Micropropagation of three *Brachystelma* species and investigations of their phytochemical content and antioxidant activity

B. pulchellum *B. pygmaeum* *B. ngomense*

By

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Research Centre for Plant Growth and Development

School of Life Sciences

College of Agriculture, Engineering and Sciences

University of KwaZulu-Natal

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Consultation took place between the student and us throughout this study. We advised the student and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the university appointed Examiners

SUPERVISOR: PROFESSOR J. F. FINNIE

CO- SUPERVISOR: PROFESSOR J. VAN STADEN

Conference contribution from this thesis

2015 17th Annual Research centre for Plant Growth and Development (RCPGD) meeting Oral presentation: *In vitro* propagation, phytochemical and pharmacological evaluation of *Brachystelma* species (N. Hlophe, J. F. Finnie, and J. Van Staden). University of KwaZulu-Natal, Pietermaritzburg.

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Potential publications from this thesis

Potential publication 1

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Potential publication 2

Hlophe, N. P., Van Staden, J., Finnie, J. F. Phytochemical profiling and antioxidant evaluation of three *Brachystelma* species.

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List of Acronyms

2,4- D	2,4-Dichlorophenoxyacetic acid
AAPH	2,2′ -Azobis(2-methylpropionamidine) dihydrochloride
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
ATM	African traditional medicine
AUC	Area under curve
BA	N^6-Benzyladenine
CaOCl	Calcium hypochlorite
CE	Catechin equivalent
DMRT	Duncan's multiple range test
DPPH	2,2 Diphenyl-1-picryl hydrazyl
DW	Dry weight
EN	Endangered
EtOH	Ethanol
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
HAT	Hydrogen atom transfer
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
iP	Isopentenyladenine
KOH	Potassium hydroxide
LC	Least concern

MeOH	Methanol
MS	Murashige and Skoog medium
***m*TR**	*meta*-Topolin riboside
NAA	1-Naphthaleneacetic acid
$NaCO_3$	Sodium carbonate
$NaNO_2$	Sodium nitrite
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NAUC	Net area under curve
NU	UKZN Bews herbarium
ORAC	Oxygen radical absorbance capacity
PGR	Plant growth regulator
PPF	Photosynthetic photon flux
RSA	Radical scavenging activity
SA	South Africa
SET	Single electron transfer
TE	Trolox equivalent
TEAC	Trolox equivalence antioxidant capacity
TRAP	Total radical-trapping antioxidant parameter
UHPLC-MS/MS	Ultra high performance liquid chromatography – tandem mass spectrometry
VU	Vulnerable

Abstract

Local communities in most African countries possess a wealth of knowledge on the uses of plants in the environment. Documentation of this knowledge is not a common practice among these communities and therefore the knowledge is in the process of disappearing as the younger generation is more inclined towards the western lifestyle. The knowledge of African communities on the use of medicinal plants has provided and continues to provide leads towards therapeutic concepts which accelerate the pace of drug discovery. This makes the use of medicinal plants an important line of research to be pursued. The use of plants, particularly medicinal plants, as a resource is generally accompanied by the concern of over exploitation. Exponential population growth along with newly emerging and resistant diseases as well as habitat destruction due to human developmental activities are among the reasons for the decline of plant biodiversity. There is therefore an urgent need to develop effective means of conservation specific to *Brachystelma* species as a medicinal group, and also to document not only the uses but also the constituents and pharmacological activity of these plants. *Brachystelma* R. Br. ex Sims is a genus of geophytic plants used traditionally in some parts of the world including East Africa, southern Africa, West Africa, northern and western India. Apart from being used as a food source, they are used for the treatment of illnesses such as colds, chest pains, wounds, and as an appetite suppressant and for enhancing fertility.

The aim of this study was to establish efficient micropropagation protocols for *Brachystelma* species, namely *B. ngomense* (Endangered - EN), *B. pulchellum* (Vulnerable - VU) and *B. pygmaeum* (Least Concern - LC), as a means of ensuring their survival, and to explore their phytochemical and pharmacological properties. A number of *Brachystelma* species have been listed as medicinal herbs. There is currently no documented approach towards the mass propagation of *Brachystelma* species and there are no available scientific studies validating the ethnomedicinal use of these plants. Traditional uses indicate that *Brachystelma* is commonly used and this is accompanied by the concern that wild populations, which are the only available ones, are under threat for various reasons. Development of optimal tissue culture protocols such as micropropagation and callus cultures may alleviate conservation concerns. In addition, these protocols would make available the possibility of utilization of the plant as a daily food and towards production of biological compounds with potential health benefits.

The development of a micropropagation protocol for the three *Brachystelma* species made use of nodal explants (~10 mm in length). The effects of different concentrations of N^6-benzyladenine (BA), 2-isopentenyladenine (iP) and *meta*-topolin riboside (*m*TR) supplemented into Murashige and Skoog (MS) (1962) media were tested over a period of 6 weeks. The specific concentrations used were 1.0, 5.0, 10 and 25 µM for each of the cytokinins without auxins. An increase in concentration of all the cytokinin treatments was found to typically result in significantly higher shoot proliferation. However, each species differed in response to each specific cytokinin, the optimal concentrations were 25 µM *m*TR, 25 µM iP and 25 µM BA for *B. ngomense, B. pulchellum* and *B. pygmaeum* respectively. The conclusion drawn from these results is that the application of BA, iP or *m*TR is beneficial for *in vitro* shoot proliferation from nodal explants of the three species. Regenerated shoots were rooted *ex vitro* after exposure to a 3 min pulse treatment using 100 mgl^{-1} indole-3-butyric acid (IBA). Shoots regenerated from a plain MS medium were typically found to have rooted prior to IBA treatment, however, these shoots as well as the ones derived from other treatments yielded poor rooting *ex vitro*. Survival of these shoots in the greenhouse was short-lived. As a result, acclimatization of all three species has been extremely limited thus the micropropagation protocol is not effective on a commercial scale.

A number of assays were used to evaluate the phytochemical content of the three species of *Brachystelma*. Vacuum filtered methanolic extracts were used for the determination of phenolics and flavonoids. Absorbance readings obtained using a spectrophotometer were further converted to concentration of compound per gram of extract. The phenolic and flavonoid contents varied between the three species. Higher phenolic and flavonoid content was found in leaf extracts. The effect of plant growth regulators (PGRs) i.e. BA, iP and *m*TR on specific phenolic acids was also observed. The cytokinin treatments were found to have a stimulatory effect on some of the phenolic acids. Pharmacological properties against oxidative stress were tested using the 2,2-Diphenyl-1-picryl hydrazyl (DPPH) and Oxygen radical absorbance capacity (ORAC) assays. The potency of the antioxidant activity of the three species was compared to the standard antioxidant, ascorbic acid. The activity of the standard antioxidant was found to not be significantly different to the plant extracts. *B. ngomense* leaf extracts (13.5 µg/ml) were more potent in comparison to ascorbic acid (14.5 µg/ml). The effect of plant growth regulators (PGRs) i.e. BA, iP and *m*TR on antioxidant

activity was also observed. In some instances, cytokinin-treated plant extracts showed an increased antioxidant capacity compared to those not treated.

Chapter 1: Introduction

1.1 Plant utilization in African Traditional Medicine (ATM)

Millions of South Africans are still dependent on traditional medicine for their primary health care needs (MANDER, 1998; WILLIAMS et al., 2013). Traditional medicine in South Africa, and Africa as a whole, remains an important part of the national health care systems, especially for those residing in remote rural areas (ABDULLAHI, 2011; DOLD AND COCKS, 2002). Among the reasons for this are availability and affordability of traditional medicine but mainly because traditional medicine is believed to address healing in a holistic manner (CUNNINGHAM, 1993; STREET et al., 2008). Holistic treatment considers various factors when addressing symptoms and diagnosing ailments. These factors include the individual's ecological, mental, physical, social and spiritual states (ABDULLAHI, 2011; COCKS AND MOLLER, 2002). On the other hand, western medicine has long been introduced to rural communities with the provision of services such as clinics (GRIERSON AND AFOLAYAN, 1999). However, the services are often found to be lacking as a result of inadequate healthcare facilities and medical equipment accompanied by the shortage of western-trained medical doctors (LIGHT et al., 2005). While 85% of the South African population is covered by 8000 doctors within the state healthcare system and the private healthcare system serving the remaining 15%, with 12 000 doctors, the traditional medical system outnumbers the western doctors by at least 10 to 1 (HAMILTON, 2004; MILLS et al., 2005; MORRIS, 2001; QUANSAH AND KARIKARI, 2016). Since the dawn of western medicine, the traditional medicine system has been labelled as an informal alternative. It has been suggested that the lack of trust, by the African communities in rural areas, in the western medical care system spurs the relentless use of traditional herbal medicines (GRIERSON AND AFOLAYAN, 1999). From personal experience, I am of the opinion that African traditional medicine will always remain relevant as there is a continued belief in "cultural illnesses" where modern medicine cannot possibly be thought of or recognised as an option. In South Africa, there is cultural diversity which translates to a variety of extensive indigenous knowledge and expertise among communities, and hence a greater utilisation of different medicinal plants (LIGHT et al., 2005; NIGRO et al., 2004).

Traditional medicines generally comprise of both animal and plant material as well as soil minerals, however within the African ethnomedicinal systems, plants are the main ingredients

used (CUNNINGHAM, 1997; DOLD AND COCKS, 2002). Southern African traditional medicine is supported by the rich and diverse variety of plants found in the region (AFOLAYAN AND ADEBOLA, 2004). These plants form a portion that accounts for about 25% of the total number of higher plants in the world (VAN WYK, 2008). By 2008, a total of 22 755 plant species were reported to occur in southern Africa (VAN WYK, 2008). A checklist of traditional medicinal plants of southern Africa recorded a total of 3481 plant taxa that are used for traditional medicine of which 2942 are administered to humans only (VAN WYK, 2008). In South Africa alone, there are approximately 2062 indigenous plant species that are reportedly used in traditional medicine (WILLIAMS et al., 2013). Of these, 82 species are threatened with extinction at a national level. A further 100 species are of conservation concern as they are harvested at a seemingly unsustainable rate. There is an estimated 70 000 tonnes of medicinal plant material used by South Africans each year (WILLIAMS et al., 2013). The trade of medicinal plants and related products generates at least 134 000 employment opportunities. There are a number of large *muthi* markets throughout South Africa (CUNNINGHAM, 1993). The most documented are found in Eastern Cape, Gauteng, KwaZulu-Natal, and Mpumalanga (CUNNINGHAM, 1997; DOLD AND COCKS, 2002; MULHOLLAND AND DREWES, 2004; NDAWONDE et al., 2007; NDHLALA et al., 2011; WILLIAMS et al., 2013). The use of wild plant populations as a primary source of health care has always been a cause for concern but more so recently as there is a seemingly growing demand (ABDULLAHI, 2011). There have already been a number of species that are pointing to unsustainable harvesting practices. Some of the signs of unsustainable harvesting are in the unavailability of some plants in certain markets, the decrease in size (especially bulbs) and number of traded components, and the longer distances taken to reach the remaining sources (CUNNINGHAM, 1997; WILLIAMS et al., 2013).

1.2 Micropropagation in plant conservation

The rich floral biodiversity of South Africa includes endemic medicinal geophytes, which are often popular, slow reproducing and habitat specific (AFOLAYAN AND ADEBOLA, 2004; AMOO et al., 2009). These geophytes are among the plants that are most likely affected by harvest pressures. Attempts made by authorities to regulate harvesting of medicinal plants have been unsuccessful as the harvesting of medicinal plants is a mode of self-employment and a source of livelihoods in both rural and urban communities (AFOLAYAN AND ADEBOLA, 2004; AMOO et al., 2009; CUNNINGHAM, 1997; NIGRO et al., 2004). It is highly unlikely that medicinal

plant traders will discontinue practicing their trade while any amount of resources are still available. There is, therefore, an urgent need for conservation of wild populations, especially those threatened with overharvesting and extinction.

It has been reported that a number of traditional medicine practitioners have indicated a willingness to utilize cultivated medicinal plants (CUNNINGHAM, 1997; DOLD AND COCKS, 2002). On the other hand, some practitioners are unwilling as they are concerned that cultivated plants are "weak" or ineffective (STREET et al., 2008). At some stage of course, one has to question the efficacy of cultivated medicinal plants. Nevertheless, *in vitro* techniques are currently among the most utilized methods of biodiversity conservation. Micropropagation, in particular, has been accepted as a biotechnological tool that can reduce the decline of medicinal plants in South Africa, and around the globe (AFOLAYAN AND ADEBOLA, 2004; DEBNATH et al., 2006; REED et al., 2011). It is an effective method for regeneration and proliferation of whole plantlets that are genetically identical to the parent plant and are pathogen-free (DEBNATH et al., 2006; ENGELMANN, 2011; JAIN et al., 2012). Micropropagation not only increases the production yield of plants but also promotes the rate of production thus eventually relieving the pressure of harvesting on wild populations (DEBNATH et al., 2006; JAIN et al., 2012; REED et al., 2011). Other available means of conservation i.e. *in situ* and other *ex situ* methods have been found to be inadequate and provisional. Seed banks, for example, are not a practical solution for all endangered species, in fact, there is a significant number of species for which seed banking is not an option (ENGELMANN, 2011; JASKOWIAK, 2014; REED et al., 2011). The application of micropropagation offers improved species conservation by overcoming the shortcomings of conventional as well as restrictive unconventional propagation methods (DEBNATH et al., 2006; REED et al., 2011; VUYLSTEKE et al., 1998).

Various accounts of the use of micropropagation as an efficient method for conservation or supplying demand are available in the literature (CHATURVEDI et al., 2007; REED et al., 2011; ROUT et al., 2000; VUYLSTEKE et al., 1998). *Nicotiana tabacum* L. (tobacco), for instance is a classic historical example of a plant successfully propagated via the use of micropropagation (BUDZIANOWSKA, 2009; CHATURVEDI et al., 2007). This particular plant is one of the first insecticides used by man and is in fact the model in plant biotechnology (BUDZIANOWSKA, 2009; GANAPATHI et al., 2004). It was while working with *N. tabacum* that Murashige and Skoog (MURASHIGE AND SKOOG, 1962) developed an *in vitro* culture nutrient medium that

became widely used for most if not all plants species to date (BUDZIANOWSKA, 2009; GANAPATHI et al., 2004; MURASHIGE AND SKOOG, 1962). Technological means of plant propagation are generally inspired by failure, of natural regeneration methods, to catch up with the speed of demand due to reasons such as poor seed viability, low germination percentage and inadequate conventional propagation (rooting cuttings) (CHATURVEDI et al., 2007; VUYLSTEKE et al., 1998). In micropropagation, improved regeneration can also be achieved through the use of callus cultures derived from somatic tissues (ALI et al., 2007; GANAPATHI et al., 2004; MURASHIGE AND SKOOG, 1962). Another example is *Catharanthus roseus* which came to be in demand due to the versatile medicinal potential of indole alkaloids produced by this plant (BAKRUDEEN et al., 2011). There arose a need to direct attention towards mass propagation either as a means of conserving the species or meeting demand (DEBNATH et al., 2006; VERMA et al., 2012). Different varieties of *C. roseus* plants have been successfully propagated, from shoot and stem meristems, and established under natural conditions (BAKRUDEEN et al., 2011; HAQ et al., 2013; KUMAR et al., 2013; ROUT et al., 2000). Successful acclimatization to the natural environment is usually indicated by continued growth of plantlets *ex vitro* accompanied by normal flowering or fruiting (ALI et al., 2007; CHATURVEDI et al., 2007). It is also evident from various studies that even though there is a common approach, no two plants are alike in their growth requirements therefore it is crucial that plant species be studied as individual entities.

1.3 Value of plant secondary metabolites in medicinal properties

It is generally understood that the primary reason behind secondary phytochemical production is for benefiting the plant. Out in the field, plants are surrounded by a range of natural threats such as herbivores, pathogens and competition (BENNETT AND WALLSGROVE, 1994; TAIZ AND ZEIGER, 2006; WINK, 1999). Therefore, these chemicals, "motivated" by the plants inability to move, serve as potent defence mechanisms. It is also understood that plant secondary compounds have a major contribution to structural support, specific colours, odours, and tastes in plants (BENNETT AND WALLSGROVE, 1994). Their classification is according to their biosynthetic pathways (BOURGAUD et al., 2001; VERPOORTE AND MEMELINK, 2002; WINK, 1999). The major plant secondary metabolites are the alkaloids, phenolics, steroids, and terpenes (BOURGAUD et al., 2001; TAIZ AND ZEIGER, 2006). A number of these major secondary metabolites are frequently accompanied by numerous

minor components which differ from organ to organ. Occasionally these differences are between individual plants but are often found between species (WINK, 1999).

For centuries, plants have been used by humans as dyes, flavours, fragrances, hallucinogens, insecticides, poisons, stimulants and therapeutic agents (KABERA et al., 2014; VERPOORTE AND MEMELINK, 2002; WINK, 1999). Over the years, there has been a shift in the level of importance of these uses as human needs changed with changing times. More recently, health care has become man's number one priority in the face of emerging diseases and drug resistance (GEARY et al., 2010; KABERA et al., 2014). This then opened a door of interest in the potential health promoting effects of plant secondary metabolites (KARUPPUSAMY, 2009; MAKKAR et al., 2007). Pharmaceuticals such as quinine and morphine were among the first known therapeutic agents (CHATURVEDI et al., 2007; CROTEAU et al., 2000; WINK, 1999). The importance of these pharmaceuticals, among other plant-derived chemicals, inspired the development of phytochemical evaluation techniques such as compound isolation (FABRICANT AND FARNSWORTH, 2001; PATIL AND BHALSING, 2016). The dual chemical-biological screening approach has been recommended as the fastest method of discovering important plant-derived bioactive compounds. The localisation of these active compounds requires the use of simple but sensitive and target specific bioassays due to the reason that plant extracts often contain low concentrations of active compounds (GÜLÇIN, 2012; IGNAT et al., 2011; NOVÁK et al., 2008).

Researchers have described the bioactivities of secondary metabolites as including properties such as anti-allergenic, anti-inflammatory, antimalarial, antimicrobial and antimutagenic (AMSCHLER et al., 1996; DELLA LOGGIA et al., 1989; IWASA et al., 1998; YAMAMOTO et al., 1993). These diverse uses in the treatment of numerous ailments have led to the development of innovative biotechnological approaches. By the early 1970's, scientists recognised the possibility of producing plant secondary metabolites in cell cultures which today affords us an alternative to chemical synthesis, even though plant biotechnologies have led to very few commercial successes (BOURGAUD et al., 2001; OKSMAN-CALDENTEY AND INZÉ, 2004). Approximately 40% or more of the pharmaceuticals used in western countries are derived or partially derived from natural sources such as vinca alkaloids (from *Catharanthus roseus*) used to treat cancers and quinine (from *Cinchona ledgeriana*) known for treating malaria (MACQUEEN, 1988; ROUT et al., 2000). Although there has been an increased interest in other

techniques, the isolation of compounds from wild or cultivated plants remains an important source of new drugs and chemical entities (NEWMAN et al., 2003).

1.4 *Brachystelma* species

1.4.1 Introduction

The genus *Brachystelma* R. Br. ex Sims is a member of the tribe Ceropegieae belonging to the sub-family Asclepiadoideae of the family Apocynaceae (BRUYNS, 2006). The family comprises of approximately 480 genera which are distributed in tropical and arid regions mainly in Africa, Asia and Australia (BRUYNS et al., 2015; DYER, 1983; KOEKEMOER et al., 2014; POOLEY, 2005). In southern Africa, this family is well-represented with 94 genera and over 650 known species (BRUYNS, 2014; DYER, 1983; KOEKEMOER et al., 2014). Among the genera occurring in southern Africa, *Brachystelma* R. Br. ex Sims is one of the most widely distributed with ±80 species out of ±120 species (BRUYNS, 1984, 2009; BRUYNS et al., 2015; POOLEY, 2005). These species are generally geophytes, although a small number have fusiform roots instead of a tuber (BRUYNS, 1984; MASINDE, 2007; MEVE AND LIEDE, 2001). Above ground, the plants are small and herbaceous, generally forming a cluster of deciduous stems with well-developed leaves (BRUYNS, 1984). The flowers are small and short lived, and the name '*Brachystelma*' meaning 'short crown' is per result of the often extremely small corona (BRUYNS, 1984; MASINDE, 2007; POOLEY, 2005).

A number of species of the Apocynaceae are known as a source of important medicinal compounds for treatment of cancer, malaria, high blood pressure, fever, gastrointestinal ailments and even psychosis (KOEKEMOER et al., 2014; SIDDIQUI et al., 2011; WONG et al., 2011). However, *Brachystelma* has been reported to be used traditionally for ailments such as coughs and colds, wounds, bodily discomfort and for enhancing fertility (CHUAKUL AND BOONPLENG, 2004; DESHMUKH AND JADHAV, 2014; MASINDE, 2007; MOTEETEE AND VAN WYK, 2011; POOLEY, 2005). Studies conducted on plant material obtained from the wild revealed the antioxidant potential of a species, *Brachystelma edulis*, belonging to this group. The bioactivity of *Brachystelma* species can be attributed to phytochemicals such as saponins and tannins that have been identified in the genus (PARE et al., 2016; RAJARAM et al., 2014; ZHANG et al., 2015). Among the Ceropegieae, *Brachystelma* is the least evaluated in terms of phytochemical use and thus poorly documented. This may be due to their small size and inconspicuous nature accompanied by general rarity. They only appear above ground during the rainy season (BRUYNS, 2009; MASINDE, 2007).

There is a paucity of information regarding their cultivation. There is a threat to most *Brachystelma* species in nature due mainly to their slow growth as well as the encroachment of their natural habitats by external factors (SANBI, 2009; SINGH, 2012; VENU AND PRASAD, 2015).

