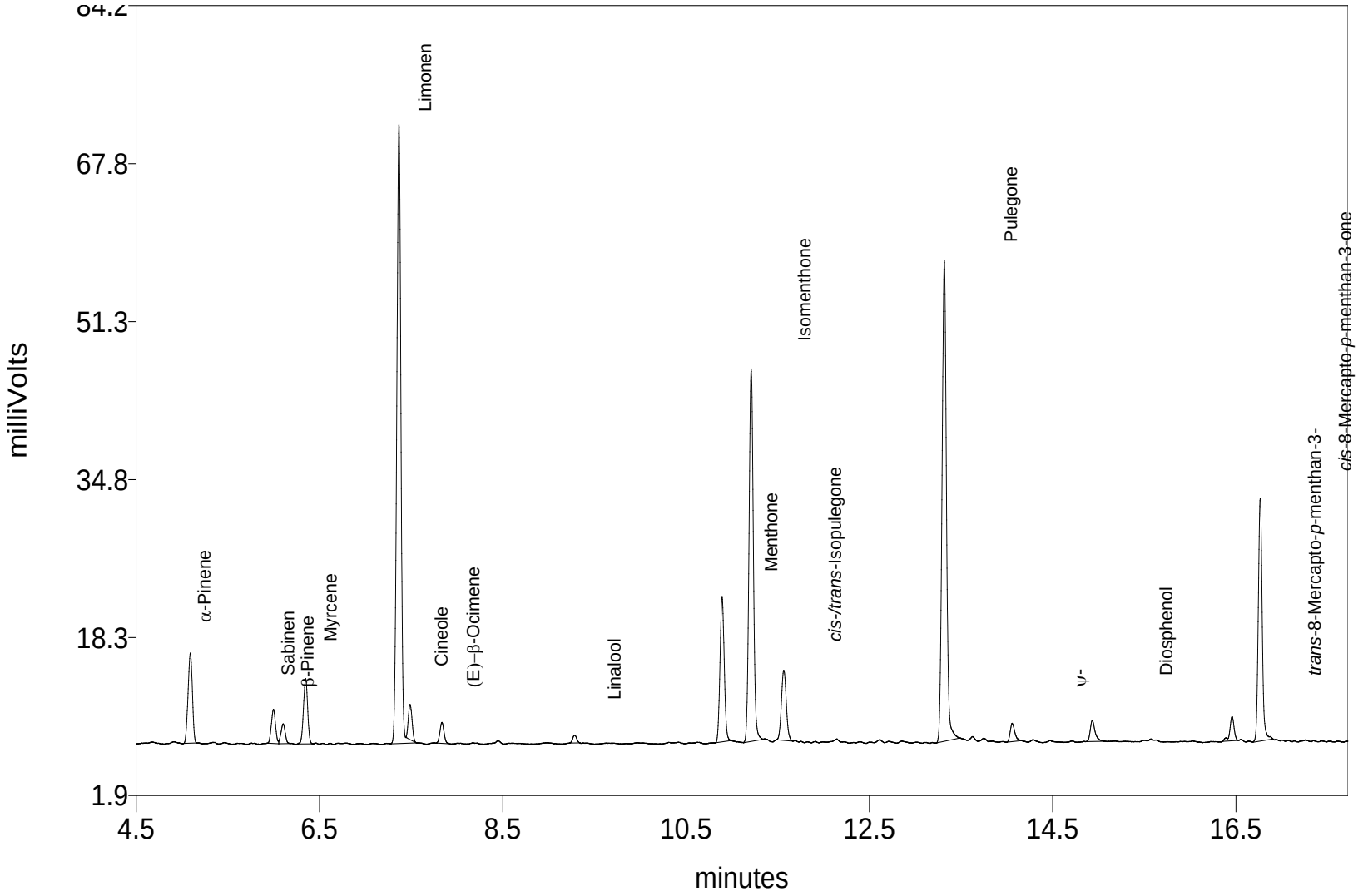
Gas chromatogram of Buchu oil Lot# HB1408N6R



