IN VITRO PROPAGATION AND LEAF ULTRASTRUCTURE OF BUSH TEA (*ATHRIXIA PHYLICOIDES*) DERIVED FROM NODAL EXPLANTS

by

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DECLARATION

I hereby declare that the work herein as a dissertation of the Master of Science in Agriculture (Horticulture) is a result of my own investigation. Work of the authors that served as sources of information has been acknowledged by references to the authors.

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ABSTRACT

Athrixia phylicoides (bush tea) is one of many plants from the Asteraceae family used as a traditional herbal medicine. With very few cultivated plants, natural growing plants currently serve as the main resource for plant material. The plant is not yet commercialised and its medicinal value is known and used only by a few people.

With the long term aim at commercial scale propagation, this study consists of three parts. Firstly we developed a protocol for *in vitro* propagation of *A. phylicoides*. Secondly, the ultrastructure and morphology of leaves were studied microscopically and thirdly, comparisons were made between *in vitro* and *ex vitro* grown plants.

Nodal segments of greenhouse plants were used to establish cultures. Better growth and less wilting was recorded on explants surface sterilised with NaOCl compared to $Ca(OCl)_2$ after establishment. The addition of growth regulators IBA (indole-3-butyric acid) and BAP (6-benzylaminopurine) to the culture medium did not seem to affect the growth response of explants during the multiplication phase.

Hyperhydricity was a problem throughout our trials. The development of hyperhydricity symptoms seems to be related to seasonal changes in the stock plant material used to initiate cultures, rather than the composition of growth medium or growth room temperatures. The occurrences of hyperhydricity symptoms were inconsistent and unpredictable.

A rooting medium with added BAP and decreased sucrose levels resulted in a higher rooting percentage compared to the control medium, free from BAP and with a higher sucrose concentration, which yielded no rooting. However, in another experiment, *in vitro* rooting occurred spontaneously after subdividing and transfer of microshoots to fresh control medium. The addition of GA_3 to the establishment medium (but not to the subsequently used multiplication and rooting media) yielded a slightly higher percentage of rooting. However, cultures initially established on GA_3 medium yielded fewer roots per explant and roots were shorter than those of explants established on hormone free medium.

The medicinal properties of plants are often linked to the production of essential oils. We hypothesised that the medicinal value of *A. phylicoides* can be linked to the production of the aromatic essential oils released by leaves. A microscopic study of leaves provided some preliminary insight of the mechanisms involved in the production of medicinally active products. Electron- and light microscopic examination of leaves were used to identify and study structures that are apparently involved in the production and secretion of essential oils. Two types of trichomes were identifyed – nonglandular and glandular trichomes. These

glandular trichomes are multicellular with a subcuticular storage space and are present only on the adaxial surface of leaves.

In the case of medicinal plants, it is essential that the medicinal properties of the plant are not altered by the method of propagation. This was our motivation for comparing the morphology and ultrastructure of leaves of plants that were grown in their natural environment to that of plants grown *in vitro*. Leaf surfaces of *in vitro* grown plants were smaller and the number of glandular trichomes per surface area was less on *in vitro* grown plants. There were no noticeable changes in the morphology of glandular trichomes.

Keywords: Asteraceae, essential oil, glandular trichome, medicinal plant

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LIST OF ABBREVIATIONS

BAP	6-benzylaminopurine
GA_3	gibberellic acid
IBA	indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	1-naphthaleneacetic acid
PAR	photosynthetically active radiation

GLOSSARY OF TERMS

acclimation	a process during which plants or other organisms become adjusted to a new climate or situation as result of a natural process
acclimatisation	a process during which plants or other organisms become adjusted to a new climate or situation where humans interceded in, or guided the adjustment process
analgesic	acting to relieve pain
aphrodisiac	a food, drink, or drug that stimulates sexual desire
ex vitro	taking place 'out of' a test tube or culture dish
explant	organ or other plant part used to establish an *in vitro* culture
growth regulators	synthetic compounds corresponding to hormones
hormones	organic compounds naturally synthesised in higher plants which influence growth and development of plants
hyperhydricity	a physiological disorder causing shoots and leaves to become brittle with a glassy, waterlogged appearance in various degrees
hypertensive	relating to or causing inflammation
in vitro	taking place in a test tube, culture dish, or elsewhere outside a living organism
inflammation	a localized physical condition in which part of the body becomes reddened, swollen, hot, and often painful, especially as a reaction to injury or infection
inflammatory	relating to or causing inflammation
microcutting	single microshoots moved to a medium to induce rooting
microshoot	shoots developed during tissue culture
narcotic	a drug which induces drowsiness, stupor, or insensibility, and relieves pain
plantlet	a plant derived from micropropagation that has both shoots and roots
purgative	strongly laxative in effect
secondary metabolites	metabolites produced by a plant which are not directly needed by the plant itself
subculture	the act of subdividing the developing explant into smaller pieces and moving them to new medium
transfer	moving the entire culture to new medium, without subdivision
vitrification	convert into glass or a glass-like substance

INTRODUCTION

The use of natural and herbal medicine is increasingly becoming a topic of study due to escalating problems with resistance to current medicines, and a widespread increase in the awareness and popularity of natural remedies over the last few decades (Salie *et al.*, 1996; Caldis, Mcleod & Smith, 2001).

Athrixia phylicoides (bush tea) belongs to the Asteraceae family and has been used as a traditional herbal medicine for many decades. Leaves are mostly used for preparing medicine. Leaves are boiled in water and the leaf extract is mostly ingested as a tea, but can also be used as a wash. The extract is used for a variety of conditions including the treatment of sores and boils, coughs and colds and throat infections. It is also used for blood purification, as a purgative and as an aphrodisiac (Roberts, 1990; Swanepoel, 1997; Van Wyk & Gericke, 2000).

Leaf extracts of bush tea have been subjected to pharmacological screening and its anti-inflammatory, antihypertensive, narcotic and analgesic properties have been confirmed. Bush tea is ideal for use against stress symptoms such as sleeplessness and hypertension. The potential of this pleasant tasting, health giving tea is currently underutilised. Despite the fact that the commercial possibilities of bush tea have been realised by some authors (Roberts, 1990; Swanepoel, 1997; Van Wyk & Gericke, 2000; McGaw, Steenkamp & Eloff, 2007), no serious efforts have been made to commercialise the product.

Like in the case of many other medicinal plants, wild population currently serve as the main source of plant material. Over exploitation of these natural resources may result in the permanent loss of biodiversity and potentially useful medicines. Propagation of bush tea for commercial use, will relief the pressure on wild plant populations. A propagation method that will produce plants which yield consistent, high quality essential oils needs to be developed for successful commercialisation.

Plant tissue culture is used for commercial scale production of many plants, including medicinal plants (Razdan, 1993; Rout, Samantary & Das, 2000). This propagation method provides a way of producing great numbers of genetically true to type plants from very little starting material (Giusti, Vitti, Fiocchetti, Colla, Saccardo & Tucci, 2002), thereby minimising the pressure on natural resources. In this study we developed a protocol for the production of plants from axillary buds on nodal explants, using tissue culture techniques.

Plants were established on ½ strength Murashige & Skoog medium (Murashige & Skoog, 1962) supplemented with 3% sucrose and solidified with Gelrite™ and were multiplied on the

same medium supplemented with an additional 1.5 mg/L indole-3-butyric acid (IBA). Rooting occurred spontaneously on the same medium plants were established on after two to three subcultures.

The active ingredients of medicinal plants are often one or more of the components of essential oils which are produced by glandular structures (Afolayan & Meyer, 1995). A microscopic investigation of the leaves of field grown bush tea revealed the presence of glandular trichomes on the adaxial surfaces. These glands seem to be the source of the aromatic oils. Essential oils are stored in a subcuticular space linked to the 4 to 5 most apical cells in the gland and are released through rupture of the glands, especially on older leaves (Möller *et al*., 2006). We hypothesised that the essential oils produced by these structures can be linked to the medicinal properties of the plant.

The anatomical, morphological and physiological changes that are often associated with micropropagated plants are especially a concern when it comes to medicinal plants (Lamproye, Hofinger, Ramaut & Gaspar, 1986; Mascarenhas, Khuspe, Nadguada, Gupta & Khan, 1988; Gaspar, 1994; Srividya, Sridevi & Satyanarayana, 1998). Possible changes in secondary metabolite production may result in plants that lack the ability to produce medicinally active compounds, or lack the capacity to produce sufficient qualities or quantities thereof (Derbessy, Touche & Zola 1989; Nalawade & Tsay, 2004).

As part of a pilot study to determine the suitability of micropropagated plants for commercial use, we compared the leaves of micropropagated plants to that of greenhouse plants microscopically. *In vitro* grown plants had smaller, lighter coloured leaves with dysfunctional stomata. No changes were noted in the structure or morphology of glandular trichomes of micropropagated plants. However, the quantity of glandular trichomes was significantly less for *in vitro* grown leaves than for greenhouse plants.

Some of the problems we encountered during this study were the unpredictable responses of explants due to changes in the biorhythm of stock plants. Stock plants entered a resting phase shortly after flowering and plant material could not be collected throughout the year. A further problem was hyperhydricity. This limited the number of subculture cycles possible since the problem escalated with time in culture. Our attempt to acclimatise *in vitro* grown plants to an *ex vitro* environment was also unsuccessful and a different strategy needs to be developed.

CHAPTER 1

PREFACE

Athrixia phylicoides is one of many plants that are traditionally used for its medicinal value by people of southern Africa (Roberts, 1990; Swanepoel, 1997; Van Wyk, Van Oudtshoorn & Gericke, 1997; Van Wyk & Gericke, 2000; Mudau, Soundy, Du Toit & Olivier, 2006). The plant has been used for many decades by many different races (Letty, Dreyer, Verdoorn & Codd, 1962; Roberts, 1990). It was probably introduced to the white colonists by the Khoi-khoi and San people (Roberts, 1990). The plant is known by many folk names such as bush tea, bushman's tea, wildetee, bostee, Zulu tea and daisy tea (Fabian & Germishuizen, 1982; Moriarty, 1982; Onderstall, 1984; Carruthers, 2000; Mudau *et al.*, 2006). These folk names indicate the common use of the plant amongst people as a tea. *A. phylicoides* is not currently commercialised, and wild plants, being the only source of plant material, are being over exploited to meet the needs of local people. The commercial potential of the plant as a pleasant tasting, health giving tea has been noted by some authors (Roberts, 1990; Van Wyk & Gericke, 2000; McGaw, Steenkamp & Eloff, 2007). Commercial propagation of bush tea will help to protect wild plants by providing alternative sources of plant material.

In this chapter, background will be given on the taxonomy, morphology, and distribution of bush tea. The medicinal and commercial potential of bush tea and other plants of the Asteraceae family will be discussed briefly. Different stages of micropropagation and some general advantages and disadvantages of tissue culture will be discussed.

1.1 Taxonomy

The genus name *Athrixia* is derived from the Greek word 'Ather' meaning an awn. This refers to the awn-like apices of the involucral bracts that are characteristic of *Athrixia* species (Letty *et al.*, 1962; Fabian & Germishuizen, 1982). *A. phylicoides* belongs to the Asteraceae family. The family Asteraceae (previously known as Compositeae) consists of 920 genera, which includes between 19 and 20 000 species. Leaves of Asteraceae are mostly alternate. The flowers are mostly small to tiny and are arranged in a closed head on a common receptacle. A typical inflorescence has a central receptacle subtended by bracts, with an outer ring of ray flowers and an inner ring of disk flowers (Figure 1.1).

A. phylicoides is one of ten *Athrixia* species that can be found in southern Africa and one of 25 species found throughout the world (Letty *et al.*, 1962). Other southern African species include *A. angustissima*, *A. arachnoidea*, *A. capensis*, *A. crinata*, *A. elata*, *A. fontana*, *A. gerrardii*, *A. heterophylla* and *A. pinifolia* (Arnold & De Wet, 1993).

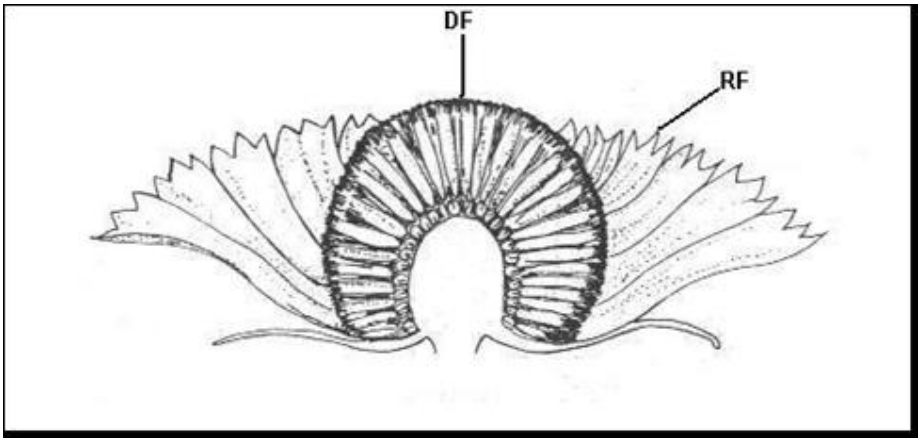

Figure 1.1: A longitudinal section through a typical composite inflorescence with the central receptacle subtended by bracts (the involucre) and with an outer ring of ray flowers (RF) and the inner cluster of disk flowers (DF) (Adapted from Baumghardt, 1998)

1.2 Morphology

Athrixia phylicoides is a dark green, branched, flowering shrub that grows to a height of between 50 and 100 cm (Roberts, 1990; Carruthers, 2000). The small, pointed leaves are dark green above and silvery grey below. The abaxial surfaces of leaves are densely covered with light coloured trichomes, giving it a felted appearance. Dark green leaves with woolly white abaxial surfaces are characteristic to most *Athrixia* species (Letty *et al.*, 1962). Younger leaves are lighter in colour and have hairs on the adaxial surfaces. The inflorescence is about 15 mm in diameter with mauve to light purple ray flowers on the outside and bright yellow disk flowers in the centre (Figure 1.2).

Literature on the time of flowering in southern Africa is inconsistent. According to Carruthers (2000) flowering season is from May to November (autumn to summer). Retief & Herman (1997) states that flowering takes place between December and September (summer to spring). A difference in flowering time between coastal and more inland regions may explain these contradictions. According to Roberts (1990), flowering time in coastal areas is from May to July, while flowering more inland occurs mid- to late summer. The stock plants kept in our greenhouse, situated in Pretoria in the Gauteng Province of South Africa, flowered from the beginning of June to late July (mid-winter). This coincides with flowering time of plants in coastal areas, even though Pretoria is situated inland. The unusual flowering time of our stock plants is likely to be a result of the altered climatic conditions inside the greenhouse. The aboveground parts of our stock plants died shortly after flowering and new growth commenced in early spring (September).

Figure 1.2: *Athrixia phylicoides* in flower, showing yellow disk flowers (DF), purple ray flowers (RF) and hairy leaves (L)

1.3 Distribution and habitat

The distribution of *Athrixia* species worldwide is largely limited to the southern hemisphere including Australia, Madagascar and Africa (Letty *et al.*, 1962). In southern Africa, *A. phylicoides* is widely distributed in South Africa, Zimbabwe, and Lesotho but is not found in Botswana and Namibia (Arnold & De Wet, 1993). In South Africa, it is especially common in the eastern parts of the country (Roberts, 1990). *A. phylicoides* is the most common and widely distributed of all the *Athrixia* species in southern Africa (Letty *et al.*, 1962). *Athrixia* is distributed over a wide range of climatic regions including woodlands, grasslands, semi-deserts and savannah wetlands. *A. phylicoides* prefers full sun and well-drained soil (Roberts, 1990). However, it is also found in areas of partial shade (Onderstall, 1984) and is often found in rocky areas with dense vegetation (Onderstall, 1984; Carruthers, 2000).

1.4 Traditional healing and medicinal value of Asteraceae in southern Africa

Traditional healing plays an important role throughout Africa. In 1995 it was estimated that between 12 and 15 million people in Africa use herbal remedies. These herbal remedies are derived from more than 700 indigenous plant species (Afolayan & Meyer, 1995). Later, in 2001, it was estimated that the use of herbal medicine is part of everyday life to almost 80% of people living in Africa (Kambizi & Afolayan, 2001). There is a worldwide increase in the awareness and use of bio-medicine (Caldis, Mcleod & Smith, 2001). In many cases the high

cost of conventional medicine (Salie, Eagles & Leng, 1996) and the limited access to medical services (Kambizi & Afolayan, 2001) necessitates the use of herbal medicine.

Species of the Asteraceae family have been used in folk medicine in many parts of the world with southern Africa being no exception (Swanepoel, 1997; Van Wyk *et al.*, 1997). Most traditional medicines used by indigenous people belong to the Asteraceae family (Salie *et al.*, 1996). In the Eastern Cape, Asteraceae is amongst the three families most often used as medicinal plants (Grieson & Afolayan, 1999) and in the Western Cape most of the plants used for their medicinal properties belong to this family (Salie *et al.,* 1996).

Despite the fact that many people depend primarily on traditional medicine for health care, knowledge on traditional healing is poorly documented. In most cases information is simply passed on, from one generation to the next, by word of mouth (Van Wyk *et al.*, 1997; Kambizi & Afolayan, 2001). With social changes in rural communities, the preservation of this knowledge is in danger. There is an urgent need for accurate documentation of the valuable knowledge and experience of traditional healers.

A further crisis we are facing with the use of conventional medicine is increased resistance of many disease-causing organisms such as *Plasmodium falciparum* (causal organism of malaria) and *Mycobacterium tuberculosis* (causal organism of tuberculosis) to the antibiotics currently used (Salie *et al.*, 1996). Screening of some traditional medicinal plants of the Asteraceae family has proved to be of great value in the development of new medicines. Extracts of *Artemisia annua* and *Bidens pilosa* (Asteraceae) have been effective in the treatment of malaria (Brandâo, Krettli, Soares, Nery & Marinuzzi; 1997; Mueller, Karhagomba, Hirt & Wemakor, 2000; Tan, Dimo & Dongo, 2000) and extracts of *Helichrysum melanacme* showed activity against *M. tuberculosis* (Lall & Meyer, 1999).

1.5 Economical value of *Athrixia phylicoides*

Athrixia phylicoides is used for a wide variety of conditions including the treatment of sores and boils, coughs and colds and throat infections, blood purification and use as a purgative and aphrodisiac (Roberts, 1990; Swanepoel, 1997; Van Wyk & Gericke, 2000). Different preparation methods and plant parts are used. Zulu people use a root decoction for treating coughs (Hutching, Scott, Lewis & Cunningham, 1996), while Xhosa people chew the leaves for cough relief and sore throat (Roberts, 1990). A wash is often used for the treatment of wounds, sore feet, sores and boils and acne and a tea made from the leaves is used as a blood purifier and for the treatment of cold symptoms (Roberts, 1990; Swanepoel, 1997).

Pharmacological screening of plant extracts has shown anti-inflammatory, anti-hypertensive (lowering of blood pressure), narcotic and analgesic (pain relief) activity (Swanepoel; 1997). A positive correlation between the pharmacological activity and the traditional medicinal use for treating of wounds, sore throats and cold symptoms can thus be made. Traditional use for the treatment of hypertension is not documented and the value of the plant in this regard may still be undiscovered.

Given the problems we face with drug-resistance, high cost and limited access to medicine, herbal medicine is becoming increasingly popular as an alternative form of health care (Rabe & Van Staden, 1997; Mueller *et al.*, 2000). Furthermore, there is a new tendency towards a more holistic approach to health and an increase in health consciousness amongst western people. In South Africa, an approach of 'sickness prevention by healthy living' is being promoted by the government and medical aid schemes.

Commercialisation of *A. phylicoides* as a health tea has great economic potential. The tea made from the leaves is pleasant tasting (Roberts, 1990). Recent chemical analyses of aqueous extracts made from leaves indicated that bush tea is high in antioxidants and is caffeine free (McGaw, Steenkamp & Eloff, 2007). Stressful and busy lifestyles are becoming a way of life to many people. Increased stress levels and insufficient physical exercise are often associated with 'lifestyle diseases'' such as hypertension, sleeplessness and headaches (Berkow, Fletcher & Beers, 1992). The narcotic, anti-hypertensive and analgesic properties of *A. phylicoides* (Swanepoel, 1997), combined with the pleasant taste of the leaf extract, without doubt, have potential for commercialisation.

1.6 History and applications of tissue culture propagation

Plant tissue culture is an alternative propagation method that is often used for commercial production of ornamentals and medicinal plants. In this study we investigated the possibilities of using this propagation method for the commercial scale production of bush tea. In the following section some general background on tissue culture, the five stages of tissue culture, and some advantages and disadvantages of using this technique for the production of bush tea will be discussed.

One of the earliest applications of tissue culture techniques was the use of meristem tip culture for the eradication of viruses on economically important crops. Its use for the propagation of ornamental plants and for industrial scale production of secondary metabolites used as pharmaceuticals, flavours and fragrances and essential oils started in the 1950's (Razdan, 1993). Meristem tip culture as means of vegetative propagation was first introduced in 1960 by Morrel (1960).

Tissue culture can be defined as the cultivation of small plants, parts of plants or plant organs, aseptically on artificial nutrient media (George, 1993). Small plants or pieces of plant material are used in this propagation method and the initial products are small plantlets. For this reason tissue culture propagation is also referred to as micropropagation. It is a laboratory method of propagation performed under sterile conditions where environmental conditions can be fully controlled (Wu, 2001).

Debergh & Read (1991) defines micropropagation as the true-to-type propagation of a selected genotype. According to Bhojwani (1990) the purpose of tissue culture is to produce carbon copies of original unique plants using pre-existing meristems. The term *in vitro* literally means 'in glass'. The term originated in the early days of micropropagation when glass containers were almost exclusively used for keeping cultures (Pierik, 1987).

1.6.1 Five steps of tissue culture

In 1974, a three-stage protocol for the micropropagation of plants was proposed by Murashige (1974). After many years of development in this field it is now agreed that successful micropropagation consists of five steps. These steps are applicable to plants in general and include: 1) mother plant selection and preparation, 2) establishment of aseptic cultures, 3) proliferation or multiplication, 4) production of self-supporting plants and 5) reestablishment in an external environment (Debergh & Read, 1991; George, 1993).

a) Preparation – Stage 0

Preparation for micropropagation starts with the selection of desirable stock material. The criteria for the selection of mother plants could be based on phenotypic or genotypic characteristics, depending on the nature and use of the propagated species. In the case of *A. phylicoides*, the quantity and quality of secondary metabolites produced should be considered when selecting stock plants. Chemical analysis that involve detailed screening of essential oils, and identification of active compounds will have to precede such a selection process. However, this was beyond the scope of this study.

The next phase of the preparative stage aims at improving hygiene. Stock material should be free from visible disease symptoms. If necessary, chemical control of pests could be performed at this stage and overhead irrigation should be avoided to prevent the development of diseases (George, 1993; Hartmann, Kester, Davies & Geneve, 1997). The hairy leaves on bush tea collected from the field necessitated pruning and removal of insect infested material and treatment of stock plants with systemic fungicides and insecticides.

Because stock material is kept in a greenhouse, the environmental conditions can be manipulated and fertilisers and growth regulators can be applied. This theoretically ensures the availability of stock material throughout the year (Debergh & Read, 1991). Our greenhouse facilities did not allow full manipulation of environmental conditions. The stock plants entered a resting phase after flowering which made year round production impossible.

b) Establishment of aseptic cultures – Stage I

The aim of Stage I is to establish plant material on a nutrient medium, free from microbial contamination. Plants grown in the field or in a greenhouse often carry fungal or bacterial contamination. Sterilising agents are used to clean the surfaces of plant material before it is introduced to growth medium. Stage I can be deemed successful if, after a short period of incubation, no signs of microbial contamination are visible, an adequate number of explants have survived and some sort of growth (e.g. shoot tip growth or callus formation) has occurred (Debergh & Read, 1991; George, 1993).

c) Proliferation / multiplication – Stage II

This stage is aimed at the multiplication of plant material. Growth is required to the extent where subculturing is possible. New plant-outgrowths (propagules) are separated from the initial culture and transferred to a new medium (Hartmann *et al.*, 1997). Each of these propagules should have the ability to give rise to a new plant. Propagules used for subculture include axillary or adventitious shoots, somatic embryos and storage or propagative organs (George, 1993). New growth is often achieved on a medium containing cytokinins.

d) Production of self supporting plantlets – Stage III

After multiplication it is essential that individual plantlets become self-supporting. At the end of Stage III, plantlets should be capable of photosynthesising and survive without a supply of artificial carbohydrates. This stage includes *in vitro* or *ex vitro* rooting of plantlets. Some species spontaneously form adventitious roots during Stage III, but usually specific measures should be adopted to induce rooting (George, 1993). Rooting is often induced by transferring plantlets to a medium that contains auxins but no cytokinins (Hartmann *et al.*, 1997). In some cases rooting will only follow after sufficient elongation (Ben - Jaacov & Langhans, 1972; George, 1993; Hartmann, 1997).

e) Reestablishment in external environment – Stage IV

Plants grown under *in vitro* conditions are poorly adapted to the external environment. Roots formed under *in vitro* conditions are not functional in soil medium. Due to the high relative humidity in culture vessels, stomata are often under-developed or non-functional. As a result, plantlets usually lack the ability to control water loss through their leaves (Smith, Roberts &

Mottly, 1990). In a growth room the temperatures are kept within a narrow range and light intensity is usually kept at a constant level. In an *ex vitro* environment, plants are exposed to a septic environment and are therefore subjected to stress from pathological organisms (Preece & Sutter, 1991). Also, *in vitro* grown plantlets need to be gradually adapted to greenhouse or external conditions in terms of lower humidity, higher light intensity and more extreme temperatures to insure an adequate survival rate (Roberts & Smith, 1990). To achieve this, plantlets are often transferred to a mist bed where the temperatures, humidity and light intensity are controlled and where new functional roots can be formed.