1.4.2 Distribution

Brachystelma is the second largest genus in the tribe Ceropegieae. It is distributed, in order of decreasing diversity, in sub-Saharan Africa, India, Southeast Asia and Australia. Ninety percent of species occur exclusively in sub-Saharan Africa (MASINDE, 2007). In South Africa, *Brachystelma* species occur across all nine provinces (SANBI, 2009). There are 85 *Brachystelma* species on the South African Red List data and out of these 73 % are endemic to South Africa. The best species representation is found in the Eastern Cape and KwaZulu-Natal (SANBI, 2009). There are 23 endemic species in the Eastern Cape and 16 endemic species in KwaZulu-Natal. Other endemic species occur in multiple provinces (SANBI, 2009). Their distribution is evidence of their variability in habitat preference. Some are found on mountain slopes with up to 3000 mm of annual precipitation while others among rock in grasslands with up to 200 mm of annual precipitation (BRUYNS, 1984; MASINDE, 2007; POOLEY, 2005; RAJARAM et al., 2014). One common factor is the requirement of well-drained soils (MASINDE, 2007). Their preferred habitat makes it difficult to locate them since they appear similar to the grasses (Figure1.1). Furthermore, their visibility is restricted because they are geophytic only appearing above ground during the rainy season (BRUYNS, 2009).

Figure 1.0: Map indicating the localities of the three species of *Brachystelma* used in this study. SANBI Redlist data, 2009.

Figure 1.1: "Enigmatic" *Brachystelma* growing among grasses, difficult to spot (*B. pygmaeum* subsp. *flavidum*) © Alison Young.

1.4.3 Botanical description

The vegetative parts of *Brachystelma* are relatively uniform. They are small perennial geophytic herbs arising from depressed-globose or discoid tubers with fibrous roots originating from the sides and the base (MASINDE, 2007; RAJARAM et al., 2014). Auxillary tubers are sometimes found branching off the main tuber (DYER, 1983). The tubers range from 15 -200 mm in diameter, and the tuber size is known to correlate with the age of the plant (MASINDE, 2007; PECKOVER, 1993; POOLEY, 2005). The inner section of the tuber consists of white tissue with a raw potato-like texture that is characteristically soft and watery (MASINDE, 2007).

From the rootstock, there arises one or generally a cluster of deciduous stems (DYER, 1983; MASINDE, 2007). The stems are herbaceous, erect or prostrate, branched or unbranched. Their leaves are simple opposite, with or without stalks, and may be extremely variable in shape, even on a single plant (BRUYNS, 1984; DYER, 1983; MASINDE, 2007). Unlike the majority of its milky sap producing family members, *Brachystelma* leaves produce a clear sap (MASINDE, 2007). The leaves are always non-succulent, and usually finely haired and inconspicuous

(BRUYNS, 1984). However, stem sub-succulence is encountered in a few species (MASINDE, 2007).

The flowers are also inconspicuous, especially in a field among other vegetation (Figure 1.1). They are often flat to cylindrical, one to several, found together on the side of the stem or rarely terminal, with short to medium length stalks. Their corolla is rotate to tubular, divided to half way or further to the base with five lobes (star-like), free at the tips and variously spreading, or united at the tips forming a cage-like structure (BRUYNS, 1984; DYER, 1983; POOLEY, 2005). The flowers usually open in succession, and the corona are in 2 series (BRUYNS, 2014; POOLEY, 2005). The colours of the flowers are generally dark (brown to maroon, or pinkish purple) and accompanied by a slightly unpleasant odour (BRUYNS, 1984, 2006; PECKOVER, 1993). Species of *Brachystelma* flower at different months throughout the year (BRUYNS, 2014; MASINDE, 2007; POOLEY, 2005; RAVI PRASAD RAO et al., 2011; WALKER, 2008). Flowers are only present for a short while and as a result, plant identification in the field is usually performed by observing leaf morphology (BRUYNS, 1984). However, the resemblance of their leaves to other plant seedlings makes it difficult to locate them.

Pollinia of most *Brachystelma* are usually ovoid and are more or less D- shaped, sub-erect to erect, near or on top of the staminal column (BRUYNS, 2014; DYER, 1983; MASINDE, 2007). The fruit are erect, long or short in fusiform pairs. Once dry, the fruit (now follicle) contains the seeds (BRUYNS et al., 2015; PECKOVER, 1993). The seeds are dark brown or light black, dorsiventrally compressed, linear-elliptic or oblong, curling lengthwise, with a very narrow pale marginal wing along the edge. Seed size ranges from 5 – 10 mm long by 1.5 – 7 mm wide. One end of the seed has a coma comprising a tuft of fine, white, fluffy hairs up to 20 mm long and this allows for dispersal by wind (DYER, 1983; MASINDE, 2007; RAJARAM et al., 2014).

The odour of the nectar is the primary attraction of pollinators which are true flies. The pollination process is affected by the deposit of pollinia on the flower's own stigmatic surfaces fused within the top of the staminal column (DYER, 1983). The arrangement of the male and female reproductive parts, united into a small columnar structure within the base of the corolla, increases the chances of self-pollination (DYER, 1983). Self-pollination inevitably affects the fertilization process due to failure of producing viable seeds (ADNAN et al., 2014;

WYATT et al., 2000). On a grand-scale, the overall effect is evident in the low number of available plants in the field with some species performing better than others.

1.4.4 Ethnobotanical uses

The tubers of several *Brachystelma* species are eaten raw or prepared by some indigenous groups in Africa, Asia and Australia (CHEIKHYOUSSEF et al., 2011; DESHMUKH AND RATHOD, 2013; MASINDE, 2007; MOTEETEE AND VAN WYK, 2011; SMITH, 1991). Based on available data, there are no reports of any *Brachystelma* species being poisonous (DYER, 1983). Five east African species, namely *B. gracile* E.A.Bruce, *B. johnstonii* N.E.Br., *B. plocamoides* Oliv., *B. simplex* Schltr. and *B. rubellum* (E.Mey) Peckover have edible tubers. Some species are reported to be particularly enjoyed by livestock herders in South Africa and East Africa (Kambaland) (DYER, 1983; MASINDE, 2007). The tubers are reported to be very effective at quenching thirst in dry areas where fresh water is scarce and they can also be a good source of starch (DYER, 1983; PARE et al., 2016; RAJARAM et al., 2014; WEHMEYER, 1986). DESHMUKH AND RATHOD (2013) conducted a study that assessed the nutritional value of *B. edulis* Collett and Hemsl. (Asian species). The authors concluded that the tuber of *B. edulis* could be a good supplement for nutrients such as fibre, protein and carbohydrates.

Some *Brachystelma* species have also been reported as medicinal herbs (DESHMUKH AND RATHOD, 2013; DYER, 1983; MASINDE, 2007; PARE et al., 2016). An east African species, *B. buchananii* N.E.Br, is regarded as a medicinal herb with healing properties. The tuber of this species is chewed and thereafter the paste is placed on a wound. In northern Uganda, the tuber of *B. johnsonii* is dried, ground and taken to relieve chest pains (MASINDE, 2007). It is however unclear in what form it is administered. DESHMUKH AND RATHOD (2013) reported on the use of *B. edulis* as a decoction for bodily discomfort as well as its use against coughs and colds. *B. foetidum* Schltr. is used exclusively for colds in children (MOTEETEE AND VAN WYK, 2011). In India (Satara district), various *Brachystelma* species are known to be used medicinally for headaches, stomach ache and colds in children (RAJARAM et al., 2014).

It is also reported that wild animals such as rodents, baboons and porcupines harvest the tubers for food purposes (DYER, 1983; MASINDE, 2007; RAJARAM et al., 2014). *Brachystelma* species fall in the category of famine food and this opens up the possibility of utilization of the tubers as daily food in rural communities (DYER, 1983; RAJARAM et al., 2014). DYER (1983) reports the use of *Brachystelma* tubers as a daily diet of the "natives" in South Africa.

The family Apocynaceae is counted amongst the top seven families, in southern Africa, with the highest number of edible species (RUITERS et al., Unpublished).

1.4.5 Conservation status

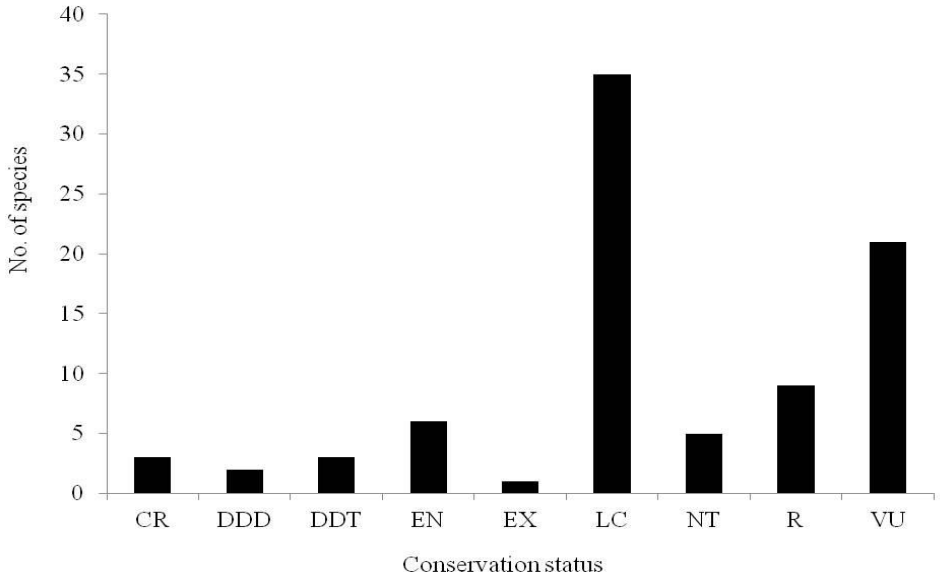

Figure 1.2: Conservation status of *Brachystelma* species occurring in South Africa (Total of 85 species). SANBI Redlist data, 2009. CR- Critically rare, DDD- Data deficient-insufficient information , DDT- Data deficient-taxonomically problematic, EN- Endangered, EX- Extinct, LC- Least concern, NT- Near threatened, R- Rare, VU- Vulnerable.

Many (54%) members of the *Brachystelma* genus have been reported to be of conservation concern on the Red List of South African plants (Figure 1.2) (RAIMONDO, 2013; RAIMONDO, 2014; RAIMONDO et al., 2009). The threat for members of this genus is not only confined to South Africa, Indian *Brachystelma* species are also under threat. SINGH (2012) has indicated *B. laevigatum* and *B. pauciflorum* as vulnerable and endangered due to use by local people as food and for their attractive foliage. RAJAKULLAYISWAMY et al. (2012) also reported on *B. volubile*, which is indicated as critically endangered. VENU AND PRASAD (2015) pointed out that, in India, the grasslands in which *Brachystelma* thrive are often regarded as "wasted or unproductive when left in the hands of nature". The grasslands are instead encroached for agriculture, and are fragmented by urbanization and invasive species.

The natural regeneration (via seed) process of *Brachystelma* is one that is uncertain and very sparingly documented. The percentage of successful fertilization varies from species to species. It is particularly low when compared to bee-pollinated species, however, there are a

few exceptions. It appears that the problem is the placement of pollinia on the somewhat hidden stigmatic surfaces (DYER, 1983). Once fertilization has been successful, the efficiency pattern changes dramatically. The development of the follicle is rapid but takes several to many weeks to mature to seeds (DYER, 1983). The most prevalent constraints with natural propagation of species belonging to the subfamily Asclepiadoideae, have been reported to be the scarcity of pollinators accompanied by poor seed setting and viability (ADNAN et al., 2014; MUTHUKRISHNAN et al., 2013). Propagation via conventional methods i.e. cuttings and seedlings is either absent or not well documented. However, conventional methods are generally known to be inefficient compared to micropropagation (KRISHNA AND SINGH, 2007; MUTHUKRISHNAN et al., 2013; SUTTLE, 2005). Therefore, efficient conservation measures are of paramount importance for the future of the *Brachystelma* genus, especially when their value to mankind has not been fully explored.

1.5 Study aims and objectives

This study was aimed at establishing an efficient micropropagation protocol as a conservation means as well as to establish a relationship between ethnomedicinal uses and scientific studies through the evaluation of the phytochemical and pharmacological properties of three *Brachystelma* species, (*B. ngomense*, *B. pulchellum* and *B. pygmaeum*).

The main objectives of the study were to evaluate:

- The effect of plant growth regulators (PGRs) on *in vitro* plant regeneration;
- The effect of PGRs on phytochemical content of *B. pulchellum* and *B. pygmaeum*; and
- The phytochemical and pharmacological properties of aerial and below-ground parts of the three plant species.

1.6 General thesis overview

Chapter 2: This Chapter, using information sourced from literature, provides a discussion on the plant micropropagation technique, gives an account of research that has been conducted regarding the propagation of *Brachystelma* species using micropropagation. The chapter also includes a general discussion on phytochemicals and their pharmacological properties, and further provides an account of phytochemical and pharmacological investigations that have been conducted regarding *Brachystelma* and related plants.

Chapter 3: This Chapter presents the micropropagation protocol of three *Brachystelma* species i.e. *B. ngomense*, *B. pulchellum* and *B. pygmaeum*. Different concentrations of three cytokinins namely BA, iP and *m*TR, were tested. Supplementation of MS medium with these cytokinins has been observed to enhance shoot proliferation leading to the conclusion that application of BA, iP or *m*TR is beneficial towards *in vitro* shoot multiplication from nodal explants of these species. The investigation of the effect of varying concentrations will also be discussed.

Chapter 4: This Chapter is based on the phytochemical and pharmacological properties of three *Brachystelma* plant species i.e. *B. ngomense*, *B. pulchellum* and *B. pygmaeum*. Wild and *in vitro*-derived plant material was used to identify and quantify the total phenolic compounds and to determine the antioxidant activity of the three *Brachystelma* species. The chapter also includes the effect of cytokinins on the phenolic compounds as well as the antioxidant activity of *B. pulchellum* and *B. pygmaeum*.

Chapter 5: The Chapter summarizes the main conclusions drawn from this study as well as makes appropriate recommendations for prospective research endeavours.

References: This section provides a list of all the cited literature, sourced from various databases e.g. Google Scholar, WorldCat and Scopus, and other material used in the construction of the thesis.

Chapter 2: Literature review

2.1 Micropropagation

2.1.1 Introduction

Micropropagation is an *in vitro* technique with the main purpose of mass propagating whole plantlets from single explants (TITOV et al., 2006). The plantlets derived from this process are often genetically identical to the source plant also known as the parent plant (VUYLSTEKE et al., 1998; ZAID AND AL KAABI, 2003; ZHAO et al., 2005b). This technique is used to achieve many objectives which have in common; the growth of healthy pathogen-free plant material (ENGELMANN, 2011; KOZAI et al., 1997; RANI AND RAINA, 2000). Among these objectives is the production of a large number of plants in a relatively short time and with minimum space used (DEBNATH et al., 2006; RANI AND RAINA, 2000; XIAO et al., 2011). In addition, micropropagation allows for the propagation of seasonal species to be carried out through the year, as well as to increase yield as a result of an increased proliferation rate (DEBNATH et al., 2006; RANI AND RAINA, 2000; ZAID AND AL KAABI, 2003). Variables such as proliferation rate are influenced by the use of different types and concentrations of plant growth regulators (PGRs), mainly auxins and cytokinins (WERBROUCK, 2010). In fact, the success or failure of micropropagation depends on the detailed and often complex interactions of a number of factors. These factors include the type and source of explants, the decontamination protocol, the type of basal medium, the concentration as well as the balance of medium constituents, and *in vitro* ecological parameters such as light and temperature (REED et al., 2011).

To complete the process of micropropagation, there are five stages which are commonly used by research and commercial laboratories globally (DEBERGH AND READ, 1991; KANE, 2005; RANI AND RAINA, 2000; VUYLSTEKE et al., 1998). The first stage involves the selection and preparation of parent stock plants (DEBERGH AND MAENE, 1981; VASIL, 1994). This is the stage in which healthy explants capable of being initiated into culture are selected from the parent plants (DEBERGH AND READ, 1991; KANE, 2005). Preparation of the stock plants may also involve growing them under controlled conditions and the use of fungicides which potentially alleviate contamination levels on the explants (DEBERGH AND READ, 1991; KANE, 2005). This stage has also been reported to increase the rate of explant survival during culture initiation (DEBERGH AND MAENE, 1981; KANE, 2005).

The second stage comprises the initiation and establishment of aseptic cultures (VASIL, 1994). Due to their totipotent ability, explants obtained from the various plant tissues have the potential to regenerate plantlets when provided with the appropriate nutrient and environment conditions (DEBERGH AND READ, 1991; JASKOWIAK, 2014; KANE, 2005; ZAID AND AL KAABI, 2003). It is also important that this environment be sterile i.e. free of any obvious infection by microbes (TENG et al., 2002). For this to be achieved, careful attention must be given to the choice of explants and their decontamination (DEBERGH AND MAENE, 1981). There are five important factors, originally listed by Murashige (MURASHIGE AND SKOOG, 1962), which are considered when choosing suitable explants (KANE, 2005). One must consider the organ that will serve as an explant source, the physiological age of the organ, the season in which the plants are obtained, the size of the explant, and the overall quality of the stock plant. In addition to explant choice, the decontamination treatments used for explants are crucial for successful initiation into culture (DEBERGH AND MAENE, 1981). The explants, when transferred into sterile media, need to be free of any contaminants in order to establish a sterile culture (KANE, 2005). Contaminated cultures often results in the death of explants because contaminants, usually fungi or bacteria, outgrow the explants in culture thereby starving them and may even produce substances that are toxic to the explants (DEBERGH AND MAENE, 1981; XIAO et al., 2011; YILDIZ, 2012).

The third stage, involving proliferation of propagules, is aimed at optimizing the production of aseptic individuals which can potentially grow into healthy whole plants (KANE, 2005; ZAID AND AL KAABI, 2003). Proliferation can be achieved through axillary shoot production, adventitious shoots as well as somatic embryogenesis (DEBERGH AND MAENE, 1981). Adventitious shoot proliferation can occur directly from the cultured explants or indirectly via callus production. A particular predetermined regeneration pathway may be characteristic for a specific tissue. However, the type and concentration of exogenous PGRs as well as the culture environmental conditions often affect and can modify the regeneration pathway (DEBERGH AND MAENE, 1981; KANE, 2005).

In the fourth stage of the micropropagation process, propagules are prepared for *ex vitro* transfer and acclimatization. The objective of this stage is to prepare regenerated shoots or plantlets for successful transfer into *ex vitro* conditions since in the previous stage they are often too small and unfit to survive in soil (DEBERGH AND READ, 1991). This stage involves the *in vitro* root induction on individual regenerated shoots, hardening of plants to impart

some tolerance to moisture stress, as well as ensuring that the plants are capable of autotrophic development (DEBERGH AND READ, 1991; KANE, 2005).

The fifth and final stage focuses on *in vivo* rooting and acclimatization for soil establishment (VASIL, 1994). It has been reported to be the most labour intensive and the most costly stage of micropropagation (DEBERGH AND READ, 1991; XIAO et al., 2011; ZAID AND AL KAABI, 2003). The root system produced *in vitro* is often non-functional ex *vitro*, therefore it is then necessary for the plantlets to form new roots after *in vivo* planting (DEBERGH AND MAENE, 1981). This often requires the plant to momentarily halt its growth process. In order to achieve optimal root formation, the presence of auxin is normally required during the induction phase (DEBERGH AND MAENE, 1981). Extra care needs to be taken during the transplanting process to avoid damage and exposure to infection (DEBERGH AND READ, 1991; KANE, 2005). Ultimately, the success of micropropagation not only lies in the production of large numbers of plantlets but also on their survival under natural conditions (CHANDRA et al., 2010; YANG AND YEH, 2008). Survival of plantlets has been reported to have a direct relationship to PGRs such as auxins which work in concert with rooting mixtures (CHANDRA et al., 2010).

2.1.2 Nutrient media

PGRs are supplemented into the nutrient media, in most cases, MURASHIGE AND SKOOG (MS) (1962) medium which is foundational in *in vitro* propagation (BEYL, 2005; CHAWLA, 2002). Nutrient medium was developed as a substrate to imitate actual soil conditions and therefore contains the elements which are labelled as essential for successful plant growth and development. Among the factors influencing explant growth and development in culture, medium constituents are usually the easiest to manipulate (BEYL, 2005). The MS medium generally successfully facilitates the maintenance of any explant that is capable of growth, however, it is not necessarily suitable for all plant material (SMITH, 2000). Failure of plant material to grow on this medium does not automatically disqualify the material as incapable of any type of growth. At this stage, the development of a suitable medium is necessary and this process is purely based on trial and error (BELL et al., 2009; BEYL, 2005; SMITH, 2000). LINSMAIER AND SKOOG (1965) adjusted the organic components of MS medium to create LINSMAIER AND SKOOG (LS) medium which was suitable for certain types of tissue (BEYL, 2005). Likewise, LLOYD AND MCCOWN (1980) created the woody plant medium (WPM) to counter salt sensitivity in some woody plants (BEYL, 2005). It is therefore worth noting that

plain MS media merely serves as a starting point. Generally, MS medium contains salts which are divided into macro- and micro-elements, supplemented with vitamins and carbohydrates accompanied by a gelling agent (BEYL, 2005; CHAWLA, 2002). Gelling or solidification agents include agar, agarose and gelrite but the most commonly used is agar (ROUT et al., 2000). Media composition, beyond plain MS media, is entirely dependent on the outcome that is desired at the time (SMITH, 2000). The desired outcome, for example shoot proliferation or root induction, is generally facilitated by media strength and the addition of PGRs, mainly auxins and cytokinins (BEYL, 2005; CHAWLA, 2002). In the development of medium, it has been established that actual and relative concentrations of various inorganic nutrients are crucial (CHAWLA, 2002). Generally, conversions of concentrations and other such calculations are part and parcel of arriving at a suitable medium (BEYL, 2005). Medium suitability also involves having appropriate pH levels. Adjustment of pH of the medium (generally between 5.6 and 5.8) is the concluding step before adding agar and is important as pH determines a number of structural aspects as well as biological macromolecule activity (CHAWLA, 2002; MURASHIGE AND SKOOG, 1962). The pH is adjusted with the use of dilute or concentrated acid or base solutions. A pH below 5.5 results in the gelling agent not hardening properly and on the other hand, a pH of 6 results in the gelling agent being too solid. Autoclaving (at 121 ^{0}C) usually results in a pH decrease of about 0.6 to 1.3 units. In addition, some plant tissue, through the production of organic acids or nitrogen utilization, causes the pH to further drop with time (CHAWLA, 2002).