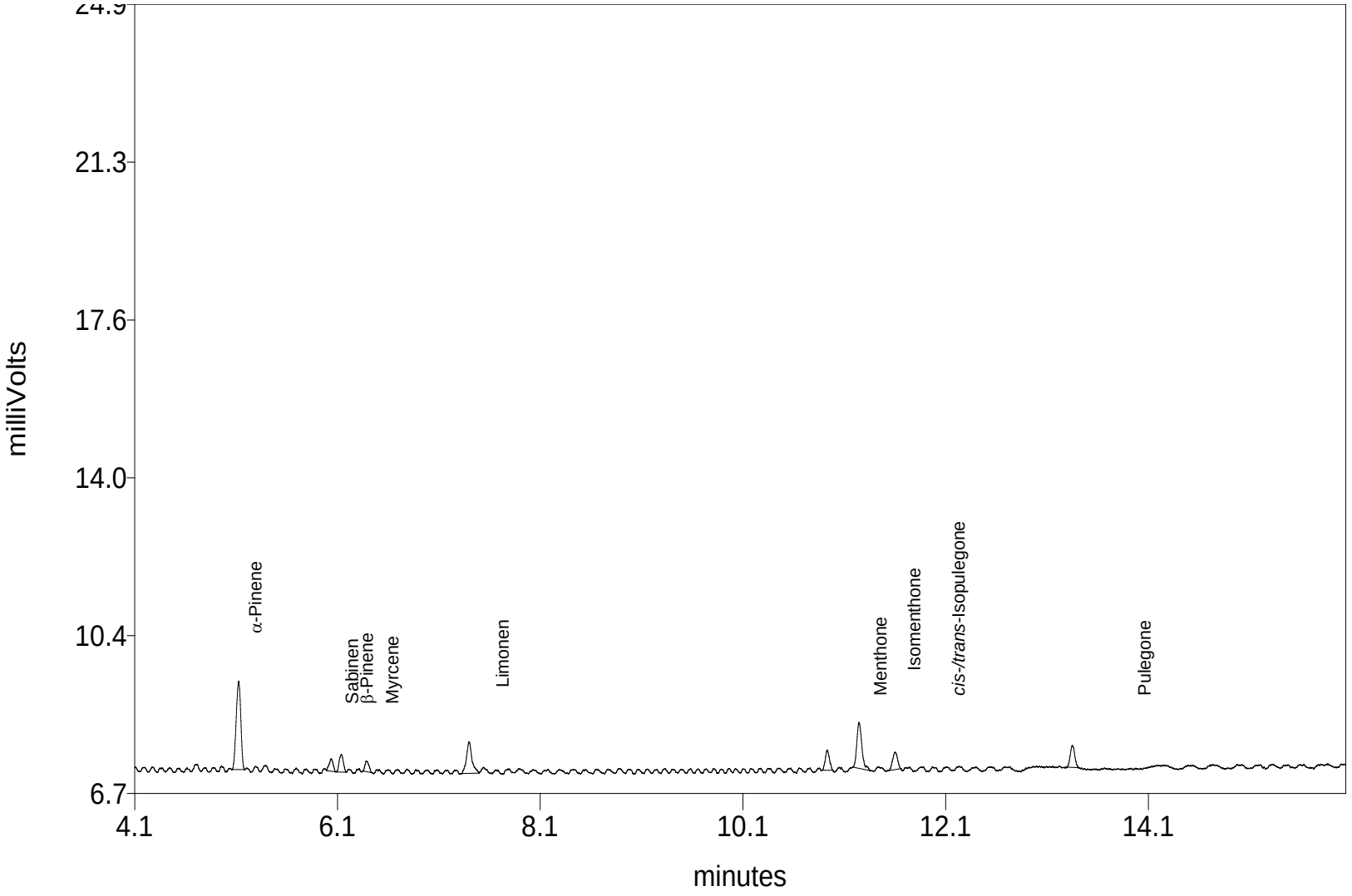
Gas chromatogram of Buchu oil Lot# HB1408N7L



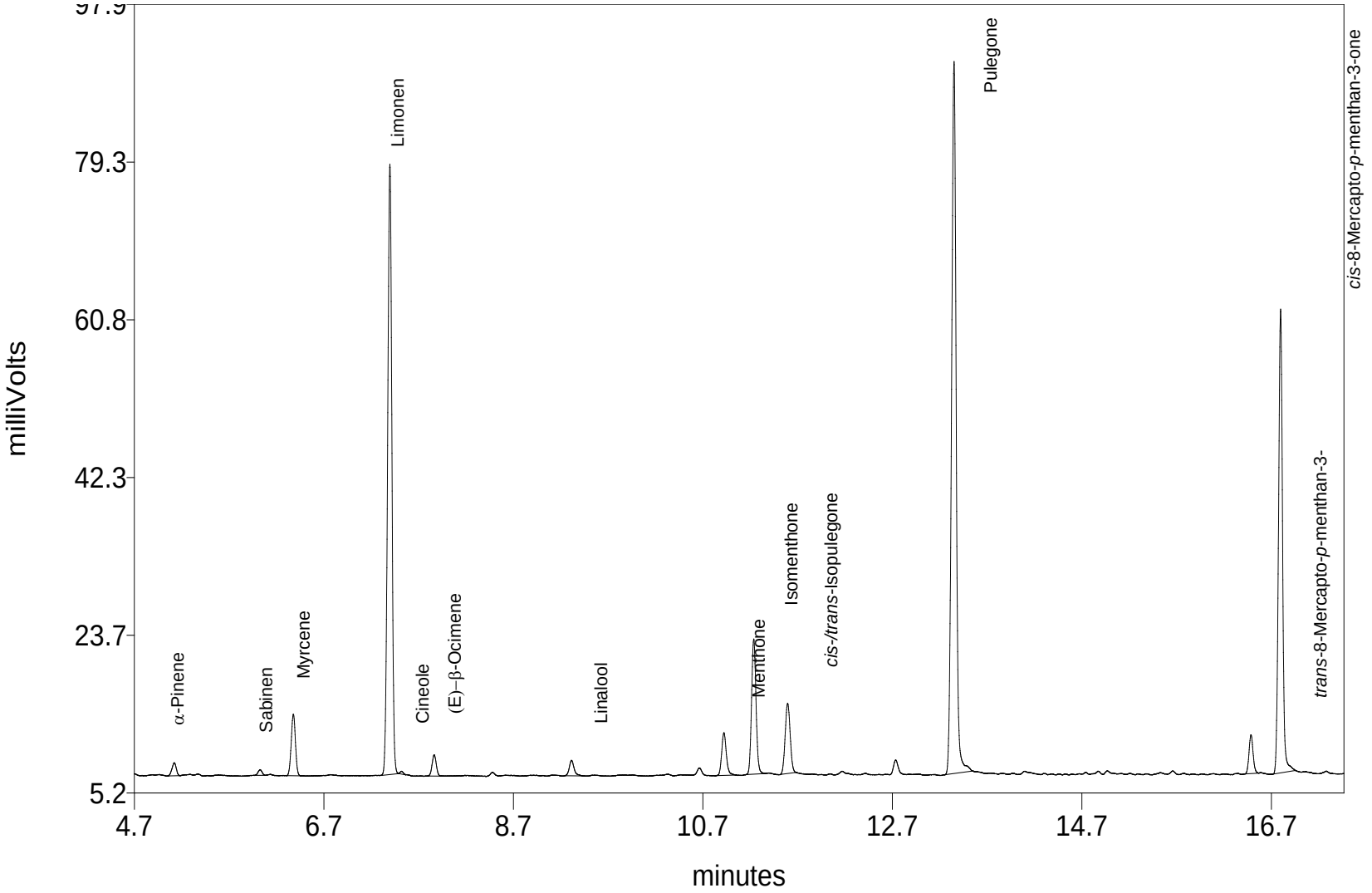