1.6.2 Advantages of tissue culture

There are many advantages to the use of micropropagation as opposed to conventional propagation methods.

a) Mass propagation

Micropropagation is often associated with mass propagation at a competitive price (Debergh & Read, 1991). Very little plant material and very little space are needed to greatly increase plant numbers (Giusti, Vitti, Fiocchetti, Colla, Saccardo & Tucci, 2002). Prasad, Sharma & Chaturvedi (1983) and Rout & Das (1997) estimated that nine million plants could be produced from a single *Chrysanthemum* stock plant in a year, using tissue culture techniques. Theoretically, plants can be multiplied at an exponential rate by consecutive multiplications (Hartmann *et al.*, 1997). It is therefore of great value where high volumes are required (Wu, 2001).

Secondary metabolites are usually produced in relatively small quantities per biomass of plant material therefore a large mass of plant material would be required for pharmaceutical use. Since natural growing plants (which are already under great pressure) will initially serve as the main source of plant material, it is crucial that plant material should be used sparingly. Once a protocol has been established for the micropropagation of *A. phylicoides*, it will be possible to provide commercial growers with large quantities of plant material at a reasonably low cost.

b) Disease-free plant material

Micropropagation plays an important role in obtaining pathogen free plant material, even from previously contaminated material (Kaul, Miller, Hutchinson & Richards, 1990; May & Trigiano, 1990). It provides a system where fungal and bacterial pathogens and even systemic viruses could be eradicated. The technique has also been used to rid plants of nematodes such as *Meloidogynae* and *Radopholus* spp. (Bhojwani, 1990).

The elimination of viruses from contaminated plant material is one of the most valuable aspects of micropropagation in the production of *Dendranthema grandiflora* (Larson, 1992). It is commercially used to supply virus-free plant material of orchid, chrysanthemums and carnations and could certainly be used, should it be necessary, to obtain virus free bush tea. The use of virus free plant material is one of the most effective ways in which to control diseases caused by viruses (Agrios, 1997).

Apart from eliminating pathogens, the system ensures that plants that are freed from contamination do not get re-infected until they are delivered to users (Hartmann *et al.*, 1997). This makes micropropagation a handy tool in the export and quarantine control of plant material (Giusti *et. al*., 2002) Commercial producers of *A. phylicoides* worldwide can benefit from this.

By eliminating pathogenic diseases from bush tea plants during initial *in vitro* propagation, growers can be supplied with healthy plants for their fields. The amount of chemicals used will be minimised, thereby ensuring a safer end product for consumers. Plant extracts made from disease free plant material is also safer for use for pharmaceutical purposes, since the risk of accidental inclusion of toxins (produced by some micro-organisms) in the extract is minimised.

c) Manipulation of environmental and growth factors

Under *in vitro* conditions, close manipulation of growth conditions is possible. These include environmental conditions such as light, temperature and gas composition, as well as the composition of the culture medium, including the types and amounts of plant growth regulators, mineral salts and carbon sources used. The ability to control the growth environments of plants is a valuable tool in both research and commercial propagation of plants.

By optimising the above mentioned growth conditions, higher than normal regeneration rates can be achieved (Rout, Samantary & Das, 2000) and the accumulation of secondary metabolites can be increased (Stafford, Morris & Fowler, 1986). According to Pierik (1987) cells in culture reach maturity faster and are able to produce secondary metabolites sooner. Micropropagation is not subjected to seasonal changes, allowing plant production throughout the year (Hartmann *et al.*, 1997) The continuous yield of secondary metabolites from micropropagated plants is therefore theoretically possible (Razdan, 1993). These factors combined makes micropropagation of many plant species that produce useful or high value secondary metabolites commercially viable.

We observed that leaves of *A. phylicoides* grown in the greenhouse became more fragrant during its flowering time (winter), indicating a likely seasonal change in the plant's production of essential oils. Our observation was confirmed by a study on the seasonal variation in the polyphenolic content of bush tea leaves, indicating that the highest polyphenol content occurred in winter (Mudau *et al.*, 2006).

By properly managing the *in vitro* growth conditions of *A. phylicoides*, we can theoretically achieve higher regeneration rates (Rout, Samantary & Das, 2000), produce cells that will reach maturity faster and produce secondary metabolites sooner (Pierik, 1987) and in higher quantities (Stafford *et al.*, 1986), and produce medicinally active secondary metabolites year round (Razdan, 1993; Hartmann *et al.*, 1997).

d) Genetic manipulation

Micropropagation provides means by which plants can be genetically manipulated through biotechnology (Hartmann *et al.*, 1997). Transgenic plants with new desirable properties can be developed and marketed. The use of strains of *Agrobacterium* to mediate genetic transformation of chrysanthemum has been investigated (De Jong, Rademaker & Wordragen, 1993; Pavingerova *et al.*, 1994). More research on the medicinally active ingredients of *A. phylicoides* will determine the need to alter this plant genetically in the future.

e) Time and space requirement

Better control over growth factors together with a prolonged growth season result in a markedly higher propagation rate compared to conventional vegetative propagation. Urban, Sherman, Moyer & Daub (1994) developed a protocol for the high frequency regeneration of three commercial cultivars of *Crysanthemum morifolium* by optimising the concentrations of growth regulators in the culture medium. Since culture vessels take up very little space, large numbers of plants can be propagated in a small area.

1.6.3 Disadvantages of tissue culture

As with most propagation methods, there are some disadvantages to micropropagation which should be considered when choosing the most suited method of propagation. The success of commercialising *A. phylicoides*, its effectiveness as a medicinal plant and the characteristics of micropropagated plants will determine whether or not tissue culture is a suited method for propagation.

a) Skill, facilities and cost

A high level of skill is required to establish cultures and precision is essential. Micropropagation necessitates the use of specialised, expensive laboratory facilities. A high level of sanitation is essential during propagation. In practice, cost is the key factor when deciding on an appropriate method of propagation (Bhojwani, 1990). Only the propagation of crops with market values significantly higher than production costs, justify the use of this propagation method. Medicinal importance will play a determining role in the case of *A. phylicoides.*

b) Genetic instability

Even though clonal multiplication is possible with micropropagation, the incessant use of this technique increases the occurrence of genetic abnormalities in plants (George, 1993). The use of callus cells for propagation might result in somaclonal variation in the offspring (Giusti *et al.*, 2002; Teixeira da Silva, 2003) The genetic uniformity of plants produced in tissue culture depends on a number of factors, the two most important being the method of induced multiplication and, to a lesser extent, the genotype (Rout *et al*. 2000). We will only be able to exclude the potential problem of genetic instability after propagating *A. phylicoides* in culture over a long period.

c) Adaptation period is required

A considerable number of micropropagated plants do not survive after being transplanted to *ex vitro* environments (Preece & Sutter, 1991). Having grown under conditions of a high relative humidity, easily accessible nutritional sources and regulated light, newly formed plantlets are highly susceptible to water loss and are not autotrophic (George, 1996). A transitional period during which plants can gradually adjust to an external environment is essential for their survival out of culture.

Plants cultured in water saturated atmospheres have been reported to be unable to control water loss, resulting in severe losses due to wilting (see 6.5). This can partly be attributed to the fact that plants cultured at 100% relative humidity (RH) lack a clearly defined cuticle compared to plants grown at 94% RH (Smith *et al.*, 1990).

d) Atypical characteristics

Plants derived from *in vitro* cultures frequently show atypical characteristics such as rejuvenation, stunted growth, dormancy, altered photosynthesis capacity, accelerated flowering and increased branching (Ziv, 1986; George, 1996). Some of these characteristics may be valuable to breeders, but in most cases it is considered a disadvantage (Bhojwani, 1990). Changes in the production of secondary metabolites have also been reported in many

plant species, including medicinal plants of the Asteraceae family such as *Artemisia alba* and *Chrysanthellum americanum* (Lamproye, Hofinger, Ramaut & Gaspar, 1986; Mascarenhas, Khuspe, Nadguada, Gupta & Khan, 1988; Gaspar, 1994; Srividya, Sridevi & Satyanarayana, 1998). Some of these abnormalities are short-lived and diminish over time while others are more permanent.

1.7 Aim of this study

The motivation for this study was firstly to develop a protocol for the *in vitro* propagation of *A. phylicoides* for commercial scale production. To our knowledge, no work has been done on the *in vitro* propagation of bush tea or any other *Athrixia* spp. Commercial propagation will alleviate stress caused by harvesting of natural plant resources. Propagated plant material can be sold for traditional medicinal use and also be introduced to a promising new market as a health tea. It can also provide the pharmaceutical industry with plant material for the development of natural, herbal medicine for which there is an increasing demand.

The second objective of this study was to investigate plant structure on a microscopic level. A study of the ultrastructure of *A. phylicoides* will, to our knowledge, be the first. Structures possibly involved in the production of aromatic oils, and thus related to the medicinal value of the plant will be identified.

Thirdly, the commercial viability of using micropropagation for medicinal purposes will be assessed preliminarily. There is a widespread concern regarding off-type plants propagated by tissue culture (Swartz, 1991). It is often stated that medicinal plants lose their medicinal properties during *in vitro* propagation. A microscopic comparison between *in vitro* propagated and greenhouse grown plants will point out any structural changes in micropropagated plants. Changes in the morphology of plants could predict alterations in the secondary metabolism and therefore affect the medicinal value of micropropagated plants.

CHAPTER 2

ESTABLISHMENT OF ASEPTIC CULTURES

2.1 Scope of this chapter

The aim of our research was to develop a protocol to establish aseptic nodal cultures of bush tea. Firstly, we investigated the role of leaf blades as a source of contamination. Secondly, stock plants were treated with fungicide to determine the effect on fungal contamination later on in culture. Thirdly, the difference between two bactericides, NaOCl and $Ca(OCl)_2$, used as part of the surface sterilisation procedure, was investigated.

2.2 Literature review

Material from plants that were grown in a greenhouse and especially material collected from the field will invariably carry contaminants such as fungi, yeasts, bacteria and viruses, both on the surface and internally (George, 1993; Hartmann, Kester, Davies & Geneve, 1997; Rout, Samantary & Das, 2000). Following the preparative stage (see 1.6.1a), the second stage (Stage I) of successful micropropagation is to establish cultures that are free from disease and contaminants. To achieve this, a sterilisation method that will effectively rid plant material of contaminants needs to be in place. Pierik (1987) and Cassells (1991) identified four main sources of infection that should be taken into account when designing a protocol for a sterilisation procedure: the plant (internal and external), the nutrient medium, the air and the research worker.

The nutrient media as potential source of infection can largely be eliminated by autoclaving media prior to use. Autoclaving is an effective way to minimise contamination via the medium and is done, almost without exception as part of proper tissue culture practice. In the *in vitro* establishment of medicinal herbs such as *Piqueria trinervia* (Saad, Diaz, Chávez, Reyes-Chilpa, Rubluo & Jiménez-Estrada, 2000), *Origanum vulgare* (Strycharz & Shetty, 2002) and *Salvia officinalis* (Avato, Fortunato, Ruta & D'Elia, 2005), nutrient medium was autoclaved at 121ºC and 100 kPa for 20 min prior to use. Contamination via air is normally prevented by working under aseptic conditions e.g. inside a laminar flow cabinet. Contact with the research worker is limited by using alcohol-sterilised and flamed instruments to handle plant material (Debergh & Read, 1991; George, 1993; Hartmann *et al.*, 1997). This leaves us with the challenge of eliminating contaminants from plant material as the last potential source of infection.

Careful selection of stock plants is an essential part of establishing healthy growing, disease free cultures. Stock plant with desirable characteristics such as vigorous growth and a history of no disease infestation should be used to establish cultures (George, 1993; Hartmann *et al.*, 1997). Where other specific morphological and physiological characteristics are required, these should be taken into account upon selection of stock plants. Avato *et al.* (2005) selected stock plants with a shrubby growth habit, vigorous and upright stems and large, thick and strongly scented leaves for establishing cultures of *Salvia officinalis* (a herbaceous perennial, cultivated for essential oil, both for pharmaceutical and food industries). Measures to reduce the level of contamination on and inside plant material should already be applied to stock material during Stage 0 (see 1.6.1a). Nalawade & Tsay (2004) emphasise the importance of keeping stock plants used for the initiation of *in vitro* cultures of various medicinal plants, in a clean controlled environment.

Choice of explants can also influence levels of contamination. A correlation exists between the size of an explant and the degree of contamination; the smaller the plant, the smaller the incidence of contamination, but the smaller the chance of successful establishment (Bhojwani, 1990). Internal structures such as meristems of buds and the inside of seeds are generally considered to be free from infection. However, in humid conditions mycelia is likely to invade plant interiors (Hartmann *et al.*, 1997). Avato *et al.* (2005) used apical and axillary buds to establish cultures of *Salvia officinalis* L (common sage) and reported contamination levels ranging between 0 – 25% on different culture media. Jain, Kanita & Kothari (2001) established *Dianthus caryophyllus* L. (Asteraceae) cultures from leaf explants. Other explants sources that have been successfully used to establish cultures of various medicinal herbs, in order of popularity, include stem tips, nodal segments, axillary meristems, apical meristems and zygotic embryos (Rout *et al.*, 2000).

Nutrient rich and humid conditions in culture vessels are beneficial to the growth and development of micro-organisms. Bacteria and fungi grow readily on medium containing organic compounds such as sugar, vitamins and amino acids (George, 1993). Removal of potential inocula from the material to be used for establishing cultures is therefore essential prior to propagation. The duration and nature of sterilisation required will vary with plants and the degree of contamination. Woody plants are often sterilised more harshly while herbaceous plants require gentle sterilisation. Furthermore, the levels of contamination vary with seasons. In temperate regions contamination is worse in spring and late summer (George, 1993). This implies that the sterilisation procedure followed may require modification to accommodate the seasonal changes.

It is important that the sterilisation procedure should not affect the plant's vigour. The ideal surface sterilants should be non-toxic to humans and plants, yet effective for the control of micro-organisms (George, 1993; Hartmann *et al.*, 1997). Another important factor that should be taken into account when choosing sterilants, especially in commercial propagation, is the cost of chemicals (George, 1993).

Surface bacteria and certain fungi can be effectively eradicated by washing plant material in appropriate sterilising agents for 10 – 25 minutes, followed by several rinses in sterile water. Generally, alcohols and hypochlorite ions are used as bactericides. Amongst the most frequently used surface sterilants are sodium hypochlorite (NaOCl) and calcium hypochlorite ($Ca(OCl)_2$), ethanol (C_2H_4OH) and mercuric chloride ($HgCl_2$) at concentrations of 5-10% (hypochlorites), 50-95% (alcohols) and 0.01 – 0.1% (mercuric chloride) respectively (Pierik, 1987; George, 1993; Rout, Samantary & Das, 2000). The sterilisation procedures and chemicals that have been used to surface sterilise plant material of some medicinal plants and some species of the Asteraceae family, prior to establishment, is summarised in Table 2.1. In most cases, plant material is first rinsed in tap water, followed by surface sterilisation with 70% ethanol in combination with either 0.1% mercuric chloride or sodium hypochlorite (2% and 15% respectively), followed again by several rinses in sterile water.

Internal infections are difficult to detect since they are often only expressed after a period of time in culture (Rout *et al.*, 2000). Development of endogenous infections are often triggered when explants are transferred to a richer medium (Debergh & Read, 1991) or become apparent when the site of infection is brought into contact with the growth medium (Pierik, 1987) during subculturing. The absence of disease symptoms and visible growth of contaminants on media is therefore not a guarantee that plants are free from contamination (Bhojwani, 1990). Eradication of internal infections is problematic and screening of stock plants for persistent fungal or bacterial contamination is essential in controlling these diseases.

Table 2.1 Surface sterilisation procedures followed in the establishments of cultures of some medicinal herbs and members of the Asteraceae family

Plant species	Plant description	Explant source	Sterilisation procedure summarised	Reference
Clitoria ternatea	Fabaceae Medicinal plant Perennial climber	Leaves	1. Wash in 2% (v/v) Teepol detergent solution. 2. Sterilise in 0.1% (w/v) aqueous $HgCl_2$ solution for 15 min. 3. Rinse 4-5 times in sterile distilled water.	Rout (2005)
Salvia ofiincialis	Medicinal herb Culinary use	Axillary buds Apical buds	4. Dip in 70% (v/v) ethanol for 5 min. 5. Sterilise in 15% NaOCl solution (4.9% active chlorine) for 20 min. 6. Rinse in sterile water.	Avato *et al*. (2005)
Dianthus caryophyllus	Asteraceae Ornamental flower	Leaves	1. Sterilise in 0.1% (w/v) $HgCl_2$ solution for 5 min. 2. Rinse in sterile distilled water x 3.	Jain *et al*. (2001)
Clitoria ternatea Linn.	Lamiaceae Perennial herb Medicinal plant Culinary use	Seeds	1. Wash with tap water. 2. Sterilise in 2% NaOCl solution and 0.1% Tween-20 for 15 min. 3. Dip in 70% ethanol for 30 sec. 4. Wash in distilled water for 10 min x 3.	Arikat *et al*. (2004)
Eclipta alba	Asteraceae Annual herb Medicinal plant	Cotyledonary Nodes	1. Wash under tap water for 30 min. 2. Treat with 2% (v/v) Teepol and 70% (v/v) ethanol for 15 sec. 3. Wash 3-5 times with sterilised distilled water. 4. Surface-disinfected with 0.1% (w/v) aqueous $HgCl_2$ for 5–6 min. 5. Rinse with autoclaved distilled water (five to seven changes).	Baskaran & Jayabalan (2005)

2.3 Origin of plant material

Plant material was collected from a field in the Thohoyandou district of Venda in the Limpopo Province, South Africa. The use of field grown plants to establish *in vitro* cultures is not ideal, both because of the strain it puts on limited natural resources, and because of high levels of contamination on field plants (George, 1993; Hartmann, 1997; Rout *et al.*, 2000). However, bush tea is not available commercially and naturally growing plants were our only resource.

Healthy looking shoots with leaves were harvested from field plants. Plant material was wrapped in wet newspaper, and kept moist during transport back to Pretoria, where trials were carried out. A soil sample was taken at the site of collection and sent for analyses. Field collected plants were multiplied by making cuttings of approximately 10 cm in length, with 6-8 nodes each, then diped into Seradix 2™ (3 g/kg indole-3-butyric acid) and planting them in sand, onto a mist bed in a greenhouse. Cuttings on the mist bed received water by mist irrigation at a frequency of 7 sec every two minutes. The greenhouse roof and walls was constructed with 6 mm semi-transparent, corrugated fibreglass panels with a 30% shade net fixed below the ventilated roof. There were no lights inside the greenhouse, and the light intensity inside the greenhouse was determined by the natural light from outside, filtered through the 30% shade net and fibreglass panels. The temperature inside the greenhouse was regulated only by natural ventilation and an overhead fogging system. Temperature inside the greenhouse, recorded for the first 2 weeks after planting of cuttings, ranged between 13ºC - 18ºC (minimum) and 20ºC - 26ºC (maximum).

After 3 weeks, approximately 30 rooted cuttings were replanted into 15 dm^3 plastic planting bags filled with a composted bark - ash mixture and moved to a temperature-controlled glasshouse, where minimum temperatures were kept above 10ºC and maximum temperatures below 26ºC, under natural day/night conditions. Plants were watered with tap water regularly.

Trials could only commence 5 months later, once the rooted cuttings had grown sufficiently to supply us with enough plant material. Limited plant material was available throughout our trials because stock plants had to be allowed to re-grow enough plant material after collecting plant material for each trial.

2.4 Surface sterilisation and initiation – preliminary trials

In the *in vitro* establishment of other medicinal herbs, sterilisation procedures normally include rinsing plant material in tap water, followed by surface sterilisation with a combination of 70% ethanol and NaOCl (Table 2.1 and references cited therein). We did some preliminary trials, based on the procedures followed in Table 2.1. In addition to C_2H_4OH, NaOCl and $Ca(OCl)_2$ used by the authors cited in Table 2.1, we included washing of plant material in soapy water, or soaking it in a fungicide solution as part of some trials.

A list of chemicals, the concentrations of chemicals and range of exposure times that we tested are summarised in Table 2.2. All our sterilisation procedure included rinsing of plant material in running tap water (Step 1), followed by submergence in ethanol (Step 2), after which one or more of the chemicals listed in Step 3 were used. In all trials, the final step was to rinse plant material in sterile distilled water as listed in Step 4 (Table 2.2).

In most cases, the sterilisation technique was either insufficient in the control of bacteria, or with longer exposure time to chemicals, or to higher concentration of chemicals, plant tissue turned brown and died after establishment. Hairs on leaves seemed to hinder proper contact with sterilants. The procedure described in 2.7.1a) gave the best results in terms of both contamination control and survival of plants.

Table 2.2. Summary of chemicals, range of concentration and exposure times to chemicals, used in preliminary trials on the surface sterilisation of *Athrixia phylcoides*

Step	Chemical or cleaning medium	Concentration	Time of exposure
1	Soapy water *and / or* Running tap water	2.5 % (v/v) -	5, 10 or 15 min 10 or 15 min
2	Ethanol	60 % or 70%	30, 60 or 90 sec
3	CaOCl *and / or* Na(OCl) *and / or* Fundazol (benomyl)	3 % (w/v) 5, 10 or 15% (v/v) 2% (w/v)	10 min 10, 20 or 30 min 0, 15 or 30 min
4	Sterile water		5 or 10 min x 3 rinses

In most of our preliminary trials, cultures were divided and incubated at both 26ºC and 30ºC and at the same radiation levels, to determine the most suitable incubation temperature. Cultures kept at 26ºC seemed more wilted that those kept at 30ºC when visually compared. The long term effect of the different temperatures could not be determined since the rate and time of survival in these preliminary trials were low.

2.5 Medium composition

We used ½ MS medium, supplemented with 3% sucrose and solidified with 3 g/L Gelrite™ as the basic medium in all our trials. Growth regulators were added, or other modifications (such as lowering of sucrose concentration) were made, according to the requirements for individual trials.

Murashige & Skoog medium (MS) (Murashige & Skoog, 1962) is the most commonly used culture media in the establishment of various medicinal plants (Rout *et al.*, 2000). It was also used to establish the medicinal herbs *Piqueria trinervia* (Saad *et al.*, 2000) and *Scrophularia yoshimurae* (Lai, Lin, Nalawade, Fang & Tsay, 2005) and other members of the Asteraceae family such as *Dianthus caryophyllus* (Jain *et al.*, 2001).

Gelrite™ is a popular gelling agent used in the establishment of various cultures (Babbar, Jain & Walia, 2005) and occasionally in the establishment of medicinal plants (Rout *et al.*, 2000). The carbohydrate requirement in culture media is usually met by the incorporation of 2–3% sucrose or, less frequently, by glucose (Rout *et al.*, 2000).

Concentrations of either 2% or 3% sucrose were also used to establish cultures of medicinal herbs such as *Salvia officinalis* (Avato *et al.*, 2005) *Scrophularia yoshimurae* (Lai *et al.*, 2005), *Origanum vulgare* (Strycharz & Shetty, 2002) and other members of the Asteraceae family such as *Piqueria trinervia* (Saad *et al.*, 2000) and *Dianthus caryophyllus* (Piqueras, Cortina, Serena & Casas, 2002).

2.6 Incubation conditions

Most *in vitro* cultured medicinal plants, as reviewed by Rout *et al.* (2000), were incubated at approximately 25ºC, and tropical species at 27ºC - 30ºC. Light is usually provided by cool white fluorescent tubes and at light intensities between 35 µmol/m^2/sec – 112 µmol/m^2/sec. The photoperiod ranges between 12h and 16h per day (Puente & Marh, 1997; Rout *et al*. 2000; Baskaran & Jayabalan 2005; Lai *et al*., 2005). We incubated the majority of our cultures at 30ºC, under cool white fluorescent light, providing 60 µmol/m^2/sec.

2.7 Removal of leaves prior to surface sterilisation

Our preliminary trials indicated that trichomes on the leaves of *A. phylicoides* prevent proper contact between sterilants and leaf surfaces, making them hard to disinfect (see 2.4). Some measures to improve contact between surface sterilants and the hard-to-reach surfaces had already been implemented in our sterilisation procedures. These include submerging plant material in ethanol for a few seconds and the use of magnetic stirrers during the submerging period (Pierik, 1987). Apart from being germicidal, submerging plant material in ethanol also improves contact with sterilants by dissolving the waxy layer on plant surfaces and by eliminating air bubbles. This allows for better penetration of plant material by successive sterilants (Pierik, 1987; George, 1993).

In spite of efforts to improve contact between leaf surfaces and sterilants, the hairy leaves of bush tea remained a source of contamination. In our preliminary trials, leaves were only removed from plant material after surface sterilisation. It is likely that leaves contributed to the contamination problem by carrying masses of contaminants, trapped between their trichomes into the sterilant solutions. We conducted a preliminary trial to evaluate the presence of leaves during surface sterilisation on the level of contamination in culture.