2.1.3 Effect of auxins and cytokinins on plant regeneration

Auxins and cytokinins are chemicals that occur naturally in plants, and they have a regulatory role on plant growth and development (BEYL, 2005; VASIL, 2008; WERBROUCK, 2010; YANG et al., 2003). These PGRs are generally active at low concentrations, however, concentrations of PGRs in plants change with time, season and developmental stage (BAI et al., 2010; CHAWLA, 2002; GABA, 2005). The synthetic PGR forms have been reported to have similar biological effects to the natural analogues (GEORGE et al., 2008; SRIVASTAVA, 2002). Auxins and cytokinins are usually a requirement in micropropagation as they regulate growth and are responsible for morphogenic changes in plants (BAI et al., 2010; GASPAR et al., 1996). As a result, PGRs are frequently added to the culture media (GABA, 2005; GEORGE et al., 2008). It has been reported that the regulatory role of the PGRs in plant growth and development is dependent on three main factors, namely concentration of PGRs present, location of the PGR

and the sensitivity or responsiveness of plant tissues (GASPAR et al., 1996; MOYO et al., 2011). The exogenous application of many PGRs may however, modify the synthesis, degradation, activation, storage or translocation of similar or different types of endogenous PGRs (GABA, 2005; GASPAR et al., 1996). Plant tissue culture essentially involves the experimental manipulation of important PGRs to achieve the desired morphogenetic response at each *in vitro* culture stage (GEORGE et al., 2008). Generally, the initial stages of bud induction from parent explants and the shoot proliferation that follows are achieved using either one type of cytokinin or using a cytokinin/auxin combination (GASPAR et al., 1996; SRIVASTAVA, 2002; WERBROUCK, 2010). The elongation of shoots is stimulated by a combination of auxins and cytokinins (BEYL, 2005) and rooting is normally achieved using auxins at different concentrations (GEORGE et al., 2008; MOYO et al., 2011).

2.1.4 The explant material

Explant material is also important for the success of tissue culture studies. There are a number of factors that affect tissue culture regeneration responses namely, explant source, explant age, explant size, explant position in donor plant, explant density, genotype and physiological stage of the donor plant (CHAWLA, 2002; YILDIZ, 2012). Almost all parts of a whole plant can be used in tissue culture, however, some, depending on specific plants, will yield better results than others (CHAWLA, 2002; YILDIZ, 2012). The initiation of micropropagation with the use of any explant not of apical or axillary bud origin has a higher probability to yield off-type plants (CHAWLA, 2002). Off-types are undesirable in the production of certain plant material, for example, in forestry where a high frequency of variation is not ideal (DEBERGH AND MAENE, 1981). Thin cell layers are also used as explants in transformation-related studies (YILDIZ, 2012). Generally, in tissue culture, the production of a whole plant is dependent on a single cell (VASIL, 2008). Even highly mature and differentiated plant cells retain the ability to change to a meristematic state and differentiate into a whole plant only if a viable nucleus and membrane system are still intact (CHAWLA, 2002). Types of explants that have been used in micropropagation of Apocynaceae are listed in **Appendix 1**.

2.1.5 Effect of *in vitro* ecological factors

Other factors to be considered, that influence growth and development, are ecological factors such as light, temperature and relative humidity (ZACCHINI et al., 1997). These factors have

to be optimized for different plants (KOZAI et al., 1997). Lighting is one of the main factors for optimization of plant growth and development in micropropagation (ROUT et al., 2000). It is critical for *in vitro* plant growth because it is the source of energy for photosynthesis (FRANKLIN, 2009; SERRET et al., 1996). Light intensity, photoperiod and spectral quality are the major important light parameters that influence plant physiological processes and growth in micropropagation (IBARAKI AND NOZAKI, 2005; ZACCHINI et al., 1997). Light energy in micropropagation is measured using photosynthetic photon flux (PPF) in the growth room (XIAO et al., 2011). Low light intensities (40-65 µmol m^{-2} s^{-1}) are generally used in micropropagation systems because exposure to high PPF levels can result in photoinhibition and photo-oxidative damage in *in vitro* plants (AMÂNCIO et al., 1999; KODYM AND ZAPATA-ARIAS, 1999; XIAO et al., 2011). Photoperiod is one of the critical ecological factors that control plant growth and development (KÜHN et al., 2009). The key advantage of micropropagation systems is that the photoperiod can be modified to suit the growth conditions for a specific plant. The normal photoperiod for most micropropagation systems is 16 h light and 8 h dark (KODYM AND ZAPATA-ARIAS, 1999; MAZUMDAR et al., 2010). Plant growth under micropropagation systems is also profoundly controlled by temperature (FRANKLIN, 2009). In particular, the biophysical and biochemical process of photosynthesis, which is a major determinant of plant growth, is controlled by temperature (KOZAI et al., 1997). Unlike under natural conditions, a constant temperature regime is maintained in micropropagation systems (IBARAKI AND NOZAKI, 2005). Growth room temperatures are usually kept at 25 ^{o}C, however, modifications are necessary for certain plants (KODYM AND ZAPATA-ARIAS, 1999; ROUT et al., 2000).

2.1.6 Challenges of contamination in micropropagtion

The first major challenge of micropropagation is that of obtaining pathogen-free plant material (TENG et al., 2002; TITOV et al., 2006; ZHANG et al., 2008). The use of underground parts such as tubers as a source of explants makes it difficult to establish uncontaminated material *in vitro* as soil is conducive to contaminants (CHANG et al., 2003). The basal media used in micropropagation provide ideal conditions for the growth of contaminants which are usually bacteria and fungi (PATIL et al., 2010; TITOV et al., 2006; ZHANG et al., 2008). In order to achieve microbe-free cultures, the growth medium and instruments to be used have to be sterilized by autoclaving at 121 ^{0}C (BEYL, 2005; PATIL et al., 2010). This is augmented by surface decontamination of explants using sterilants such as 70% ethanol (EtOH), calcium

hypochlorite (CaOCl), sodium hypochlorite (NaOCl) and mercuric chloride ($HgCl_2$) (TENG et al., 2002; YILDIZ, 2012; ZHANG et al., 2008). In other cases, contamination emanating from internal infections particularly for explants which have a vascular system can only be minimized by using antibiotics (TENG et al., 2002; ZHANG et al., 2008). On the other hand, the surface sterilization process must cause the least damage on the plant material. The explants viability and regeneration capacity should be taken into consideration when choosing the sterilant, its concentration and period of exposure (YILDIZ, 2012).

2.1.7 Micropropagation in the family Apocynaceae

During the course of this study, no other studies reporting on the micropropagation of *Brachystelma* species were found. The closest available literature is that of the *Brachystelma* family, Apocynaceae (**Appendix 1**). One of the closest members, to *Brachystelma*, in this family is the genus *Ceropegia* (BRUYNS et al., 2015; DYER, 1983). MUTHUKRISHNAN et al., (2013) gives a sufficient account of the different *in vitro* propagation approaches for *Ceropegia* species as well as *in vitro* techniques used for the production of phytochemicals found in the genus *Ceropegia*, mainly Cerpegin. *Ceropegia* species such as *C. spiralis*, *C. juncea* and *C. thwaitesii* have been propagated to the stage of successful rooting with the use of various meristems including nodal segments, axillary and apical buds (MUTHUKRISHNAN et al., 2013). *In vitro* and *ex vitro* root induction for a number of *Ceropegia* species has been shown to yield the same result, however *ex vitro* rooted shoots displaying superiority during the hardening stage. This makes *in vitro* rooting an unnecessary step and the bypassing of which would save time, resources as well as labour (MUTHUKRISHNAN et al., 2013; PHULWARIA et al., 2013). *Ex vitro* rooting, particularly in *C. bulbosa*, was obtained after regenerated shoots were treated with 100 mgl^{-1} IBA for 3 min (PHULWARIA et al., 2013). Likewise, *Catharanthus roseus* (Apocynaceae), well known for its anti-cancer potential, has also been micropropagated (KUMAR et al., 2013). Standardization of an efficient plant regeneration protocol for *C. roseus* has been achieved using axillary buds and shoot-tip explants (BAKRUDEEN et al., 2011; DEBNATH et al., 2006; KUMAR et al., 2013). For instance, the best shoot production was obtained from MS medium supplemented with 4.0 mgl^{-1}NAA and 4.0 mgl^{-1}BA (BAKRUDEEN et al., 2011). Other studies have observed the effect of auxins, cytokinins, nutrient media and explant source on callus induction and shoot multiplication of *C. roseus* (DEBNATH et al., 2006). Successful rooting has also been obtained using half strength MS medium supplemented with IBA (BAKRUDEEN et al., 2011; KUMAR et al., 2013).

These studies have also investigated morphological changes that are possibly as a result of *in vitro* or *in vivo* cultivation (DEBNATH et al., 2006). These morphological changes which may result in production of somaclonal variants are a common hindrance to the up-scaling of micropropagation techniques (KUMAR et al., 2013). The plant regeneration method performed by DEBNATH (2006) has been reported as an ideal approach for the large scale production of leaf material for alkaloid extraction.

2.2 Phytochemicals in plants and their pharmacological potential

2.2.1 Phytochemicals in plants

Plant secondary metabolites otherwise known as phytochemicals, were originally known to be a source of active pharmaceuticals among other important uses (BOURGAUD et al., 2001; TAIZ AND ZEIGER, 2006; ZHAO et al., 2005a). This knowledge of phytochemical bioactivity led to further exploration towards the unveiling of their adaptive significance. The search for adaptive significance of phytochemicals resulted in their association mainly with defence and signalling in plants (BOURGAUD et al., 2001; KENNEDY AND WIGHTMAN, 2011; ZHAO et al., 2005a). There are three main and chemically distinct classes of phytochemicals, the alkaloids, phenols, and terpenes (BOURGAUD et al., 2001; KABERA et al., 2014). The alkaloids are the group that predominantly contains basic "alkaline" nitrogen atoms (JALEEL et al., 2009; ROBERTS AND WINK, 1998). A few examples include the indole alkaloids, polyketide alkaloids and purine alkaloids (CROTEAU et al., 2000). Unlike other classes of secondary metabolites, alkaloids are characterised by a huge difference in their structures thus there is no uniform classification for this group (KABERA et al., 2014). The closest form of classification is in the similarity of their carbon skeleton. The ecological function of alkaloids is typical of most phytochemicals as they play a role directed more towards plant protection rather than nutrient supply (CROTEAU et al., 2000). Many alkaloids such as caffeine, codeine and quinine have been found to have a bitter taste which is suggested to be an important feature to deter feeders (KABERA et al., 2014; ROBERTS AND WINK, 1998). In this regard, alkaloids are found to have a direct interaction with molecular targets within the nervous system of plant consumers. This interaction has been useful in the development of clinical applications towards modification of the functioning of the central nervous system (KENNEDY AND WIGHTMAN, 2011).

Polyphenols are reported to be the most numerous and structurally diverse group (BALASUNDRAM et al., 2006; BENNETT AND WALLSGROVE, 1994). A few typical examples are

the flavonoids, phenolic acids, and tannins (BALASUNDRAM et al., 2006; BARROS et al., 2012). The phenols are characterised by one or more hydroxyl groups which are bound directly to an aromatic hydrocarbon group (IGNAT et al., 2011; KABERA et al., 2014). Most phenolic compounds are synthesised precursors resulting from the phenylpropanoid pathway (BALASUNDRAM et al., 2006; GRUZ et al., 2011; IGNAT et al., 2011). In addition to their role in plant defence, some contribute to symbiotic relationships with insects and soil bacteria (KABERA et al., 2014; TAYLOR AND GROTEWOLD, 2005). Although there is a diverse array of important health benefits associated with this class and other phytochemicals in general, very little is understood about their cellular targets in plants or animals (BALASUNDRAM et al., 2006; BENNETT AND WALLSGROVE, 1994; KABERA et al., 2014).

The terpenes (terpenoids) are classified according to the number of isoprene units as they are isoprene derivatives (CROTEAU et al., 2000; PATIL AND BHALSING, 2016). Their original synthesis is from acetate by means of the mevalonic acid pathway (KABERA et al., 2014; KENNEDY AND WIGHTMAN, 2011). In the process of terpene formation, there is a head to tail link between the isoprene units which ultimately determines their classification (CROTEAU et al., 2000). A few typical examples are the carotenoids, gibberellins and steroids (PAVARINI et al., 2012; VERPOORTE AND MEMELINK, 2002). Terpenes have been recognized as feeding deterrents with lethal potential on plant feeding insects and other predators (BENNETT AND WALLSGROVE, 1994; CROTEAU et al., 2000). Some terpenes are hydrophobic i.e. non-volatile whereas the remainder are extremely volatile (PAVARINI et al., 2012). Volatility mainly provides a means of communication between a plant and its competitors, pollinators, predators etc. via the atmosphere (KENNEDY AND WIGHTMAN, 2011; VERPOORTE et al., 2002). This is a trait that has been useful in agricultural applications. A typical example of some non-volatile terpenes is the limonoids which are found mainly in citrus fruit. Limonene, belonging to the limonoids, has played a role in the pharmaceutical industry as a dietary anti-carcinogen (BENNETT AND WALLSGROVE, 1994; KABERA et al., 2014).

2.2.1.1 Phytochemicals of the Apocynaceae

Members of the Apocynaceae typically possess secondary metabolites such as cardiac glycosides, flavonoids, tannins and terpenoids (GURIB-FAKIM, 2006; HENEIDAK et al., 2006; PURUSHOTHAMAN et al., 2016). However, they are better known for the potent pharmacological activity of their alkaloid content. *Catharanthus roseus* (*Vinca rosea*) is a

classical example that was found to be the source of over 150 alkaloids. A number of these were found to be indole alkaloids including dimeric and bis-indole. Further isolation led to the characterisation of vinblastine and vincristine which are known to treat breast cancer, childhood leukaemia and Hodgkin's disease with a high success rate (GURIB-FAKIM, 2006; MACQUEEN, 1988; SIDDIQUI et al., 2011). In addition, *C. roseus* has been used for centuries to treat a wide variety of diseases globally.

KADIYALA et al. (2013), reported on the versatility of isolated phytochemical compounds of *Calotropis gigantiea* in treating a wide spectrum of diseases. This versatility is once again evident from the phytochemical compounds of *Tabernaemontana* species (SATHISHKUMAR AND BASKAR, 2014; WONG et al., 2011). The large amount of isolated compounds from the Apocynaceae species and other plant groups can be viewed as an indication of the value of their potential in the pharmaceutical industry. From the few reported studies, it is evident that the Apocynaceae members have in common the potential to treat diabetes and cancer related diseases which are often associated with oxidative damage (AYOOLA et al., 2008; GURIB-FAKIM, 2006; KADIYALA et al., 2013; SATHISHKUMAR AND BASKAR, 2014; WONG et al., 2011).

2.2.1.2 Phytochemicals in *Brachystelma*

Preliminary phytochemical studies of *Brachystelma* were conducted on the basis of reported use of this genus by native communities. Most studies search first for alkaloids as they are known among the Apocynaceae to possess a range of pharmacological activity including anti-bacterial, anti-cancer and anti-malarial activities (GURIB-FAKIM, 2006; KABERA et al., 2014; ROBERTS AND WINK, 1998; WONG et al., 2011). Some *Brachystelma* species have been observed to have a fibrous stem covered by bark that appears red in colour. Red bark has been reported to be characteristic of chromoalkaloids located in the cellular vacuoles of sub-epidermal tissue (PATIL AND BHALSING, 2016). Phytochemical analysis of *Brachystelma edulis* indicated the presence of alkaloids, cardiac glycosides, phenols, saponins and tannins (RAJARAM et al., 2014). Similarly, *Brachystelma bingeri* was shown to contain saponins, triterpenes and sterols (PARE et al., 2016).

Generally, these phytochemicals i.e. alkaloids, cardiac glycosides, phenols, saponins, triterpenes and sterols have been identified as useful in one way or another (ABDELGADIR AND VAN STADEN, 2013; GURIB-FAKIM, 2006; KABERA et al., 2014). Tannins, for example,

in addition to pharmaceutical use, have also been reported to have a diverse range of uses in other industries such as the food industry (KHANBABAEE AND VAN REE, 2001). The dependency of man on phytochemicals as an important source of pharmaceuticals has been known for a long time (KABERA et al., 2014; ROBERTS AND WINK, 1998). A more detailed understanding of phytochemical function as pharmaceuticals is gained through continued investigations which hastens the process of new drug discovery. Evidently, for *Brachystelma* species, this is an area that still requires extensive investigation particularly in South Africa where a notable portion of the genus occurs.

2.2.2 Pharmacological activity

Pharmacology is generally understood to be a branch of medicine concerned with the uses, effects and modes of action of drugs. The therapeutic action of plant material was observed in various traditional medicine practices which led to further investigation (SATHISHKUMAR AND BASKAR, 2014). Medicinal plants have been an area of interest since ancient times, however, work on experimental pharmacology was born in the early 19th century (GROVER AND YADAV, 2004; TANAKA et al., 2009). Extensive biological investigations of the various classes of plant secondary metabolites have since unveiled a broad range of pharmacological properties (NCUBE et al., 2012). Some of which have become very useful in medicinal applications. For instance, the terpenes exhibit anti-hypertensive, anti-microbial, anti-carcinogen and hepaticidal properties (KABERA et al., 2014; KENNEDY AND WIGHTMAN, 2011). Bilobalide and ginkgolides, and ginsenosides are some terpenes found in *Ginkgo biloba* and *Panax ginseng* that are known to be active against dementia, and have neuro-protective and anti-depressant properties (BARNES AND PRASAIN, 2005; KENNEDY AND WIGHTMAN, 2011; WINK, 1999). Phenolics have demonstrated antioxidant activity, UV protection and chemoprotective properties among others (PATIL AND BHALSING, 2016; TANAKA et al., 2011; ZOVKO KONČIĆ et al., 2010). Curcuma obtained from *Curcuma longa* (L.), is a typical example of a phenol known for its oestrogenic and anti-oestrogenic properties. The alkaloids have anti-malarial, anti-cancer, anti-inflammatory and anti-allergenic properties. For instance, morphine and taxol found in *Papaver somniferum* and *Taxus baccata* are known to function as analgesics and anti-cancer agents, respectively (WINK, 1999).

2.2.2.1 Pharmacological activity of the Apocynaceae

A number of species in the family Apocynaceae have been documented for their pharmacological uses (**Appendix 2**). Pharmacological evaluations of the Apocynaceae reveal a wide range of therapeutic potential some of which have been isolated as novel chemicals (SRIVASTAVA, 2014). Anti-microbial and antioxidant activities are among the commonly found biological activities in the Apocynaceae group. The ethnobotanical use of *Brachystelma* species as medicinal herbs for treatment of bacterial and fungal borne illnesses sustains the motion towards investigating potential anti-microbial activity. Even though anti-microbial activity has not been reported in *Brachystelma* species, members of the *Brachystelma* tribe, Ceropegieae, are commonly known for their use in the treatment of bacterial and fungal infections (DOUGHARI AND OBIDAH, 2008; HERRERA et al., 1996; MUTHUKRISHNAN et al., 2013). The lack of documented studies on the pharmacological investigations of *Brachystelma* species is an invitation for more comprehensive studies. Therefore, further investigations are encouraged including toxicological tests to assess their safety.

Earlier studies have reported antioxidant activity in members of the Apocynaceae (DHIR AND SHEKHAWAT, 2014; WOODE et al., 2007). A number of researchers have recognised the antioxidant activity of *Ceropegia* species (CHAVAN et al., 2013; CHAVAN et al., 2014a; DHIR AND SHEKHAWAT, 2014) (**Appendix 3**). Species of *Ceropegia* are not only found within the same tribe as that of *Brachystelma* but also have similar morphological features (**Appendix 5**) (MEVE AND LIEDE, 2001). DHIR AND SHEKHAWAT (2014) conducted a study, using methanolic extracts, from leaves of both the wild and *in vitro* grown *Ceropegia* plants. Total antioxidant capacity was found to be highest in *in vitro*-derived plants. Other studies have also reported obtaining higher levels of antioxidant activity from the *in vitro*-derived *Ceropegia* species (CHAVAN et al., 2013; CHAVAN et al., 2014a). Antioxidant activity has been reported in some *Brachystelma* species, with limited or no reports of investigations validating the isolation of bioactive compounds with antioxidant activity. DESHMUKH AND JADHAV (2014) conducted a study on the antioxidant bioactivity of wild edible tubers among them was *B. edulis* which demonstrated good antioxidant capacity. Thus indicating that *Brachystelma edulis* is a potential source of natural antioxidants, and further supporting evaluation of the entire genus of mostly endemic species in order to uncover potential

medicinal uses. These evaluations are also necessary to scientifically support and secure traditional ethnobotanical knowledge.

2.2.2.2 Antioxidant activity

Oxidative stress is considered to be involved in the "normal" process of aging but also in the development and progression of various human degenerative diseases (COSTA et al., 2012; OU et al., 2001; YOO et al., 2008). These include cardiovascular and neurodegenerative diseases as well as infections and tumours (AYOOLA et al., 2008; BECKER et al., 2014; DAKAH et al., 2014). Most of these conditions involve a large production of reactive oxygen species (ROS) which are released as part of an anti-proliferative strategy during pro-inflammatory immune responses (FAWOLE et al., 2010; ZOVKO KONČIĆ et al., 2010). Antioxidants have beneficial effects in this regard, most of which are considered to be derived from their influence on the immune system (DESHMUKH AND JADHAV, 2014; SHARMA AND BHAT, 2009; SULAIMAN et al., 2011). The antioxidant activity of plant preparations may be important in the prevention of oxidation processes and in counteracting the activation of redox-regulated signalling pathways (ADNAN et al., 2014; BECKER et al., 2014; GÜLÇIN, 2012).

The idea of antioxidant activity is based on the availability of electrons to neutralize any free radicals (COSTA et al., 2012). As such there are a number of antioxidant activity screening methods. These methods have common ideal requirements among which are simplicity, a defined endpoint and chemical mechanism, and adaptability for assay of both hydrophilic and lipophilic antioxidants as well as making use of different radical sources (GÜLÇIN, 2012). Based on the reaction mechanism, the methods are either hydrogen atom transfer (HAT) or single electron transfer (SET) (COSTA et al., 2012; TIJERINA SÁENZ et al., 2009). Examples of methods involving HAT reaction include the chemiluminescent assay, oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP). On the other hand, SET methods include ferric ion reducing antioxidant power (FRAP) assay, trolox equivalence antioxidant capacity (TEAC) assay and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (GÜLÇIN, 2012; TIJERINA SÁENZ et al., 2009). Among the most commonly used methods are ORAC and DPPH as they have certain advantages in the area of ideal requirements (DÁVALOS et al., 2003; JABERIAN et al., 2013; WOJDYŁO et al., 2007; WONG et al., 2011). When screening, it is also ideal to make use of a combination of different

types of assays i.e. one that addresses radical scavenging and one that is associated with lipids (COSTA et al., 2012; TIJERINA SÁENZ et al., 2009). In addition, the universal use of specific or standard methods enables easy comparison of data among researchers.