Gas chromatogram of Buchu oil Lot# HB1408N7S



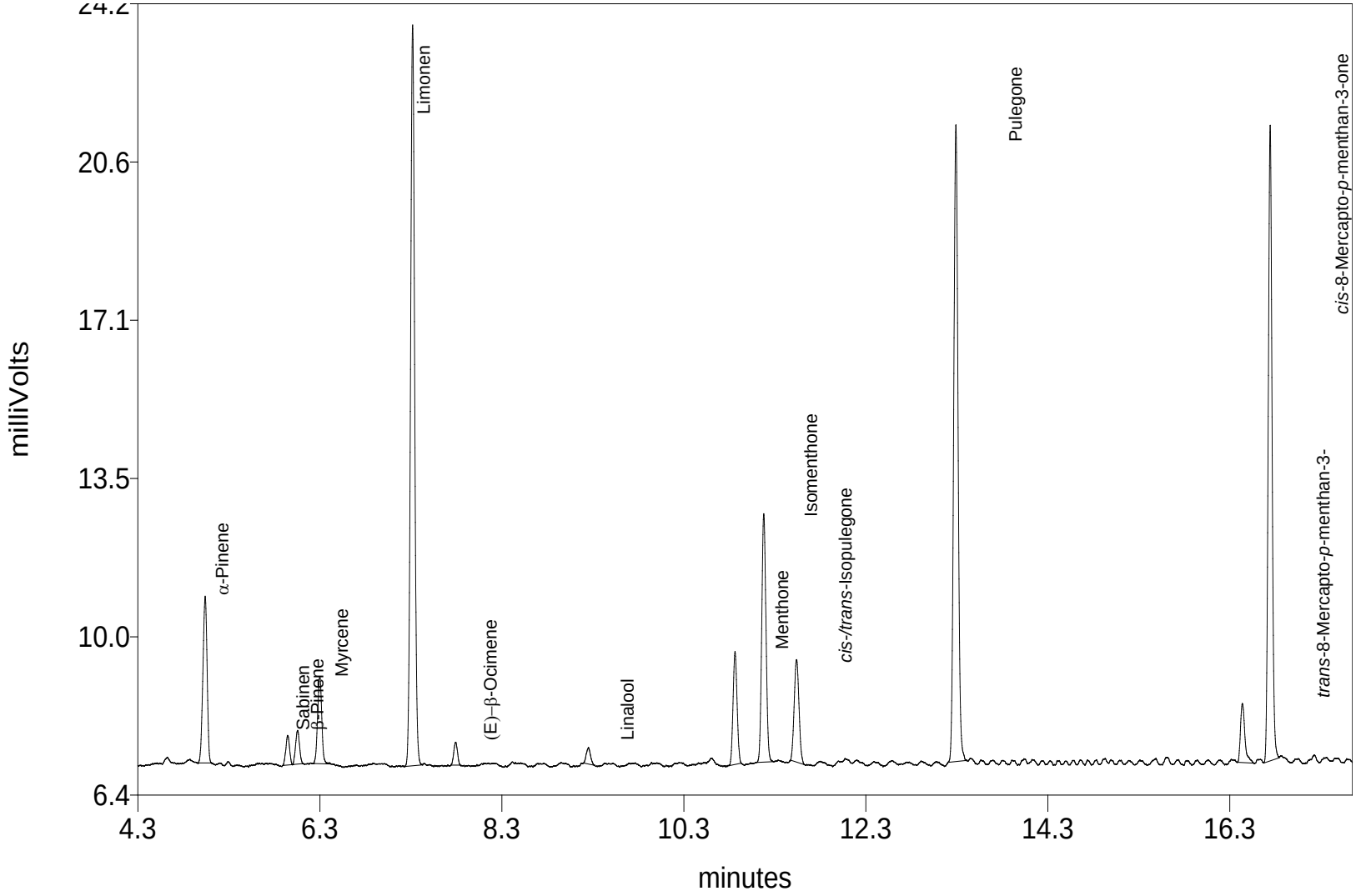
Gas chromatogram of