2.7.1 Materials and Methods

The trial was conducted in the first week of August (late winter). Our stock plants were greenhouse plants grown from field collected cuttings (see 1.1). Shoots that appeared similar in size, physiological stage and vigour, were collected from greenhouse stock plants and cut into segments of approximately 10 cm.

a) Surface sterilisation

The sterilisation method used in this trial was chosen based on the results of preliminary sterilisation trials (see 2.4).

The plant material was rinsed in running tap water for 10 minutes. The 10 cm shoot segments were placed in a large glass beaker and covered with mesh to prevent it from being flushed out of the beaker. Water was poured off and cuttings were placed on sterile blotting paper inside a laminar flow cabinet. All equipment used from this point forward were sterilised, either by flaming it with 100% alcohol (forceps, scalpels and surgical blades) or by autoclaving it prior to use (beakers, distilled water used for dilution, paper towels and blotting paper). The magnetic stirrers used were sprayed with 100% alcohol and wiped with autoclaved paper towels before placing them in the laminar flow cabinet.

Shoot segments were divided into two treatments of 20 replicates each (due to the limited amount of plant material available, the trial consisted of a small number of replicates, see 1.1). In Treatment 1, leaves were abscised at the petiole using a surgical blade, while the leaves in Treatment 2 were kept intact.

Both treatments were then surface sterilised according to the following procedure: Shoot segments were soaked in 70% ethanol for 90 seconds after which they were transferred to a beaker containing 10% (v/v) household bleach (3.5% active NaOCl) on a magnetic stirrer for 10 minutes. The used bleach solution was carefully poured off into a container, taking care that the plant material stayed in the beaker. It was then placed onto several layers of sterile blotting paper from where it was transferred to a beaker containing a large volume of sterile, distilled water on a magnetic stirrer. After 5 minutes, the explants were transferred to another beaker containing sterile distilled water for another 5 minutes. Shoot pieces were removed from the sterile water and placed on several layers of sterile blotting paper to dry off excess water.

b) Initiation

The exposed, water-soaked ends of the shoots segments were removed and shoot segments were cut into 3 node explants (approximately 2.5 cm long), using a surgical blade. Nodal explants were planted vertically onto 10 ml ½ MS medium, supplemented with 3% sucrose and solidified with 3 g/L Gelrite™ (based on the composition of growth media commonly used in the establishment of other medicinal herbs and plants of the Asteraceae family (see 2.5). Medium was poured into 15 cm long test tubes. The growth medium was prepared according to the procedure described in 2.7.1d). Each test tube was closed with a plastic cap and cling wrap was wrapped around the test tube where the cap and test tube joined to seal cultures.

c) Growth medium

Cultures were established in ½ MS medium, supplemented with 3% sucrose and solidified with 3 g/L Gelrite™, containing no growth regulators (see 2.5).

d) Preparation of growth medium

All growth media used throughout our experiments were prepared less than 2 weeks prior to initiation of trials and kept in the dark until needed. Distilled water was boiled and approximately 800 ml was poured into a large beaker on a magnetic stirrer with a heat-plate. The appropriate amounts (specified in ‘Materials and Methods’ of each individual trial) of MS salts, sucrose and growth regulators were mixed with small amounts of water and gradually added to the warm water (in the order listed) and stirred until it was dissolved. Distilled,

boiled water was added to the medium to make up the final volume, after which the pH was adjusted to 6.5 with either KOH or HCl (corresponding to the pH value of the soil sample taken at the site where plants were collected, see 1.1). Medium was solidified with 3 g/L Gelrite™ and autoclaved at 100 kPa for 20 min (Saad *et al.*, 2000; Strycharz & Shetty, 2002; Avato *et al.*, 2005).

e) Incubation conditions

Plants were kept in a growth room at 30°C ± 2 (temperature indicated during our preliminary trials, see 2.4) with 8h dark: 16h light intervals under cool white fluorescent lights providing 60 µmol/m^2/sec photosynthetically active radiation (PAR).

Treatments were compared after 16 days in culture.

2.7.2 Results and Discussion

By removing leaves from plant material prior to surface sterilisation, the level of contamination visible in the growth medium was reduced from 10% to 0% but, removal of leaves resulted in poor growth. No growth was recorded in Treatment 1 (abscised leaves) while explants of Treatment 2 (leaves) showed growth of 0.75 axillary buds per explant on average (Table 2.3). The total removal of leaf blades caused exposure of axillary meristems to chemicals and as a result almost 100% of meristems died. From similar research conducted by Debergh & Read (1991), both a decrease in the level of contamination by removal of leaves and damage of unprotected meristems by exposure to chemicals was reported. In all trials that followed, we abscised leaves only partly, leaving a piece of the leaf blade, approximately 2 mm long, on plants to cover the axillary buds. This effectively reduced contamination and minimised exposure of meristems to chemicals.

Table 2.3: The effect of removing leaves from explants prior to surface sterilisation on contamination and growth of *Athrixia phylcoides* after 16 days in culture

Treatment	Number of buds out of a possible 3, showing growth (mean)	Cultures contaminated (%)
Leaves	0.75 [a]*	10%
Leaves removed	0 [a]	0%

* Values that are not significantly different at 5% probability level are indicated by the same letter

2.8 Treatment of stock plants with fungicide

During our preliminary trials on surface sterilisation (see 2.4), plant material was treated with 2 g/L Benomyl (a fungicide) as part of the surface sterilisation procedure. The sterilisation procedure that we followed was insufficient in the control of fungal contamination and required revising. Adjusting our procedure by exposing plant material to higher concentrations of chemicals, or exposing it to chemicals for longer periods of time, resulted in death of the plant material and a different approach needed to be investigated. The use of chemicals during Stage 0 to reduce the level of contamination is encouraged by many authors such as George (1993) and Hartmann *et al.* (1997) and is considered an essential part of tissue culture. The aim of this trial was to evaluate the effectiveness of a systemic fungicide applied to bush tea stock-plants prior to *in vitro* establishment.

2.8.1 Materials and Methods

Fundazol™ 2 g/L(active ingredient Benomyl) was applied to stock plants using a spray applicator, 3 weeks prior to establishing cultures. Shoots (that appeared similar in size, physiological stage and vigour) were collected from the same stock plant and cut into segments of approximately 10 cm. Shoot segments were subjected to one of two surface sterilisation procedures. Both treatments consisted of 20 replicates and differed only by an additional 10-minute treatment with fungicide in Treatment 1. Sterilisation procedures were as follows: The plant material was rinsed in running tap water for 10 minutes, after which it was moved to a laminar flow cabinet where the rest of the surface sterilisation procedure was continued. All equipment used from this point onwards were sterilised, either by flaming it with alcohol (forceps, scalpels and surgical blades) or autoclaving it prior to use (beakers, distilled water used for dilution and blotting paper).

Shoot segments were divided into two treatments. The initial sterilisation procedure for the two treatments were similar; plant material of both treatments was soaked in 70% ethanol for 90 seconds followed by 30 g/L $Ca(OCl)_2$ for 10 minutes. The used $Ca(OCl)_2$ solution was carefully poured off into a container, taking care that the plant material stayed in the beaker. Shoot segments were shaken onto several layers of sterile blotting paper.

Plant material of Treatment 2 was transferred directly to sterile distilled water and that of Treatment 1 was placed in 2 g/L Fundazol™ for 10 minutes, and again removed by pouring off the chemical solution and shaking segments onto sterile blotting paper, before transferring them to water. In both treatments, shoot segments were rinsed in a large volume of sterile distilled water on a magnetic stirrer. After 5 minutes, the explants were transferred to other beakers containing sterile, distilled water for another 5 minutes. Shoot pieces were

removed from the water and placed on several layers of sterile blotting paper to absorb excess water.

Explants of three nodes each were planted vertically onto 10 ml ½ MS medium supplemented with 3% sucrose and solidified with 3 g/L Gelrite™ in 15 cm glass test tubes according to the procedure described in 2.7.1b). The growth medium was prepared according to the procedure described in 2.7.1d) and both treatments were kept in a growth room at growth conditions described in 2.7.1e).

2.8.2 Results and Discussion

After 21 days in culture, 0% contamination was recorded in both treatments. No fungal or bacterial contamination was visible in the growth medium or on any of the 40 explants (20 per treatment). However, 100% of the cultures had turned brown and died and no growth was recorded. The cause of death of explants was unclear, but fungal or bacterial contamination did not seem to be responsible, since no signs of contaminants were visible on growth the medium. In a follow-up trial on the use of alternative sterilants, the same sterilisation procedure described above was followed for one of the treatments and death of plant material did not occur (see 2.9). This also ruled out the possibility of over exposure to chemicals as cause of death.

Treatment of stock plants with a systemic fungicide prior to the collection of plant material seemed to effectively eliminate fungi from plants and the additional treatment with fungicide during the sterilisation procedure was superfluous and omitted in all the treatments that followed. Treating plants with fungicide during Stage 0 is a convenient alternative and saves time during initiation and reduces the time plant material is exposed to liquids. Minimising exposure of plant material to liquids during sterilisation helps to prevent problems with hyperhydricity, which became a problem later on (see CHAPTER 5).

2.9 The effect of NaOCl versus $Ca(OCl)_2$ as bactericide on contamination and vegetative growth

Preliminary trials indicated that *A. phylicoides* is very sensitive to lengthy exposure to liquid and disinfectants (see 2.4). Exposure resulted in browning of tissue and plants soon became waterlogged. This also contributed to problems with hyperhydricity later on (see CHAPTER 5). It was therefore important to develop a sterilisation procedure where exposure time to liquid and harsh chemicals was kept to a minimum.

Mercuric chloride ($HgCl_2$), NaOCl (sodium hypochlorite) and $Ca(OCl)_2$ (calcium hypochlorite) are amongst the most popular and commonly used disinfectants. $HgCl_2$ is extremely toxic to both plants and animals and is expensive. Since *A. phylicoides* already showed sensitivity in preliminary trials, this chemical was eliminated from the trial. Pierik (1987) suggested the use of $Ca(OCl)_2$ on plants that are sensitive to NaOCl. Both NaOCl and $Ca(OCl)_2$ are affordable, toxic to micro-organisms but relatively non-toxic to plant material (Hartmann *et al.*, 1997).

The bactericidal action of both NaOCl and $Ca(OCl)_2$ can be related to the oxidising capacity of HOCl (hypochlorous acid) and OCl^- ions that form as products when NaOCl and $Ca(OCl)_2$ are mixed with water (
Equation **2-1**). Both HOCl and OCl^- ions have bactericidal properties, with HOCl being the most active (George, 1993; Tchobanoglous, Burton & Stensel, 2003).

Equation 2-1: Hypochlorite reaction in water (adapted from **Tchobanoglous *et al*., 2003**)

$$NaOCl + H_2O \leftrightarrows 2HOCl + Ca(OH)_2 \quad \text{.....[a]}$$
$$Ca(OCl)_{2+} H_2O \leftrightarrows HOCl + NaOH \quad \text{.....[b]}$$
$$HOCl + H_2O \leftrightarrows H^+ + OCl^- \quad \text{.....[c]}$$

The sterilisation efficiency of both NaOCl and $Ca(OCl)_2$ are best in a slightly acidic solution of pH 6-7 (George, 1993). Lower pH values favour the conversion of hypochlorite to HOCl, the more active bactericide, above OCl^- (Figure 2.1). However, pH values lower than 4 would be detrimental to most plants and pH 6-7 is therefore recommended. When surface sterilising plant material, it should be kept in mind that even a slight variation in the pH of the sterilant solution, especially between pH 6.5 and pH 8.5, may lead to differences in sterilisation efficiency (Figure 2.1).

We conducted a trial in which NaOCl and $Ca(OCl)_2$ were evaluated for their bactericidal action and their effect on vegetative growth.

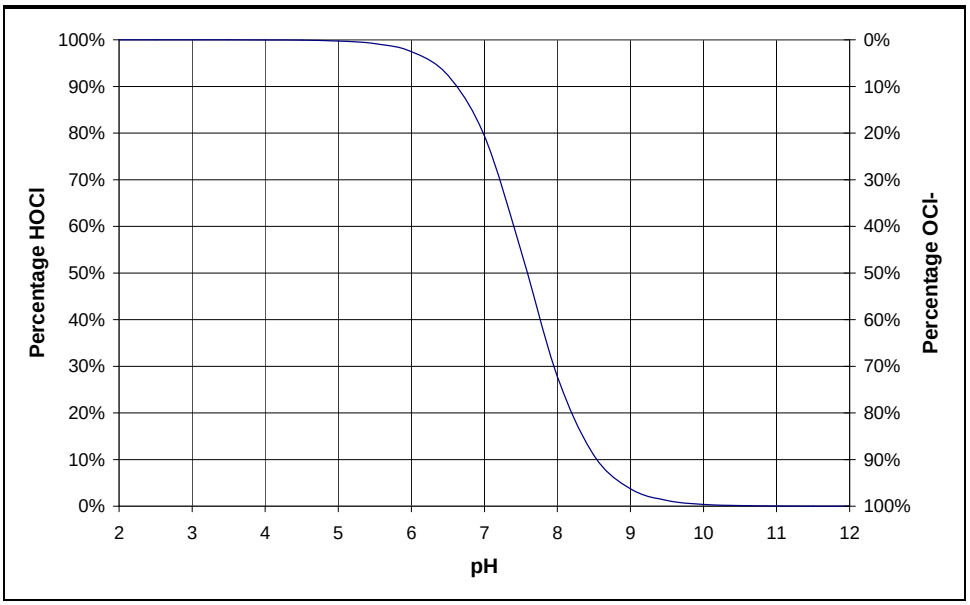

Figure 2.1: Percentage distribution of hypochlorous acid and hypochlorite in water as a function of pH determined at 20ºC (Tchobanoglous et al., 2003)

2.9.1 Materials and Methods

This trial was conducted during the second week of March (late summer). Greenhouse stock plants were sprayed with 2 g/L Fundazol™ (active ingredient Benomyl) 14 days prior to *in vitro* initiation (see 2.8). Shoots (that appeared similar in physiological stage and vigour) of between 30 and 40 cm were collected from different stock plants. Leaf blades were partly abscised, approximately 3 mm from the axils, leaving the axillary buds covered. Shoots were cut into smaller segments of 11-nodes for surface sterilisation.

The sterilisation procedure followed were the same as described in 2.7.1a) with the exception of one step: After removing plant material from ethanol, it was divided into two treatments and placed in either a 10% household bleach solution (3.5% active NaOCl) (Treatment 1) or 35 g/L $Ca(OCl)_2$ (Treatment 2) for 10 minutes. Chemical solutions were in beakers and were constantly stirred on magnetic stirrers. The pH of both solutions was adjusted to 6.5 using hydrochloric acid (HCl) to ensure optimum bactericidal action of hypochlorite (see 2.9) and to exclude possible variation in bactericidal effect due to differences in the pH of solutions. Shoot segments were removed from chemicals and rinsed according to the procedure described in 2.7.1a).

The shoot segments were cut smaller into 3-node segments on sterilised blotting paper using sterile surgical blades and forceps. For each treatment, twenty explants were planted onto 10 ml ½ MS medium supplemented with 3% sucrose and solidified with 3 g/L Gelrite™, devoid

of growth regulators (basic medium), in 15 cm glass test tubes, according to the procedure described in 2.7.1b). The medium used was prepared according to the procedure described in 2.7.1d) and cultures were kept in growth conditions described in 2.7.1e).

Plants were monitored and data collection was done on a weekly basis for four weeks. Data collected included the degree of wilting on a scale from 1 – 5, and the number of axillary buds showing growth and contamination. Grading on the degree of wilting was done using the criteria specified in Table 2.4.

Table 2.4: Criteria used to grade the degree of wilting on a scale of 1 – 5 on *Athrixia phylcoides* explants in culture

Wilting degree	Description
1	No signs of wilting
2	Slightly wilted
3	Wilted with less than 20% of explants showing browning
4	Wilted with 20% - 70% of explants showing browning
5	Completely wilted with more than 70% of explants showing browning

The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test using Statistical Analysis System (SAS) (Cary, 2004).

2.9.2 Results and Discussion

a) Bactericidal effect of NaOCl versus $Ca(OCl)_2$

Both $Ca(OCl)_2$ and NaOCl treatments successfully inhibited the growth of bacteria and very little (maximum 3%) contamination was recorded. Explants sterilised with NaOCl showed a slightly higher incidence of bacterial contamination after 21 days compared to plants sterilised with $Ca(OCl)_2$. However, the difference was not statistically significantTable 2.5). According to Pierik (1987) $Ca(OCl)_2$ penetrates plant material slower than NaOCl. With exposure to equal amounts of active ingredient and similar exposure time, one would expect better bacterial control with NaOCl than $Ca(OCl)_2$. This is contradictory to our findings.

Table 2.5: Contamination of *Athrixia phylcoides* explants surface sterilised with $Ca(OCl)_2$ and NaOCl respectively after 21 days in culture

Treatment	Number of explants contaminated (%)
NaOCl	3^{a*}
$Ca(OCl)_2$	2^{a}

*Values that are not significantly different at 5% probability level are indicated by the same letter

b) The effect of NaOCl and $Ca(OCl)_2$ on wilting and vegetative growth

The effect of the different bactericides on wilting and vegetative growth of plants was noticeably different after two weeks (no growth was recorded in the first week). Plants treated with NaOCl showed significantly better bud growth, both on day 14 and 21. The difference in bud growth was still significant on day 28 even though there was very little increase since day 21 (Figure 2.2). The degree of wilting in NaOCl treated explants was also significantly less than for $Ca(OCl)_2$ treated explants after 14 days in culture. By day 21, the $Ca(OCl)_2$ treatment showed slightly higher levels of wilt, but the difference between the two treatments was no longer statistically significant (Figure 2.3). After 21 days in culture, NaOCl sterilised plants appeared, in general, greener and healthier than $Ca(OCl)_2$ sterilised plants, even though symptoms of hyperhydricity were visible on most plants (Figure 2.4). After day 28, most plants were wilted and symptoms of hyperhydricity had increased. The transferable shoots were subcultured and the trial was terminated.

These results were in contradiction with our expectations, since $Ca(OCl)_2$ is generally considered to be a more gentle disinfectant. According to Pierik (1987) and George (1993), $Ca(OCl)_2$ is less likely to induce tissue browning and injury of sensitive plants than NaOCl and is used in the case of sensitive plants. George (1993) attributes this to the high concentration of Ca^{+} in solution. Calcium is an important structural component of plant cell walls (Salisbury & Ross, 1992).

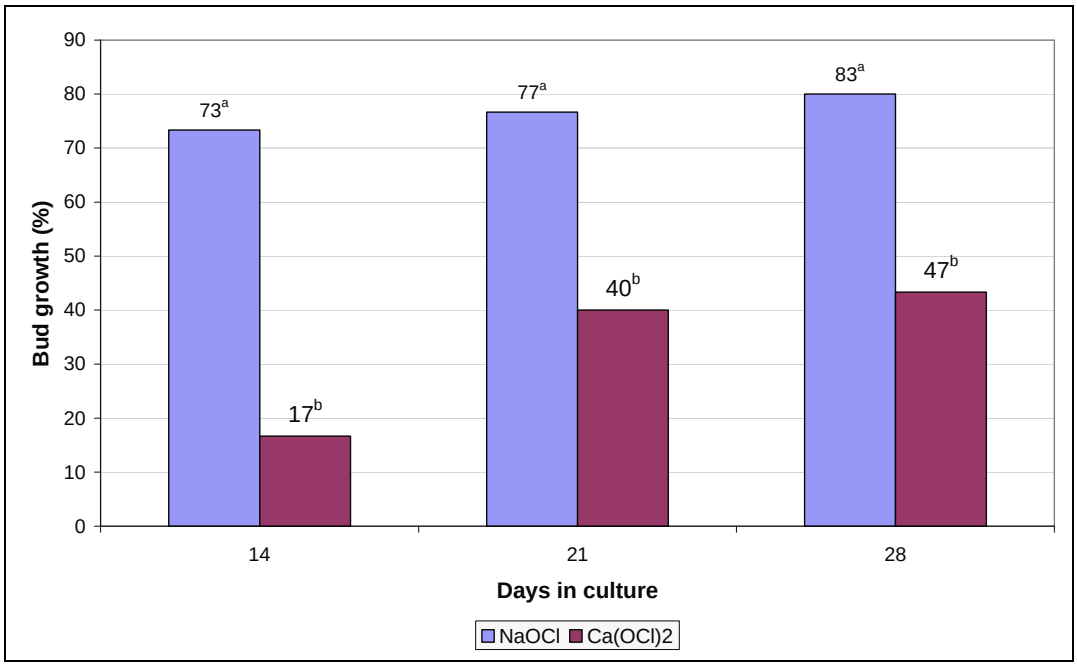

Figure 2.2:Percentage of axillary buds showing signs of growth on 3-node *Athrixia phylicoides* explants sterilised with NaOCl and $Ca(OCl)_2$ after 14, 21 and 28 days in culture

*Values that are not significantly different at 5% probability level are indicated by the same letter

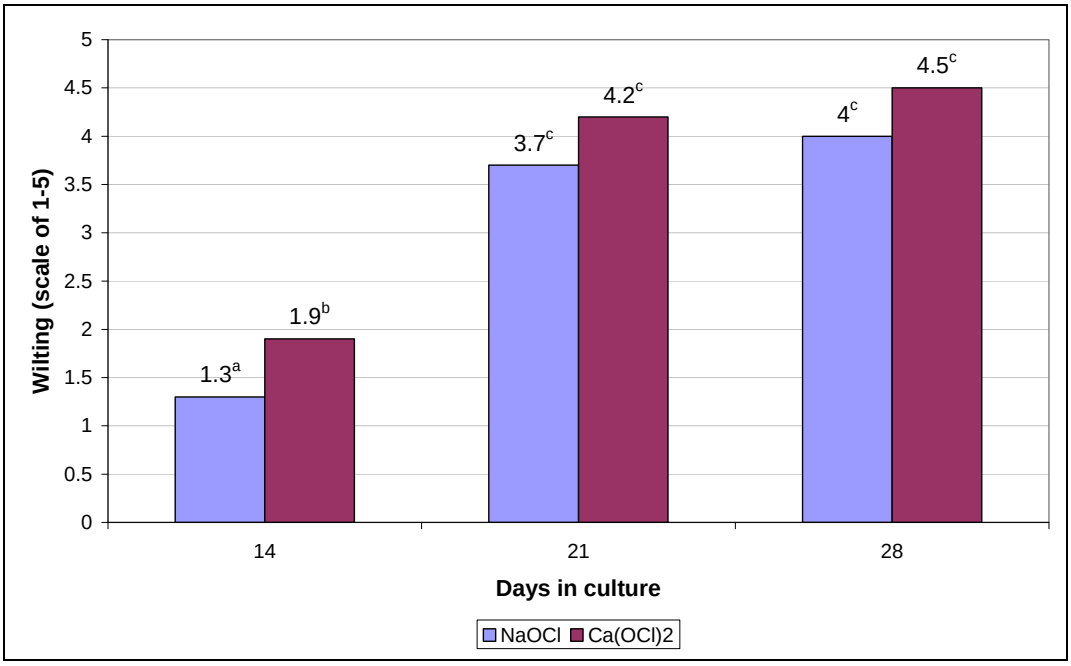

Figure 2.3:The degree of wilting on a scale of 1 (not wilted) to 5 (completely wilted) of *Athrixia phylicoides* explants sterilised with NaOCl and $Ca(OCl)_2$ after 13, 21 and 28 days in culture

*Values that are not significantly different at 5% probability level are indicated by the same letter

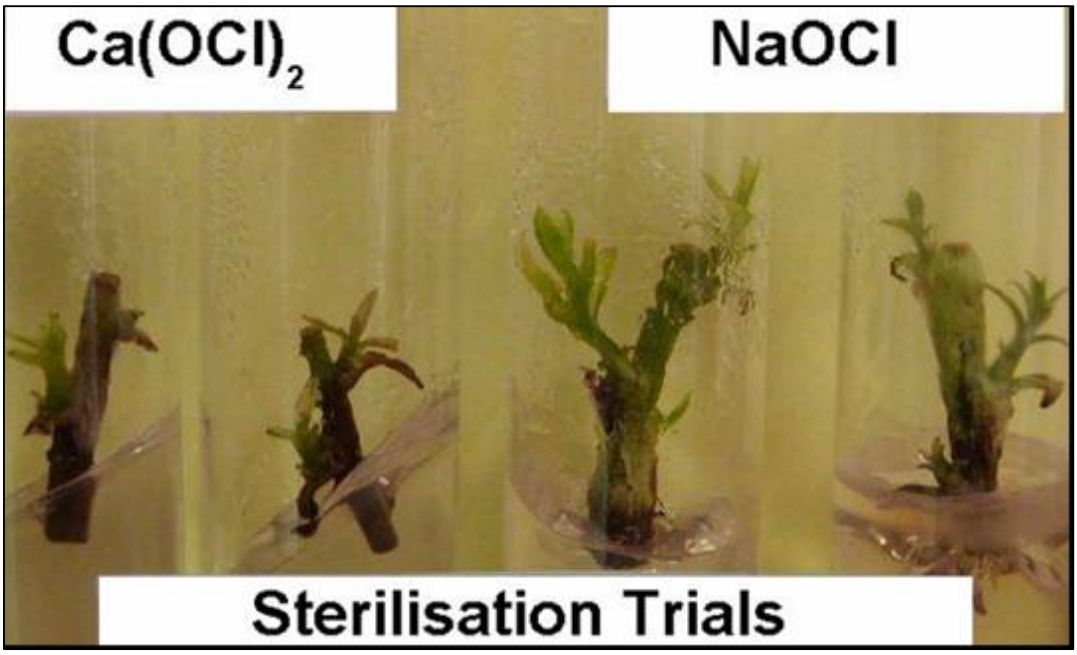

Figure 2.4 Effect of the bactericides $Ca(OCl)_2$ and NaOCl on the in vitro axillary bud growth on *Athrixia phylicoides* explants

2.10 Conclusions

Total removal of leaf blades (a great source of contamination due to the abundant numbers of trichomes) from plant material prior to surface sterilisation effectively reduced contamination, but caused damage to meristems by exposing them to harsh chemicals. The problem was solved by partial abscission of leaf blades. This decreased contamination by 10%, without damaging the axillary meristems. Application of a systemic fungicide in the greenhouse effectively controlled fungal disease in our cultures. This also saves time during surface sterilisation and reduces the time plant material is exposed to liquid. This is beneficial to plants like *A. phylicoides* that show sensitivity to liquid exposure.