2.3 Conclusions

Plants, both in their raw and altered form, have benefited man in countless ways. One of the most outstanding being their pharmaceutical potential. Medicinal plants are an important line of research as they provide leads towards therapeutic concepts which accelerate the pace of drug discovery. A lot of work has been done in the area of "medicinal" plant use as pharmaceutical resources. Over the years researchers have made many discoveries through phytochemical and pharmacological investigations. However, only a portion of the available plant resources have been investigated. In the area of plant based medicine, micropropagation holds great potential as a propagation tool and has proven to be reliable for the rapid clonal propagation of various plant species.

Chapter 3: Micropropagation of three *Brachystelma* species

3.1 Introduction

Over the past few decades, plant science has seen advancement through the development and use of new methods for crop improvement. These methods mainly involve the manipulation of plant cells, tissue and organs (RANI AND RAINA, 2000). Biotechnological propagation methods in particular have been used as alternative techniques as they are faster in achieving optimum yield and production rates compared to conventional methods (MUTHUKRISHNAN et al., 2013; VUYLSTEKE et al., 1998; XIAO et al., 2011). Globally, plant biotechnology, in particular plant tissue culture via micropropagation, has remained valuable in its beneficial role in conservation and mass production of plants, and plant-based resources (CHAVAN et al., 2014b; CRUZ-CRUZ et al., 2013; PATHAK AND ABIDO, 2014). Plant tissue culture is the practice of aseptic cultivation of seeds, and plant parts including embryos and single cells on nutrient media *in vitro* (ZAID AND AL KAABI, 2003). Micropropagation is one of the most used components of tissue culture (GASPAR et al., 1996). Its main objective is the production of clonal plants that are true-to-type (VASIL, 1994; ZAID AND AL KAABI, 2003). The achievement of successful micropropagation is mainly dependent on plant growth regulators (PGRs) among other factors (see Chapter 2). Synthetic PGRs, having similar physiological activities and biological effects as the endogenous PGRs, are used to regulate plant growth and development. For instance, the initial application of synthetic auxin and cytokinin was found to significantly benefit root and shoot formation in *Nicotiana tabacum* (MURASHIGE AND SKOOG, 1962). Biotechnological techniques have since been used and are constantly being improved by several commercial and research facilities globally (CAPONETTI et al., 2005).

Brachystelma R. Br. ex Sims (Apocynaceae) is a genus of geophytic perennial herbs. They are chiefly found in sub-Saharan Africa, many endemic to South Africa (SA). A large portion of *Brachystelma* species, in general, are reported to be of conservation concern. Fifty-four percent of those found in South Africa are under threat. The species of interest in this study are *Brachystelma ngomense* (EN), *Brachystelma pulchellum* (VU) and *Brachystelma pygmaeum* (LC), two of which are of conservation concern (**Figure 3.1**). The natural

regeneration of *Brachystelma* species is via seed, however, due to scarcity of pollinators accompanied by poor seed set and poor seed viability, natural regeneration is low. Also, the fruits are often subjected to aphid attack (**Figure 3.2D**) which is a factor likely contributing to the poor seed set as well as low viability. Propagation via conventional methods such as seedlings and cuttings is either apparently not practised or there has been lack of documentation. Some *Brachystelma* species have been reported for their use as food and as medicinal herbs mainly in countries in Africa and India. An efficient propagation method for *Brachystelma* species is therefore desirable so as to ensure their diversity.

Despite their use in traditional medicine, there is, according to the best of available knowledge, no documented report on the micropropagation of *Brachystelma* species. However, there are a number of studies on the micropropagation of *Ceropegia* species (Apocynaceae). *Ceropegia* is a genus closely-related and morphologically similar to *Brachystelma* for which there has been reports of successful micropropagation using different explants (CHAVAN et al., 2011; MUTHUKRISHNAN et al., 2013; NIKAM AND SAVANT, 2009; PALAWAT AND LODHA, 2014). Callus culture and *in vitro* flowering protocols have also been established for species of *Ceropegia* (NIKAM AND SAVANT, 2009). However, no two plants or plant groups are the same especially genotypically. Although there is an available standard approach, this approach interacts within biological determinants which produce a response. Therefore, it is necessary to develop protocols that will be effective for the *Brachystelma* genus. The establishment of successful propagation protocols would potentially decrease the use of the threatened wild populations. All the studies conducted on *Brachystelma* species have reported using plants obtained from the wild.

This study was aimed at establishing an efficient micropropagation protocol as a means to alleviate conservation concern of *Brachystelma* species. The effect of varying concentrations of auxins and cytokinins on explants of *B. ngomense, B. pulchellum* and *B. pygmaeum* was investigated.

Figure 3.1: *Brachystelma* species investigated. A- *B. pygmaeum* fruit. B- *B. pulchellum* fruit © Adam Shuttleworth. C- *B. ngomense* flowers. D- *B. ngomese* tuber. E- *B. pulchellum* flowers. F- *B. pulchellum* tuber. G- *B. pygmaeum* flowers. H- *B. pygmaeum* tuber. Scale bar = 10 mm

3.2 Materials and methods

3.2.1 Explant decontamination and bulking up of experimental material

Stock plants of *B. ngomense, B. pulchellum* and *B. pygmaeum* were obtained (March and April, 2015) from the Botanical garden at the Univesity of KwaZulu- Natal, Pietermaritzburg where they were kept in a shade house. Voucher specimens (*B. ngomense* R. Br. *A. Shuttleworth 335* (NU), *B. pulchellum* R. Br. *N. Hlophe* 20 (NU) and *B. pygmaeum* R. Br. *A. Shuttleworth 322* (NU)) can be found at the UKZN Bews Herbarium (NU). Young stem and tuber explants were excised from the stock plants and thoroughly cleaned with running tap water. In the laboratory, the plants were further washed with liquid detergent followed by thorough rinsing with tap water. In the process of the surface decontamination treatment,

stem and tuber explants were submerged in 1% Benlate® (w/v) for 30 min followed by a solution of 70% ethanol (v/v) for 60 s. This process was accompanied with frequent agitation of the solution to ensure maximum contact of explants with the sterilant. The plant material was then rinsed three times, with distilled water. Thereafter, varying concentrations of two sterilants, namely sodium hypochlorite (NaOCl) (v/v) and mercuric chloride ($HgCl_2$) (w/v) were used as independent treatments for the surface decontamination of the explants. The sterilant solutions were supplemented with a few drops of the surfactant, Tween 20. The plant material was kept in this solution for varying periods of time, during which the solutions were frequently agitated to allow for optimum contact between explants and solution. The explants were once again thoroughly rinsed, three times, with sterile distilled water under sterile conditions in a laminar flow bench. Surface decontaminated explants were further divided into lengths of ~10 mm each, and inoculated into culture tubes containing 10 ml of full strength MURASHIGE AND SKOOG (MS) (1962) basal medium supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol and solidified with 8 g/L bacteriological agar. The agar was added after the pH of the medium was adjusted to 5.8 using either HCl or NaOH (Sigma-Aldrich) solutions. The medium was dispensed into culture tubes (100 mm x 25 mm, 40 mL) followed by autoclaving for 20 min at 121°C and 103 kPa. Sealed cultures were incubated in a growth room set at 25 ± 2°C under 16 h light/ 8 h dark photoperiod and PPF 40-50 µmol m^{-2} s^{-1} provided by fluorescent (OSRAM) lamps. The number of explants for initial culture of each plant was approximately 50 depending on availability of plant material. After 4 weeks in culture, the number of sterile explants per treatment was recorded as a percentage. The *in vitro*-derived aseptic shoots obtained from the sterilization stage were continually sub-cultured until sufficient material was available to conduct subsequent experiments.

3.2.2 *In vitro* shoot proliferation

Upon obtaining sufficient plant material, an experiment was conducted to investigate the effects of N^6-benzyladenine (BA), isopentenyladenine (iP) and *meta*-topolin riboside (*m*TR) on shoot proliferation and other shoot growth parameters. Full strength MS basal medium was used. The MS medium was supplemented with 30 g/L sucrose, 0.1g/L myo-inositol and different concentrations (1, 5, 10 and 25 µM) of BA, iP and *m*TR. The control for this experiment was MS basal medium without PGRs. Nodal explants were excised to a length of ~10 mm each, and inoculated into culture tubes. The cultures, 25 replicates per treatment, were incubated under controlled environmental conditions in a growth room set at 25 ± 2°C

and 16 h light / 8 h dark photoperiod and PPF 40-50 µmol m^{-2} s^{-1} provided by fluorescent lamps. Data on frequency of shoot proliferation, number of shoots per explant, length of longest shoot (mm), number of nodal segments, fresh weight (g), number of roots and root length (mm) were recorded after an incubation period of 6 weeks. This experiment was done simultaneously for all three species and was repeated.

3.2.3 *In vitro* rooting

This experiment was conducted to investigate the effect of the auxins, indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), on root induction and/or development. Surface decontaminated nodal explants of *B. pygmaeum* were excised and inoculated onto MS medium supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol and varying concentrations (0.5, 5, 10, 15 and 25 µM) of IAA, NAA and 2,4-D. The control was MS medium without PGRs. The length of each explant was ~ 10 mm and tubes were used as culture vessels. The cultures, 25 replicates per treatment, were incubated under controlled environmental conditions in a growth room set at 25 ± 2^{o}C and 16 h light / 8 h dark photoperiod and PPF 40-50 µmol m^{-2} s^{-1} provided by fluorescent lamps. Data on frequency of the number of roots and root length (mm) were recorded after an incubation period of 4 weeks. The period of incubation was based on "standard" tissue culture minimal incubation procedures. This experiment was performed only on *B. pygmaeum* based on its availability at the time. The experiment was repeated. A soil mix of 1:1 (v/v) vermiculite:sand was used for potting shoots that were transferred to *ex vitro* conditions. Prior to the vermiculite mixture, a soil mixture of 1:1 (v/v) rough sand: garden soil was tested but resulted in the death of all the plantlets.

3.2.4 *Ex vitro* rooting and acclimatization

The *in vitro* regenerated shoots (> 20 mm) were washed in water to remove any traces of agar. *Ex vitro* rooting was first investigated with the use of a 3 min pulse treatment using 492.1 µM indole-3-butyric acid (IBA). A second pulse treatment investigation was performed using 492.1 µM of IBA for 3, 12 and 21 min. After treatment with IBA, the shoots were potted in plastic planting trays (45 mm x 15 mm per well) containing 1:1 (v/v) vermiculite:sand mixture. The potted shoots were irrigated with ¼ strength 492.1 µM of IBA and incubated in a mist house in which high relative humidity (90-100%) was maintained using a high pressure fog system. The plantlets were kept in the mist house, during mid-

winter, under natural 12 h light / 12 dark photoperiod conditions for 3 weeks. Thereafter, the plantlets were transferred to a greenhouse with natural temperature (midday PPF of approximately 1000 µmol m^{-2} s^{-1}) and natural photoperiodic conditions. The plantlets were watered with tap water every second or third day. Survival rate (%) was monitored once every week for a period of 5 weeks in the greenhouse. The period for which survival rate was monitored was completely dependent on how long the plantlets survived under natural conditions. The acclimatization procedure was similar for all experiments.

3.2.5 Effect of type of culture vessel on *Brachystelma pygmaeum* growth

Nodal explants were cultured onto full strength MS medium without PGRs in two types of culture vessels i.e. culture tubes (100 mm x 25 mm, 40 mL) and culture jars (110 mm x 55 mm, 300 mL). MS medium was prepared as stated in section 3.2.1. Single nodal explants were inoculated onto 10 mL (tubes) and 30 mL (jars) of the MS media. The cultures, twenty-five replicates per treatment, were incubated under controlled environmental conditions in a growth room set at 25 ± 2^{o}C and 16 h light / 8 h dark photoperiod and PPF 40-50 µmol m^{-2} s^{-1} provided by fluorescent lamps. Results were recorded after 6 weeks in culture.

3.2.6 Effect of plant density

Nodal explants were cultured onto culture jars containing 30 mL of full strength MS medium without PGRs. The preparation of MS medium was as per section 3.2.1. The number of explants per jar was 1, 2, 3 or 4. The culture vessels were incubated under controlled environmental conditions in a growth room set at 25 ± 2^{o}C and 16 h light / 8 h dark photoperiod and PPF 40-50 µmol m^{-2} s^{-1} provided by fluorescent lamps. Results were recorded after 6 weeks in culture.

3.2.7 Data analysis

A complete randomized experimental design was used for all experiments. Collected data were analyzed using one-way analysis of variance (ANOVA). Where there were statistical significances, mean values were further separated using Duncan's multiple range test (DMRT) using SPSS for Windows (IBM SPSS Statistics 24, USA). Significant treatment effects were accepted at $p \leq 0.05$. Reported values are mean ± standard error. Graphic representations were created using Sigma plot 8.0.

3.3 Results and discussion

3.3.1 Explant decontamination and bulking up of plant material

The treatments used to surface sterilize *B. pygmaeum* stem and tuber plant material are represented in **Table 3.1**. Plant material that was exposed to these treatments showed no indication of detrimental effects caused by the sterilants. The 30 min 1.75% sodium hypochlorite (NaOCl) treatment was found to be most effective as it resulted in the highest (67%) decontamination frequency. This treatment was used for the sterilization of all plant material in subsequent experiments. Upon further use, the treatment was found to be equally effective for the other two *Brachystelma* species. Contaminants in tissue culture can cause huge economic losses by either making the explant unfit for subculture or resulting in its death (CASSELLS, 1991; PATIL et al., 2010; TITOV et al., 2006). Even though the 30 min 1.75% NaOCl treatment was found to be the most effective for surface sterilization, a notable number (±40%) of explants were lost. Among the likely reasons contributing to the contamination, in this particular study, were the aphids which were feeding on the stock plants (**Figure 3.2D**). Plant feeders or pests such as aphids do not only contribute to surface contamination but also internal contamination which is an even bigger challenge when attempting to obtain aseptic plants (CASSELLS, 1991; ROUT et al., 2000; TENG et al., 2002). Thus, for the purpose of optimizing decontamination success rate, it is necessary to ensure that stock plants are well protected prior to use so as to avoid huge losses (KANE, 2005).

At this culture initiation stage, nodal explants, of all three species, were observed to have new growth either in the form of shoots or callus, however, a notable number of these explants showed no response. RANI AND RAINA (2000) have reported lack of response from meristematic tissue to be due to bud dormancy or failure in stem elongation. In the current study, some nodal explants taken from various parts of the stem showed no growth response i.e. no elongation or shoot production. Thus it is suggested that the reason for this lack of growth response is not based on a specific position on the stem but likely due to the physiological state of the plant material. The physiological state of an explant determines its biological response, therefore, some explants are more responsive than others at a given time. Callus was more frequently observed in *B. pygmaeum* compared to *B. pulchellum* and *B. ngomense*. Callus cultures of *B. pygmaeum* were observed to develop adventitious shoots after a period of 6-8 weeks (**Figure 3.2B**). On the other hand, tuber explants of *B. pygmaeum* did not show any indication of new growth in the four weeks in culture. It is likely that there

is an absence of meristematic potential in *Brachystelma* tuber tissue. There have been reports that totipotency is highly likely to be influenced by the presence of PGRs if it does not occur spontaneously (ASCOUGH et al., 2009; CAPONETTI et al., 2005; ZAID AND AL KAABI, 2003). Failure to perform subsequent experiments on *B. pygmaeum* tubers and to evaluate tubers of the other two *Brachystelma* species was due to the unavailability of starting plant material. Secondary explants obtained from initiated cultures were used for bulking up of plant material. This took several months.

Table 3.1: Surface decontamination of *Brachystelma pygmaeum* plant material using mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) for different time durations.

Treatment	Duration (min)	Explant	Decontamination success (%)
$HgCl_2$ (0.1%)	5	Nodal	0
$HgCl_2$ (0.1%)	10	Nodal	9
$HgCl_2$ (0.2%)	30	Tuber	0
NaOCl (1.75%)	30	Nodal	67
NaOCl (3%)	10	Nodal	20

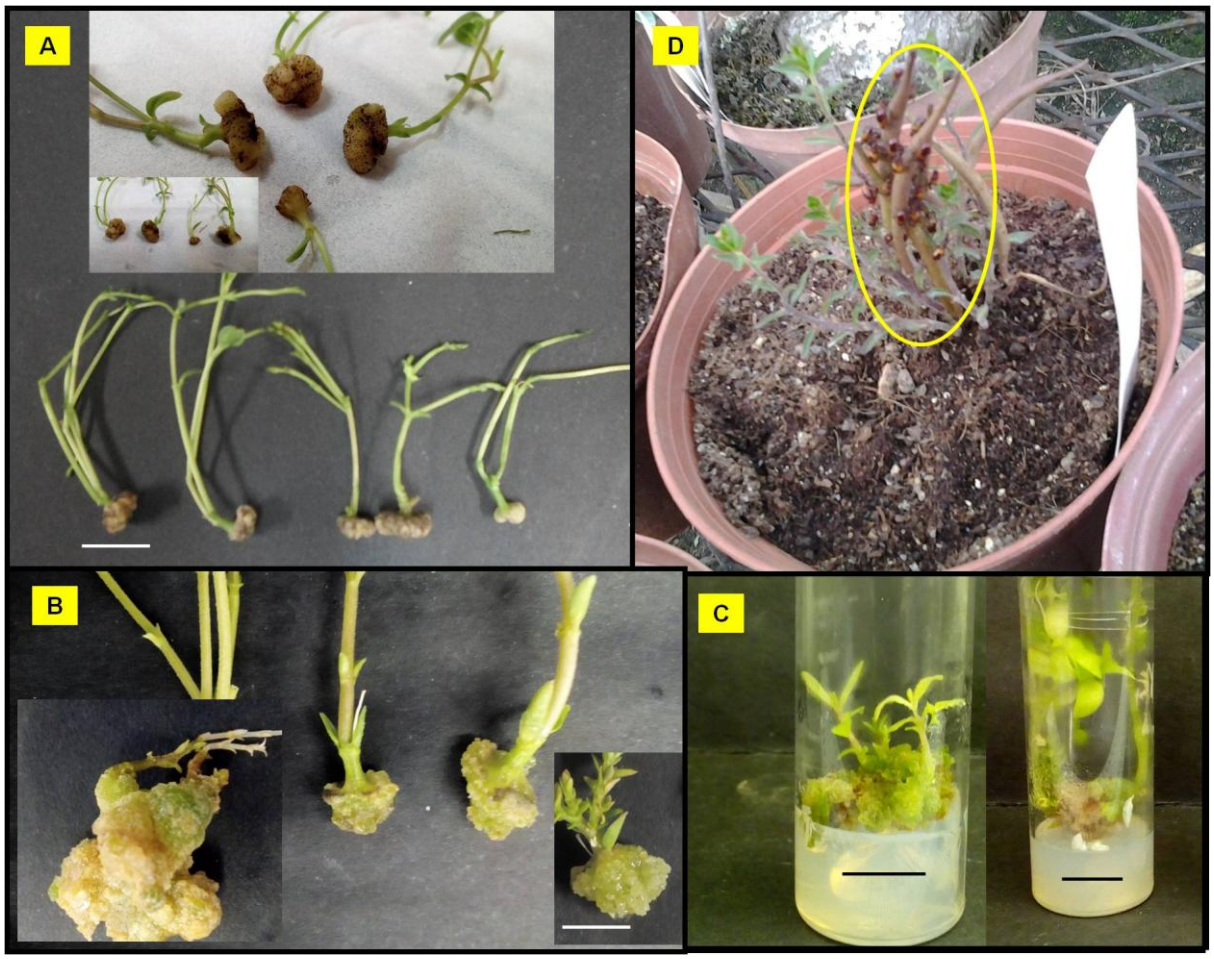

Figure 3.2: Observations during explant decontamination and bulking up of starting plant material. A-Miniature *in vitro* derived tubers, B- Callus (distiguishable from tubers) ,C-hyperhydric shoots derived from *B. pygmaeum* callus cultures on MS medium without plant growth regulators, D- Aphids feeding on *B. pygmaeum* fruit contributing to contamination. Scale bar = 10 mm.

3.3.2 Effect of cytokinins on shoot proliferation and other shoot growth parameters

The three species varied in their response to the different BA, iP and *m*TR concentrations. Depicted in **Figure 3.3** are the effects of cytokinin treatments on shoot proliferation after six weeks in culture. The cytokinin treatments are shown to generally improve shoot production in all three species. The number of shoots produced per explant for *B. ngomense*, were higher with increasing concentrations of BA, iP and *m*TR compared to the control (**Figure 3.3A**). The highest shoot production (4.44 ± 0.41) was observed at 25 µM *m*TR in *B. ngomense*. On the other hand, *B. pulchellum* (**Figure 3.3B**) responded in a slightly different manner. There was no particular trend to show the effect of increasing cytokinin concentration with BA and *m*TR. However, iP did show a trend of increased shoot production with increasing

concentration. The highest shoot production (2.04 ± 0.20) was observed at 25 µM iP and was also significantly different from the control. For *B. pygmaeum* (**Figure 3.3C)**, all treatments displayed a significantly higher shoot production per explant compared to the control with the exception of 1 µM iP and *m*TR. BA treatments showed a significant increase in shoot production with increasing concentration while iP and *m*TR had a fluctuating response. In all three treatments, the higher concentrations resulted in better shoot production. The highest shoot production (2.57 ± 0.26) was observed at 25 µM BA. Increase in shoot numbers as a response to cytokinin treatments has been observed in various plants regenerated via *in vitro* methods (AMOO et al., 2011). This observation is thought to reflect the effect of increasing concentrations of cytokinins during micropropagation (D'ARTH et al., 2002; ZHAO et al., 2005b).