Buchu oil Lot# HB1408N7R



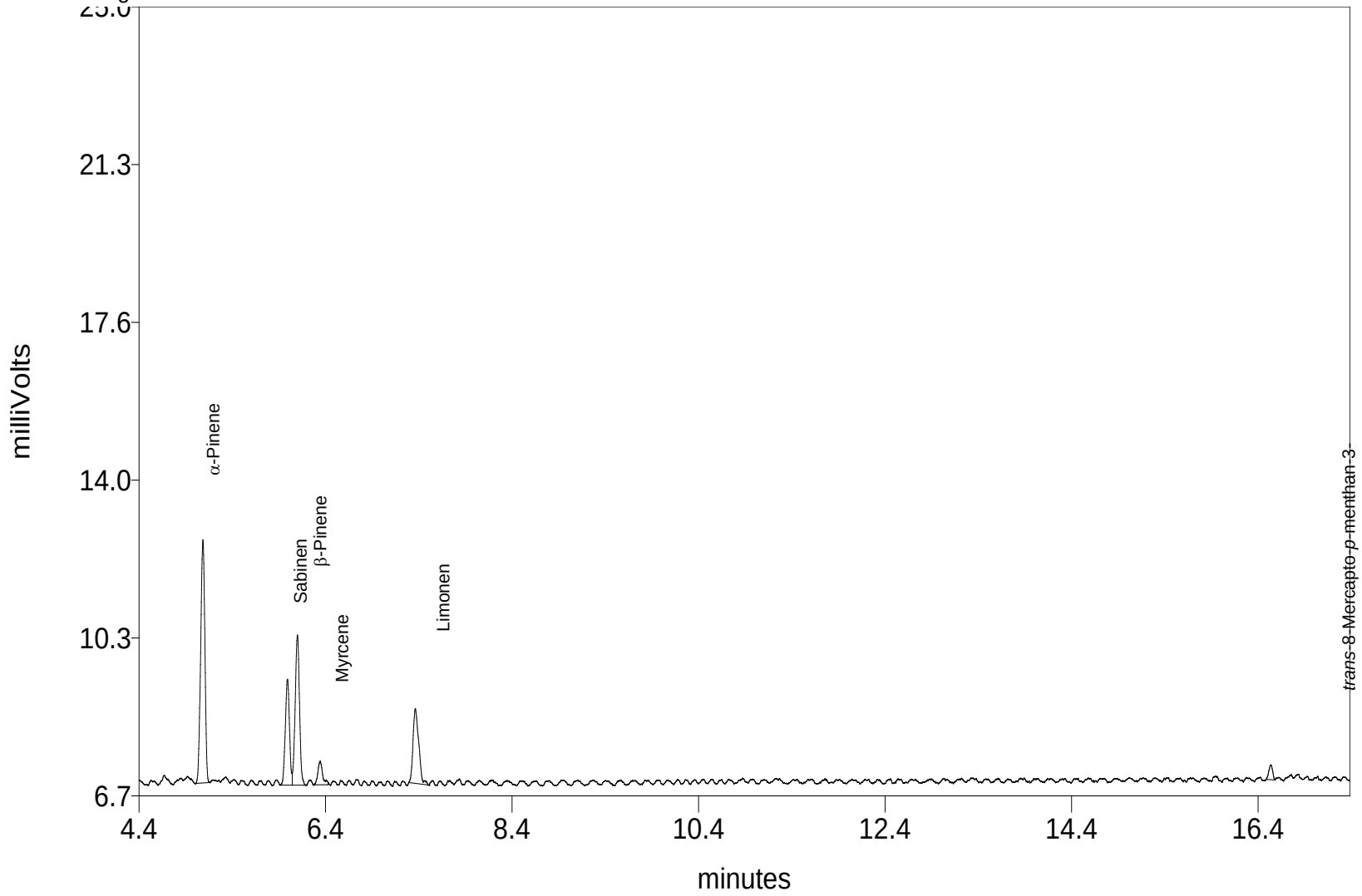
Gas chromatogram of Buchu oil Lot# HB1408N8L