NaOCl is a more suitable sterilant for use on bush tea than $Ca(OCl)_2$. The bactericidal action of these sterilants is similar and equally effective in controlling contamination. Despite this, bush tea explants responded differently to the sterilants in terms of wilt and vegetative growth. Explants sterilised with $Ca(OCl)_2$ showed less growth of axillary buds and showed more severe wilting than plants sterilised with NaOCl.

Contamination on nodal explants of bush tea was effectively controlled by a combination of control measures including the treatment of stock plants with a systemic fungicide, partial removal of hairy leaf blades and a 5-step surface sterilisation procedure. The sterilisation procedure can be summarised as follows: Rinse plant material in running tap water (10 min),

submerge it in 70% ethanol (90 sec), soak in 10% (v/v) household bleach (3.5% active NaOCl) (10 min) and rinse twice in sterile water (5 min each).

Following the contamination control measures listed above, cultures could be established on basic medium. The first signs of bud growth were visible after two weeks in culture. Despite this, the physical condition of plants deteriorated quickly after 21 days in culture due to the development of hyperhydricity, and subculture of newly formed microshoots should therefore be done as soon as possible.

CHAPTER 3

MULTIPLICATION

3.1 Scope of this chapter

After the successful establishment of nodal explants, as discussed in the previous chapter, our aim was to promote the formation of axillary shoots from nodal explants by addition of different growth regulators to our growth medium. We studied the effect of two growth regulators indole-3-butyric acid (IBA), an auxin, and 6-benzylaminopurine (BAP), a cytokinin, at concentrations of 1.5 mg/L, alone and in combination, on axillary shoot formation.

3.2 Literature review

The rapid multiplication rate that is theoretically possible with tissue culture is one of the motivations for choosing this propagation method above conventional methods (Hartmann, Kester, Davies & Geneve, 1997; Rout & Das, 1997; Rout, Samantary & Das, 2000; Giusti, Vitti, Fiocchetti, Colla, Saccardo & Tucci, 2002; Baskaran & Jayabalan, 2005). The objective of the multiplication stage (also referred to as the proliferation stage) is to bring about new plant outgrowths which can be separated from the culture and, when transferred to a new medium, have the capacity to give rise to new plants (George, 1996). Unless a specialised multiplication procedure is required (which is the case in some species), the medium used in this stage is mostly similar to that used in Stage I (Hartmann *et al.*, 1997).

The number of subcultures possible, as well as the period of time plants can remain in culture, seems to differ with species. Some plant species can be kept in culture for extensive periods without apparent changes. Gavidia, Agudo & Perez-Bermudez (1996) reported that the long-term *in vitro* culture of *Digitalis obscura in vitro* did not affect its genetic stability. Cultures of *Scrophularia yoshimurae* (a medicinal herb) were kept in culture for over 2 years without any changes in morphogenetic potential (Nalawade & Tsay, 2004). Where species are prone to develop hyperhydricity, the time it can be kept in culture is limited. This poses a potential problem for *in vitro* propagation of bush tea, unless a successful method for prevention is found (see CHAPTER 5).

According to Rout *et al.* (2000), the age of cultures can influence the incidence of abnormalities in the progeny. Debergh & Meane (1981) and Debergh & Read (1991) reported that, after repeated subculturing, explants of various ornamental plants showed changes in growth response. Plants that originally produced axillary shoots, started to produce more abundant adventitious shoots. The reported effects of repeated subculturing

on rooting are conflicting. Adventitious root formation on repeatedly subcultured plants of the Rosaceae declined, while the rooting percentage on Jonathan apple cultivar increased with increasing numbers of subcultures (Pierik, 1987). The rate of rejuvenation of plant material tends to increase after a number of subcultures (Pierik, 1987; Webster & Jones, 1989). Other responses to prolonged time in culture and/or repeated subcultures include loss of vigour, deterioration and dormancy (Hartmann *et al.*, 1997).

Various plant organs can be used for multiplication, including newly formed axillary or adventitious shoots, somatic embryos, miniature storage organs, cell suspension cultures, etc. Choice of multiplication material will influence the morphogenetic and regenerative potential of cultures (Nalawade & Tsay, 2004) and even its ability to produce secondary metabolites. Derbessy, Touche & Zola (1989) reported that only cells derived from apical meristems of *Santolina chamaecyparissus* were able to produce essential oil. Microshoots are relatively easy to distinguish and handle, and are popular proliferation organs. Depending on the grower's requirements, adventitious or axillary shoots (or both) can be used. Adventitious shoot regeneration provides a fast *in vitro* multiplication method for various plant types (Debergh & Read, 1991; George, 1996; Giusti *et al.* 2002), but the progeny is not genetically stable (Rout *et al.*, 2000; Giusti *et al.*, 2002; Nalawade & Tsay, 2004). Axillary shoot regeneration, on the other hand, results in a much slower rate of production (George, 1996; Giusti *et al.* 2002), but plants derived from these shoots are genetically stable (Pierik, 1987; George, 1996; Hartmann *et al.*, 1997; Rout *et al.*, 2000).

Multiplication through adventitious shoots usually involves the growth of clusters of microshoots from a basal mass of callus-like tissue. These shoots are separated and subcultured. After sufficient growth, these secondary microshoots can again be used as a source of material for subsequent subcultures (Jain, Mathur, Gupta, Singh, Verma, Gupta & Kumar, 1996), or be rooted (George, 1996). The genetic variety obtained through this technique may yield unique plants with beneficial qualities. However, a high proportion of plants that have been regenerated from adventitious shoots (or other organs derived from callus), show structural or physiological abnormalities (Corneanu, Corneanu, Copacescu, 1990; Mangolin, Prioli & Machado, 1994). In contrast to this, George (1996) stated that, in some instances, callus can be semi-organised and may result in genetically stable progeny.

Where plants are cultivated for medicinal use, plants should ideally be homogenous in their capacity to produce secondary metabolites (Nalawade & Tsay, 2004; Avato, Fortunato, Ruta & D'Elia, 2005), and should therefore be genetically similar. If *A. phylicoides* is to be cultivated commercially, plants with high quality essential oils and a high production capacity will be selected and cloned. Because of their genetic stability (Pierik, 1987; George, 1996;

Hartmann *et al.*, 1997; Rout *et al.*, 2000), the development of axillary shoots will in this case, be preferred over adventitious shoots, despite their much lower rate of production (George, 1996; Giusti *et al.* 2002). Growth regulators can be adjusted to suppress the development of adventitious shoots, thereby ensuring the genetic purity of the next generation (George, 1996). Induction of axillary shoots should be a suitable *in vitro* multiplication method in the case of bush tea.

3.3 The effect of IBA and BAP on axillary shoot development

The level and type of plant growth regulators supplemented to culture medium determine the growth response of plants to a great extent. Auxins generally cause cell division, cell elongation and formation of adventitious roots while cytokinins overcome apical dominance (Pierik, 1987). Gibberellins stimulate growth of organs but generally do not favour organ initiation (Rout *et al.*, 2000). The optimum type and relative concentrations of plant growth regulators need to be determined according to the type of growth desired and for each species.

The ratio between auxin and cytokinin closely regulates root and shoot initiation and differentiation from unorganised callus (Skoog and Miller, 1957; Ammirato, 1983; Bajaj, 1988; Rout & Das, 1997). Auxin: cytokinin ratios of ~ 4 favour the development of shoots (Murashige, 1980). A combination of cytokinin and auxin are usually used to promote multiple shoot formation (George, 1996; Hartmann *et al.,* 1997; Puente & Marh, 1997; Dhingra, Rao & Narasu, 2000; Giusti *et al.* 2002; Lai, Lin, Nalawade, Fang & Tsay, 2005; Rout, 2005). Cytokinins were shown to be the most critical growth regulators for shoot elongation of many medicinal plant species (Jha & Jha, 1989; Sharma, Chandel & Paul, 1993; Chen, Hu & Huang, 1995; Saxena, Rout & Das, 1998; Rout *et al.*, 2000).

A trial was conducted to investigate the effect of indole-3-butyric acid (IBA) and 6-benzylaminopurine (BAP) on axillary shoot growth of nodal explants from bush tea. These growth regulators are amongst the most effective and most commonly used growth regulators in micropropagation of medicinal plants (Rout *et al.*, 2000; Rout, 2005; Avato *et al.*, 2005; Baskaran & Jayabalan, 2005; Thomas & Maseena, 2006) and other plants of the Asteraceae family (Ueno, Cheplick & Shetty, 1998).

3.3.1 Materials and Methods

Plant material was collected from four different stock plants during July (winter season and flowering time). Shrubs were fairly small, and the amount of plant material that could be collected from a single plant was limited (see 2.3). All the stock plants were kept in the same greenhouse and were regularly treated with Fundazol™ 2 g/L (active ingredient Benomyl) to

control fungus infection as part of preparation (see 2.8). Shoots that appeared similar in size, physiological stage and vigour were selected.

The basic medium used in all treatments was ½ MS medium supplemented with 3% sucrose, solidified with 3 g/L Gelrite™ and pH adjusted to 6.5. For each of the four treatments, the medium was supplemented with one of four growth regulator combinations: Treatment 1 was fortified with 1.5 mg/L indole-3-butyric acid (IBA), Treatment 2 with 1.5 mg/L 6-benzylaminopurine (BAP), Treatment 3 with both IBA and BAP at 1.5 mg/L and Treatment 4 - contained no growth regulators (control). Only one concentration of growth regulators could be tested due to the limited availability of plant material.

Shoots were surface sterilised, cut into 3 node explants and established according to the procedure described in 2.7.1a) and 2.7.1b). Nodal explants were planted vertically onto one of the 4 growth media as described above. The growth media used was prepared according to the procedure described in 2.7.1d). Cultures were stored at the same growth conditions described in 2.7.1e).

All cultures were monitored on a weekly basis and the number of axillary buds on each explant showing growth was recorded. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test using Statistical Analysis System (SAS) (Cary, 2004).

3.3.2 Results and Discussion

The addition of IBA and BAP at a concentration of 1.5 mg/L did not seem to promote axillary shoot growth. In the first two weeks of the trial, axillary shoot growth on BAP treatments was slightly higher than that on the IBA and BAP/IBA combination treatments, but not higher than the control treatment (Figure 3.1). After 21 days, the BAP treatments showed 7% more axillary bud growth than the control treatment, but the difference between the treatments was not statistically significant. The addition of IBA, alone or in combination with BAP, seemed to suppress axillary shoot growth to some extent. The IBA treatment showed the least axillary bud growth throughout the trial and the second lowest growth response was observed on the BAP/IBA combination treatment. However, none of the differences between treatments were statistically significant.

Our results were also influenced by fungal and bacterial contamination. Due to severe contamination, most explants ceased to grow after Day 14, and a total of 34 explants (out of 160) were dead after 21 days. More than 60% of cultures were contaminated by Day 21 and

the trial was terminated. Our results were based on the total number of replications initiated, and the deaths due to contamination are reflected in the decrease in growth from Day 14 onward (Figure 3.1).

The effect of contamination escalated with time. The contamination observed originated from within the plant material. Seasonal variation in the level of contamination has been reported. According to George (1993) contamination is normally more severe in spring and late summer. This is in contradiction with the high incidence of contamination in this trial. This trial was conducted during the winter and more effective contamination control was achieved, using same sterilisation procedure, during our previous trial in summer (see CHAPTER 2). The high levels of contamination in this trial may be related to flowering. Flowers often attract insects that may serve as vectors for contaminants. Even though plants were kept in a greenhouse and insect pests were controlled, it is possible that contamination levels during flowering season may have been elevated due to the presence of insects on plants. Specific literature on the seasonal variation of contamination levels of other Asteraceae and medicinal plants could not be found.

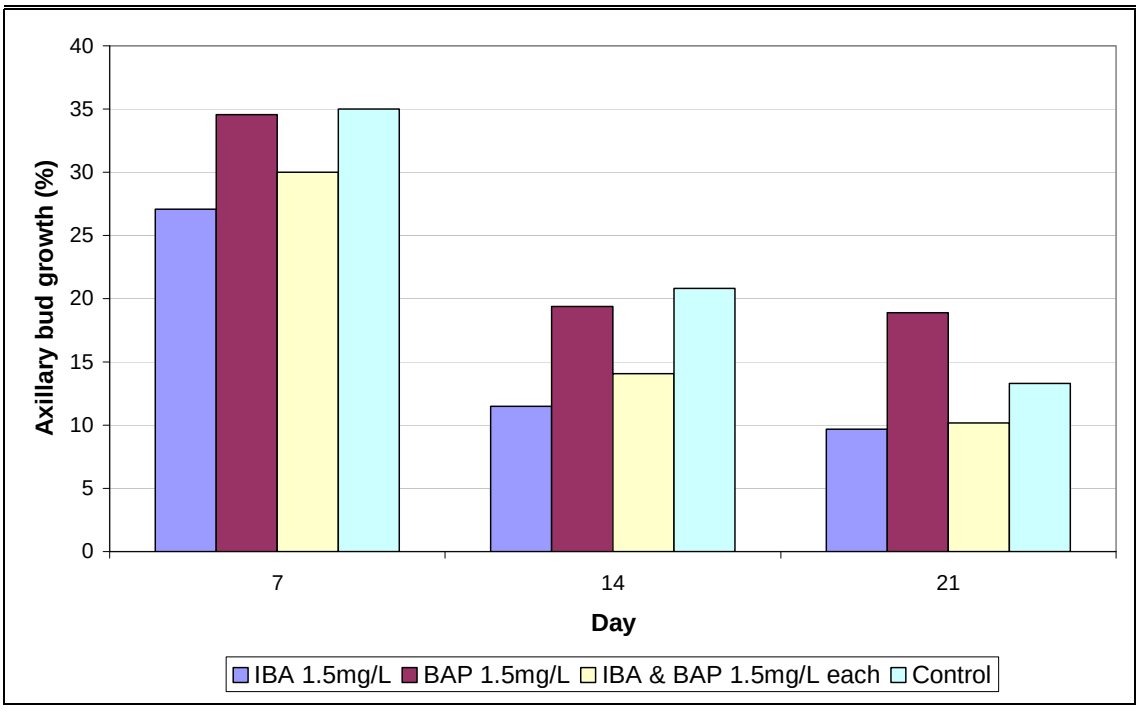

Figure 3.1 Effect of IBA (1.5 mg/L) and BAP (1.5 mg/L) separately, or combined, on the percentage axillary shoot growth of nodal explants of *Athrixia phylicoides*

3.4 Inconsistent growth responses

Seasonal changes and the physiological growth stage of plants are often associated with differences in the levels of endogenous nutrients (Mudau, Soundy, Du Toit & Olivier, 2006), secondary metabolites (Giannouli & Kintzios, 2000; Nalawade & Tsay, 2004; Mudau *et al.*, 2006) and growth regulators in plants. The biochemical composition of the stock plants at the time of collection of plant material may influence the response of explants in culture. We observed changes in growth response in our different trials over a period of two years.

The following section is a summary of visual observations made during our trials. These observations could not be statistically supported, since they appeared unexpectedly and proper data collection was not done. This section serves to lay a foundation for possible future investigations.

3.4.1 Callus formation

Adventitious shoot regeneration can be achieved directly or indirectly via callus formation. Multiplication by means of adventitious shoots offers a much higher proliferation rate than axillary shoots, and genetic variety is preserved. Genetic variety in medicinal plants is potential new sources of useful bio-pharmaceuticals, and may produce plants that are better adapted to changing environments in the long term (Nalawade & Tsay, 2004). On the down side, genetic variety results in inconsistent production and quality of bio-pharmaceuticals.

Callus formation on *in vitro* propagated Asteraceae and some medicinal plants have been achieved by including a combination of auxin and cytokinin in growth media. Auxin: cytokinin ratios of ~ 10 yield rapid growth of undifferentiated callus (Murashige, 1980). Callus has been induced on *Dianthus caryophyllus* L. (Jain, Kanita & Kothari, 2001), *Cardiospermum halicacabum* (Thomas & Maseena, 1996) and *Artemisia annua* (Dhingra *et al.*, 2000) on MS media containing 2,4-D and BAP at concentrations ranging between 0.2 – 5 mg/L. Also, a combination of 1 mg/L BAP and 0.5 - 1 mg/L naphthalene acetic acid (NAA) in MS-medium induced callus formation on *Piqueria trinervia*, a medicinal plant from the Asteraceae family (Saad, Diaz, Chávez, Reyes-Chilpa, Rubluo & Jiménez-Estrada, 2000) and on American safflower (*Carthamus tinctorius* L.) (Orlikowska & Deyer, 1993). Baskaran & Jayabalan (2005) reported that mild callus formation occurred on *Eclipta alba* microshoots with all types of auxins in full-strength MS medium, and that reducing MS salt concentration by one-half reduced callusing. Increasing the concentration of either BA (above 17.8 mM) or kinetin (18.6 mM) in the culture medium resulted in basal callusing of the growing shoots of *Clitoria ternatea* (Rout, 2005).

a) Materials and Methods

The relevant microplants were part of a preliminary hyperhydricity reversal trial, initiated during the month of February (summer). They were established on ½ MS medium fortified with 3% sucrose, solidified with 3 g/L Gelrite™ and pH adjusted to 6.5. The growth medium was enriched with one of four growth regulator combinations viz. 1.5 mg/L BAP, 1.5 mg/L IBA, a combination of BAP and IBA, both at 1.5 mg/L, or a hormone free medium. The same surface sterilisation and initiation techniques and culture conditions described in 2.7.1 applied. Transferable microshoots were subcultured between 3 and 4 weeks after initiation, to culture medium with the same composition as that on which explants were first established.

b) Results and Discussion

Callus formation, in various degrees, was observed in some of the cultures, both on BAP and on IBA-BAP combination media, but not on IBA alone or on control medium, 4 weeks after subculturing. In some cases, rooting occurred from the callus (Figure 3.2). BAP included in the growth medium seemed to promote callus formation. However, in another trial on hyperhydricity (see 5.3), where similar growth media was used, callusing did not occur. The growth response of cells in culture is a result of both endogenous and exogenously hormone stimulus and culture conditions (Rout *et al*., 2000). The endogenous hormone levels, produced by explants, may have induced callus formation in this case (Pierik, 1987).

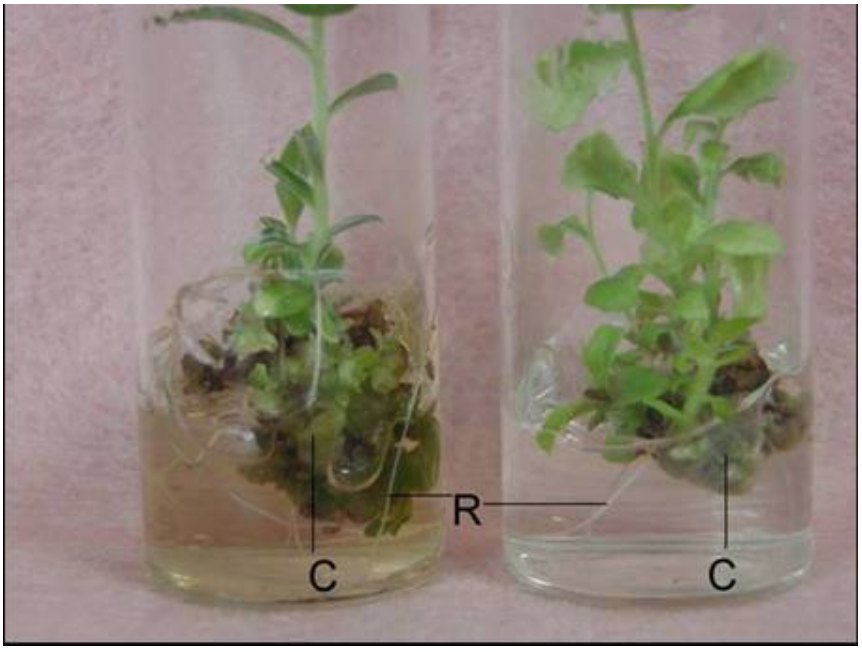

Figure 3.2: Callus (C) formation and rooting (R) from basal callus on *Athrixia phylicoides* explants on MS medium supplemented with 1.5 mg/L BAP after 8 weeks in culture

3.4.2 Hyperhydricity

The occurrence of hyperhydricity in many of our cultures, and in many different trials was a great hindrance during the multiplication stage. The occurrence of hyperhydricity was unpredictable and symptoms appeared on cultures in all the different growth media tested. The symptoms varied considerably in severity, and time of appearance throughout our trials over the two year period. In some cases, cultures were completely killed and trials had to be terminated within 4 weeks after initiation, while symptoms in other cases only occurred at a much later stage, or in some cases, not at all. In most cases, the symptoms of hyperhydricity became more severe with time in culture, and microshoots had to be transferred to fresh medium as soon as they reached a suitable size. Hyperhydricity on bush tea is discussed in more detail in CHAPTER 5 .

3.5 Conclusions

Bush tea cultures can be successfully multiplied by means of axillary shoot formation. This can be achieved on ½ MS medium devoid of growth regulators and within four weeks after establishment. The addition of 1.5 mg/L BAP to the growth medium did not promote axillary shoot formation, and the effect of BAP at a higher concentration, or the use of a different cytokinin may still be investigated. The length of time cultures could be kept on the same medium was limited by the occurrence of hyperhydricity. The problem was partly controlled by transplanting newly formed axillary microshoots to fresh medium as soon as possible. The cause of inconsistent growth responses such as callus formation and the occurrence of hyperhydricity are still unclear. Variation in endogenous hormone levels of stock plants or in cultures may be, at least partly, involved (Pierik, 1987).

CHAPTER 4

ROOTING AND REESTABLISHMENT

4.1 Scope of this chapter

In this part of our study we aimed to produce rooted acclimatised plantlets that are suitable for transfer to a greenhouse and ultimately to field conditions. To our knowledge, no research on *in vitro* rooting of *Athrixia phylicoides* has been documented, and investigations described in this chapter will provide new information on this topic.

We conducted two trials on *in vitro* rooting. In the first trial, the combined effect of lowered sucrose concentrations and the presence of IBA (indole-3-butyric acid) in the growth medium were investigated. In the second trial the effect of GA_3 (gibberellic acid) included in the establishment medium on subsequent rooting was studied.

our trial on acclimatisation was only partly conducted because of failure in the early stages of the trial. We intended to transfer plantlets, with their *in vitro* formed roots, either removed or intact, to greenhouse conditions and induce the formation of new roots in their new growth medium. We also attempted to gradually expose cultured plantlets to greenhouse conditions.

4.2 Literature review

The last steps in *in vitro* propagation are rooting, acclimatisation and reestablishment of cultured plants. The purpose of these steps is to prepare microplants for the new environment in which they will be cultivated. This is achieved by gradual exposure of plantlets or microcuttings to their new growth conditions in terms of climatic conditions, nutrient availability and other environmental stress factors such as pathogens (Smith, Roberts & Mottley, 1990a; Smith, Roberts & Mottley, 1990b; Preece & Sutter, 1991; Hartmann, Kester, Davies & Geneve, 1997).

Inside culture vessels, plants are protected against many stress factors (Preece & Sutter, 1991). The relative humidity (RH) inside culture vessels is close to 100% (Piqueras, Cortina, Serena & Casas, 2002), resulting in very little water stress. Macro- and micronutrients and carbon (normally in the form of sucrose) are provided through the nutrient medium and are easily accessible. Light intensity, photoperiod and temperature are controlled to create desirable growth conditions and the aseptic environment in which plants are kept, reduces the risk of infection and stress caused by pathogens. Outside culture vessels, plants will be exposed to significantly lower RH, higher light intensities and variable photoperiods.

Temperatures will be more extreme and plants will be exposed to pathogens. Plants will also need to obtain macro- and micronutrients and other minerals from soil and produce carbons through photosynthesis (Smith, Roberts & Mottley, 1990a; Smith, Roberts & Mottley, 1990b; Preece & Sutter, 1991; Hartmann, Kester, Davies & Geneve, 1997). Plants produced under *in vitro* conditions are anatomically and physiologically unsuited to survive the above mentioned external conditions (Preece & Sutter, 1991) and need to undergo changes to improve their chance of survival in their new environment.

In order to change from heterotrophic to autotrophic, plant need to undergo both physiological and anatomical changes. Because plants in culture derive their carbon and other nutrients mainly from their growth medium, there is a reduced need for photosynthesis (Preece & Sutter, 1991; Hartmann *et al.*, 1997). As a result, leaves that are formed in culture may have a reduced capacity for photosynthesis (Ziv, 1986; Grout & Donkin, 1987), or are completely unable to photosynthesise (Preece & Sutter, 1991; Nalawade & Tsay, 2004). Because of the accessibility of nutrients and radically different compositions of culture medium compared to soil, roots that are formed *in vitro* are not functional in soil-like medium (Debergh & Read, 1991). These roots often lack root hairs and vascular connections (Preece & Sutter 1991; George, 1996).