In the current study, among the three *Brachystelma* species, treatment with iP generally gave the better (not significantly) response in terms of shoot growth parameters when compared to BA and *m*TR. However, iP did not necessarily result in the highest performance. For instance, shoot proliferation in *B. ngomense* was generally better with the iP treatment while the highest shoot proliferation was obtained from 25 µM *m*TR (**Figure 3.3A**). Even though the results of the current study have not particularly shown a significant difference between the different cytokinins (BA, iP and *m*TR), other studies have shown the typical differences that result from treatment with cytokinins belonging to different groups mainly the aromatic and isoprenoid (ARINAITWE et al., 2000; DOBRÁNSZKI et al., 2006; IVANOVA AND VAN STADEN, 2008). Differences are also observed even within the cytokinin groups (BOGAERT et al., 2006; DOLEŽAL et al., 2006). Cytokinins, or PGRs in general, have different mechanisms of stimulation depending on factors such as plant species and plant organ. I*n vitro* multiplication rate and biomass are typically the primary indicators of cytokinin treatment stimulation effects (AREMU et al., 2012a). Aromatic cytokinins, including BA and *m*TR, are generally known to have a greater influence on developmental processes mainly those related to morphogenesis and senescence whereas isoprenoids, including iP, are suggested to be more involved in growth processes concerning the continuation of the cell cycle (BAROJA-FERNÁNDEZ et al., 2002; HOLUB et al., 1998; IVANOVA et al., 2006). N^6-benzyladenine (BA), synthetic cytokinin, remains the most widely used exogenously applied aromatic cytokinin in commercial micropropagation due to its effectiveness and affordability but BA is also known to have deleterious effects on *in vitro* cultures (AMOO et al., 2011; DOLEŽAL et al., 2006;

VALERO-ARACAMA et al., 2010). Isopentenyladenine (iP), a natural isoprenoid cytokinin, is also reported to cause physiological abnormalities and to have weak activity in the *in vitro* propagation of some plant species, especially in comparison to BA (ARINAITWE et al., 2000; STROSSE et al., 2008). Thus, other aromatic cytokinins such as the *meta*-topolins, hydroxylated 6-benzyladenine derivatives, have been identified as possible alternatives (GENTILE et al., 2014).

Meta-topolins, including *m*TR, have generally been observed to not only result in improved shoot proliferation but also result in minimal physiological abnormality (AREMU et al., 2012b; BAIRU et al., 2007; BOGAERT et al., 2006; VASUDEVAN AND VAN STADEN, 2011; VINAYAK et al., 2009; WERBROUCK et al., 1996; WOJTANIA AND GABRYSZEWSKA, 2001). For instance, BAIRU et al (2008) obtained higher shoot multiplication rates from a number of *meta*-topolin derivatives at different concentrations compared to BA. On the other hand, some studies have reported lower multiplication rates from *meta*-topolins compared to BA (BOGAERT et al., 2006; NIEDZ AND EVENS, 2010; ROSALES et al., 2008). Meanwhile, some studies show no significant difference between treatments (DE DIEGO et al., 2010; SALVI et al., 2002). Thus, it is evident that responses to PGR treatments can be species specific. Nevertheless, the better performance of the *meta*-topolins has been attributed to their structure (AMOO et al., 2011; AREMU et al., 2012b; MOYO et al., 2012). The advantage of the topolin structure is due to the presence of hydroxyl groups on the benzyl ring which, during cytokinin metabolism, increase the chances of the formation of *O*-glycosides instead of the deleterious *N*-glycosides found in BA treated plantlets (BAIRU et al., 2011). The *O*-glycoside metabolites are considered only stable at times when they are not required by the plant but are rapidly converted to active cytokinin bases when required (AMOO et al., 2011). This then allows the continuous availability of physiologically active cytokinin over an extended period of time, thus resulting in high shoot formation *in vitro* (AMOO et al., 2011). On the other hand, the chemical stability of *N*-glycoside is the reason for their implication in the deleterious effect of BA in plant tissue culture extending all the way to the acclimatization stage (AREMU et al., 2012b).

In addition to shoot production, other parameters measured and recorded were shoot length, number of nodal segments, fresh weight, number of roots and root length. The shoot lengths and number of nodal segments of *B. ngomense, B. pulchellum,* and *B. pygmaeum* showed no significant difference between treated explants and the control (**Figure 3.5-6**) with a couple

of exceptions. The shoot length in *B. ngomense* (**Figure 3.6A**) was significantly increased by 5 µM iP and the number of nodal segments in *B. pulchellum* (**Figure 3.5B**) and *B. pygmaeum* (**Figure 3.5C**) were significantly increased by 5 µM *m*TR and 5 µM BA, respectively. Fresh weight on the other hand, in all three species, to some degree generally benefited from the application of cytokinins however, it was not in line with the shoot production response (**Figure 3.4**). The effect of BA, iP and *m*TR on fresh weight of *B. ngomense* (**Figure 3.4A**) was observed to be a significant increase in comparison to the control, with some exceptions. The highest fresh weight (0.18 ± 0.02) was observed at 25 µM *m*TR. For *B. pulchellum* (**Figure 3.4B**), treatment with BA had no significant effect on fresh weight while specific concentrations of iP and *m*TR treatments significantly increased fresh weight compared to the control. The highest fresh weight (0.10 ± 0.01) was observed at 5 µM *m*TR. In *B. pygmaeum* (**Figure 3.4C**), fresh weight was significantly increased by all cytokinin treatments compared to the control with the exception of 1 µM BA and 1 µM *m*TR. The highest fresh weight (0.23) was achieved at 5 µM BA and 5 µM iP, respectively.

Although the primary aim of this particular experiment was to observe the effect of cytokinins on *Brachystelma* shoot growth parameters, rooting was also observed. The highest number of roots as well as longest root length was observed in the control (**Figure 3.7-8**) which may be due to the influence of endogenous auxins in the explants. On the other hand, the treatments with BA, iP and *m*TR had an antagonistic effect on rooting in all three *Brachystelma* species. It is likely that the presence of exogenous cytokinins over-powered the action of endogenous auxins. In *B. pygmaeum*, however, the lower concentrations of BA were found to have the least inhibiting effect on rooting (**Figure 3.7B**). No roots were induced in the regenerated *B. pulchellum*, the control inclusive. Nevertheless, the poorly-rooted or root-lacking shoots were transferred to *ex vitro* conditions. This was motivated by the observation of "spontaneous" *ex vitro* rooting, as a result of endogenous auxin activity, in some micropropagated plants (DEBERGH AND READ, 1991). The plants rooting response to a "natural" substrate is beneficial in micropropagation systems as it enables the bypassing of the root induction stage which in turn decreases labour and saves resources (HLOPHE et al., 2015; KANE, 2005). The shoots of *B. ngomense*, *B. pulchellum* and *B. pygmaeum*, however, did not form "spontaneous" roots under *ex vitro* conditions. A survival rate of 0% was observed after 2 weeks in the mist house and one week in the green house.

In vitro rooting is controlled by both endogenous and exogenous auxins in plant tissue (AREMU et al., 2012b). Regardless of the absence of exogenous auxins in the medium, cultured plantlets are generally able to produce roots particularly in medium without PGRs. However, medium supplemented with high concentrations of cytokinins has inhibitory effects. A study by VALERO-ARACAMA et al., (2010) has reported higher concentrations of BA and *m*TR to inhibit rooting. Similar observations have been made in the current study where treatment with higher concentrations of BA has inhibited *in vitro* rooting (**Figure 3.7B**). On the other hand, iP and *m*TR, irrespective of concentration, inhibited *in vitro* rooting. Treatment with BA is typically known to reduce acclimatization competence (VALERO-ARACAMA et al., 2010; WERBROUCK et al., 1995), however, in the current study, acclimatization competence was lacking regardless of the cytokinin treatment.

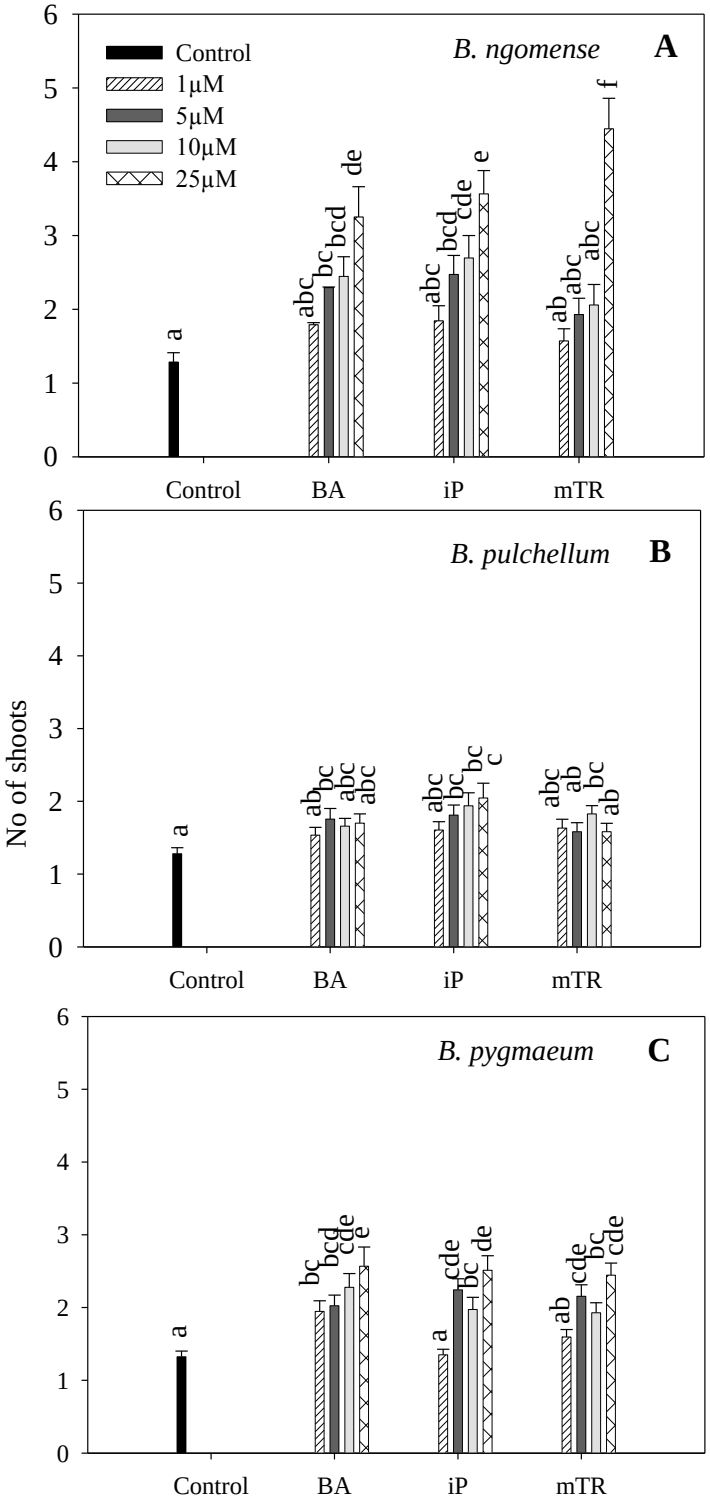

Figure 3.3: Effect of different concentrations of cytokinins on shoot production in A-*Brachystelma ngomense*, B-*Brachystelma pulchellum* and C-*Brachystelma pygmaeum* after six weeks in culture. In each graph, different letter(s) on bars show significant differences according to Duncan's multiple range test (DMRT) ($p \leq 0.05$).

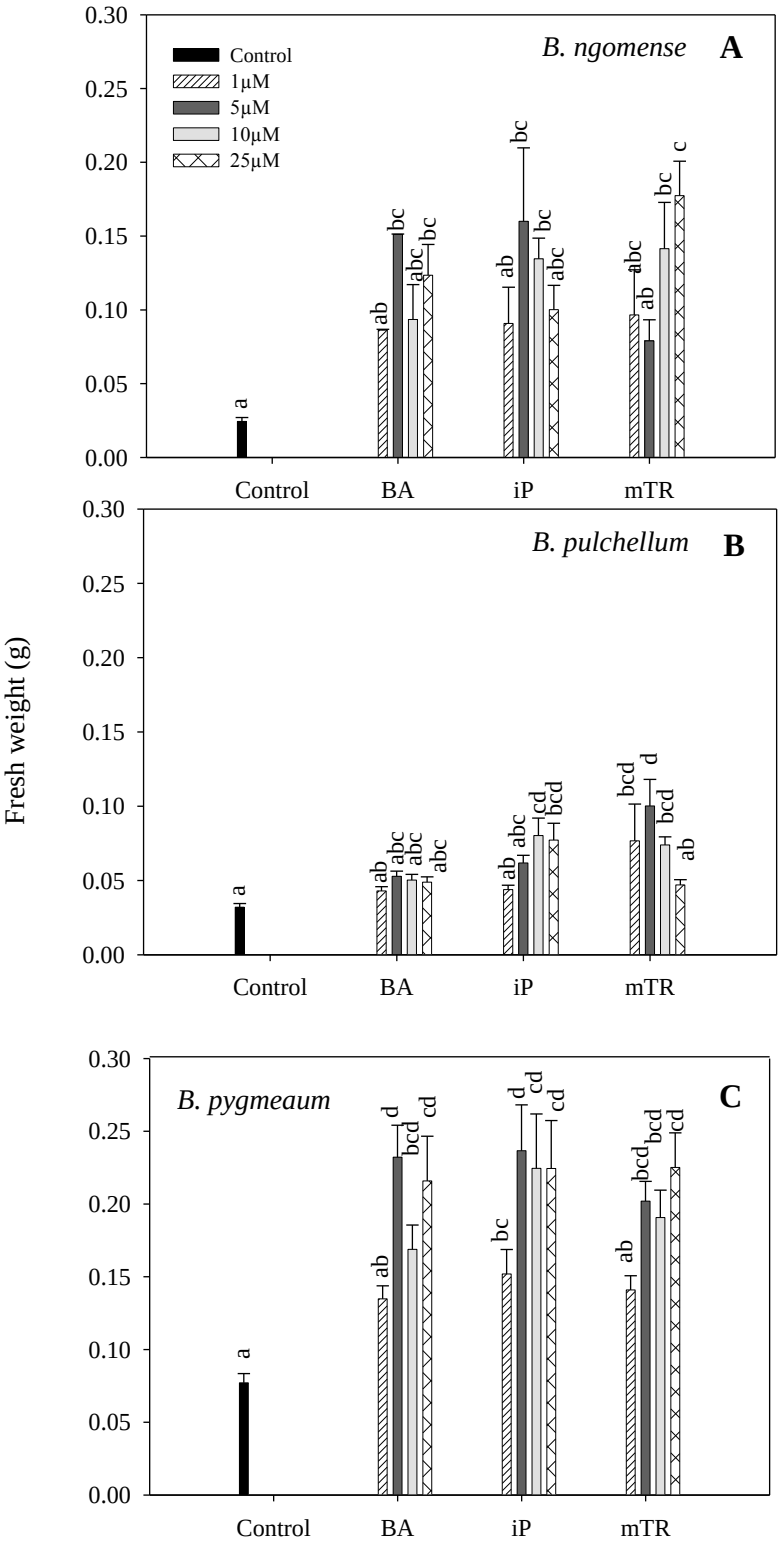

Figure 3.4: Effect of different concentrations of cytokinins on fresh weight (g) in A-*Brachystelma ngomense*, B-*Brachystelma pulchellum* and C-*Brachystelma pygmaeum* after six weeks in culture. In each graph, letter(s) on bars show significant differences according to Duncan's multiple range test (DMRT) ($p \leq 0.05$).

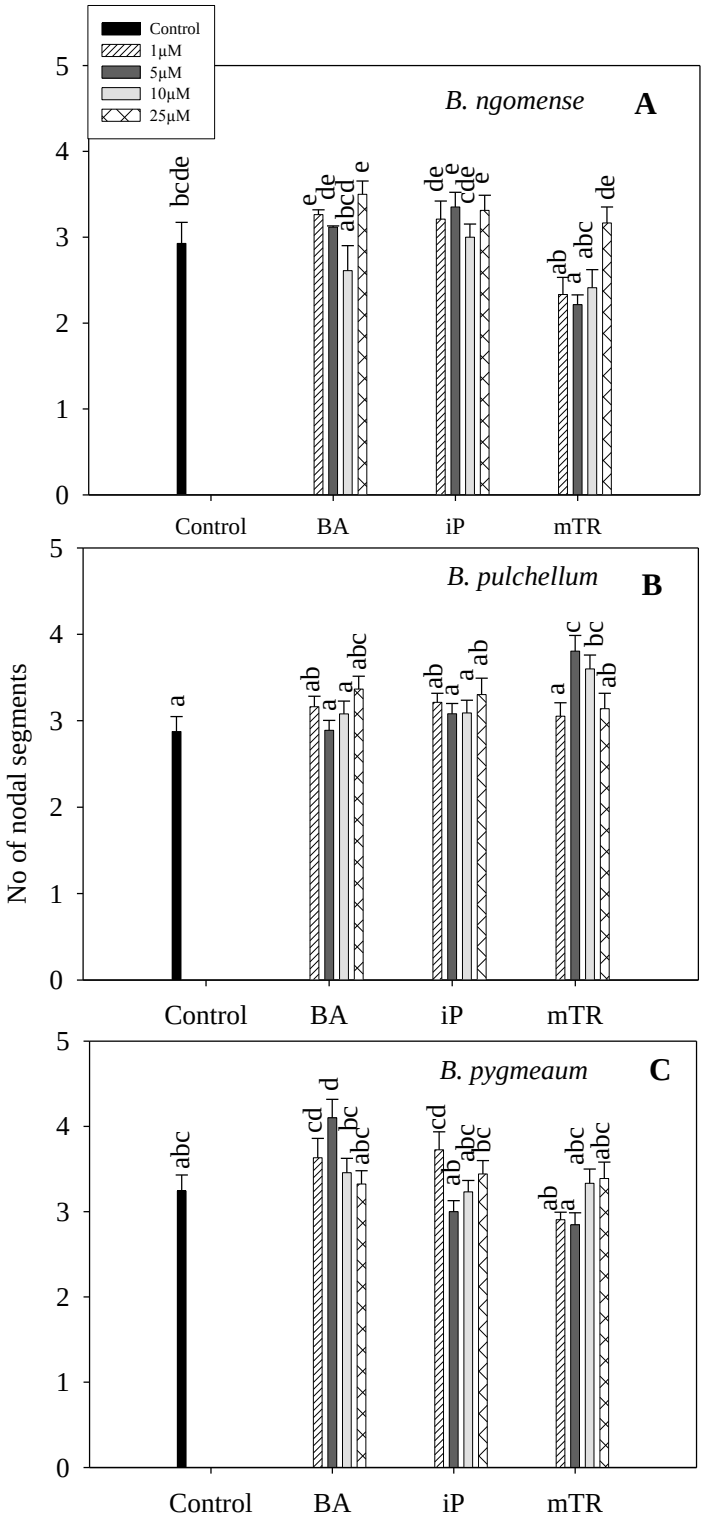

Figure 3.5: Effect of different concentrations of cytokinins on the number of nodal segments in A-*Brachystelma ngomense,* B-*Brachystelma pulchellum* and C-*Brachystelma pygmaeum* after six weeks in culture. In each graph, different letter(s) on bars show significant differences according to Duncan's multiple range test (DMRT) ($p \leq 0.05$).

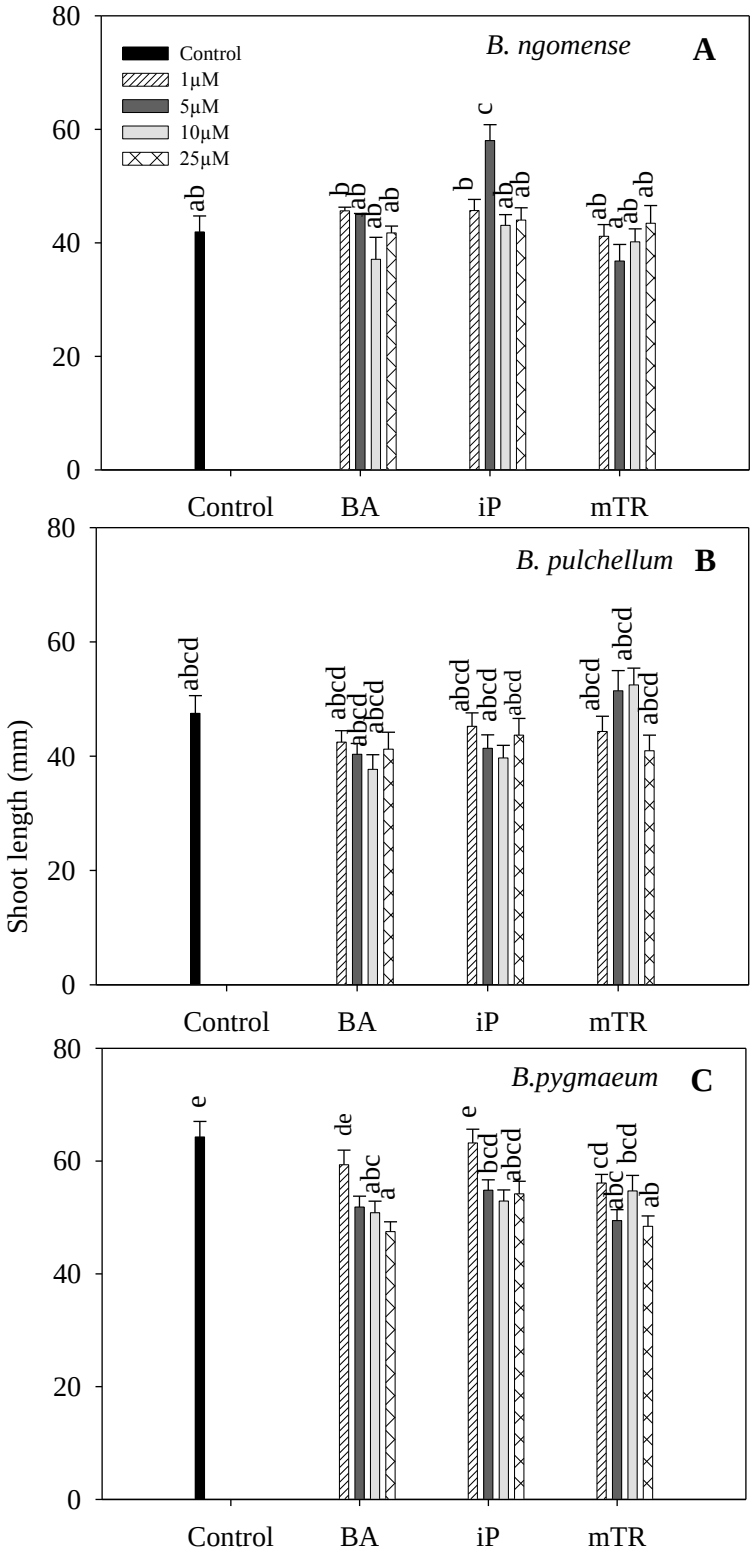

Figure 3.6: Effect of different concentrations of cytokinins on the shoot length (mm) of A-*Brachystelma ngomense*, B-*Brachystelma pulchellum* and C-*Brachystelma pygmaeum* after six weeks in culture. In each graph, different letter(s) on bars show significant differences according to Duncan's multiple range test (DMRT) ($p \leq 0.05$).