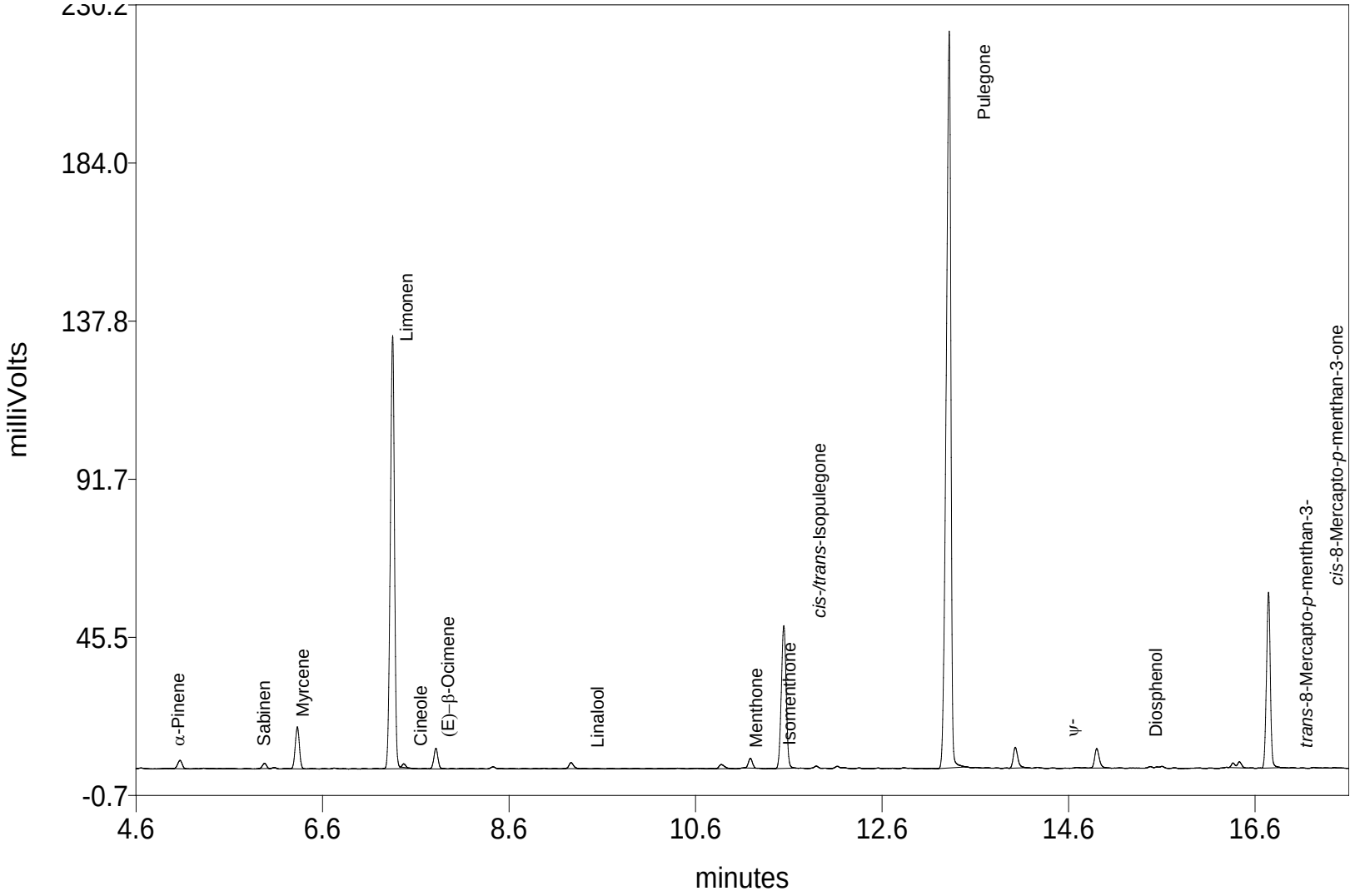
Gas chromatogram of Buchu oil Lot# HB1408N8S