In order to obtain nutrients necessary for survival under external conditions, a plants needs to develop a root system that will absorb minerals and other vital nutrients from the new growth medium. New leaves that are capable of photosynthesis need to be formed, or existing leaves need to adapt and become photosynthetically active.

A second challenge is to adjust plantlets to climatic differences. In a greenhouse and especially in the open field, plants will be exposed to more extreme temperatures, variable photoperiods, higher light intensities and a substantially lower RH (Preece & Sutter, 1991). Stomata of *in vitro* grown plants are often unable to close in response to water loss (Wardle, Dobbs & Short, 1983; Short, Ziv, 1986; Warburton & Roberts, 1987; Smith *et al.*, 1990a; Smith *et al.*, 1990b). Studies on cabbage, cauliflower and carnation revealed that the cuticle layers of *in vitro* grown plants differed chemically and structurally from field and greenhouse plants, allowing more water permeability (Sutter & Langhans, 1979; Grout, 1975; Sutter & Langhans, 1982; Sutter, 1984; Ziv, 1986). As a result, plants transferred from the high RH in a culture vessel to the much lower RH outside, lose vast amounts of water through transpiration and evaporation from leaf surfaces (Smith *et al.*, 1990a: Smith *et al.*, 1990b; Preece & Sutter, 1991).

Microscopical investigation of the leaf surfaces of *in vitro* grown bush tea plantlets, indicated that their stomata are not responsive to water loss (see 6.5). Wilting and damage due to water loss can therefore be expected when transferring micropropagated bush tea plantlets to external conditions.

4.3 *In vitro* rooting

The rooting potential of plant material is determined by numerous factors such as the age and developmental stage of a plant, position on a plant, plant species and cultivars, wounding, the number of subcultures, oxygen supply, light, temperature and the composition of the growth medium (pH, levels of hormones, minerals and sugars etc.) (Pierik, 1987; Rout, Samantary & Das, 2000).

Numerous studies on adventitious root formation led to the process being divided into several successive interdependent phases (Gaspar, Kevers, Hausman, Berthon & Ripetti 1992; Gaspar, Kevers, Hausman, Ripetti, 1994). Jarvis (1986) and Moncousin (1987) identified three phases viz. induction, initiation, and expression. Rooting can either be accomplished *in vitro* or outside vessels in a mist bed. *In vitro* rooting is often achieved by transferring plantlets to a suitable rooting medium, or in the case of liquid medium, adjusting the medium to favour or stimulate rooting (Roest & Bokelmann, 1975; Bush, Earle & Langhans, 1976; Hartmann *et al.*, 1997).

There are advantages and disadvantages to both *in vitro* and *ex vitro* rooting methods. Some of the advantages of *in vitro* rooting are that it is quick and provides a way to root plants that are difficult to root *ex vitro* (George, 1996). In some species, the survival rate of rooted plantlets is higher, more axillary shoots are formed and they have a higher growth rate after transfer (Preece & Sutter 1991; George, 1996). *In vitro* rooting offers the greatest degree of control over rooting (George, 1996). Disadvantages of *in vitro* rooting include the high cost and labour intensiveness (George, 1996). Furthermore, roots that were formed *in vitro* are not adapted to function in a soil-like medium and new roots need to be formed (Debergh & Read, 1991; Hartmann *et al.*, 1997). Also, transplanting rooted plantlets without injuring them can be problematic (Ben-Jaacov & Langhans, 1972). Injury to fragile roots during transplant increase the risk of infection by soil borne pathogens and may result in root or stem diseases (Debergh & Read, 1991; Preece & Sutter, 1991).

The composition of culture media has been shown to have an effect on *in vitro* rooting of various plant species. Media composition includes factors such as salt concentration, levels of minerals and sugars, pH (Tian *et al.*, 1993; Gaspar, 1994; Rout *et al.*, 2000), the presence and relative concentration of growth regulators (GR's) such as cytokinin, auxin and GA_3

(gibberellic acid) (George, 1993; Gaspar, 1994; Pierik, 1987; Rout *et al.*, 2000) and the type and amount of gelling agent used (Babbar, Jain & Walia, 2005). Incubation conditions such as light quality and intensity, photoperiod, oxygen supply and temperature are also known to affect *in vitro* rooting (Pierik, 1987; Rout *et al.*, 2000). Documentation mostly deals with the combined effect of two or more of the above mentioned factors.

In some plant species rooting occurs spontaneously without the presence of growth regulators. Kaul, Miller, Hutchinson & Richards (1990) reported rooting of 12 different chrysanthemum cultivars (Asteraceae) in hormone free ½ MS medium (Murashige & Skoog, 1962). These and other plants seem to respond to an elongation period between Stages II and III rather than the presence of growth regulators (Hartmann, Kester & Davies, 1990; Hartmann *et al.*, 1997). Rooting of various medicinal plants such as *Datura insignis, Clerodendrum colebrookianum* and *Psoralea corylifolia* has also been achieved on MS medium in the absence of growth regulators (Cristina, dos Santos, Esquibel & dos Santos, 1990; Mao, Wetten, Fay & Caligari, 1995; Saxena, Rout & Das 1998).

The effect of growth regulators on rooting vary between species. Avato, Fortunato, Ruta & D'Elia (2005) reported rooting of *Salvia officinalis* (a medicinal plant of the Asteraceae family) without the addition of auxin, while auxin seems to be a requirement for rooting for many other *Salvia* species. Ben–Jaacov & Langhans (1972) reported rooting of *Dendranthema grandiflora* only after some elongation of shoots in a medium containing both IAA (indol acetic acid) and BAP (6-benzylaminopurine). Pierik (1987) showed that the rate of rejuvenation increases with an increase in the number of subcultures. Juvenile plant material has a greater potential for rooting than mature material and according to Pierik (1987), spontaneous rooting after repeated subculturing may therefore be a result of rejuvenation.

4.4 The effect of IBA and lowered sucrose concentration on *in vitro* rooting

The effect of externally added growth regulators on *in vitro* rooting varies greatly between plant species. The difference in response may partly be explained by the fact that some explants produce sufficient levels of hormones themselves (Pierik, 1987) which may mask or influence the effect of externally added growth regulators. The optimum concentration of growth regulators required for rooting needs to be determined for each plant species.

The presence of auxin in a medium is generally accepted to promote rooting while cytokinin usually suppresses rooting (Pierik, 1987; George, 1993; Gaspar, 1994; Hartmann *et al.*, 1990; Hartmann *et al.*, 1997; Rout *et al.*, 2000). According to Pierik (1987), the presence of low concentrations of auxin will promote adventitious root formation, while high

concentrations will suppress rooting, and instead promote the formation of callus in general. Rooting on *Dendranthema grandiflora* was achieved by increasing the levels of the auxin IAA in medium (Ben-Jaacov & Langhans, 1972). Jain *et al*. (2001) achieved *in vitro* rooting of carnation on MS medium supplemented with either 1-naphthaleneacetic acid (NAA) (1 mg/L) or IBA (2 mg/L). Rooting of many *Salvia* species (medicinal Asteraceae) could only be achieved by the addition of auxin to the growth medium (Avato *et al.*, 2005).

The effect of changes in the concentrations of salts and sugar in the medium seems to vary. According to Pierik (1987), a high concentration of sugar is needed for adventitious shoot formation, especially in woody plants. Roest & Bokelmann (1975) reported rooting of *Chrysanthemum morifolium* by increasing both the levels of sucrose and IAA. In contradiction to this, lowering both or either of the concentrations of salts or sugar in the growth medium, has been reported to promote rooting by many authors and on different plant species, including 12 different species of *Dendranthema grandiflora* (Kaul *et al.*, 1990) and various medicinal plants reviewed by Rout *et al.* (2000). The combined effect of salts, sugars and growth regulators has also been documented. Gaspar (1994) achieved *in vitro* rooting of *Chrysanthellum americanum* (medicinal plant of the Asteraceae family) by diluting the salt concentration of the growth medium four times in a medium devoid of cytokinin, containing either IBA or NAA (naphthalene acetic acid) as a rooting auxin.

We conducted a trial to investigate the combined effect of a lowered sucrose concentration and addition of auxin on *in vitro* rooting of bush tea.

4.4.1 Materials and Methods

Plantlets used in this trial were established on 10 ml ½ MS medium supplemented with 3% sucrose and solidified with 3 g/L Gelrite™ in 15 cm glass test tubes, using the technique described in 2.7.1a). A total of 30 cultures were initiated. Cultures were incubated in a growth room at 30°C ± 2ºC with 8h dark: 16h light intervals under cool white fluorescent lights providing 60 µmol/m^2/sec photosynthetically active radiation (PAR). The incubation conditions were determined by previous trials (see 2.7.1e) and 5.3.2c)).

After 21 days in culture, the 30 plantlets were removed from the test tubes under sterile conditions and all the transferable shoots were cut off and transferred to test tubes containing 10 ml of either rooting medium or a control medium. The rooting medium consisted of ½ MS medium supplemented with 1.5% sucrose as well as 1.5 mg/L IBA and solidified with 3 g/L Gelrite™. The control medium was identical to the medium plantlets were first established on (½ MS medium containing no growth regulators, supplemented with 3% sucrose and solidified with 3 g/L Gelrite™). Each treatment consisted of 11 replicates (the 30

cultures initially established yielded only 22 transferable microshoots). Cultures were returned to the same incubation conditions described for establishment, and rooting was monitored on a weekly basis for a period of 6 weeks after transplanting (since rooting in our other trials (see 4.6.1a) only occurred after 3 weeks). A value of 1 was assigned to replicates showing any degree of rooting and 0 to those not showing signs of rooting. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test using Statistical Analysis System (SAS) (Cary, 2004).

4.4.2 Results and Discussion

After 7 days in culture roots became visible on 27.3% of the transferred microshoots in the rooting medium. By the end of week 6, no further rooting was observed in either of the treatments. The combined effect of the lowered sucrose concentration and addition of IBA to the growth medium seemed to promote *in vitro* rooting to a degree. The difference in rooting between the treatments was however not statistically significant (Table 4.1). Only one concentration of IBA was tested due to the limited number of transferable microshoots obtained, and higher concentrations of IBA may have a more pronounced effect on rooting.

From the combined results of this trial and the trial in 4.6.1a) it would seem that cultures that do not show rooting within 6 weeks after transplanting onto a rooting medium, will fail to form *in vitro* roots altogether.

Table 4.1: The combined effect of lowered sucrose and IBA on in vitro rooting of *Athrixia phylcoides*

Growth medium	Mean number of microshoots rooted after 6 weeks	Percentage of microshoots rooted after 6 weeks
Control	0.00 a*	0%
Rooting medium (50% less sucrose and 1.5 mg/L IBA)	0.27 a	27.3%

*Values that are not significantly different at 5% probability level are indicated by the same letter

4.5 Effect of GA_3 in the establishment medium on *in vitro* rooting

Gibberellins such as GA_3 (gibberellic acid) are very seldom used in *in vitro* culture of higher plants (Pierik, 1987). This group of compounds generally induce elongation of internodes or may be used to break dormancy in seeds or embryos (Pierik, 1987). The presence of GA_3 in the current growth medium usually inhibits adventitious root formation (George, 1993; Pierik,

1987). However, including GA_3 in a medium used in a previous growth stage (e.g. establishment or multiplication stage), has been reported to promote rooting in a later stage, in medium free from GA_3, in various plants (George, 1993).

We conducted a trial in which GA_3 was included in the establishment medium, but not in the multiplication medium, to investigate its effect on *in vitro* rooting of bush tea. Literature on the use on gibberellins in *in vitro* culture is scarce. Another motivation for testing the effect of GA_3 in the case of bush tea, was the hyperhydricity problems we encountered throughout our trials, and the potential of using this hormone to reverse such symptoms (see 5.4).

4.5.1 Materials and Methods

Plantlets were surface sterilised and established according to the techniques described in 2.7.1, on either ½ MS medium supplemented with 3% sucrose and solidified with 3 g/L Gelrite™(control) or on the same medium supplemented with an additional 1 mg/L GA_3 (treatment). The concentration of GA_3 used in this experiment was chosen because of its success in reversing hyperhydricity symptoms on *Dianthus caryophyllus* L. (Jain, Kanita & Kothari; 2001). Hyperhydricity on bush tea cultures was one of the problems we were continuously faced with (see 5.3.1a). Each treatment consisted of 20 cultures that were incubated in a growth room under the same growth conditions described in 2.7.1e).

After 21 days in culture, the growth of newly emerged axillary shoots had slowed down and cultures started to show symptoms of hyperhydricity. The plantlets established on both the control medium and GA_3-supplemented medium, were removed from the test tubes under sterile conditions. All the transferable microshoots were cut off and transferred to test tubes containing 10 ml growth regulator (GR) free medium identical to the control medium previously described. The 20 cultures established on control medium yielded 32 transferable microshoots and those on GA_3-supplemented medium yielded 23. Cultures were returned to the same growth conditions they were established in (see 2.7.1e).

Rooting was monitored for 6 weeks after transfer. A value of 1 was assigned to plantlets showing any degree of rooting and 0 to those not showing signs of rooting. Data were collected and analysed. No analysis of variance (ANOVA) was carried out in this case.

4.5.2 Results and Discussion

By the end of Week 6, microshoots obtained from the cultures established on GA_3-supplemented medium showed 65.2% rooting compared to 56.3% rooting of microshoots obtained from cultures established on control medium (Table 4.2). Based only on rooting percentage, our results correlate with the findings of George (1993) that GA_3 in a previous

medium promotes subsequent rooting in a new medium. However, despite the 8.9% higher rooting percentage, microshoots from cultures established on the GA_3-supplemented medium yielded shorter roots and fewer roots per plant (Figure 4.1 A) than shoots from control medium (Figure 4.1 B). After establishment, these cultures also yielded less transferable shoots (23) than those established on hormone free medium (32), thus showing less vigour in general (Table 4.2). This lack of vigour was only a visual observation and was not supported with data on total root dry mass or root volume. Further trials during which the data on total root mass and volume is recorded may give a better indication of the effect of GA_3 on rooting. Since the effect on rooting in this trial was not statistically significant, and GA_3 was unsuccessful in reversing symptoms of hyperhydricity (see 5.4.2), no further trials on the use of GA_3 were conducted.

Table 4.2: Effect of GA_3 in establishmet medium on shoot production and subsequent rooting of *Athrixia phylcoides* microshoots

Establishment medium	**Percentage rooting after 6 weeks**	**Total number of transferable shoots yielded afer 6 weeks**
GA_3 supplemented medium	65.2%	23
Control medium	56.3%	32

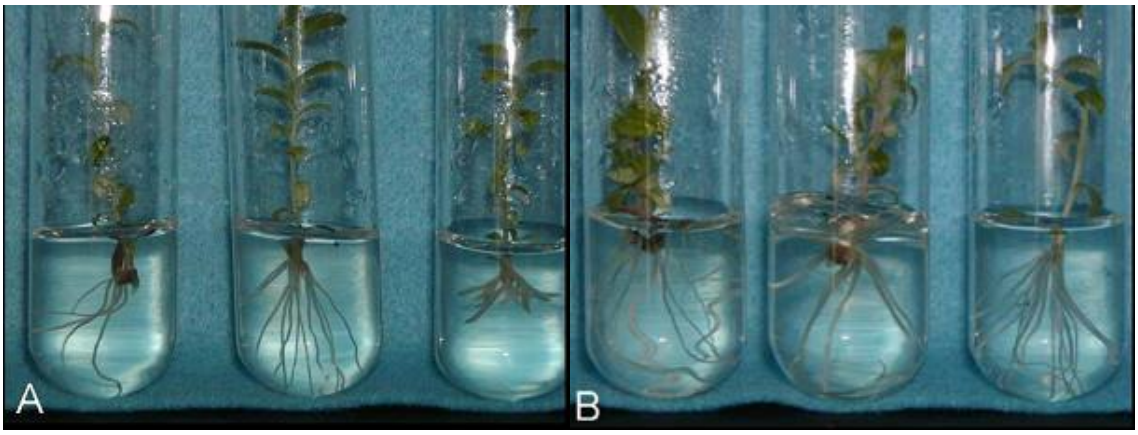

Figure 4.1: In vitro rooting of *Athrixia phylicoides* shoots transferred from a medium containing GA_3 (A) and a hormone free medium (B)

In this experiment, expression of rooting occurred on hormone-free medium, while initiation and induction of roots occurred on either hormone-free medium or medium supplemented

with 1mg/L GA_3. These results contradict our findings in the previous rooting experiment (see 4.4) where no rooting occurred on hormone free medium and under the same incubation conditions (Table 4.1). In this case, rooting occurred spontaneously after transfer to a medium containing no growth regulators, and after the first subculture following establishment. Rooting after transfer to a hormone free medium has been confirmed by George (1996) on various plant species. It also correlates with the findings of Ben-Jaacov & Langhans (1972); Kaul *et al.* (1990) and Hartmann *et al.* (1997) that, in some species, rooting will only occur after sufficient elongation. Rooting as a result of rejuvenation induced by repeated subculture (Pierik, 1987) as mentioned earlier, is unlikely in this case since it occurred after the first subculture that followed establishment.

A possible explanation for the apparent spontaneous rooting during this trial (and failure to root in previous trials, see 4.4) may lie in the seasonal changes in the chemical composition of stock plants. A study by Mudau *et al.* (2006) indicated that the polyphenol content of bush tea leaves differed significantly from season to season. A similar study on the internal hormone levels of leaves may indicate seasons that are more favourable for rooting.

4.6 Transfer of *in vitro* grown plantlets to external environment

The final stage of *in vitro* propagation is the transfer of *in vitro* grown plantlets to external conditions. After successful establishment, multiplication and rooting of *A. phylicoides*, this was our last challenge. If bush tea is to be grown on commercial scale, as a health tea, microplants will have to be transferred to a greenhouse until they reach a proper size for transplanting to an open field. In order to survive greenhouse, and ultimately field conditions, microplants need to adapt to their new environment by gradual exposure to new growth conditions (see 4.1).

According to Hartmann *et al.* (1997), success in this stage depends on three key aspects: induction of rooting, shoot and root acclimatisation and physiological hardening of tissue. Physiological hardening can be achieved *in vitro* by increasing light intensity, reducing the temperature to 19ºC and increasing the sucrose concentration in the medium to encourage lignification (Hartmann *et al.*, 1990, Hartmann *et al.*, 1997). After transfer to *ex vitro* conditions, plants should be kept at very high humidity and shoots should be actively growing (Hartmann *et al.*, 1990; Hartmann *et al.*, 1997).

Plantlets are normally transferred to a high humidity, shaded greenhouse, under mist or fog (Hartmann *et al.*, 1990; Debergh & Read, 1991; George, 1996). Agar is washed from plantlets to prevent contamination and the plantlets are planted with or without roots in soil, sand or other growth mixture. Shoot and root acclimatisation involve the formation of new

shoots, leaves and roots after transfer. Light intensity is gradually increased, and humidity is gradually decreased. Newly formed roots will be functional in the new soil-like medium (Hartmann *et al.*, 1990) and newly formed leaves will be better adapted to photosynthesise (Preece & Sutter, 1991).

The sensitivity of plants to environmental changes varies considerably between species. Jain *et al.* (2001) successfully acclimatised rooted plantlets of *Dianthus caryophyllus* by planting them into pots covered with polyethylene bags, and moving them directly to field conditions, while rooted plantlets of *Clerodendrum colebrookianum* (medicinal plant) required a minimum of 2 weeks in a mist tunnel, before they could be transferred to greenhouse conditions successfully (Mao *et al.*, 1995). *Bowiea volubilis* plantlets required an incubation period of 4 weeks at 24–38ºC for hardening prior to transfer to field conditions (Jha and Sen, 1985). Avato *et al*. (2005) acclimatized microprpagated plantlets of *Salvia officinalis* (medicinal herb) by transplanting them into pots filled with nutrient enriched peat mixture and moving them to greenhouse conditions at 18ºC on a misting-bed system where humidity level were reduced from 90% to 60% over 3 weeks.

Since our microscopical investigations indicated that the stomata of *in vitro* propagated bush tea plantlets were unresponsive (see 6.5), great water losses were expected after removing plantlets from their *in vitro* environment. Furthermore, plantlets that show symptoms of hyperhydricity are more difficult to acclimatise to outdoor conditions (Strycharz & Shetty, 2002). This can be attributed to the abnormalities such as irregular epidermal tissue, thin cell walls, thin cuticle or no cuticle and abnormal stomata that are often associated with hyperhydric plants (Picoli, Otoni, Figueira, Carolino, Almeida, Silva, Carvalho & Fontes, 2001). Since hyperhydricity was a common problem throughout our trials, this could pose further problems during acclimatisation.

We aimed to find a protocol for the gradual exposure of rooted bush tea plantlets to external conditions, especially in terms of RH, during which water losses would be minimised.

4.6.1 Materials and Methods

a) Preparation of plant material

A. phylicoides stock plants were pre-treated with systemic fungicide (see 2.8). Shoots were harvested from various stock plants and cut into pieces of approximately 10 cm. Leaf blades were partly removed (see 2.6 and 2.7) before the plant material was surface sterilised and initiated using the procedure described in 2.7.1a). Fifty nodal cuttings were established on hormone free ½ MS medium supplemented with 3% sucrose and solidified with 3 g/L

Gelrite™. Medium was prepared according to the method described in 2.7.1d). Plants were incubated in a growth room at conditions described in 2.7.1e). After 28 days in culture, all the transferable microshoots were subcultured to fresh medium with the same composition as the medium they were established on. A total of 85 microshoots were subcultured. The first rooting occurred 20 days after subculturing to the fresh medium. Of the 85 shoots, a total of 37 plantlets rooted after 40 days. The remaining unrooted shoots became brown and wilted and were discarded.

b) Ex vitro growth environment

The 37 rooted plantlets were moved to a mist bed in a greenhouse inside closed culture vessels. The greenhouse roof and walls were constructed of 6 mm semi-transparent, corrugated fibreglass panels with a 30% shade net fixed below the ventilated roof. There were no lights inside the greenhouse, and the light intensity inside the greenhouse was determined by the natural light from outside, filtered through the 30% shade net and fibreglass panels. The temperature inside the greenhouse was regulated only by natural ventilation and a fogging system. Temperatures recorded in the first week of the trial ranged between a minimum of 17ºC (night) and 28ºC (day).

c) Proposed acclimatisation process

Plantlets were moved to the greenhouse and placed on the mist bed in the approximate position where they would be planted, in closed test tubes, for a period of 7 days. The RH close to the sand-surface was regulated at 70%-80% by mist sprinklers spraying intermittently for 15 sec every 2 min. The caps of test tubes were loosened for 5 days (at this point trials were terminated due to severe wilting that resulted in death of plantlets and the remaining part of the protocol described could not be carried out). After this, caps of test tubes would be removed completely for another 5 days. Plantlets would then be removed from test tubes and divided into two treatments. In the first treatment, roots would be removed from plantlets before planting. In the second treatment, agar would be washed from roots, and rooted plantlets planted into the silica sand in mist-beds. The rate of survival would be compared between the two treatments.

4.6.2 Results and Discussion

The trial was terminated 10 days after plantlets were transferred to the greenhouse. Plants in test tubes showed severe wilting after the initial 7 day period (after being moved to the mist bed in closed test tubes) and died within 3 days after the caps of test tubes were loosened.

Wilting due to water loss, even though stomata were unable to respond to water loss, is unlikely in this case. Since the test tubes remained closed for the first 7 days, the RH inside

the culture vessels should have remained close to 90% and therefore did not differ drastically from conditions in the growth room.

According to Griffin, Hennen & Oglesby (1983), *in vitro* grown leaves resemble shade leaves and will become necrotic or scorched in too high light intensities. The semi-transparent fibreglass panels on the roof together with the 30% shade net reduced the light intensity inside the greenhouse significantly compared to natural light outside and damage caused by too high light intensities is therefore also unlikely.

Night temperatures in the greenhouse dropped to 17ºC compared to the continuous temperatures of 30°C ± 2 in growth rooms. Night temperatures were approximately 8ºC cooler than minimum temperatures in growth rooms. Even though field grown bush tea can tolerate much lower temperatures in its natural environment, the sudden variation in temperature may have caused the more sensitive *in vitro* grown plantlets to wilt. In most of the literature we cited, the acclimatisation protocols are either poorly documented or omitted. No literature could be found to support the theory that lower night temperatures could have caused death of plantlets.

Another possible explanation for the death of explants was hyperhydricity. Even though no visual symptoms of hyperhydricity were present on the plantlets used in this trial, plants may have been in a less severe, undetected state of hyperhydricity. This would make them exceptionally vulnerable to environmental changes (Picoli *et al*., 2001; Strycharz & Shetty, 2002).

Another likely cause of failure in this trial is physiological and biochemical changes in the stock plants, preceding flowering, and dormancy. Plant material used to initiate the cultures used in this trial was collected shortly before stock plants started flowering. In trials on *Salvia officinalis,* Avato *et al.* (2005) concluded that cultures keep the age of maternal tissue in spite of the physiological stage of their own development. Seasonal biochemical changes in *A. phylicoides* have also been confirmed by Mudau *et al*. (2006) and Chabeli, Mudau, Mashela and Soundy (2008). Even though cultures were established from material collected before flowering, the physiological and biochemical changes that precede dormancy in stock plants may have already been present in material used to establish cultures in this trial. According to Hartmann *et al.* (1997) shoots transferred to *ex vitro* environment should be actively growing in order to achieve successful acclimatisation. The sudden deterioration of plantlets after moving them to the mist bed, may have been a result of commencing dormancy.