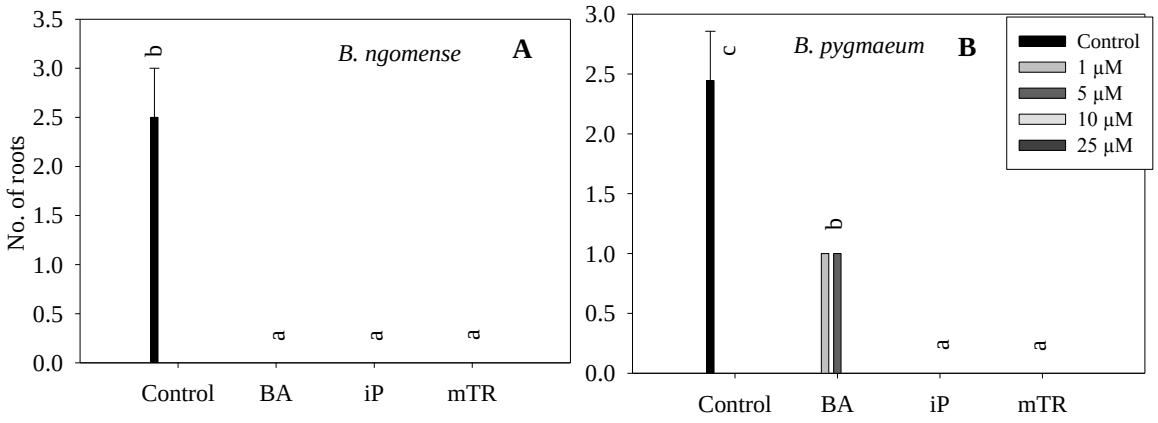

Figure 3.7: Effect of different concentrations of cytokinins on the number of roots in A-*Brachystelma ngomense*, and B-*Brachystelma pygmaeum* after six weeks in culture. In each graph, different letter(s) on bars show significant differences according to Duncan's multiple range test (DMRT) ($p \leq 0.05$).

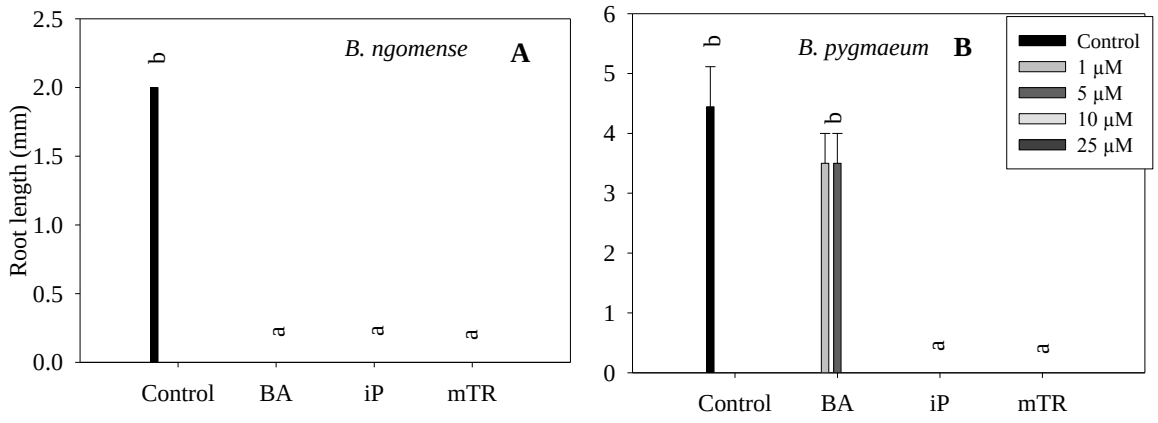

Figure 3.8: Effect of different concentrations of cytokinins on the root length (mm) in A-*Brachystelma ngomense*, and B-*Brachystelma pygmaeum* after six weeks in culture. In each graph, different letter(s) on bars show significant differences according to Duncan's multiple range test (DMRT) ($p \leq 0.05$).

3.3.3 *In vitro* rooting of *Brachystelma pygmaeum*

Brachystelma explants treated with a cytokinin-only treatment gave rise to shoots that had either an absence of roots or rooted poorly, both *in vitro* and *ex vitro*. The effect of different concentrations of IAA, NAA and 2,4-D on rooting of *B. pygmaeum* is shown in **Figure 3.9**. After 4 weeks in culture, there was no significant difference between the treatments and the control for both the number of roots induced and root length with the exception of 5 µM 2,4-D (**Figure 3.9B**) which yielded the highest root length (6 ± 0.9). On the other hand, the highest number of roots (3.33 ±0.33) resulted from 0.5 µM 2,4-D (**Figure 3.9A**). however,

higher concentrations of 2,4-D were found to reduce and further inhibit root induction. Similarly, higher concentrations of IAA and NAA were found to also reduce root induction (**Figure 3.9A**). A study by MOYO et al. (2011) indicated that an increase in IAA concentration reduced root induction in *Sclerocarya birrea*. Other studies have shown that high concentrations of auxins such as IAA and NAA will slightly inhibit the number of roots as well as their length when compared to the control (ASCOUGH et al., 2011; DE KLERK et al., 1999; MOYO et al., 2012). Treatment with 15 µM NAA was observed to inhibit root induction whereas 10 µM and 25 µM NAA were found to produce rooting. This result highlights the interaction between endogenous and exogenous auxins. A certain concentration of exogenous auxin has the ability to complement endogenous auxin resulting in better rooting as in the case of 0.5 µM 2,4-D (DE KLERK et al., 1999; FOGAÇA AND FETT-NETO, 2005; GABA, 2005).

During acclimatization, the shoots derived from this experiment had an extended survival period of 7 weeks as opposed to the 3 week survival of those treated only with cytokinins. There were no surviving plants by the end of 7 weeks. The failure to survive under green house conditions is assumed to be a direct reflection of the poor root production both *in vitro* and *ex vitro*. Additionally, the fragile nature of *Brachystelma* species in general is likely to be a contributing factor. It was also observed, from *in vitro* subcultured material used to maintain stocks, that extending the time spent in culture by two or three fold improves root induction. It has been reported that consistency in growth rate and shoot multiplication in herbaceous perennials such as the *Brachystelma* species is achieved only after multiple subcultures of anywhere between 3 to 24 months on initiation medium (DEBERGH AND READ, 1991; KANE, 2005).

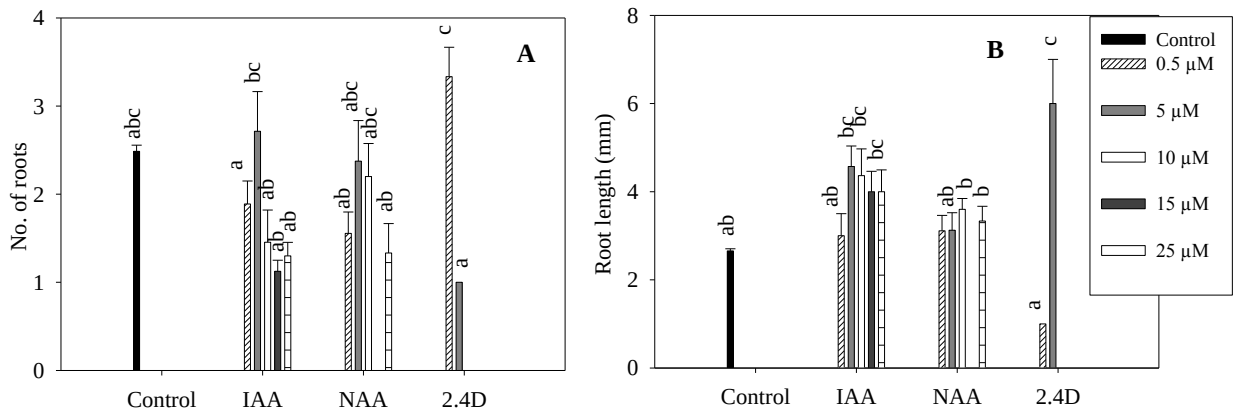

Figure 3.9: Effect of different concentrations of auxins on the number of roots (A) and root length (mm) (B) of *Brachystelma pygmaeum* after four weeks in culture. In each graph, different letter(s) on bars show significant differences according to Duncan's multiple range test (DMRT) ($p \leq 0.05$).

3.3.4 *Ex vitro* rooting and acclimatization of *B. ngomense*, *B. pulchellum* and *B. pygmaeum*

Pulse treatment of regenerated shoots with IBA (100 mg/L) for 3 min showed an improved green house survival compared to shoots previously potted directly after *in vitro* root induction. During acclimatization, the shoots derived from this experiment had an extended survival period of beyond 10 weeks as opposed to the 7 week survival of the *in vitro* root induction treatments. This extension can be attributed to the *ex vitro* root induction that was observed by the fourth week under green house conditions. The survival rate after 4 weeks was 42%, 35% and 30% for *B. ngomense*, *B. pulchellum* and *B. pygmaeum*, respectively. By the end of the tenth week, survival rate was 5%, 0% and 3% for *B. ngomense*, *B. pulchellum* and *B. pygmaeum*, respectively. The decrease in survival, in this current study, is primarily attributed to the poor rooting which is commonly observed in micropropagation systems (DEBERGH AND READ, 1991; XIAO et al., 2011). The few surviving plants were observed to maintain a healthy green appearance with no obvious morphological abnormalities for both the above and below ground organs (**Figure 3.10 C&F**). The healthy green appearance was perceived as an indication of functional photosynthetic capacity. Subsequently, *B. pygmaeum in vitro* derived shoots were used to test the effect of pulse treatment with 100 mg/L of IBA at different time intervals (3, 12 and 21 min). By the end of 8 weeks, survival rate was 5% for 3, 12 and 21 min while there was no survival (0%) for the control. Thus, the exposure time had no effect on *ex vitro* root induction and overall survival of shoots remained low. Some studies

have shown that increasing exposure time promotes rooting (MADHULATHA et al., 2004). In a study by PHULWARIA et al. (2013), pulse treatment with IBA (100 mg/L) for 3 min was particularly effective in *ex vitro* root induction for *Ceropegia bulbosa*. Thereafter, 100% rooting was observed which was followed by successful hardening and transfer to the field (PHULWARIA et al., 2013). In some instances, the same treatment will not reproduce the same result and there are many possible reasons for this which are not only limited to the differences of plant species. For example, MAKUNGA et al. (2005) found that the exact same IBA treatment used on the same plant (*Thapsia garganica*), possibly derived from one stock plant, reproduced a completely different result. Even though the pulse treatment used in this study did not yield a positive result, *ex vitro* rooting is known to be more advantageous in comparison to *in vitro* rooting especially for plants that are difficult to root (DEBERGH AND READ, 1991; DOBRÁNSZKI AND TEIXEIRA DA SILVA, 2010; PHULWARIA et al., 2013).

The sequence of root and tuber induction is variable in the *in vitro* and *in vivo* conditions. In all the *in vitro* culture experiments, miniature tubers (**Figure 3.2A**) formed on cultured shoots often preceding root induction regardless of media composition whereas tubers and roots occur simultaneously *in vivo*. It is not clear at present whether the induction of *in vitro* tubers in the absence of a root system might have hindered root induction. Therefore, this "tuber before root" *in vitro* observation could well be a growth/morphological abnormality. Morphological abnormalities are a common occurrence in micropropagated plants (SMULDERS AND DE KLERK, 2011; VANDEMOORTELE, 1999; WU et al., 2009; ZHAO et al., 2005b). *In vitro* abnormalities observed in this study include hyperhydricity (**Figure 3.2C**) and "bushiness" (**Figure 3.10J**) in the form of an abundance of small leaves on a short node section (D'ARTH et al., 2002; WHITEHOUSE et al., 2002). Hyperhydricity mainly affected adventitious shoots derived from callus. Shoots derived from nodal explants were seldom affected by hyperhydricity. No further morphological differences were observed between the parent plants and regenerants.

Concerning acclimatization incompetence, there are a couple of factors that have been reported as possible contributors. Light stress is one of the possible contributing reasons for this short-lived survival as transfer of plantlets to *in vivo* conditions of higher light intensities is known to have an overwhelming effect (CHANDRA et al., 2010; DEBERGH AND READ, 1991). Humidity is another factor that changes drastically, thus affecting plantlet survival (CHANDRA et al., 2010). Successful establishment of plantlets *ex vitro*, as a concluding step,

is crucial in micropropagation because many biotechnological applications are dependent on plant regeneration efficiency (TITOV et al., 2006; XU et al., 2009; YANG AND YEH, 2008; ZHANG et al., 2001). Poor survival during acclimatization is often attributed to the heterotrophic mode of nutrition under which the plantlets develop morpho-physiological disorders such as poor control of water loss (DEBERGH AND READ, 1991; XIAO et al., 2011; YANG AND YEH, 2008). However, *Brachystelma* has been observed to be a naturally fragile group of plants which might very well be a factor contributing to their poor performance in root development and acclimatization. Perhaps a study involving seed germination may yield better results and also shed some light on the rooting process of *Brachystelma* species. During this study, it was quite challenging to obtain *Brachystelma* seeds. A few seed companies and nurseries such as Blackwood's (Pietermaritzburg), Lifestyle seeds (Bloemfontein), Seedroom (SANBI) (Kirstenbosch), and Silverhill (Cape Town) were consulted but they had no supply of any *Brachystelma* seeds.

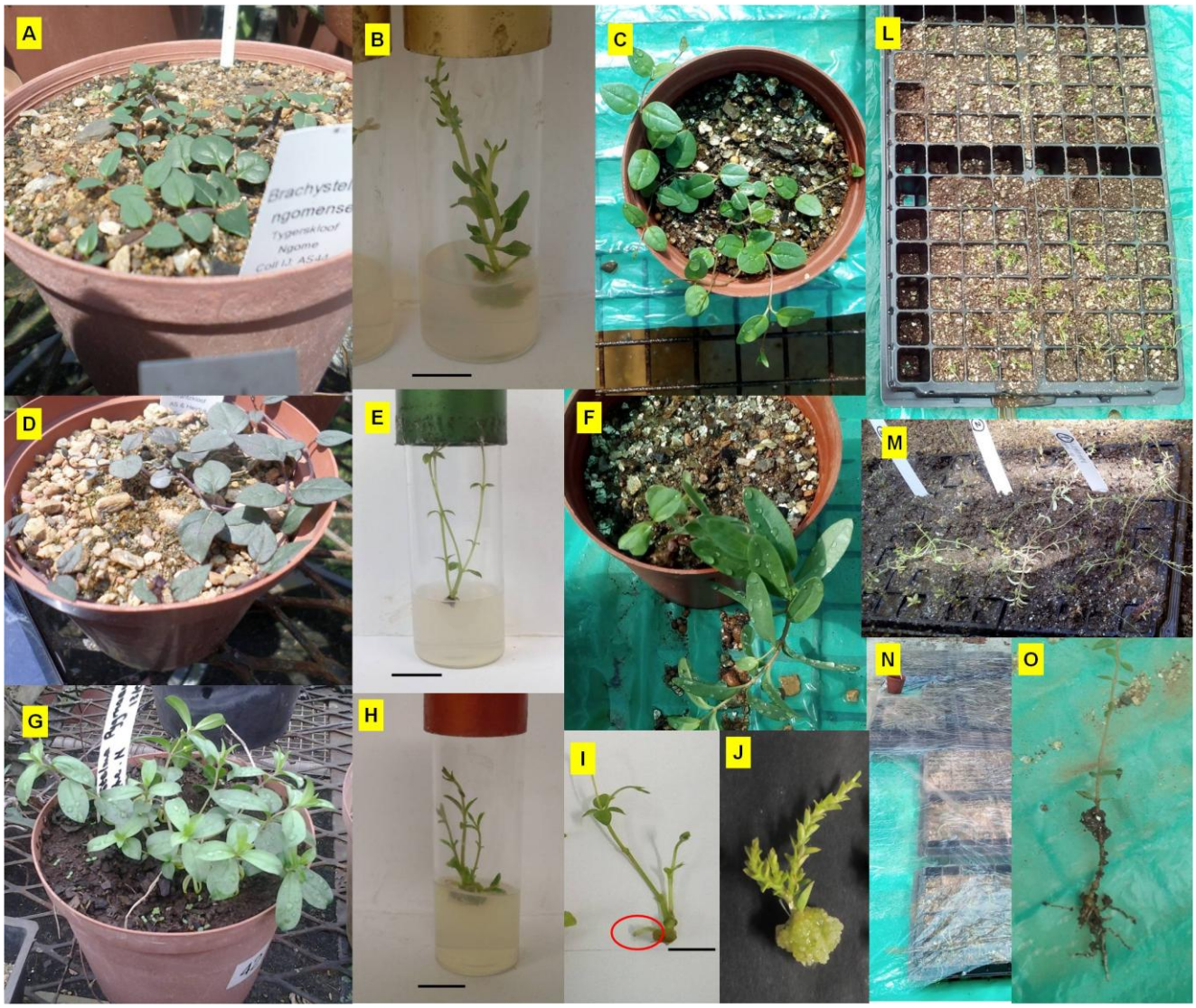

Figure 3.10: Micropropagation process of A-*B. ngomense*, D-*B. pulchellum* and G-*B. pygmaeum*. A, D & G-Stock plants. B, E & H- *In vitro* establishment of *B. ngomense*, *B. pulchellum* and *B. pygmaeum* .C- Fully acclimatized *B. ngomense*. F- Fully acclimatized *B. pygmaeum*. I- *In vitro* rooting. J- Morphological abnormality of *in vitro* grown *Brachystelma*. L- *Brachystelma* species in potting trays in mist house. M- *Brachystelma* species growing in green house. N- Humidity cover retaining moisture during first week of acclimatization in green house. O- *Ex vitro* derived roots. Scale bar = 10 mm.

3.3.5 Effect of type of culture vessel on *Brachystelma pygmaeum* growth

The type of culture vessel was found to have a significant effect on growth of *B. pygmaeum*. Relative to the tubes, explants grown in culture jars had increased productivity with the exception of root parameters (**Table 3.2**). It has been suggested that the type of vessel closure as well as the larger vessels, the culture jars in this case, affects the gaseous composition

which is involved in plant growth and development in culture (CASANOVA et al., 2008; KOZAI et al., 1997). In this study, culture tubes were closed using aluminium caps and sealed with parafilm whereas, the culture jars were closed using clear screw-caps alone. The type of vessel closure not only affects air exchange but also the light component (BAROJA-FERNÁNDEZ et al., 2002; KOZAI et al., 1997), thus the lower performance of the plants grown in tubes (**Table 3.2**) was not only related to tube size but also less light availability due to the aluminium cap covering and the likely lower air exchange due to the parafilm seal. The number of roots and root length observed from both the culture tubes and culture jars were not significantly different from each other. KOZAI et al., (1997) reports that the *in vitro* environment is directly and indirectly influenced by the *ex vitro* environment, however, the aerial *in vitro* environment is more directly influenced by the *ex vitro* environment than the root zone *in vitro* environment which is a possible explanation for the differences in the results obtained for the shoot and root parameters of this study.

Table 3.2: Effect of culture vessel on *in vitro* growth of *Brachystelma pygmaeum*

Culture vessel	No. of shoots	Shoot length (mm)	Nodal segments	Fresh weight (g)	No. of roots	Root length (mm)
Tube	1.32 ± 0.08	64 ± 2.75	3.24 ± 0.19	0.08 ± 0.01	0.59 ± 0.19	1.08 ± 0.35
Jar	1.78 ± 0.15	80 ± 8.47	5.44 ± 0.29	0.24 ± 0.04	0.11 ± 0.11	2.22 ± 2.22
p value (=)	0.013	0.028	0.005	0.005	0.378	0.246

3.3.6 Effect of plant density

The number of explants per vessel was observed to have no significant effect on plant growth (**Figure 3.11**). This suggests that sharing of resources among *B. pygmaeum* nodal explants cultured in groups of 2 to 4 has no effect on their productivity performance. This observation can further be used as a means to save space in growth rooms where space is often a challenge. The use of culture jars is "time and resource friendly" as it reduces the labour required. STANDAERT-DE METSENAERE (1991) stated that growth of many explants in a single vessel, provided that productivity is maintained, would not only save space and labour but also decrease total cost in commercial tissue culture thus improving profit. High planting density is an important factor for successful micropropagation, especially at a commercial level (ZOBAYED et al., 2004).