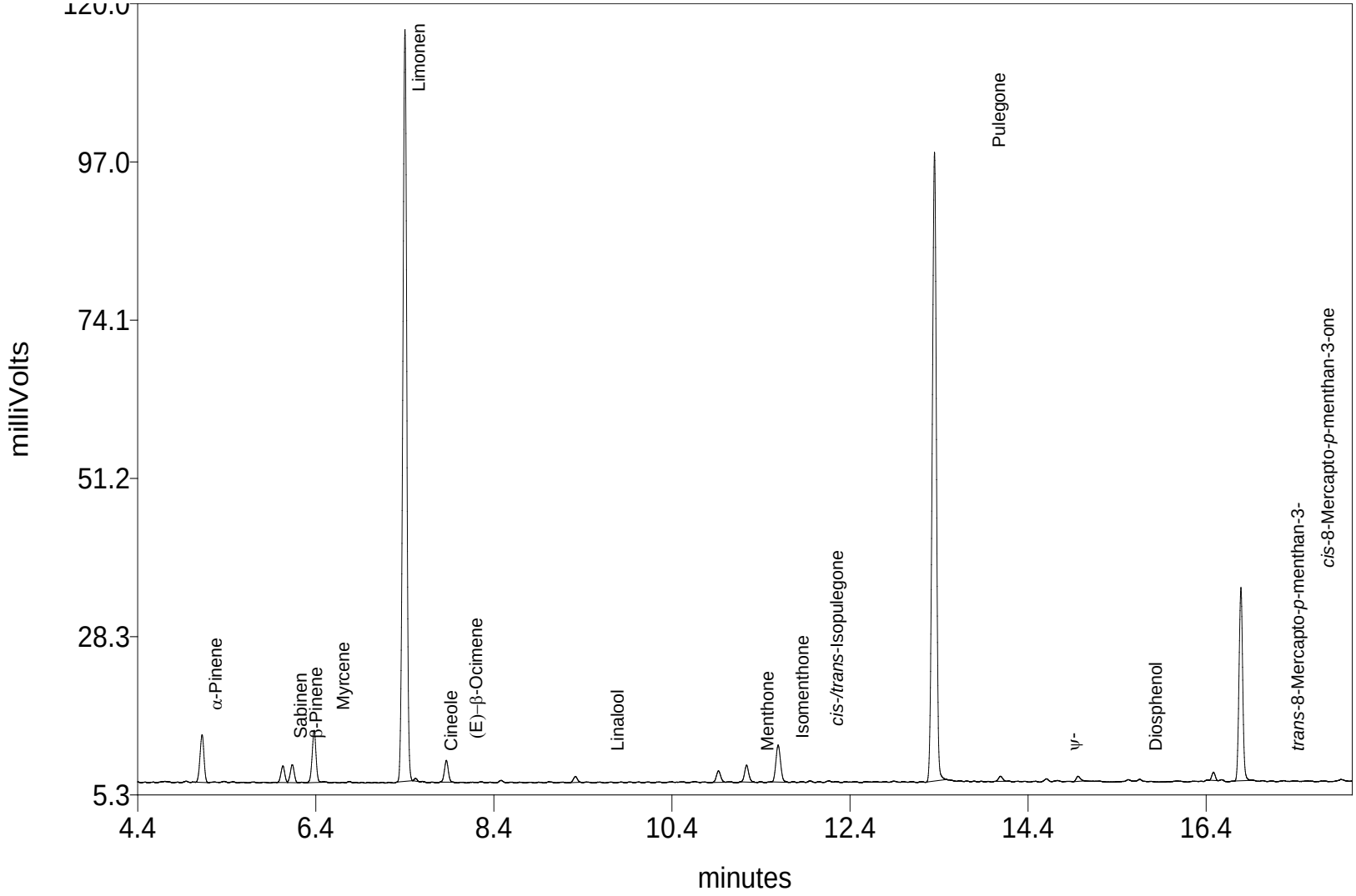
Gas chromatogram of Buchu oil Lot# HB1408N8R



Gas chromatogram of Buchu oil Lot# HB2308N1L



Gas chromatogram of Buchu oil Lot# HB2308N1S



Gas chromatogram of Buchu oil Lot# HB2308N1R