Shortly after the failure of this trial, stock plants started to flower. Plants died back after flowering season and remained dormant throughout the winter. Due to this, no plant material was available to conduct more trials in the available time. Further acclimatisation trials, in a phytotron environment where light intensity and temperature can be closely regulated and gradually adjusted to resemble field conditions, may be useful to clarify the possible effects of low night temperatures and too high light intensities on deterioration of plantlets after transfer to *ex vitro* conditions. Also, repetition of this trial with plantlets that are definitely free from hyperhydricity and at a time when stock plants are actively growing, will clarify the possible effects that hyperhydricity, or the physiological stage of the mother plants could have had on rapid plantlet deterioration.

After establishing a protocol for successfully moving plantlets to field conditions, the acclimatized plants will need to be monitored for any abnormalities for a few seasons. Their capacity to produce secondary metabolites need to be established and the chemical composition of essential oils produced by leaves need to be determined and compared to that of field grown plants in order to evaluate the success of this propagation method.

4.7 Conclusions

The combined effect of lowering sucrose levels and the addition of IBA (1.5 mg/L) to medium promoted rooting to some extent. The control medium in this trial yielded no rooting. Only one concentration of IBA was tested due to the limited number of axillary microshoots available, and higher concentrations of IBA may have a more pronounced effect on rooting.

The presence of GA_3 in the establishment medium did not promote rooting as reported by George (1993). In this experiment, rooting occurred in both treatments, after transfer to hormone free medium. The hormone free medium was identical to the control medium used in the IBA / lowered sucrose experiment that previously yielded no rooting.

Since rooting in both of the trials revered to above, was attempted on the same growth medium, in the same incubation conditions, and over the same period of time, the difference in rooting response may be a result of the stock plants used to establish cultures. It is likely that the plant material for these trials were collected from different mother plants. The *in vitro* rooting ability of plants is determined, amongst other factors, by characteristics of the mother plant (Pierik, 1987; George, 1993; Rout *et al*., 2000) and may also be influenced by levels of endogenous hormones that are , in some cases, produced by cultures (Pierik, 1987).

Our attempt at the acclimatisation of bush tea plantlets was not successful. The initial part of the trial indicated that plantlets were probably sensitive to changes in their growth

environment, but further trials in a more controlled environment will have to be done to confirm this theory. Furthermore, a method to successfully prevent hyperhydricity in plantlets need to be established in order to ensure that healthy plantlets are available for further trials on acclimatisation. Our attempts to find ways to prevent or reverse hyperhydricity so far, have been insufficient (see CHAPTER 5).

The failure of our acclimatisation trial may also have been attributed to the fact that stock plants were approaching flowering, followed by dormancy, around the time cultures were established. A repetition of this acclimatisation trial with cultures that were established at a time when stock plants were actively growing will indicate the effect of physiological stage of the stock plants, at the time of establishing cultures, on acclimatisation.

CHAPTER 5

HYPERHYDRICITY (VITRIFICATION)

5.1 Scope of this chapter

Throughout our trials on the *in vitro* propagation of *Athrixia phylicoides* (bush tea), we were faced with the unpredictable occurrence of hyperhydricity, and in various degrees. In this chapter we aimed to identify possible causes of hyperhydricity by investigating the effect of the growth regulators IBA (indole-3-butyric acid) and BAP (6-benzylaminopurine) in the growth medium, the presence of leaves on explants and the effect of growth room temperatures. We also investigated the potential effect of GA_3 (gibberellic acid) on reversing of hyperhydricity symptoms.

5.2 Literature review

During the *in vitro* propagation of *A. phylicoides* we were faced with the problem of hyperhydricity (also known as vitrification) - a physiological disorder causing shoots and leaves to become brittle with a glassy, waterlogged appearance in various degrees (Figure 5.1). Other terms that have been used to describe this condition are wet, vitreous, translucent and succulent (Ueno, Cheplick & Shetty, 1998; Piqueras, Cortina, Serena & Casas, 2002). The term vitrification literally means 'to turn into glass or a glasslike substance'. Debergh, Aitken-Christie, Cohen, Grout, Von Arnold, Zimmerman & Ziv (1992) suggested the term 'hyperhydricity' as a more correct term to describe this condition.

According to literature, plants that were grown *in vitro* often show abnormal anatomical, morphological or physiological characteristics, when compared to plants grown in an external environment (Picoli, Otoni, Figueira, Carolino, Almeida, Silva, Carvalho & Fontes, 2001; Giusti, Vitti, Fiocchetti, Colla, Saccardo & Tucci, 2002). Piqueras *et al.* (2002) attribute these changes to the high relative humidity, accumulation of ethylene and poor gaseous exchange, typically associated with the inside of culture vessels. Various abnormalities in micropropagated plants result in poor regeneration and decrease the survival rate of affected plants (Kevers, Coumans, Coumans-Gilles & Gaspar, 1984). Hyperhydricity, in particular, has contributed losses of up to 60% in commercial laboratories (Piqueras *et al.*, 2002).

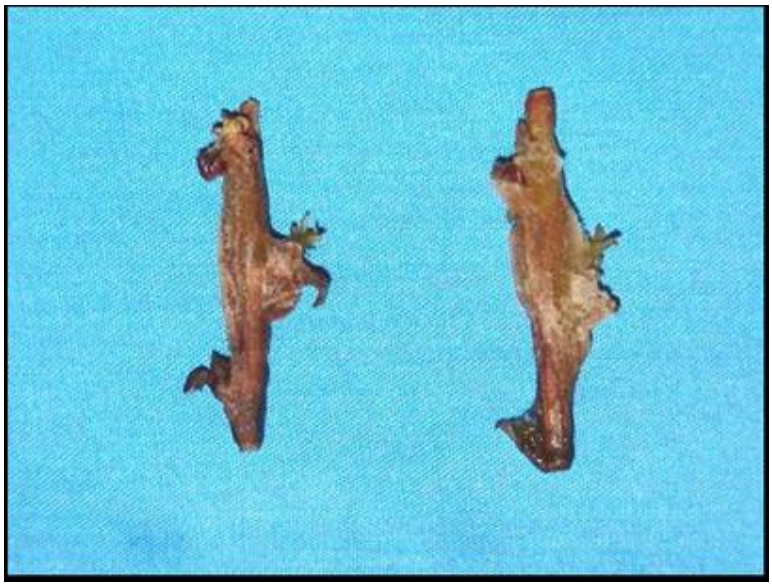

Figure 5.1: Severe symptoms of hyperhydricity on *Athrixia phylicoides* explants

Hyperhydrated plants show physiological, anatomical and morphological changes compared to normal plants. These include reduced levels of lignification in tracheids and vessels, decreased cellulose levels, thin or no cuticles, abnormal stomata, chlorophyll deficiencies, reduced number of palisade cell layers, irregular epidermal tissue, large intercellular spaces in the mesophyll cell layer, thin cell walls and reduced vascular tissue (Kevers *et al.*, 1984, John, 1986; George, 1993; Ueno *et al.*, 1998; Picoli *et al*., 2001). As a result of these abnormalities, the success rate in acclimatising hyperhydric plants to *ex vitro* environments is very low (Picoli *et al*., 2001; Strycharz & Shetty, 2002).

In S*itka spurce* and *Dianthus caryophyllus* cultures, hyperhydricity resulted in loss of apical dominance, followed by enhanced axillary and adventitious growth (John, 1986; Werker & Leshem, 1987). Other side effects are oxidative stress, mineral deficiencies and alteration of some biosynthetic processes (Piqueras *et al.*, 2002). Alteration of biosynthetic processes, as a result of hyperhydricity, even after reversal of symptoms, is a concern in the case of *A. phylicoides* as this may alter the plant's capacity to produce secondary metabolites and result in the loss of its medicinal properties.

Although some authors have reported successful reversion of the symptoms by treatments such as bottom cooling on carnation (Piqueras *et al.*, 2002), cool storage of cultures at 3-4ºC (Rugini & Verma, 1983) the use of bactopeptone and GA_3 (gibberellic acid) on *Dianthus* (Sato, Hagimori & Iwai 1993; Jain, Kanita & Kothari. 2001) and consecutive subcultures to fresh medium (Teixeira da Silva, 2003), the symptoms of hyperhydricity are in many cases non-reversible. John (1986) recorded spontaneous reverting between normal and

hyperhydrated cultures of *Sitka spruce*, both from normal to hyperhydrated and back to normal again.

5.3 Possible causes of hyperhydricity

Many factors that may induce hyperhydricity have been identified. These include incubation conditions such as low light intensity, high temperatures, the use of cytokinins - especially BAP (6-benzylaminopurine), the presence of GA_3 in culture medium, too high concentrations of micro and macronutrients, high relative humidity, the use of activated charcoal, poor gaseous exchange and vessel aeration and the type and concentration of gelling agent used (Upadhyay, Arumugam & Bhojwani, 1989; George, 1993; Picoli *et al*., 2001; Giusti *et al.*, 2002; Avato, Fortunato, Ruta & D'Elia, 2005). High relative humidity has been implied as one of the most important environmental causal factors (George, 1993; Jain *et al.*, 2001; Piqueras, 2002). The occurrence of hyperhydricity has to a greater or lesser extent been controlled by avoiding the above conditions.

Another promising method to control hyperhydricity is the inoculation of cultures with certain naturally occurring soil bacteria such as *Pseudomonas* spp. Ueno & Shetty (1997) successfully reduced hyperhydricity on oregano (*Origanum vulgare*) by inoculating cultures with polysaccharide-producing *Pseudomonas* spp. Following the promising results on oregano, the technique has also been applied to prevent hyperhydricity on anise (*Pimpinella anisum*) (Bela, Ueno & Shetty, 1998), four commercial clonal lines of raspberry (*Rubus* sp.) (Ueno *et al*., 1998), and more clonal lines of oregano (Stykhaz & Shetty, 2002). The effect of the *Pseudomonas* spp. in the control of hyperhydricity was further demonstrated by restoring hyperhydricity to non-hyperhydrated oregano cultures by the addition of antibiotics to remove *Pseudomonas* from cultures (Perry, Ueno & Shetty, 1999).

The reported effects of BAP in growth media on hyperhydricity seem to be species dependant. Picoli *et al*. (2001) found no correlation between the occurrence of hyperhydricity in eggplant and concentration of BAP in growth medium. Jain *et al*. (2001) reported that BAP at concentrations of 0.5 or 1 mg/L in medium promoted differentiation of vitrified shoots in carnation (*Dianthus caryophyllus*). In contrast to this, Leshem (1986) reported that BAP in medium reduced shoot tips that developed into vitrified plantlets on carnation. In melon, hyperhydricity was induced by BAP, in a dosage-dependent manner (Leshem, Shaley & Izhar, 1988).

The use of low concentrations of gelling agents in the growth media has often been implicated as one of the causal factors of hyperhydricity. Symtoms of hyperhydricity have been recorded on *Dianthus caryophyllus* (Olmos & Hellín, 1998; Piqueras *et al*. 2002),

Origanum vulgare (Strycharz & Shetty, 2002), and *Solanum melongena* (Picoli *et al.,* 2001), all cultured on MS media solidified with agar at concentrations between 3 g/L - 0.9 g/L. Debergh (1983) stated that hyperhydricity of cultures, in some cases, can be reduced by increasing the agar concentration in medium, thereby changing the water relations *in vitro*. In agreement to this, Kim *et al*. (1988) reported that the use of high concentration of agar (greater than 15 g/L) in the medium, reduced the frequency and extent of hyperhydricity in carnation plantlets.

The effect of the type of gelling agents used in culture media differs for plant species and symptoms of hyperhydricity has been reported on growth media containing various types of gelling agents. Franck *et al*. (2004) induced hyperhydricity of *Prunus avium* shoots by replacing agar as gelling agent by Gelrite™ into the culture medium. According to Franck *et al*. (2004), the physical structure of Gelrite™ appears to allow an increased absorption of substances suspected to be responsible of hyperhydricity. However, symptoms of hyperhydricity also commonly occur on plants cultured in media solidified with agar (Olmos & Hellín, 1998; Picoli *et al.* 2001; Piqueras *et al*. 2002; Strycharz & Shetty, 2002).

Two kinds of hyperhydricity exist according to Pâques (1991). The first is a result of passive diffusion of water into tissue and the second is an active process as a result of a disturbance in the metabolic processes of the plant. The reported induction of hyperhydricity in cultured plants by submergence supports the theory of passive diffusion (John, 1986). Hyperhydricity is often limited to, or most severe in the parts of the explants touching the growth medium (George, 1993).

Severe symptoms at the base of explants were observed in some cases during propagation of *A. phylicoides* (Figure 5.2). This implies that a passive diffusion of water into the plant is likely to be, at least partly, responsible for symptoms in this case. Other possible contributing factors were also investigated.

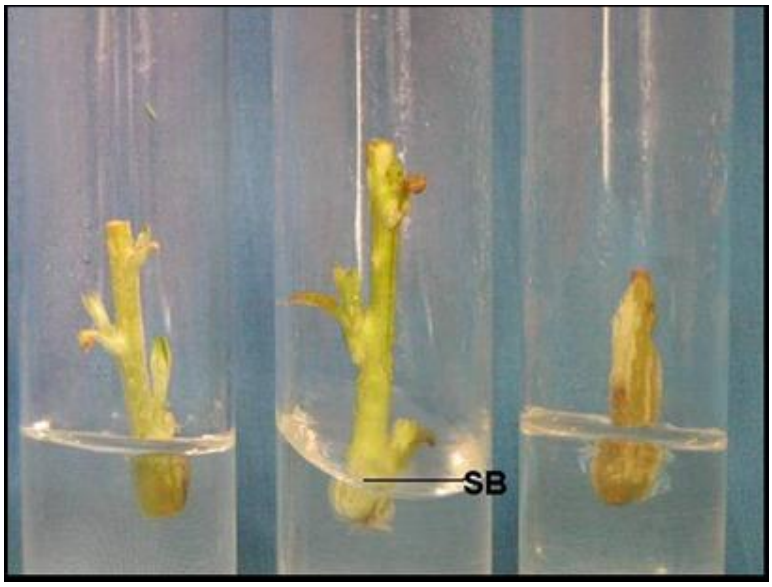

Figure 5.2: Passive diffusion of water into explants of *Athrixia phylicoides* causes swelling at the base (SB) of plants

5.3.1 Materials and Methods

Data from different initiation trials that were completed over a period of 10 months were compared to try to determine the factors that are most likely responsible for hyperhydricity. We compared data on levels of exogenously applied growth regulators, the effect of leaves on explants and growth room temperatures to the occurrence of hyperhydricity in these trials.

a) Growth regulators

Data from 2 trials, initiated within one week from each other, were used to determine the effect of growth regulators on hyperhydricity. The trial layout and treatments for Trials 1 and 2 were identical. Each trial consisted of 4 treatments with 10 replicates in each. The plant material used in each trial was collected from the same stock plant. Trials 1 and 2 were done from two different stock plants (A and B) that appeared similar in vigour, size and growth stage.

Surface sterilisation was done using the procedure described in 2.7.1a). The basic medium used in each treatment was ½ MS medium (Murashige & Skoog, 1962) supplemented with 3% sucrose, solidified with Gelrite™ 3 g/L and one of four growth regulator combinations viz. 1.5 mg/L IBA (indole-3-butyric acid), 1.5 mg/L BAP (6-benzylaminopurine), both IBA and BAP at 1.5 mg/L and a control treatment containing no growth regulators.

Plants were kept in a growth room with a 8h dark: 16h light cycle under cool white fluorescent lights providing 60 µmol/m^2/sec photosynthetically active radiation (PAR) at 30ºC

± 2. Data on hyperhydricity symptoms were collected 15 days after initiation. A value of 1 was allocated if plants showed symptoms of hyperhydricity, while 0 was allocated for those not showing symptoms. The percentage occurrence of hyperhydricity in each treatment was calculated and graphed using Microsoft Excel.

b) Leaves on explants

This trial consisted of 2 treatments with 5 replicates each. In Treatment 1, leaves were abscised 3 mm from the petiole (see 1.1) prior to sterilisation while the leaves in Treatment 2 were left on the plants. The sterilisation procedure described in 2.7.1a) was used to sterilise plant material. The growth medium used was ½ MS supplemented with 3% sucrose and no growth regulators. Plants were kept in a growth room at the same growth conditions described in 2.7.1e). Data on hyperhydricity symptoms were collected 14 days after initiation. A value of 1 was allocated if plants showed symptoms of hyperhydricity while 0 was allocated for those not showing symptoms. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test using Statistical Analysis System (SAS) (Cary, 2004).

c) Growth room temperature

The trial consisted of 2 temperature treatments with 20 replicates each. Plants were surface sterilised and initiated according to the procedure described in 2.7.1. The growth medium used was ½ MS supplemented with 3% sucrose and no growth regulators. Plants were kept in two different growth rooms both with 8h dark: 16h light cycle under cool white fluorescent lights providing 60 µmol/m^2/sec PAR. One room was kept at 26ºC ± 2 (Treatment 1) and the other at 30ºC ± 2 (Treatment 2). Data on hyperhydricity symptoms were collected 15 days after initiation. A value of 1 was allocated to plants that showed symptoms of hyperhydricity while 0 was allocated to those not showing symptoms. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test using SAS (Cary, 2004).

5.3.2 Results and Discussion

a) Growth regulators

The effect of growth regulators (GRs) on hyperhydricity were inconsistent (Figure 5.3). Symptoms of hyperhydricity appeared in all treatments including the control. Plants became water soaked, swollen and brittle in various degrees. In both trials the occurrence of hyperhydricity was higher in the IBA treatment than the BAP treatment (3% and 13% higher

in Trials 1 and 2, respectively). In the first trial the control plants showed a relatively low percentage (30%) of hyperhydricity, while 100% hyperhydricity was recorded for control plants in the second trial. This was significantly higher than both the combined IBA /BAP treatment and the BAP treatment. In both trials the IBA and BAP combination treatment resulted in a low incidence of hyperhydricity (30% and 11%, respectively).

These results indicated that IBA, more so than BAP, is associated with the occurrence of hyperhydricity. However, IBA in combination with BAP seemed to have the opposite effect. The high occurrence of hyperhydricity in the presence of auxin and the fact that the combined IBA/BAP treatment (therefore the highest total concentration of growth regulators) showed relatively low levels of hyperhydricity is contradictory to the findings in literature that indicate a correlation between high levels of growth regulators and the occurrence of hyperhydricity (George, 1993; Jain *et al.*, 2001). The high occurrence of hyperhydricity (100%) in the control treatment of the second trial also contradicts this.

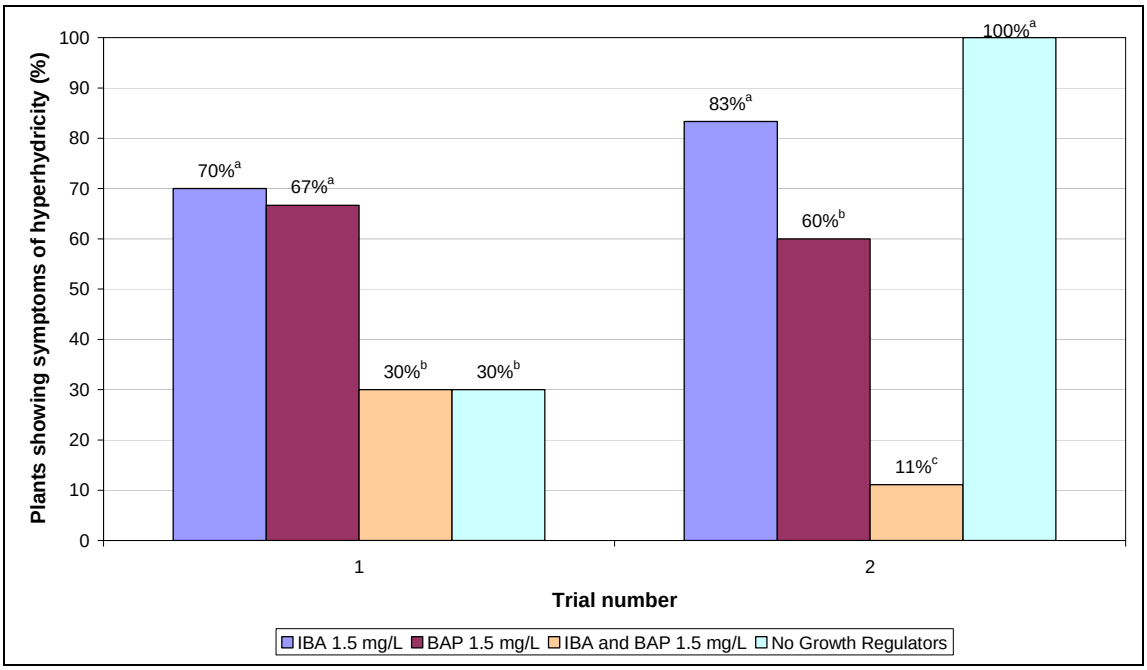

Figure 5.3: Effect of growth regulators on hyperhydricity on *Athrixia phylicoides* explants after 15 days in culture

*Values that are not significantly different at 5% probability level are indicated by the same letter

A possible explanation for our inconsistent results may be differences in endogenous hormone levels produced by explants (Pierik, 1987). The effect of exogenously applied growth regulators may be masked by that of endogenous hormones, in cases where explants produce sufficient levels of hormones. From these trials it would seem that hyperhydricity is

best controlled on medium that contains both IBA and BAP. However, the conflicting results on control medium indicate that the occurrence of hyperhydricity can not be linked exclusively to the levels of exogenously applied growth regulators.

b) Leaves on explants

After 14 days in culture, plants with their leaves still attached showed a slightly higher incidence of hyperhydricity than plants without leaves however, the difference was not statistically significant (Table 5.1). Explants with leaves tended to become more swollen than those without leaves (Figure 5.4). Stock plant leaves present on explants may alter the endogenous hormone levels, raising it compared to those without leaves. In the trials on the effect of exogenous growth regulators (see 3.3 and 3.4) we stated that the endogenous hormone levels present in explants may have masked the effect of exogenously applied growth regulators. The slightly higher incidence of hyperhydricity in plants with leaves supports the theory that endogenous hormone levels may be responsible for hyperhydricity.

Table 5.1:Hyperhydricity symptoms on *Athrixia phylcoides* explants with and without leaves respectively

Treatment	Number of plants showing symptoms of hyperhydricity (mean)	Percentage plants showing symptoms of hyperhydricity (%)
Leaves abscised	0.56[a]*	11.2
Leaves on explants	0.64[a]	12.8

*Values that are not significantly different at 5% probability level are indicated by the same letter

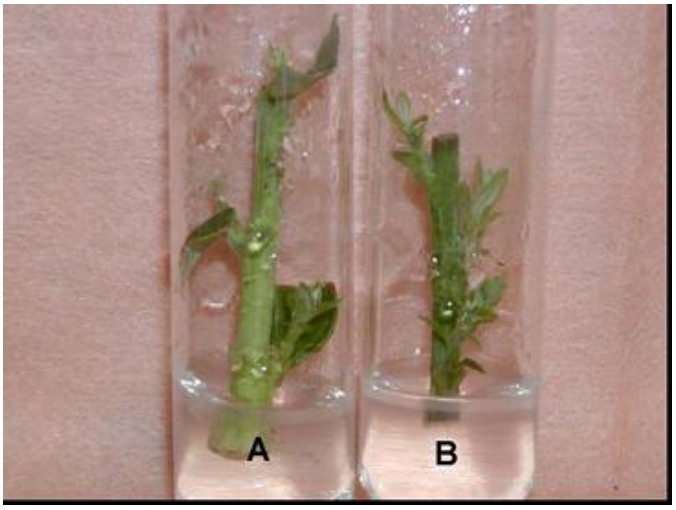

Figure 5.4: Hyperhydricity symptoms on explants with leaves (A) compared to that on explants with no leaves (B)

c) Growth room temperatures

After 15 days in culture, plants kept at 26ºC showed a slightly higher incidence of hyperhydricity than plants kept at 30ºC. However, the difference was not statistically significant (Table 5.2). According to literature high incubation temperatures are often associated with hyperhydricity (George, 1993; Jain *et al.*, 2001; Piqueras, 2002). The difference between treatments in this case is almost negligible (1.25%) and this trial consisted of very few replicates due to a shortage of plant material at the time. A further trial with more replicates is needed to make a reasonable conclusion. From these results it seems unlikely that the incubation temperatures are the only causal factor of hyperhydricity symptoms on bush tea plantlets.

Table 5.2: Hyperhydricity symptoms on *Athrixia phylcoides* explants cultured at 26ºC and 30ºC respectively

Temperature treatment	Mean number of plants showing symptoms of hyperhydricity	Percentage of plants showing symptoms of hyperhydricity (%)
26ºC	0.40[a]*	2
30ºC	0.15[a]	0.75

*Values that are not significantly different at 5% probability level are indicated by the same letter

5.4 Effect of GA_3 on the reversal of hyperhydricity symptoms

Jain *et al.* (2001) successfully reversed the symptoms of hyperhydricity on *Dianthus caryophyllus* by transferring hyperhydrated plantlets to a medium containing 1 mg/L GA_3. We investigated the effect of GA_3 on the reversal of hyperhydricity symptoms on *A. phylicoides* explants.