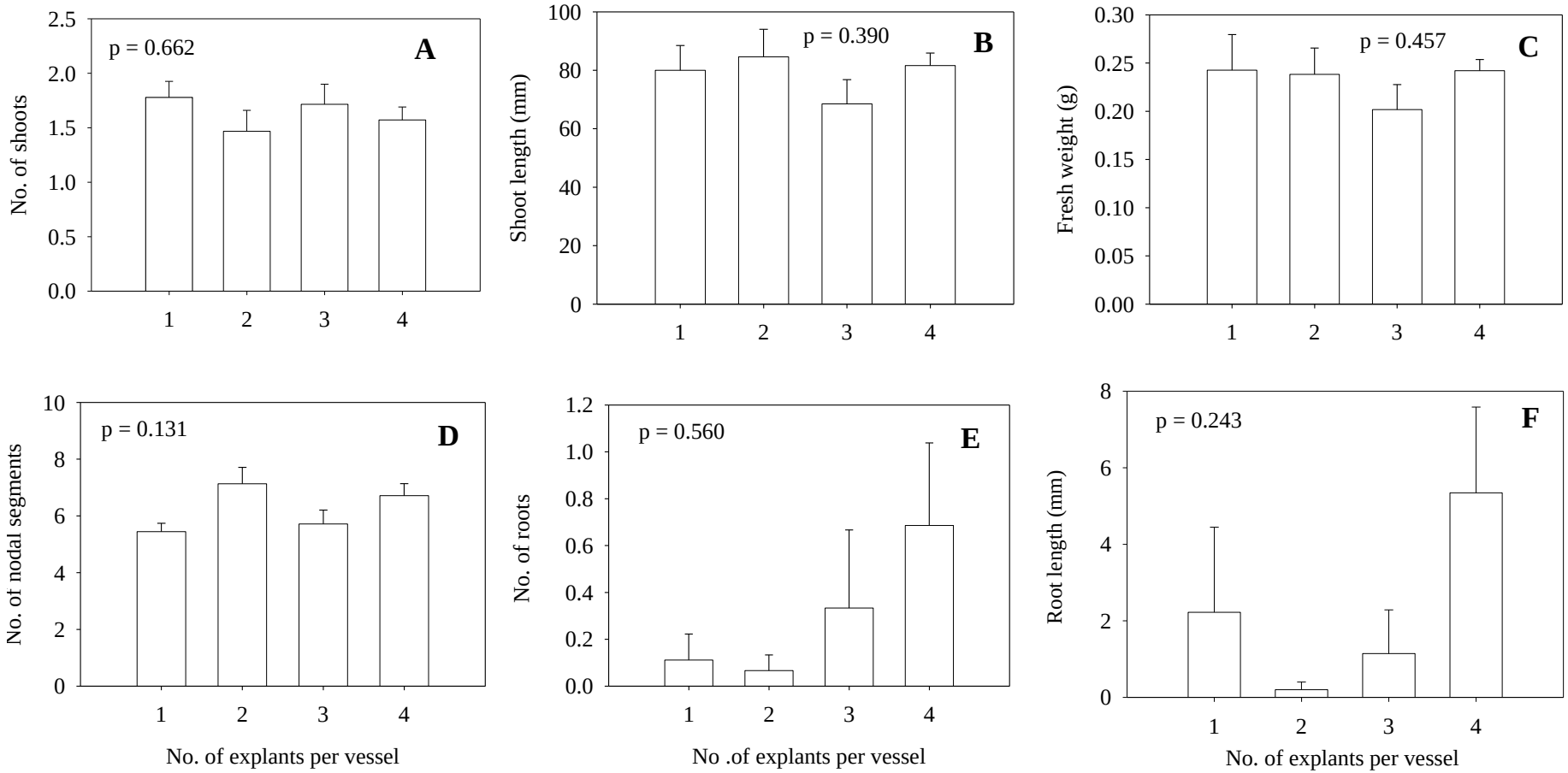

Figure 3.11: Effect of number of *Brachystelma pygmaeum* nodal explants in a single vessel on plant growth.

3.4. Conclusions

The results of this study have demonstrated that *Brachystelma* plants can be manipulated under *in vitro* culture conditions, though not optimally at present. The manipulation of the culture media with various auxins and cytokinins, and the use of controlled environmental conditions have moderate effects in the development of micropropagation protocols for the *Brachystelma* species used in this study. Further, acclimatization of *B. ngomense*, *B. pulchellum* and *B. pygmaeum* has been extremely limited due to the poor rooting that lead to short-lived survival under green house conditions. The concern of decline in the wild *Brachystelma* populations calls for an improvement of the available standard micropropagation protocol which can be specifically used in their conservation. Once established, these protocols can also be used on other biotechnological applications involving *Brachystelma*. The results obtained in the current study provide a stepping stone for subsequent research towards the successful rapid clonal propagation of *Brachystelma* species which is much needed especially in the absence of efficient alternative conventional propagation techniques.

Chapter 4: Phytochemical profiling and antioxidant evaluation of three *Brachystelma* species

4.1 Introduction

It is extremely difficult to imagine a world without plants. Mankind has benefited tremendously from plants as a wellspring of their continued survival (BARNES AND PRASAIN, 2005; NCUBE et al., 2012). The relevance and role of plant-related health resources is gaining importance in modern society. Generally, plant natural products and their biological properties are better recognised and appreciated due to an understanding of their novelty (CALIXTO, 2000; FENNELL et al., 2004). Natural plant products, particularly, secondary metabolites also referred to as phytochemicals are as a response to biotic and abiotic stresses including herbivory, light intensity, microbial attack and moisture (NCUBE et al., 2012; TAYLOR AND GROTEWOLD, 2005). Their synthesis is often subject to tight regulation and is restricted to certain plant tissue or developmental stages (CROTEAU et al., 2000). Synthesis can also be induced in response to a stimulant for instance, microbial attack (NCUBE et al., 2012). There are also restrictions among groups of plants in their ability to synthesize specific classes of phytochemicals (CROTEAU et al., 2000). Based on the biosynthetic pathways, the three main classes are alkaloids, phenolics and terpenes (BOURGAUD et al., 2001; CROTEAU et al., 2000). Being a part of the plants defence system, some of these phytochemicals are potentially highly toxic in their crude form (BARNES AND PRASAIN, 2005; CALIXTO, 2000; CROTEAU et al., 2000). The toxicity of certain phytochemicals is an important factor concerning the safety of plants used as medicine. The use of medicinal plants as a source of relief from illness began with various indigenous groups around the globe (CROTEAU et al., 2000; FENNELL et al., 2004; SATHISHKUMAR AND BASKAR, 2014). Plant natural products have since been considered as an important source of active pharmaceuticals (BOURGAUD et al., 2001; CALIXTO, 2000).

Even though there has only been a handful of cases of direct use of secondary metabolite-derived drugs, these metabolites have been useful models for synthetic drugs (BARNES AND PRASAIN, 2005; WINK, 1999). The natural environment in which the plant exists plays a role in the type and amount of bioactive chemical produced and thus this relationship has been

used to identify specific bioactivity. For example, a plant that flourishes in an environment with extreme levels of microbial organisms does so by releasing antimicrobial chemicals (BARNES AND PRASAIN, 2005; BOURGAUD et al., 2001). Pharmacological screening methods have revealed a range of bioactivity such as anti-amoebic, anti-inflammatory, anti-proliferative, antioxidant and neurotropic activity (FENNELL et al., 2004; SATHISHKUMAR AND BASKAR, 2014). Screening methods include various assays that make use of standard drugs to ensure the plant's effectiveness (GÜLÇIN, 2012; OU et al., 2002; ROBBINS, 2003). Some of these assays are regarded as simple, sensitive and have the capacity to rapidly localize specific activities of plant extracts (GÜLÇIN, 2012; IGNAT et al., 2011; NOVÁK et al., 2008). Only a small percentage (± 6%) of the worlds plants have been evaluated for their potential pharmacological use (FABRICANT AND FARNSWORTH, 2001; MAHESH AND SATISH, 2008). Thus, it is important to continuously screen available plants especially those with restricted distribution. Plant groups such as *Brachystelma*, which are very small in size and have a creeping habit, can be easily overlooked and remain under conservation concern.

The aim of this study was to investigate the phytochemical content and antioxidant potential of three *Brachystelma* species. In addition, the effect of varying concentrations of cytokinins on the phytochemical content and antioxidant activity of *in vitro* grown plants was investigated.

4.2 Materials and methods

4.2.1 Collection and preparation of plant material

Whole plant material of *Brachystelma ngomense*, *Brachystelma pulchellum* and *Brachystelma pygmaeum* were collected (October, 2016) from a shade house at the Botanical Garden of the University of KwaZulu-Natal (UKZN), Pietermaritzburg campus. Voucher specimens (*B. ngomense* R. Br. *A. Shuttleworth 335* (NU), *B. pulchellum* R. Br. *N. Hlophe* 20 (NU) and *B. pygmaeum* R. Br. *A. Shuttleworth 322* (NU)) can be found at the UKZN Bews Herbarium (NU). After washing, leaf and stem parts were separated from the tubers. The tubers were cut into small cubes, and thereafter the leaf and tuber material was freeze-dried using a VirTis freeze-dryer (SP Scientific, USA). Once dried, the material was ground into fine powders using a ZM 200 (Retsch ®, Germany) centrifugal mill. The ground material was temporarily stored in tight-seal glass containers in the dark at room temperature.

The phenolic acid profile and oxygen radical absorbance capacity (ORAC) of *B. pulchellum* and *B. pygmaeum* was evaluated using plant material harvested from 6-weeks-old *in vitro* cultures. These cultures were treated with different concentrations i.e. 1, 5, 10 & 25 µM of N^6-benzyladenine (BA), isopentenyladenine (iP) and *meta*-topolin riboside (*m*TR). Harvested plant material was cleaned of any agar residue and freeze-dried. Once dried, the plant material was ground into fine powders using a pestle and mortar, and liquid nitrogen. The plant material was kept in sealed Eppendorf tubes until analysis. Preparation of extracts towards phenolic acid profiling and ORAC as well as the quantification of specific phenolic acids were conducted in the Palacký University and Institute of Experimental Botany ASCR, Czech Republic.

4.2.2 Phytochemical investigations

4.2.2.1 Extract preparation

The ground plant material was extracted using the method described by MAKKAR (2000) with slight modification. Ground material (0.1 g) was extracted using 10 ml of 50% (v/v) aqueous methanol (MeOH). This mixture was kept in a sonication bath for 40 min and maintained at a low temperature with the use of ice. The extract was filtered using Whatman™ No. 1 filter paper under vacuum using a Büchner funnel attached to a conical vacuum filtering flask. The filtrate was used immediately to determine flavonoid and phenolic contents.

4.2.2.2 Flavonoid determination

Flavonoids were measured following the method described by ZHISHEN et al., (1999). The reaction mixtures were prepared under dim light. Each reaction vessel consisted of 250 µl of extract, 1000 µl distilled water, 75 µl of 5% sodium nitrite ($NaNO_2$), 75 µl of 10% aluminium chloride ($AlCl_3$), and 500 µl of sodium hydroxide (NaOH). The reaction vessel was gently swirled to allow mixing and thereafter, absorbance was measured at 504 nm using a Cary 60 UV-visible spectrophotometer (Varian, Australia). A mixture with 50% MeOH in place of a sample extract was used as a blank. Each treatment had three replicates with the exception of standard treatments having only two replicates. Catechin (Sigma-Aldrich) was used for the preparation of standards for the calibration curve. The concentration of flavonoids in the sample extracts was expressed in mg/g catechin equivalents (CE).

4.2.2.3 Phenolic acid determination

Based on the methods of MAKKAR (2000), phenolics were quantified using a modified Folin-Ciocalteu's assay with gallic acid as a standard. Each reaction vessel consisted of 50 µl of extract, 950 µl distilled water, 500 µl of 2 N Folin-Ciocalteu's phenol reagent and 2.5 ml of 2% sodium carbonate (Na_2CO_3). The mixture was kept at room temperature for 30 min prior to measuring absorbance. Absorbance was measured at 725 nm using a Cary 60 UV-visible spectrophotometer (Varian, Australia). A mixture with 50% (v/v) MeOH in place of a sample extract was used as a blank. Each treatment had three replicates with the exception of standard treatments having only two replicates. Conversion of absorbance readings to phenolic compound concentrations per gram of extract was performed with the use of a gallic acid equivalents (GAE) standard curve.

4.2.2.4 Phenolic acid profile

4.2.2.4.1 Extract preparation

In a 1.5 ml Eppendorf tube, ground *in vitro* shoot material (30 mg) was extracted with 750 µl of 80% (v/v) MeOH and 20 µl of the internal standard (10^{-4} M salycilic acid and 4 – hydrobenzoic acid) aided by a metal bead. The content in the Eppendorf tube was homogenized for 3 min at a frequency of 27 $1s^{-1}$ using an oscillatory ball mill homogenizer (MM 301, Retsch, Haan, Germany). Thereafter, the metal bead was removed and the mixture was centrifuged for 10 mins at 17000 rpm. The supernatant of the extract was removed and transferred into a 2 ml Eppendorf. Again, 750 µl of 80% MeOH was added into the 1.5 ml Eppendorf tube, for re-extraction followed by vortexing and sonication for 10 min. The mixture was again centrifuged at 17000 rpm for 10 min. The supernatant of the extract was again removed and added into the same 2 ml Eppendorf tube followed by evaporation using a nitrogen evaporator.

The extract residue was dissolved in 500 µl of 0.1 M formic acid followed by vortexing and sonication for 5 min. Under a fume hood, 750 µl of diethyl ether was added and the mixture was homogenized at a frequency of 10 $1s^{-1}$ for 5 min. The mixture was then centrifuged for 2 min at 10 000 rpm followed by removal of the diethyl ether layer into an Eppendorf tube. Again, 750 µl of diethyl ether was added for re-extraction and homogenized for 5 min. The mixture was again centrifuged for 10 min at 10 000 rpm followed by the second removal of diethyl ether. The combined diethyl ether fractions were evaporated using a nitrogen

evaporator. The remaining residue was dissolved in 200 µl of 30% MeOH followed by vortexing and sonication for 5 min. The sample mixture was filtered through 0.45 µm nylon microfilters (Alltech, Breda, Netherlands) and pipetted into vials with inserts. The filtrate was used to identify and quantify specific phenolic acids.

4.2.2.4.2 Specific phenolic acid quantification

Ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC – MS/MS) was used to measure phenolic acids. Each treatment had three replicates. The UHPLC–MS/MS system used was an ACQUITY Ultra Performance LC™ (Waters, Milford, MA, USA) linked simultaneously to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro microTM API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source operating in negative mode. MassLynx^{TM} software (version 4.0, Waters, Milford, MA, USA) was used to control the instruments, and for data acquisition and processing. Further proceedings i.e. sample injection into column were performed as described by GRUZ et al. (2008) (**Appendix 4**).

Phenolic acids and deuterium- labelled internal standards were detected in multiple reaction monitoring (MRM) mode using mass-to-charge transitions of precursor and product ions. Identification of phenolic acids was achieved by matching retention times and mass spectral data with the calibration standards. Analyte concentrations in the samples were derived from analyte:average internal standard peak area ratios, using the formula below:

(Analyte concentration) = IS_c × slope × A × $2(IS_1 + IS_2)^{-1}$ + *c* (**Equation 4.1**).

IS_c is the concentration of internal standard in the sample; A is the peak area of the analyte; IS_1 is the peak area of 4-hydroxybenzoic acid; IS_2 is the peak area of salicylic acid; slope and *c* are linear regression parameters. The concentrations were expressed in µg/g DW.

4.2.3 Antioxidant activity

4.2.3.1 Extract preparation

2,2 Diphenyl-1-picryl hydrazyl (DPPH): Dried plant material was extracted using 40 ml of 80% aqueous MeOH for 60 min in a sonication bath containing ice-cold water. The sonication bath was maintained at a low temperature with the use of ice. The extract was filtered using Whatman™ No. 1 filter paper into a conical flask. The filtrate was left to dry

under a stream of cool air. The residue was used to conduct the DPPH assay for antioxidant activity.

Oxygen radical absorbance capacity (ORAC): Extract preparation was conducted as prescribed in section 4.2.2.4.1 with a slight difference at the very end. The extract supernatant in the 2 ml Eppendorf was used for analysis after it was diluted with buffer solution (75 mM potassium phosphate-pH 7.4).

4.2.3.2 2,2 Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity

Radical scavenging activity of *B. ngomense*, *B. pulchellum* and *B. pygmaeum* was determined using a modified DPPH assay described by KARIOTI et al., (2004). For each plant, different concentrations of 15 µl methanolic extracts were diluted with 735 µl of 50% MeOH and thereafter, 750 µl of a 0.1mM DPPH solution was added under dim light conditions. Each of the extracts were tested in triplicate and incubated in the dark at room temperature for 30 min. After incubation, absorbance readings were measured at 517 nm using a Cary 60 UV-visible spectrophotometer (Varian, Australia). As a blank 50% MeOH was used. The absorbance of extracts without DPPH was measured to correct for the DPPH colour on samples. These measurements were subtracted from corresponding readings containing DPPH. Ascorbic acid was used as a positive control. A mixture containing only 50% MeOH and methanolic DPPH was used as a negative control. The free radical scavenging activity (RSA), as determined by the decolouration of the DPPH solution, was calculated according to the formula below:

$$\textbf{\% RSA} = 100 \times (1 - (A_E / A_D)) \quad \textbf{(Equation 4.2)}.$$

A_E is the absorbance of the reaction mixture containing the standard antioxidant or extract. A_D is the absorbance of the negative control. The half maximal effective concentration (EC_{50}) values were calculated and the results were represented as EC_{50} means ± standard error.

4.2.3.3 Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity (ORAC) was performed according to the method described by OU et al., (2001). Briefly, 100 µl aliquot of 500 mM fluorescein along with 25 µl of diluted extract were pipetted into individual wells on a 96 well microplate which was pre-incubated at 37 ^{0}C. Thereafter, 25 µl of 250 mM 2,2′ -Azobis (2-methylpropionamidine) dihydrochloride (AAPH) was immediately added and the microplate was shaken for 5 s in a

microtiter plate fluorometer Multiskan Ascent instrument (Labsystems, Helsinki, Finland). Each of the samples had three replicates. The fluoresecence (Ex. 485 nm, Em. 510 nm) was read every 3 min over 90 min. The calibration curve was calculated by using standard of trolox (hydrophilic derivative of vitamin E) at concentrations ranging from 5 to 250 µM. 75 mM potassium phosphate buffer (pH 7.4) was used as a blank. The net area under curve (NAUC) was used to calculate antioxidant capacity which was expressed in micromole trolox equivalents (TE) per gram of DW (µmol TE/g). The area under curve (AUC) was calculated using the formula:

AUC = 1 + $f_1/f_0 + f_2/f_0 + \ldots + f_{20}/f_0$ (**Equation 4.3**).

Where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. NAUC was obtained by subtracting the AUC of the blank from that of the sample. The relative ORAC value (trolox equivalents) was calculated using the calibration curve (NAUC vs. concentration).

4.2.4 Data analysis

GraphPad Prism® version 5.02, Microsoft Excel and SigmaPlot® version 8.0 were used in conjunction to analyze data. Appropriate formulas were applied on Microsoft Excel and GraphPad from which final data representation values, expressed in the appropriate units, were obtained. Graphic data representations were obtained with the use of GraphPad Prism® version 5.02 and SigmaPlot® version 8.0. After analysis with the various softwares, the data were further analyzed using one-way analysis of variance (ANOVA). Where there were statistical significances, mean values were further separated using Duncan's multiple range test (DMRT) on SPSS for Windows (IBM SPSS Statistics 24, USA). Significant treatment effects were accepted at $p \leq 0.05$. Reported values are mean ± standard error.

4.3 Results and discussion

4.3.1 Phytochemical investigations

4.3.1.1 Total phenolic and flavonoid content of wild *Brachystelma* species

The total phenolic content of leaves and tubers of three *Brachystelma* species is presented in **Figure 4.1A**. The concentration of phenolics varied from 1.85 to 8.69 mg GAE/g DW among the three species. In all three species, the leaves had significantly higher levels of phenolics than the tubers. *B. pulchellum* leaves had the highest phenolic content (8.69 mg GAE/g DW).

A study by RAJARAM et al., (2014) screened the phytochemical content of *Brachystelma edulis* using a number of extraction solvents. Their study showed that the use of different extraction solvents can achieve a more detailed phytochemical screening investigation. Their study also indicated the presence of phenolics in *B. edulis* methanolic tuber extracts. The observation of phenolic content in the investigated species is an indication that the *Brachystelma* group has therapeutic potential seeing that phenolic compounds have been associated with pharmaceutical potential such as anti-microbial and antioxidant activity (AMOO AND VAN STADEN, 2013; ANDARWULAN AND SHETTY, 1999; BARROS et al., 2012; MOYO et al., 2013). This phenolic observation also serves as support for the use of *Brachystelma* species by communities in Africa and India for the various ailments. The use of tubers of certain *Brachystelma* species for medicinal purposes such as wound healing (*B. buchananii*) and chest pains (*B. johnsonii*) (MASINDE, 2007) can be evaluated as there is likelihood that the leaves of these species have a higher potency compared to their tubers which would also benefit conservation of the species.

The flavonoid content of *B. ngomense, B. pulchellum* and *B. pygmaeum* ranged from 0.29 to 2.59 mg CE/g DW (**Figure 4.1B**). Flavonoid content of tubers of all three species was significantly lower compared to the leaves. *B. pulchellum* leaves were observed to have the highest flavonoid content (2.59 mg CE/g DW). The observation of higher phenolic and flavonoid content in leaf extracts is significant where conservation is concerned as the use of above ground parts is often less detrimental to the survival of the plant compared to using the bulbs. Leaf extracts of wild plant species are usually found to have higher phenolic compounds compared to other plant parts (THIEM et al., 2013; ZOVKO KONČIĆ et al., 2010). In a study by CHAVAN et al., (2013), leaf extracts of some *Ceropegia* species were found to have higher phenolic and flavonoid content (15-18 fold higher) compared to those of tuber extracts. Phenolics consist of a number of specific compounds such as flavonoids, lignins, phenolic acids, and tannins (BALASUNDRAM et al., 2006; GÜLÇIN, 2012; ROBBINS, 2003). Phenolics are known to delay or inhibit oxidative degradation of lipids by scavenging free radicals such as hydroxyl and peroxyl radicals (BARROS et al., 2012; GÜLÇIN, 2012). Phenolic scavenging activity results in the formation of phenolic radicals that have lower energy which is not sufficient to promote lipid oxidation at biologically significant rates, thus significantly reducing cell damage (KIM et al., 2006; MPOFU et al., 2006). Oxidative damage is implicated in the processes of diseases such as asthma, cancer, inflammatory conditions and liver disease

(AYOOLA et al., 2008; COSTA et al., 2012; GÜLÇIN, 2012). Research on phenolics, for example flavonoids, suggests their use in treatment and preventing some chronic diseases (AYOOLA et al., 2008). Thus quantification of phenolic compounds of the plant species used in this study is necessary and further leads to identification and quantification of specific phenolics which can be useful in establishing the individual phenolic compounds responsible for the biological activity observed.