5.4.1 Materials and Methods

Greenhouse plants used as stock plants were regularly treated with a systemic fungicide to reduce the level of contamination (see 2.8). All shoots were collected from a single stock plant. Shoots were cut into 10 cm pieces and leaves were abscised approximately 3 mm from the petiole using a surgical blade (see 1.1). Plant material was then surface sterilised using the same procedures as described in 2.7.1a). It was then cut into 3 node explants and initiated onto basic medium (10 ml ½ MS medium supplemented with 3% sucrose and solidified with 3 g/L Gelrite™) in 15 cm glass test tubes. Cultures were kept in a growth room at 26°C with a 8h dark: 16h light cycle under cool white fluorescent lights providing 60 µmol/m^2/sec PAR. A total of 50 replicates were initiated.

After 26 days in culture, more than 70% of the 50 initiated explants showed symptoms of hyperhydricity. Data were collected on the hyperhydricity-status of each plant (0 = no symptoms and 1 = hyperhydricity symptoms). Transferable shoots were randomly transferred, either to basic medium (control) or to the basic medium supplemented with an additional 1 mg/L GA_3 (treatment). Altogether 23 shoots were transferred to GA_3-supplemented medium and 28 to control medium. Cultures were incubated under the same conditions as mentioned above. Data on hyperhydricity status of shoots were collected after 8 days. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test using SAS (Cary, 2004).

5.4.2 Results and Discussion

The percentage of explants showing symptoms of hyperhydricity decreased notably 8 days after subculture in both the treatments. There were no significant differences between the treatments (Table 5.3). Since both treatments showed a noticeable (but not statistically different) reduction in hyperhydricity after transplant, the reduction in hyperhydricity seemed to be related to the subculture and transfer to fresh medium rather than the effect of GA_3 in the medium.

Table 5.3: Hyperhydricity symptoms on in vitro propagated *Athrixia phylcoides* explants upon subculture (26 days after establishment) and 8 days after subculture

Medium	Hyperhydricity on day of subculture (%)	Hyperhydricity 8 days after subculture (%)	Reduction in Hyperhydricity (%)
Control	73.9	26.1	47.8[a]
GA_3	75	28.5	46.5[a]

* Values that are not significantly different at 5% probability level are indicated by the same letter

5.5 Conclusions

The cause of hyperhydricity in *A. phylicoides* cultured plants is still unclear. The presence of different combinations of exogenously applied growth regulators at the levels we tested could not be related to the occurrence of hyperhydricity. Our trials on the effect of temperature as a possible cause of hyperhydricity resulted in a slightly higher incidence of hyperhydricity at 26ºC than at 30ºC, and it seems unlikely that temperature alone is the causal factor.

We found a positive correlation between leaves on explants and the occurrence of hyperhydricity symptoms. We hypothesise that the leaves present on explants may alter the levels of endogenous hormones, thereby inducing hyperhydricity. The results did not show a statistically significant difference, and therefore further investigation is needed to conclude the study.

There is an urgent need for further investigations to clarify the causes of hyperhydricity in order to prevent it. Physical ways in which hyperhydricity may prevented in future trials is worth investigating, these include the use of bottom cooling (Piqueras *et al.*, 2002) or aeration of vessels (Piqueras *et al.*, 2002; Lai *et al*., 2005). The use of a different gelling agent such as agar (Franck *et al*., 2004), or higher concentrations of gelling agent (Kim *et al*., 1988) may also alleviate the problem. Due to time limitations no further trials could be conducted.

CHAPTER 6

MICROSCOPICAL INVESTIGATION

6.1 Scope of this chapter

Electron and light microscopic examination of leaves were used to identify and study structures that are apparently involved in the production and secretion of essential oils. The glandular trichomes we identified, were compared between *in vitro* and *ex vitro* grown plants, both morphologically and quantitatively. Morphological differences between leaves of greenhouse and *in vitro* grown plants were made. Part of the research done in this chapter was published in a scientific journal (Möller *et al*., 2006).

6.2 Literature review

Athrixia phylicoides (bush tea, bos tee, daisy tea) is one of many plants from the Asteraceae family used as a traditional herbal medicine (Van Wyk, Van Oudtshoorn & Gericke, 1997). The plant is widely distributed throughout southern Africa (Arnold & De Wet, 1993). However, due to its medicinal use, it is facing the danger of becoming over exploited. The most common preparation for medicinal use is to make an infusion from the leaves and ingest it as a tea. Leaf extracts have been subjected to pharmacological evaluation and have shown anti-inflammatory, antihypertensive, narcotic and analgesic (pain relief) properties (Swanepoel, 1997).

The medicinal properties of plants are often linked to the production of essential oils. Mono-, sesqui-, di- and triterpenes, lactones and flavones are often present in essential oils and have been shown to have an antimicrobial activity towards some organisms (Wagner, 1991). Epicuticular flavonoids have been indicated as medicinally active ingredients in other plants that belong to Asteraceae, such as *Helichrysum aureniotens* (Afolayan & Meyer, 1995), *Helichrysum nitents* (Tomas-Barberan, Iniesta Sanmartan, Tomas-Lorente & Rumbero, 1990) and *Bidens pilosa* (Brandão, Krettli, Soares, Nery & Marinuzzi, 1997). The flavonoids in *H. aureniotens* are believed to be secreted and synthesised by glandular trichomes present on the leaves of the plant (Afolayan & Meyer, 1995). We hypothesised that the medicinal value of *A. phylicoides* could also be linked to essential oils produced by the leaves.

It is often a concern amongst supporters of the use of medicinal plants, that plants propagated under *in vitro* conditions are inferior to field grown plants in terms of their ability or capacity to produce medicinally active secondary products. The *in vitro* production of

secondary metabolites seems to differ between cultivars, and in some cases production is more than field grown plants and in other cases, less (George, 1993).

In the case of *Azadiracha indica* the production of secondary metabolites was higher in *in vitro* propagated plants than in field plants (Srividya, Sridevi & Satyanarayana, 1998). In contrast to this, Socorro, Tárrega & Rivas (1998) reported that micropropagated plants of O*riganum bastetanum* produced less essential oils and the composition of essential oil differed slightly form that of wild plants. Gaspar (1994) reported that *in vitro* grown plantlets of *Chrysanthellum americanum* (a medicinal plant of the *Astaraceae* family) produced a smaller range of flavonoids than field grown plants. A change in the production pattern of secondary metabolites between micropropagated and greenhouse plants has also been reported in *Artemisia alba* by Lamproye, Hofinger, Ramaut & Gaspar (1986). In the case of *Eucalyptus citriodorae* leaves that are harvested for the extraction of oil, leaves from micropropagated plants had the same oil content as the stock plants, but only after being planted in the field for a 6 month period. Oil concentrations in these leaves only reached a stable level after 2 to 3 years (Mascarenhas, Khuspe, Nadguada, Gupta & Khan, 1988).

The differences in secondary metabolite production between micropropagated and field grown plants should not be solely attributed to the different propagation methods used, as other factors such as cultivation period, season of collection, plant to plant variability and levels of nutrition will also affect the accumulation of secondary products in plant, both *in vitro* and in the field (Stafford, Morris & Fowler, 1986; Nalawade & Tsay, 2004).

The influence of environmental factors such as light, temperature and nutrition, on the chemical composition of leaf extracts of *A. phylicoides* has been demonstrated by Mudau, Soundy, Du Toit & Olivier (2006) and Chabeli, Mudau, Mashela & Soundy (2008). They reported that the leaf phenol and tannin contents of *A. phylicoides* varied with season, as well as types and levels of nutrition, specifically nitrogen (N), phosphorus (P) and potassium (K). Avato *et al.* (2005) reported that the composition of essential oil in *S. offincialis* is also influenced by environmental factors such as light and temperature.

Another factor that may contribute to both morphological and physiological differences between micropropagated and field grown plants is rejuvenation of plants in culture. Avato *et al*. (2005) attributed the increase of peltate glandular hairs on micropropagated plants of *Salvia officinalis* to rejuvenation. They also stated that the content of essential oils in *Salvia* changes with the age of the plants. It is therefore likely that the secondary metabolite production of some plant species in which changes have been reported may only resemble that of field grown plants after the acclimatised, micropropagated plants reach maturity,

similar to the reports on *Eucalyptus citriodora* mentioned before (Mascarenhas, Khuspe, Nadguada, Gupta & Khan, 1988).

6.3 Aim of study

The aim of this study was firstly to familiarise ourselves with the ultrastructure and morphology of *A. phylicoides*, in order to identify the structures that are most likely involved in the production and secretion of epicuticular flavonoids (that may contain medicinally active ingredients).

Essential oils are produced by a wide range of plant species. These oils are mostly produced and stored in specialised structures on the surfaces of plants or in the plant tissue (Svoboda, Hampson & Hunter, 1998). With the long-term aim at commercial scale propagation of *A. phylicoides*, an understanding of the mechanisms involved in the production of medicinally active products will be of great value.

To our knowledge, no literature other than our own publication (Möller *et al*., 2006) is available on the morphology of glandular structures on leaves of *A. phylicoides*. However, literature on other species of *Athrixia* indicated that *A. arachnoidea* has stalked glands present on the margins of leaves. *A. elata* has also shown the presence of glandular hairs on leaf margins and *A. fontana* has long, gland tipped hairs present on the lower surfaces of leaves (Retief & Herman, 1997).

The second objective of our study was to compare these glandular structures on *in vitro* and *ex vitro* grown plants morphologically and quantitatively. Changes in the morphology of these structures may be indicative of an inability to produce secondary products. A decrease in the number of glandular structures is likely to be related to a decrease in the quantities of active ingredients produced.

In the case of medicinal plants, it is essential that the medicinal properties of the plant are not altered by the method of propagation. By comparing the morphology and ultrastructure of plants that have been grown in their natural environment to that of plants grown *in vitro* we can get an indication of the economical possibilities of propagating this plant *in vitro* on a commercial scale.

6.4 Structures involved in secretion of essential oils

6.4.1 Materials and Methods

a) Plant material

Plants used in this study were collected from the field in the Thohoyandou district of Venda in the Limpopo Province, South Africa. Plants were kept in a temperature-controlled greenhouse, where minimum temperatures were kept above 10ºC and maximum temperatures below 26ºC, under natural day/night conditions for 5 months. Three leaf samples of three different ages were collected from stock plants for examination under the light and scanning electron microscopes. Sampled leaves were grouped into one of three age groups based on the time since emersion: Young leaves (younger than one month), middle age (between 1 and 3 months old) and old leaves (between 6 and 9 months). Sampled leaves were removed from the explants less than one hour before preparation started.

b) Preparation for scanning electron and light microscopy

The first steps in preparation for scanning electron microscopy (S.E.M) and light microscopy were similar. Sections from each of the sampled leaves were fixed in 2.5% glutaraldehyde in 0.75 M phosphate buffer at pH 7.5 for 1 h at room temperature. Plant material was rinsed 3 times for 5 minutes, at a time, in 0.075 M phosphate buffer, followed by fixation in 0.25% aqueous osmium tetroxide for 1 h. Leaf sections were removed from the solutions and rinsed with distilled water 3 times for 5 minutes at a time. They were then gradually dehydrated in a series of 30%, 50%, 70%, 90% and 100% acetone respectively for 5 minutes each. After this step, sections to be used for light microscopy and S.E.M were separated.

Sections for S.E.M were dried to critical point in liquid CO_2, mounted and sputtered with gold. Specimens were examined with a JSM 840 scanning electron microscope.

Sections used for light microscopy were infiltrated with 30% quetol, followed by infiltration with 60% quetol in ethanol for 1h. They were then infiltrated with 100% quetol for 4h and polymerised at 65ºC for 24 h. Ultra-thin sections were cut using a Reichert-Jung Ultracut E™ microtome. These sections we placed on glass microscope slides and stained with toluidine blue.

To determine the distribution of trichomes on leaves, S.E.M photos of 200 x enlargement were used. For both *in vitro* (Treatment 1) and greenhouse (Treatment 2) grown plants,

photos were taken at random positions on different leaf surfaces. Three photos were taken adjacent to the central vein and another three photos approximately halfway between the central vein and leaf margin for each treatment (thus, 6 photos per treatment). The number of glands on each photo was counted and recorded for the different ages and positions on the leaf surfaces. Analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test using Statistical Analysis System (SAS) (Cary, 2004).

6.4.2 Results and Discussion

We observed that mature leaves of *A. phylicoides* are more aromatic than juvenile leaves. We also noted that handling of plant material promote the secretion of aromatic oil. These observations suggest the presence of glandular structures on or near the leaf surface, which are involved in accumulation and/or secretion of essential oils, especially in mature plant material.

This was confirmed by a S.E.M. study of the leaves of *A. phylicoides* that showed the presence of at least two types of trichomes on the surfaces of leaves, one of which appears to be glandular and the other nonglandular. No glandular trichomes were present on the adaxial surfaces of leaves, while nonglandular trichomes were present, but in very small numbers (A). On the abaxial surfaces of leaves, glandular trichomes were scarce and were distributed randomly across the leaf surface with a higher density near the central vein of the leaf (Table 6.1). Nonglandular trichomes were abundant and they cover the leaf surface almost entirely giving them a hairy appearance (Figure 6.1).

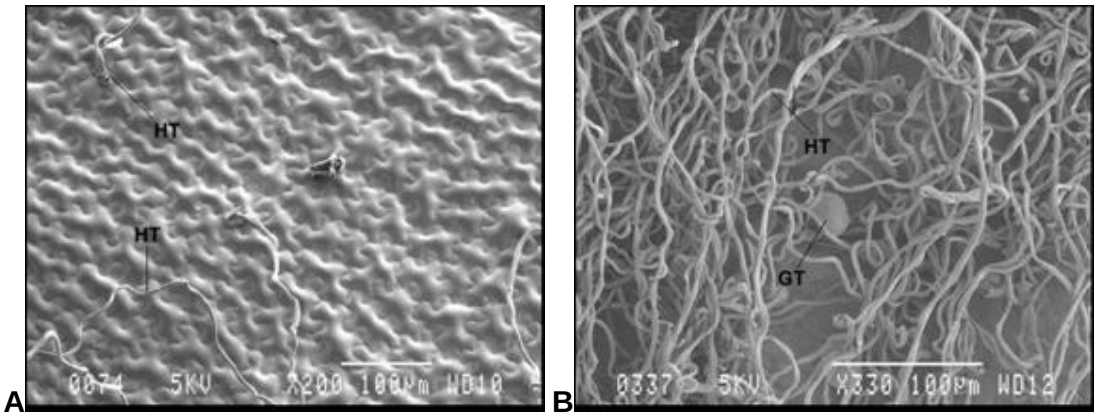

Figure 6.1: S.E.M image of surfaces of young leaves of *Athrixia phylicoides*: A) the adaxial surface showing the presence of a small number of nonglandular hairy trichomes (HT) and B) the abaxial surface covered by abundant numbers of nonglandular, hairy trichomes (notice the presence of the glandular trichome (GT)

Table 6.1: Distribution of glandular trichomes on the leaf surfaces of *Athrixia phylcoides*

Number of glandular trichomes / mm^2	
Area adjacent to central vein	**Middle of leaf surface**
7.14^a	5.28^b

* Values that are not significantly different at 5% probability level are indicated by the same letter

A mature nonglandular trichome consists of 2 cells, a basal cell containing a single nucleus and an elongated apical cell (Figure 6.2). On young leaves, the apical cell appears bulbous at its base and both the basal and apical cells were alive (Figure 6.3 A). On older leaves the basal cell remains alive while the greatly elongated apical cell dies and becomes fibrous, causing it to coil in a helix-like manner (Figure 6.3 B).

Young leaves tend to have more nonglandular trichomes than older leaves. Similarly, a higher density of peltate glandular hairs were reported on juvenile leaves of *Salvia officinalis* by Avato *et al*. (2005).Their abundant numbers and distribution over the entire surface of the leaf indicate that these hairs are likely to be involved in protecting leaf surfaces against insects and other pests during early development (Afolayan & Meyer, 1995). These light coloured, nonglandular trichomes may also be involved in protecting leaves against temperature extremes and water losses by increasing light reflectance (Wagner, 1991).

The glandular trichomes are peltate club shaped and multicellular. The gland consists of 8 – 10 cells with a single cell forming the base and the remaining cells, arranged in a biserial

manner, forms the head of the gland. The 4 to 5 most apical cells are linked to a subcuticular space where a secretory product is stored (Figure 6.4). The apical cells adjacent to the subcuticular space are probably responsible for producing the essential oils stored inside the space. It is widely accepted in literature that the cells in glandular trichomes, rather than epidermal cells in the vicinity, produce the secreted product in subcuticular spaces. Substantial evidence for this theory has been gained from studies on mint and tobacco (Keene & Wagner, 1985; Kandra & Wagner, 1988; Wagner, 1991).

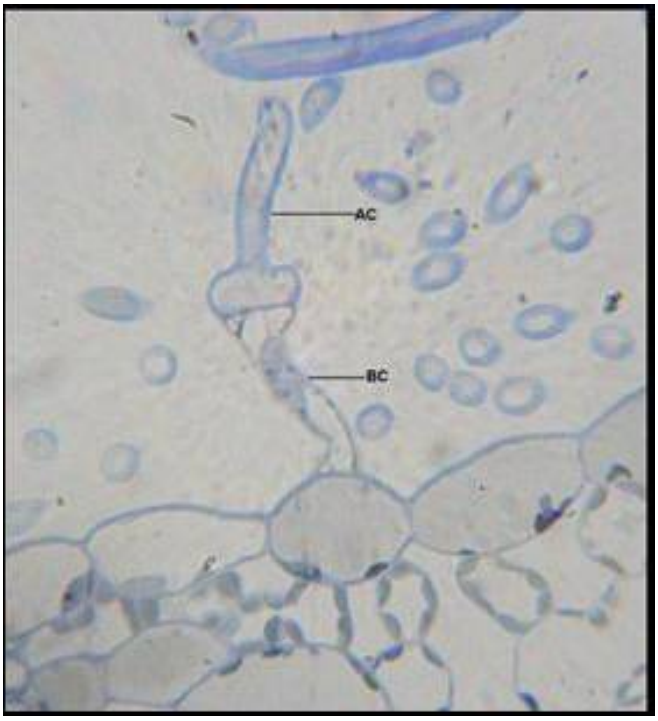

Figure 6.2: Light microscope image (100 x 12.5 magnification) of a section through a nonglandular trichome on a mature leaf of *Athrixia phylicoides* showing the basal cell (BC) and elongated apical cell (AC)

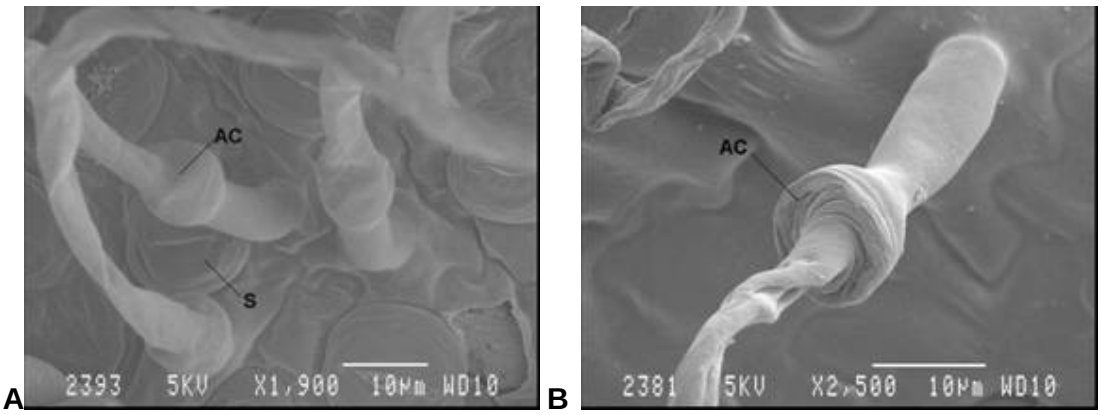

Figure 6.3: S.E.M images of nonglandular trichomes on leaves of *Athrixia phylicoides*: A) a young leaf showing the bulbous base of the apical cell (AC) and B) an old leaf showing coiling of the dead apical cell (AC)

The absence of pores on glands indicates that essential oil in bush tea leaves is released either through diffusion or through rupture of the cuticle layer. We observed that glands on mature leaves are often ruptured while those on young leaves are mostly intact. This correlates well with the observations that old leaves are more aromatic than young leaves. Rupture of the cuticle is probably caused by external pressure, hence the observation that leaves become more aromatic after being handled.

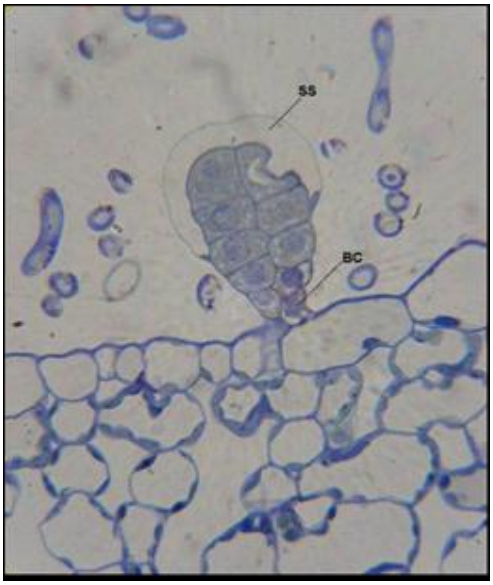

Figure 6.4: Light microscope image (100 x 12.5 magnification) of a section trough a glandular trichome on a mature leaf of *Athrixia phylicoides*, showing the subcuticular space (SS) and single cell forming the base (BC)

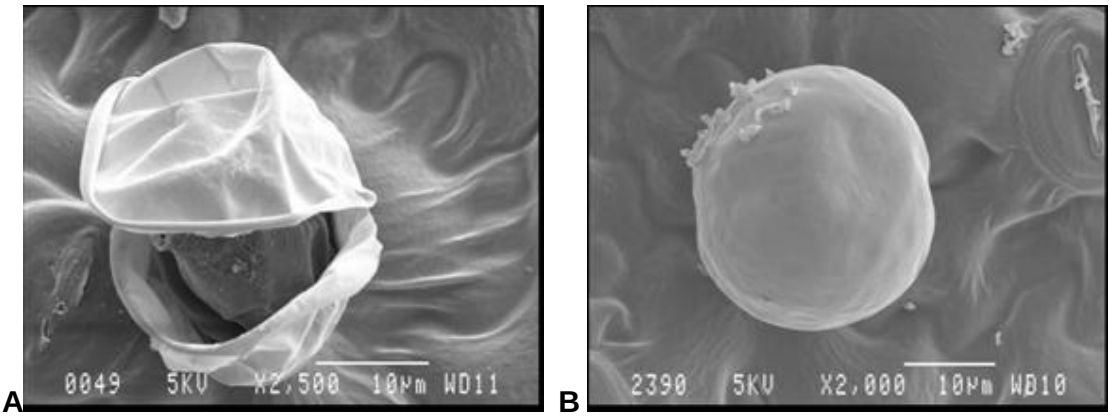

Figure 6.5: Glands on *Athrixia phylicoides* leaves: A) ruptured gland on a mature leaf and B) intact gland on a young leaf

6.5 Morphological differences between greenhouse and *in vitro* grown plants

6.5.1 Materials and Methods

Leaves used in this trial were collected from 3-month-old, third generation, micropropagated plants. Plants were established on a ½ MS medium (Murashige & Skoog, 1962), supplemented with 3% sucrose and 1,5 mg/L indole-3-butyric acid (IBA) and solidified with 3 g/L Gelrite™ . Plants were initiated in 15 cm glass test tubes and kept in a growth room at 26ºC ± 3ºC under white fluorescent light with a light intensity of 60 µmol/$m^{2/}$sec and an 8h dark: 16h light, cycle. After sufficient growth, micro-shoots were subcultured and transferred to fresh medium for three generations before rooting occurred. Leaves were collected from well rooted plantlets approximately 10 cm in height at 8 am, the same time as collection of leaves from greenhouse plants used in this study.

Plant material was prepared for S.E.M examination as described in 6.1.2 b. The general appearance of glandular trichomes, nonglandular trichomes and stomata were photographed and examined. Photos of the abaxial leaf surface (three or four near the central vein and three or four in the middle of the leaf surface) at 150x magnification were used to calculate the number of glandular trichomes per mm^2. Structures and the number of glandular trichomes were compared to that on newly emerged leaves from plants in the greenhouse.

Leaf area was determined using a sample of 10 newly emerged leaves. Cuttings of greenhouse material were placed on a mist bed where shoot formation was monitored to determine the age of individual shoots. Microshoot formation on first generation *in vitro*

established plants were monitored in the same way. Area of individual leaves was traced onto 1 mm^2 grid paper and individual areas calculated.

6.5.2 Results and Discussion

There were noticeable differences between leaves of greenhouse and micropropagated plants when visually compared. Leaves of greenhouse plants were 40% bigger than leaves of micropropagated plants, and were darker in colour and more rigid (Table 6.2). Furthermore, greenhouse plants had a white, woolly appearance while trichomes on *in vitro* grown leaves were less noticeable. The lighter colour of leaves can be explained by the lower content of chlorophyll in the micropropagated plants, since they are not initially photosynthetically active, and only become autotrophic, to a lesser degree, at a later stage (Rout *et al*., 2000).

Leaves of micropropagated plants are often smaller than normal leaves. This is often, but not always a result of juvenility in plants. Plants may start producing normal adult phase leaves soon after reestablishment in external conditions. However, in some cases the atypical shape persists for several years (George, 1996).

Table 6.2: Leaf surface areas (cm^2) of *Athrixia phylcoides* grown in vitro compared to greenhouse grown plants

	Greenhouse leaves	***In vitro* leaves**
Total leaf surface area	10.2	4.1
Means	1.02^a	0.41^b

* Values that are not significantly different at 5% probability level are indicated by the same letter

Our microscopic comparisons showed no noticeable differences in the morphology of glandular trichomes. However, greenhouse plants had significantly more glandular trichomes present on their leaf surfaces than micropropagated plants (Table 6.3). This is in contrast to the higher number of glandular trichomes that were observed on leaves of micropropagated *Salvia officinalis* plants (Avato *et al*., 2005). Secondary metabolites are believed to be involved in warding off of predators, attraction of pollinators and combating infectious diseases (Razdan, 1993). The mechanisms involved in the production of secondary metabolites may be under-developed in plant grown under *in vitro* conditions, and free from contamination and the threat of predators and disease.

Even if we assume that the potential of each individual gland to produce essential oil is unchanged in micropropagated plants, a decrease in the number of glandular trichomes is still a concern as it may be indicative of a decreased potential of micropropagated plants to produce essential oil.

A more accurate comparison between the quantity of glandular trichomes of micropropagated and field plants, would be one in which acclimatised, re-established micropropagated plants are compared to field grown plants. Since plants in culture often become rejuvenated (Avato *et. al*, 2005), the physiological ages of the plants was most likely different, and thus influenced the number of trichomes present on leaves.

Unfortunately, our efforts at acclimatising plantlets were so far not successful (see 4.6), and therefore, such a comparative study could not be undertaken.

Table 6.3: Density of glandular trichomes on the leaf surfaces of *Athrixia phylcoides* grown in vitro and in a greenhouse

Number of glandular trichomes / mm^2	Area adjacent to central vein	Middle of leaf surface	Total Means
Greenhouse leaves	7.14	15.18	7.86^{a}
***In vitro* leaves**	5.29	6.50	4.57^{b}

* Values that are not significantly different at 5% probability level are indicated by the same letter

S.E.M images of leaves of *in vitro* propagated plants showed almost 100% open stomata, compared to less than 5% open stomata on images of greenhouse leaves. Stomata on *in vitro* leaves seemed unable to close in response to a change in atmospheric conditions. (Figure 6.6 A). The inability of stomata to regulate water loss through leaves is often the case with micropropagated plants (George, 1993; Hartmann, Kester, Davies, 1997). The water-saturated atmospheres in a culture vessel have been implicated in the inability of plants to control water loss after transplantation to soil (Wardle, Dobbs & Short, 1983; Short, Warburton & Roberts, 1987). This results in poor adaptation of these plants to natural conditions and great plant losses after transplanting.

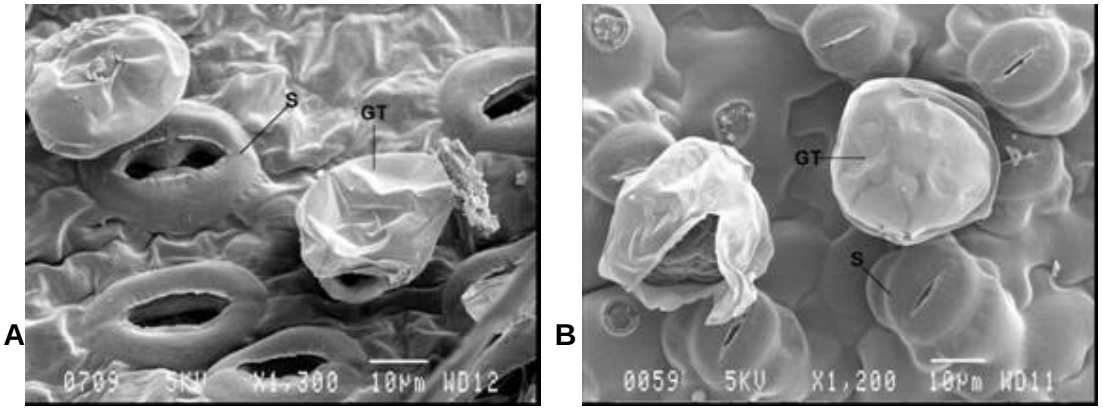

Figure 6.6: Glandular trichomes (GT) and stomata (S) of *Athrixia phylicoides* on A) a three month old leaf of a micropropagated plant (Note that stomata are all open) and B) a three month old leaf of a greenhouse plant (Note that stomata are all closed)

6.6 Conclusions

Essential oil production by *A. phylicoides* can probably be linked to the medicinal value of the plant. The synthesis of essential oils is believed to take place in cells in the glandular trichomes present on the surfaces of leaves. These glandular trichomes are peltate, multicellular structures with a subcuticular cavity at the apical end where the secreted product is stored. The essential oils are released from glands on mature leaves by rupture of the cuticle caused by external pressure.

The lighter colour of micropropagated leaves is not a concern, since it can be explained by lower levels of chlorophyll in leaves, because of a reduced need for photosynthesis of plants in culture. (Preece & Sutter, 1991; Hartmann *et al.*, 1997). The inability of stomata to close in response to water loss is not an unusual phenomenon in micropropagated plants (George, 1993; Hartman, Kester & Davies, 1997). Even so, dysfunctional stomata can be expected to result in severe wilting of plants after transfer to *ex vitro* environments.

The lower density of glandular trichomes on micropropagated leaves may indicate a reduced capacity to produce essential oils. Yet, it is possible that the number of glandular trichomes on leaves of acclimatised, re-established plants may resemble those on field grown plants more closely (Mascarenhas *et al*., 1988) and further studies will need to be conducted to clarify this. Despite their lower density, glandular trichomes on micropropagated plants were morphologically similar to those on greenhouse grown plants. Their ability to function normally will need to be confirmed by chemical analyses of the essential oils they produce.

CHAPTER 7

GENERAL DISCUSSION

7.1 Establishments of aseptic cultures

We managed to establish disease free bush tea cultures by applying a combination of methods to reduce and control the levels of contamination on the plant material used to establish cultures. These included the regular application of a systemic fungicide to stock plants, the partial removal of leaf blades prior to surface sterilisation and a five-step surface sterilisation procedure.

Our preliminary trials indicated that leaves on plants contribute to the high levels of contamination. Leaves were hard to surface sterilise due to the abundant numbers of hairy trichomes on their surfaces. These hairs resulted in poor contact between the leaf surfaces and sterilants, even with the use of magnetic stirrers and submergence of plant material in ethanol. Pierik (1987) and George (1993) suggested these methods to improve contact between plant material and sterilants.

Deberg & Read (1991) stated that the levels of contamination in culture could be reduced by removing leaves from explants prior to surface sterilisation. In our case, the contamination problem could be alleviated by the removal of leaf blades prior to surface sterilisation. However, the complete removal of leaf blades apparently caused damage to axillary meristems by exposing them to chemicals. Damage of unprotected meristems by chemicals during surface sterilisation has also been reported by Deberg & Read (1991). We obtained the best results by partially removing leaf blades, thereby removing the source of contamination, but at the same time, preventing exposure of meristems to chemicals.

Even with leaf blades removed, the levels of contamination in our cultures were still too high, and additional means of contamination control were needed. Our trial on the use of a systemic fungicide on stock plants, underlined the importance of maintaining healthy stock plants in order to obtain disease free cultures. Reducing the levels of contamination on stock plants is encouraged by authors such as George (1993) and Hartmann *et al*. (1997). After first treating our stock plants with the systemic fungicide 3 weeks prior to establishing cultures, the levels of contamination in our cultures was reduced by 10%. By regularly applying the systemic fungicide to our stock plants, fungal contamination was controlled even more effectively in the trials that followed.

Bush tea also showed sensitivity to long exposure to the chemicals we used in the surface sterilisation procedures. During surface sterilisation, plants became waterlogged and wilted, and completely failed to grow after establishment. We needed to reduce the time of exposure to chemicals during surface sterilisation. By applying fungicide to stock plants, we were able to eliminate the use of a fungicide from our surface sterilisation procedure, and thereby reduced the time and number of chemicals the plant material had to be exposed to.

Due to the sensitivity of bush tea to chemicals, we conducted a trial in which the effect of calcium hypochlorite ($Ca(OCl)_2$) was compared to that of sodium hypochlorite (NaOCl) as part of the surface sterilisation procedure. Pierik (1987) suggested the use of $Ca(OCl)_2$ in cases where plants show sensitivity towards NaOCl. In contrast to the findings of Pierik (1987), our results indicated that the use of $Ca(OCl)_2$ as an alternative to NaOCl, resulted in more severe wilt and a lower rate of axillary shoot production in cultures. Therefore, NaOCl is a more suitable surface sterilant in the case of bush tea.

The most effective control of contamination, and best growth response of explants was achieved with the following surface sterilisation procedure: Rinse plant material in running tap water (10 min), submerge it in 70% ethanol (90 sec), soak in 10% (v/v) household bleach (3.5% active NaOCl) (10 min) and rinse twice in sterile water (5 min each). In all our sterilisation trials, cultures were established on ½ MS medium, supplemented with 3% sucrose and solidified with 3 g/LGelrite™.

7.2 Multiplication

A combination of auxin and cytokinin is usually used to induce multiple shoot formation in cultures (George, 1996; Hartmann *et al.,* 1997; Puente & Marh, 1997; Dhingra, Rao & Narasu, 2000; Giusti *et al.* 2002; Lai, Lin, Nalawade, Fang & Tsay, 2005; Rout, 2005) We evaluated the effect of two growth regulators, indole-3-butyric acid (IBA) and 6-benzylaminopurine (BAP), on the *in vitro* multiplication rate of bush tea cultures.

Multiplication trials were done on four different growth media. The basic medium used in these trials was ½ MS medium supplemented with 3% sucrose and solidified with 3 g/LGelrite™. The basic medium served as a control and for treatments, it was supplemented with either 1.5 mg/L IBA, 1.5 mg/L BAP or a combination of IBA and BAP both at 1.5 mg/L. Our results indicated that IBA in the medium, alone or in combination with BAP, seemed to suppress axillary shoot formation to some extent. BAP in the medium did not notably promote axillary shoot formation and yielded the same amount of axillary shoots as the control medium. The effect of BAP at higher concentrations or the use of different cytokinins to promote axillary shoot formation may still be investigated.

We concluded that bush tea cultures can be successfully multiplied by means of axillary shoot formation, within four weeks after establishment. This was achieved on ½ MS medium, supplemented with 3% sucrose, solidified with Gelrite™ (3 g/L) devoid of growth regulators.

More cultures were initiated, and on the same 4 growth media described above, in different trials over our 2 years of research. The growth response of cultures was not always consistent. In some trials, explants on all 4 media failed to form axillary microshoots altogether, while those formed in other trials were mostly hyperhydrated. The formation of callus was occasionally recorded on cultures established on media containing either a combination of IBA and BAP, or BAP alone.

In trials where hyperhydricity occurred, the time cultures could be kept on the establishment medium before they died, was limited. By transplanting the newly formed axillary microshoots to fresh media as soon as possible, symptoms of hyperhydricity could be alleviated to some extent. The causes of inconsistent growth responses such as callus formation and the occurrence of hyperhydricity are still unclear. Variation in endogenous hormone levels of stock plants may be, at least partly, involved.

7.3 Rooting

The presence of auxin in a medium is generally accepted to promote rooting (George, 1993; Gaspar, 1994; Hartmann *et al.*, 1990; Hartmann *et al.*, 1997; Pierik, 1987; Rout *et al.*, 2000) *In vitro* rooting of *Dendranthema grandiflora* (Kaul *et al.*, 1990) and various medicinal plants (Rout *et al.*, 2000) has been achieved by addition of auxin to medium. According to George (1993), the presence of GA_3 in growth medium used in an earlier growth stage, has been reported to promote rooting at a later stage, in a different growth medium, free from GA_3, while GA_3 in the present growth medium usually suppresses rooting.

In one of our rooting experiments, the combined effects of lowering sucrose levels and the addition of IBA (1.5 mg/L) to medium, promoted rooting to some extent. The control medium in this trial yielded no rooting. Only one concentration of IBA was tested due to the limited amount of plant material available. Higher concentrations of IBA may have a more pronounced effect on rooting.

In a different rooting trial, the presence of GA_3 in the establishment medium had no effect on rooting after the transfer of microshoots to a GA_3 free medium later on. In the same experiment, rooting occurred in both treatments, after transfer to hormone free medium. The

hormone free medium was identical to the control medium used in the IBA / lowered sucrose experiment revered to above, that yielded no rooting.

Since rooting in both of the trials was attempted on the same growth medium, in the same incubation conditions, and over the same period of time, the difference in rooting response may be a result of the stock plants used to establish cultures. It is likely that the plant material for these trials were collected from different mother plants. The *in vitro* rooting ability of plants is determined, amongst other factors, by characteristics of the mother plant (Pierik, 1987; George, 1993; Rout *et al*., 2000).

7.4 Acclimatisation

Only a single attempt could be made to acclimatise bush tea plantlets to greenhouse conditions. Cultures used in this trial were established shortly before our stock plants started flowering. Stock plants died back after flowering season and remained dormant throughout the winter. Due to this, no plant material was available to conduct more trials in the available time.

Our attempt at acclimatisation failed at an early stage. Some possible causes of damage to the plantlets after transfer to *ex vitro* conditions include, water loss, damage by high levels of irradiation (Griffin, Hennen & Oglesby, 1983), temperature damage, hyperhydricity (Picoli *et al*., 2001; Strycharz & Shetty, 2002) and the physiological stage of stock plants at the time of establishing cultures.

We ruled out the possibility that damage could have been caused exclusively by excessive water loss since wilting already occurred before test tubes were opened, and the RH inside culture vessels should therefore not have been drastically different to that in incubated conditions. Damage due to high levels of irradiation is also unlikely since the light intensity inside the greenhouse was reduced by the semi transparent fibreglass panels, as well as a 30% shade net fitted below the greenhouse roof.

Night temperatures in the greenhouse dropped to 17ºC compared to the continuous temperatures of 30°C ± 2 in growth rooms. Night temperatures were approximately 8ºC cooler than minimum temperatures in growth rooms. The sudden variation in temperature may have caused damage to plants, but this theory could not be supported by literature.

It is possible that hyperhydricity influenced the response of plantlets after transfer to greenhouse conditions. Even though no symptoms of hyperhydricity were observed on plantlets at the time of transfer to *ex vitro* conditions, less visible changes associated with

hyperhydricity, may have been present, making them exceptionally vulnerable to environmental changes (Picoli *et al*., 2001; Strycharz & Shetty, 2002). A method to successfully prevent hyperhydricity in our cultures need to be established in order to ensure that healthy plantlets are available for further trials on acclimatisation.

Another possible cause of failure in this trial was the physiological and biochemical changes in the stock plants, preceding flowering, and especially dormancy. Even though cultures were established from material collected before flowering, the physiological and biochemical changes that preceded dormancy in stock plants may have already been present in material used to establish cultures in this trial. Avato *et al.* (2005) confirmed that *Salvia officinalis* cultures keep the age of maternal tissue, in spite of the physiological stage of their own development. Seasonal biochemical changes in *A. phylicoides* have also been confirmed by Mudau *et al*. (2006) and Chambeli *et al*. (2008). The sudden deterioration of plantlets after being moved to the mist bed may have been a result of plantlets approaching a state of dormancy 'inherited' from the mother material.

Further acclimatisation trials in a phytotron environment where light intensity and temperature can be closely regulated and gradually adjusted to resemble field conditions, may be useful to determine the possible effects of low night temperatures and of too high light intensities on the deterioration of plantlets after their transfer to *ex vitro* conditions. Repetition of this trial with non-hyperhydrated plantlets, and at a time when stock plants are actively growing, will clarify the possible effects that hyperhydricity, or the physiological stage of the mother plants, could have had on the rapid deterioration of plantlets.

After establishing a protocol for successfully moving plantlets to field conditions, the acclimatized plants will need to be monitored for any abnormalities over a few seasons. Their capacity to produce secondary metabolites will need to be established and the chemical composition of essential oils produced by leaves need to be determined and compared to that of field grown plants in order to evaluate the success of this propagation method.

7.5 Hyperhydricity

The cause of hyperhydricity in *A. phylicoides* cultured plants is still unclear. Even though the use of BAP in culture medium has been reported to induce hyperhydricity in species such as carnation (Jain *et al*., 2001), we found no correlation between the use BAP or IBA in medium and the occurrence of hyperhydricity.

In contradiction to literature (George, 1993; Piqueras, 2000; Jain *et al*., 2001), our trials indicated that a cooler incubation temperature (26ºC) resulted in a slightly higher incidence of

hyperhydricity than the higher temperature (30ºC). It seems unlikely that temperature alone is the causal factor.

We found a positive correlation between leaves on explants and the occurrence of hyperhydricity symptoms. We hypothesise that the leaves present on explants increase the levels of endogenous hormones, thereby inducing hyperhydricity. The results did not show a statistically significant difference, and therefore further investigation is needed to conclude the study.

The inconsistent occurrence of hyperhydricity suggests that it is caused by a combination of factors rather that a single one. Hyperhydricity caused great losses during our trials, and thereby influenced the results of many trials, especially where only a few replicates could be used due to limited plant material. In the long run, hyperhydricity may even affect acclimatisation of plantlets. There is an urgent need for further investigations to clarify the causes of hyperhydricity in order to prevent it. Physical ways in which hyperhydricity may be prevented in future trials is worth investigating, these include the use of bottom cooling (Piqueras *et al.*, 2002) or aeration of vessels (Piqueras *et al.*, 2002; Lai *et al*., 2005). The effect of agar as an alternative gelling agent (Franck *et al*., 2004) or the effect of higher concentrations of gelling agent on hyperhydricity (Kim *et al*., 1988) might be worth investigating. Due to time limitations no further trials could be done.

7.6 Microscopical investigation

The medicinal properties of *A. phylicoides* can probably be linked to the essential oils produced by the plant's leaves. Microscopical studies of the leaves of greenhouse grown bush tea plants revealed the presence of two types of trichomes viz. glandular and nonglandular. The synthesis of essential oils is believed to take place in the cells of the glandular trichomes present on the leaf surfaces. These glandular trichomes are peltate, multicellular structures with a subcuticular cavity at the apical end where the secreted product is stored. The essential oils are released from the glands on mature leaves by rupture of the cuticle.

In a comparative study, four differences were observed between the leaves of greenhouse and micropropagated plants. Firstly, on macroscopic level, micropropagated leaves were lighter in colour, suggesting the presence of lower chlorophyll levels in leaves. This is not unexpected, since plants in culture are mainly autotrophic, and the reduced need for photosynthesis will naturally result in less chlorophyll synthesis (Preece & Sutter, 1991; Hartmann *et al.*, 1997). Leaves that are formed in culture may have a reduced capacity for photosynthesis (Ziv, 1986; Grout & Donkin, 1987), or in some cases, are completely unable

to photosynthesise (Preece & Sutter, 1991; Nalawade & Tsay, 2004). Secondly, micropropagated leaves were much smaller than greenhouse leaves of similar age. This may be a result of rejuvenation in culture (Avato *et al*., 2005).

On microscopic level, two further differences were observed. Firstly, the stomata on the leaves of the micropropagated plants seemed unable to close in response to water loss. Stomata on micropropagated plants are often not functional (George, 1993; Hartman, Kester & Davies, 1997) and as a result, these plants lose vast amounts of water when they are transferred to an *ex vitro* environment where the RH is much lower than in culture. Secondly, the density of glandular trichomes was lower on micropropagated leaves than on greenhouse leaves. A decrease in the number of glandular trichomes will, without doubt, result in a decrease in quantities of essential oils produced. However, it is possible that the number of glandular trichomes on the leaves of acclimatised, re-established plants may resemble those of field grown plants more closely. Further studies will need to be conducted to clarify this.

Our comparative study revealed no noticeable changes in the morphology of the glandular trichomes on micropropagated plants that may suggest their inability to function normally. However, the ability of these glands to function normally will have to be confirmed by analysing the chemical composition of essential oils, and by comparing the chemical profiles of essential oils produced by micropropagated, greenhouse and field grown plants respectively.

SUMMARY

Although bush tea (*Athrixia phylicoides*) has been used as a traditional herbal medicine for many decades, its value as medicinal herbal tea is known and used only by a small number of individuals. Pharmacological screening of leaf extracts confirmed its anti-inflammatory, antihypertensive, narcotic and analgesic properties (Swanepoel, 1997). The pleasant tasting leaf extract is also free from caffeine and high in antioxidant activity, making it an ideal commercial herbal health tea (McGaw, Steenkamp & Eloff, 2007).

Despite its promising commercial possibilities, no serious efforts have been made to commercialise bush tea, or to propagate the plant on commercial scale (Roberts, 1990; Swanepoel, 1997; Van Wyk & Gericke, 2000). Wild population currently serve as the main source of plant material. Apart from a few studies on the chemical composition of leaf extracts (Swanepoel, 1997; McGaw, Steenkamp & Eloff, 2007; Chabeli, Mudau, Mashela & Soundy, 2008) and the effects of fertilisers (Mudau, Soundy, Du Toit & Olivier, 2006), little research on A. phyliciodes have been documented. With the knowledge gained from this study we hope to assist in the commercialisation of this valuable herbal tea and at the same time, provide means of obtaining plant material for commercial propagation, without adding to the existing pressure on our valuable natural resources.

This study consisted of three parts. Firstly, we developed a protocol for the production of plants from axillary buds on nodal explants, using tissue culture techniques. Secondly, a microscopic study was done to learn more about the structures and mechanism involved in the production of medicinally active essential oils. Thirdly, a preliminary study was carried out to determine the suitability of micropropagated plants for commercial use, by comparing the leaves of micropropagated plants to that of greenhouse plants on macroscopic and microscopic level.

We managed to establish disease free bush tea cultures by applying a combination of methods to reduce and control the levels of contamination on the plant material used to establish cultures. These included the regular application of systemic fungicide to stock plants, the partial removal of leaf blades prior to surface sterilisation and a five-step surface sterilisation procedure.

Regular treatment of stock plants with a systemic fungicide was essential to ensure sterile cultures. Leaf blades on starting material contributed to high levels of contamination in cultures after establishment. The levels of contamination were effectively reduced by partial removal of leaf blades prior to surface sterilisation.

Plant material showed sensitivity towards chemicals used during surface sterilisation. The use of calcium hypochlorite ($Ca(OCl)_2$) during surface sterilisation resulted in more severe wilt and a lower rate of axillary shoot production in cultures than sodium hypochlorite (NaOCl). Surface sterilisation was achieved by rinsing the plant material in running tap water for 10 minutes, followed by soaking it in 70% ethanol for 90 seconds, followed by soaking it in 10% (v/v) household bleach (3.5% active NaOCl) on a magnetic stirrer for 10 minutes, and finally two rinses in sterile water, on a magnetic stirrer, for 5 minutes each.

Cultures were established on ½ Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), supplemented with 3% sucrose and solidified with 3 g/L Gelrite™. We investigated the effect of indole-3-butyric acid (IBA) and 6-benzylaminopurine (BAP) in growth medium on axillary shoot formation. IBA alone or in combination with BAP seemed to suppress axillary shoot formation. BAP in medium did not promote axillary shoot formation notably and yielded the same amount of axillary shoots as the control medium. In most cases, bush tea cultures could be successfully multiplied by means of axillary shoot formation within four weeks after establishment.

The combined effects of lowering sucrose levels and the addition of IBA (1 mg/L) to medium promoted *in vitro* rooting to some extent. The presence of gibberellic acid (GA_3) in the establishment medium had no effect on rooting later on. In many experiments, rooting occurred spontaneously on the same medium in which plants were established on after two to three subcultures.

Our attempt at acclimatisation failed at an early stage due to severe wilting and death of plantlets after transfer to *ex vitro* conditions and further trials on acclimatisation is needed.

Microscopical studies of the leaves of greenhouse grown bush tea plants revealed the presence of two types of trichomes viz. glandular and nonglandular. The synthesis of essential oils is believed to take place in cells in the glandular trichomes present on the leaf surfaces. These glandular trichomes are peltate, multicellular structures with a subcuticular cavity at the apical end where the secreted product is stored. The essential oils are released from glands on mature leaves by rupture of the cuticle.

In a comparative study between the leaves of greenhouse and micropropagated plants, four differences were observed. Firstly, on macroscopic level, micropropagated leaves were lighter in colour, suggesting the presence of lower chlorophyll levels in leaves. Secondly, micropropagated leaves were much smaller than greenhouse leaves of similar age. On microscopic level, two further differences were observed. Firstly, the stomata on the leaves

of the micropropagated plants seemed unable to close in response to water loss. Secondly, the density of glandular trichomes was lower on micropropagated leaves than on greenhouse leaves. Our comparative study revealed no noticeable changes in the morphology of the glandular trichomes on micropropagated plants that may suggest their inability to function normally.

Throughout our study we were faced with the inconsistent appearance of hyperhydricity. The cause of hyperhydricity in *A. phylicoides* cultured plants is still unclear. We found no correlation between the use of BAP or IBA in medium and the occurrence of hyperhydricity. Our trials on the effect of temperature on hyperhydricity, indicated that a cooler incubation temperature (26ºC) resulted in a slightly higher incidence of hyperhydricity than at the higher 30ºC. We also found a positive correlation between leaves on the explants and the occurrence of hyperhydricity symptoms. There is an urgent need for further investigations to clarify the causes of hyperhydricity in order to prevent it.

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