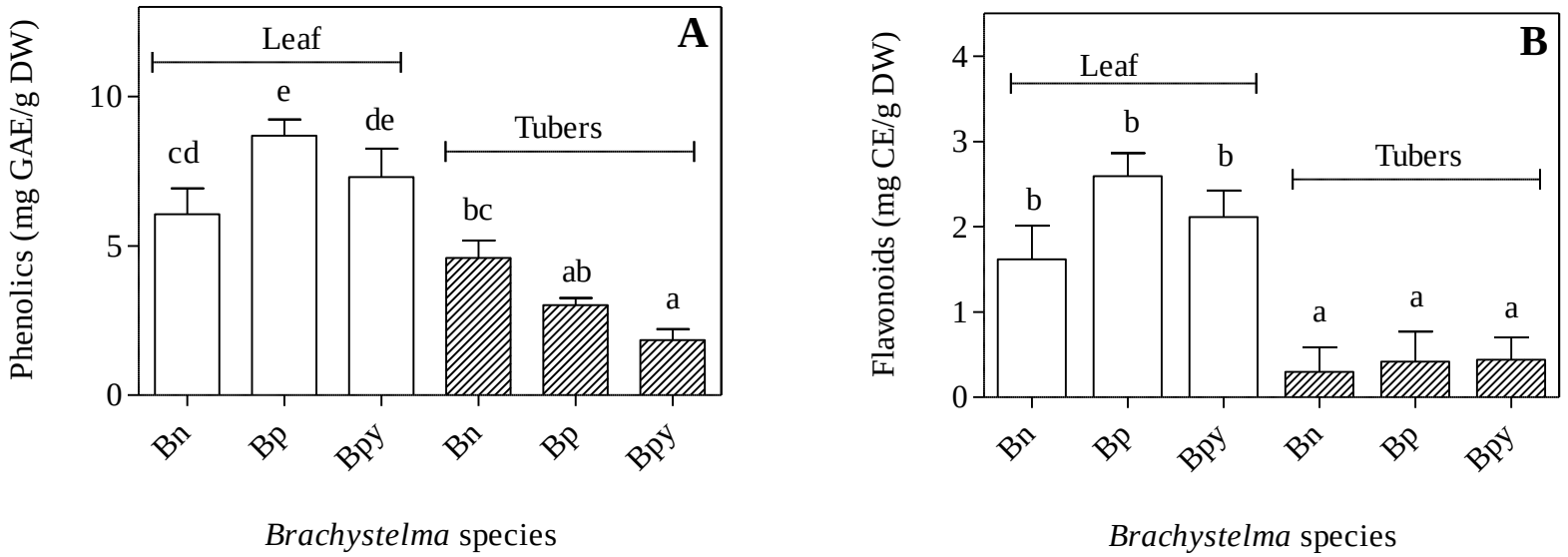

Figure 4.1: Phenolic and flavonoid content of three wild *Brachystelma* species. In each graph, bars represent means with standard error and different letter(s) indicate significant differences ($p \leq 0.05$) among the tested species. Bn- *B. ngomense*, Bp- *B. pulchellum* and Bpy- *B. pygmaeum*. DW-dry weight, GAE-gallic acid equivalent and CE-catechin equivalent.

4.3.1.2 Specific phenolic acid determination

Seven benzoic acid derivatives were quantified from *in vitro* derived shoots of *B. pulchellum* (**Figure 4.2**) and *B. pygmaeum* (**Figure 4.3**). These phenolic acid profile results showed that the concentrations of some benzoic acid derivatives varied significantly in *in vitro* derived shoots from BA, iP and *m*TR treatments. For *B. pulchellum*, the amount of gallic acid was found to significantly increase with increasing iP concentration (**Figure 4.2A**). Similarly, an increase in *m*TR concentration also significantly increased gallic acid in *B. pulchellum* shoots however this effect decreased after the 10 µM concentration. Gallic acid in *B. pygmaeum* was increased by different concentrations of BA, iP and *m*TR (**Figure 4.3A**). The highest gallic acid amount (0.56 µg/g DW) resulted from the 25 µM iP treatment.

All three cytokinins had no stimulatory effect on the concentration of vanillic acid produced in shoots of both *B. pulchellum* (**Figure 4.2B**) and *B. pygmaeum* (**Figure 4.3B**). The only exception was 1 µM BA which had a significant effect on vanillic acid present in *B. pulchellum* (**Figure 4.2B**), this treatment also yielded the highest vanillic acid content (1.80 µg/g DW). In *B. pygmaeum*, syringic acid (**Figure 4.3C**), protocatechuic acid (**Figure 4.3D**), and *p*-hydroxybenzoic acid (**Figure 4.3E**) content were generally not significantly improved by the different cytokinin treatments. However, the concentration of *p*-hydroxybenzoic acid was increased by 25 µM iP and 1 µM *m*TR with 25µM iP being the highest (1.06 µg/g DW). In *B. pulchellum*, syringic acid was significantly improved by specific concentrations of all cytokinin treatments i.e. 10 µM BA, 1 µM iP, and 10 µM *mTR*. The highest amount (3.10 µg/g DW) of syringic acid was observed with 10 µM BA (**Figure 4.2C**). On the other hand, protocatechuic acid (**Figure 4.2D**) and *p*-hydroxybenzoic acid (**Figure 4.2E**) were generally not significantly improved by cytokinin treatments with the exception of 1 µM BA and 10 µM *m*TR, respectively.

Only iP had a significant and clear effect on *m*-hydroxybenzoic acid levels of both *B. pulchellum* (**Figure 4.2F**) and *B. pygmaeum* (**Figure 4.3F**). The highest concentration of *m*-hydroxybenzoic acid in *B. pulchellum* (1.16 µg/g DW) and *B. pygmaeum* (1.59 µg/g DW) was obtained with 25 µM iP. For *B. pulchellum*, salicylic acid levels were only significantly increased by 1 and 5 µM *m*TR (**Figure 4.2G**). On the other hand, salicylic acid in *B. pygmaeum* was significantly affected by most concentrations of BA, iP and *m*TR (**Figure 4.3G**). An increase in *m*TR concentration resulted in a higher salicylic acid content.

The cinnamic acid derivatives in the extracts were sinapic acid, ferulic acid, caffeic acid and *p*-coumaric acid. Sinapic acid in both *B. pulchellum* (**Figure 4.4A**) and *B. pygmaeum* (**Figure 4.5A**) was generally significantly improved by cytokinin treatments. For *B. pulchellum*, the concentration of sinapic acid increased with increasing iP concentration with the highest amount (58.4 µg/g DW) observed in 10 µM iP regenerants. Similarly in *B. pygmaeum*, the highest concentration (97.8 µg/g DW) of sinapic acid was obtained from the 10 µM iP treatment. None of the cytokinins had a beneficial effect on the concentration of ferulic acid in both *B. pulchellum* (**Figure 4.4B**) and *B. pygmaeum* (**Figure 4.5B**) with the exception of 10 µM BA particularly in *B. pulchellum*. In *B. pulchellum*, ferulic acid was highest (54.9 µg/g DW) in 10 µM BA regenerants while the control had the highest amount of ferulic acid (43 µg/g DW) in *B. pygmaeum*.

The cytokinin treatments decreased the concentration of caffeic acid in *B. pulchellum* as they were mostly significantly lower compared to the control (**Figure 4.4C**). However, BA and iP treatments had a significant effect on the concentration of caffeic acid in *B. pygmeaum* (**Figure 4.5C**). An increase in BA and iP concentrations generally did not lead to a gradual increase or decrease in caffeic acid. The highest concentration of caffeic acid (1.28 µg/g DW) was obtained at 10 µM iP regenerants. Treatment with 10 µM BA and 25 µM iP significantly influenced the concentration of *p*-coumaric acid in *B. pulchellum* (only 10 µM BA) (**Figure 4.4D**) and *B. pygmaeum* (**Figure 4.5D**). At some of the treatments, specific concentrations, were found to not be significantly different from the control. *m*TR was particularly the treatment frequently observed to result in lowest phenolic acids in comparison to all treatments and the control. For example, sinapic acid in *B. pulchellum*, ferulic acid in both species, *p*-hydroxybenzoic acid in *B. pygmaeum*, protocatechuic acid in both species, syringic and vanillic acid in *B. pygmaeum*.

Cytokinins have been found to stimulate the production of secondary metabolites in various plant species (NCUBE et al., 2011; YU et al., 2002). For instance, BA, iP and *m*TR were shown to increase the accumulation of important specific phenolic acids under *in vitro* conditions (COSTA et al., 2013). NCUBE et al. (2011) observed micropropagated *Tulbaghia violacea* plants (five months old) to have higher total phenolic and flavonoid concentrations compared to those that were outdoor grown (12 years old). The demonstrated ability of the plants to produce higher levels of secondary metabolites *in vitro* suggests that *in vitro* environmental conditions, particularly with the presence of PGRs, are more favourable

compared to the natural outdoor environment (CHAVAN et al., 2013; CHAVAN et al., 2014a; COSTA et al., 2013; NCUBE et al., 2011). Phenolic acids in particular, are associated with various health benefits and thus the ability to effectively accumulate phenolic acids is highly favourable in this regard (COSTA et al., 2012; MOYO et al., 2010; NCUBE et al., 2011). For both species evaluated in this study, the cytokinin treatments applied generally increased the concentration of gallic, salicyclic and sinapic acids. Of these, ferulic and sinapic acids were the two most abundant in both *B. pulchellum* and *B. pygmaeum* regardless of treatment. Ferulic and sinapic acids belong to the cinnamic acid derivatives which are known to have a higher antioxidant activity in comparison to benzoic acid derivatives due to the structural differences between these two groups (GÜLÇIN, 2012; KIM et al., 2006; ROBBINS, 2003). Benzoic acid derivatives are usually found as minor phenolics, particularly in edible plants (GRUZ et al., 2011). Nevertheless, both hydrobenzoic and hydrocinnamic acid derivatives are associated with anti-cancer activity, and have also shown significant potential as anti-diabetic and cardioprotective agents (SPILIOTI et al., 2014).

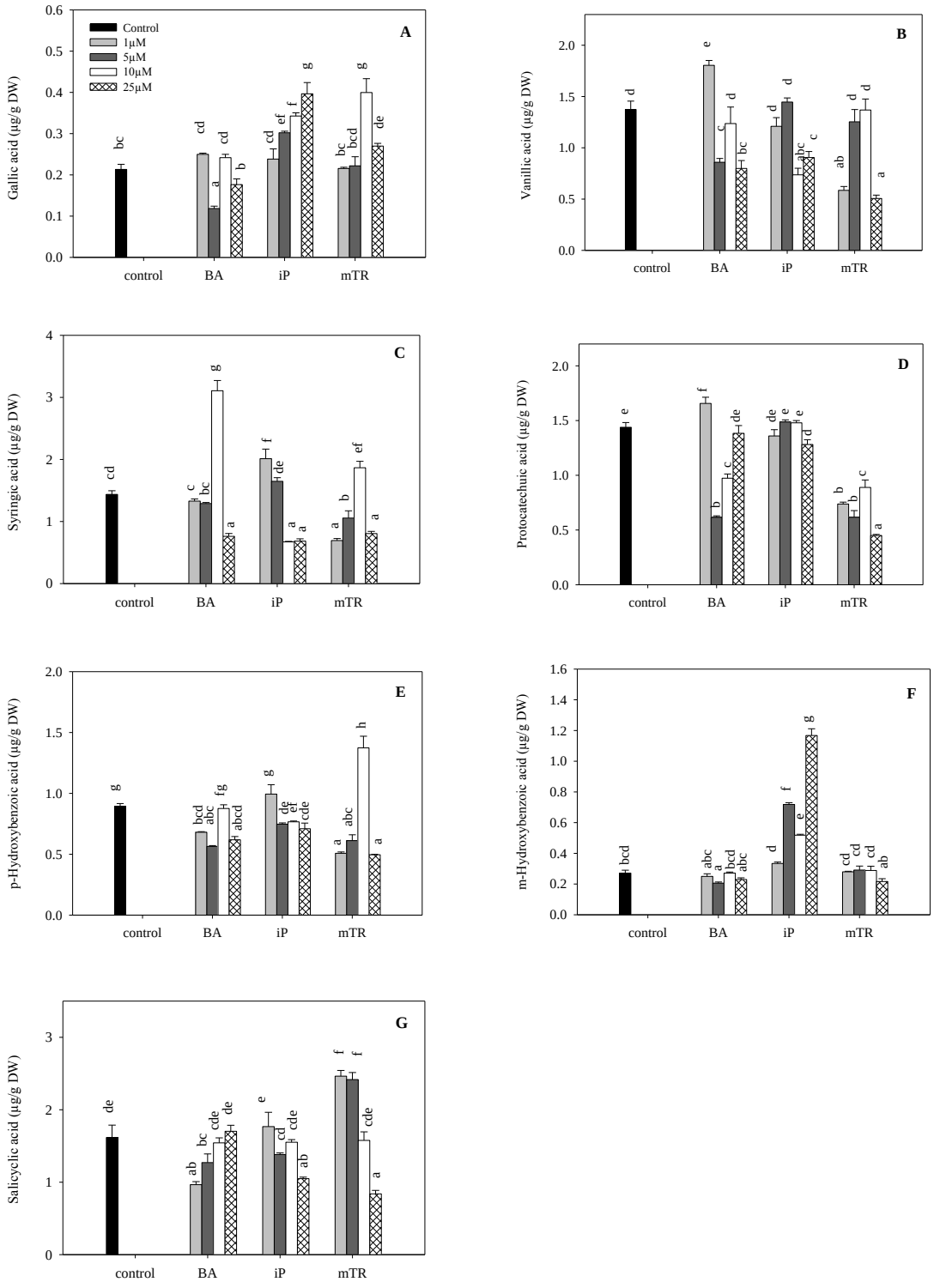

Figure 4.2: Effect of cytokinins on benzoic acid derivatives in *Brachystelma pulchellum*. In each graph, bars represent means with standard error. Different letter(s) indicate significant differences ($p \leq 0.05$) among concentrations and cytokinin types. BA= N^6-benzyladenine, iP= isopentenyladenine and *m*TR= *meta*-topolin riboside.

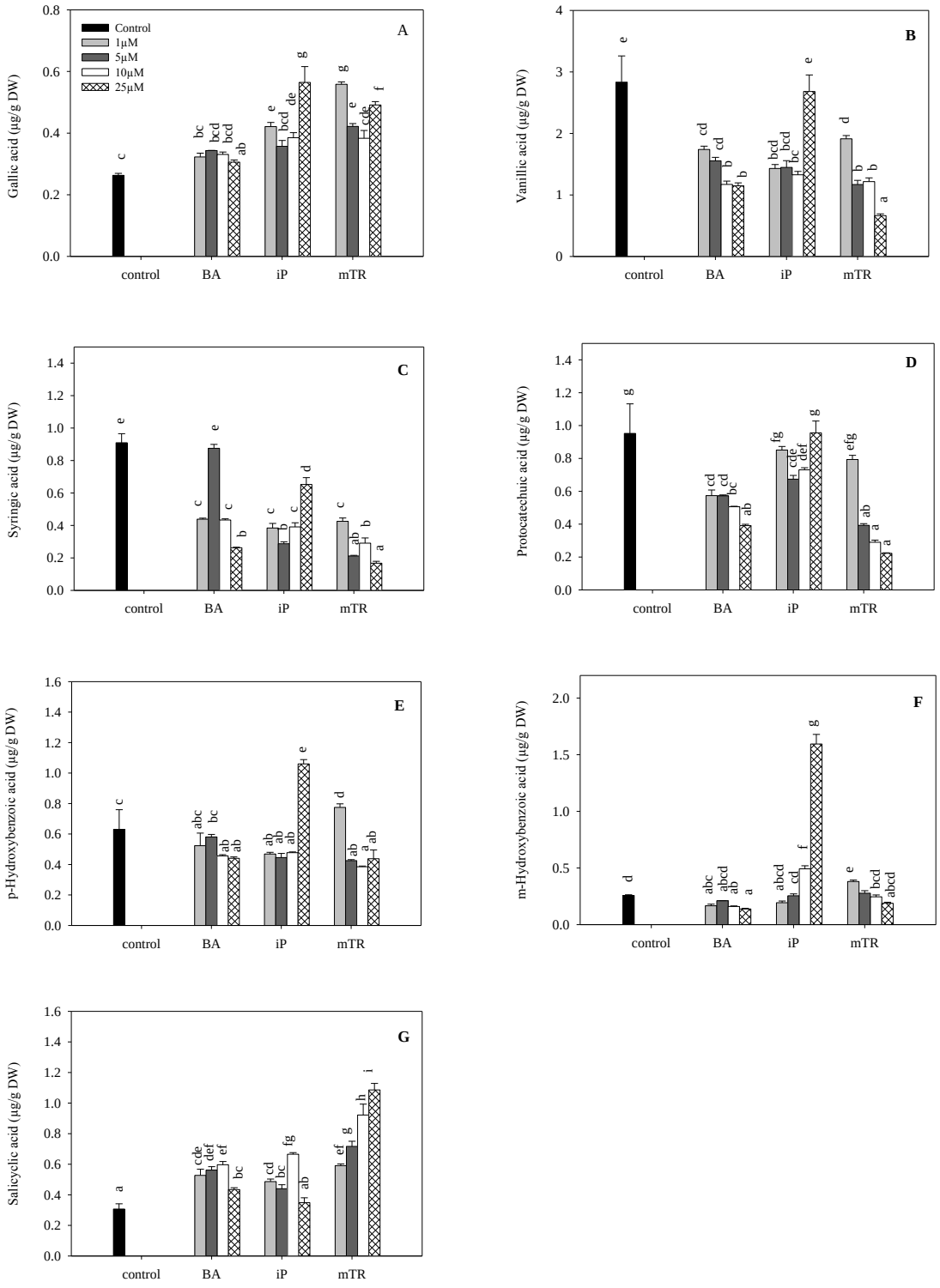

Figure 4.3: Effect of cytokinins on benzoic acid derivatives in *Brachystelma pygmaeum*. In each graph, bars represent means with standard error. Different letter(s) indicate significant differences ($p \leq 0.05$) among concentrations and cytokinin type. BA= N^6-benzyladenine, iP= isopentenyladenine and *m*TR= *meta*-topolin riboside.

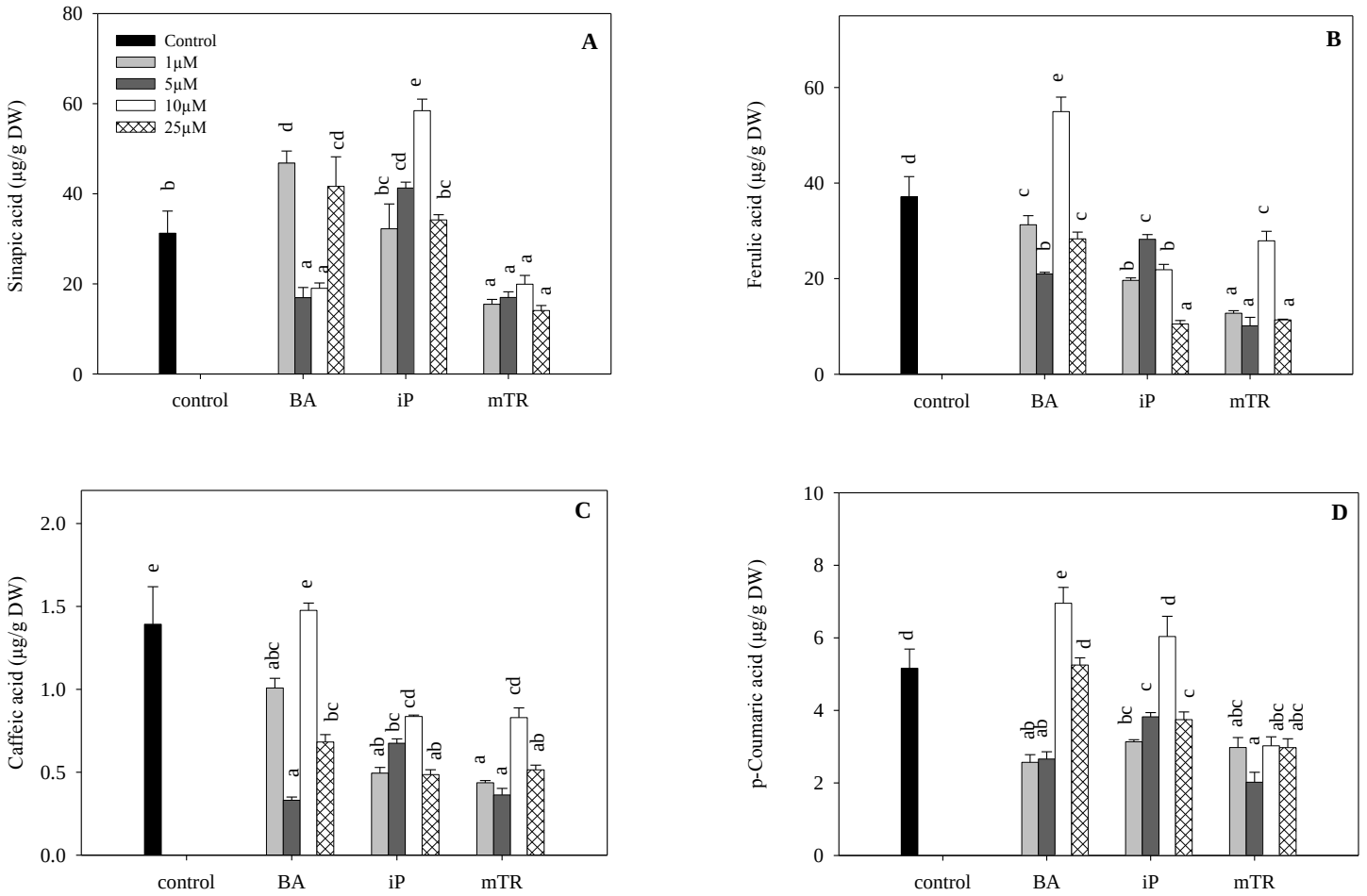

Figure 4.4: Effect of cytokinins on cinnamic acid derivatives in *Brachystelma pulchellum*. In each graph, bars represent means with standard error. Different letter(s) indicate significant differences ($p \leq 0.05$) among concentrations and cytokinin types. BA= N^6-benzyladenine, iP= isopentenyladenine and *m*TR= *meta*-topolin riboside.

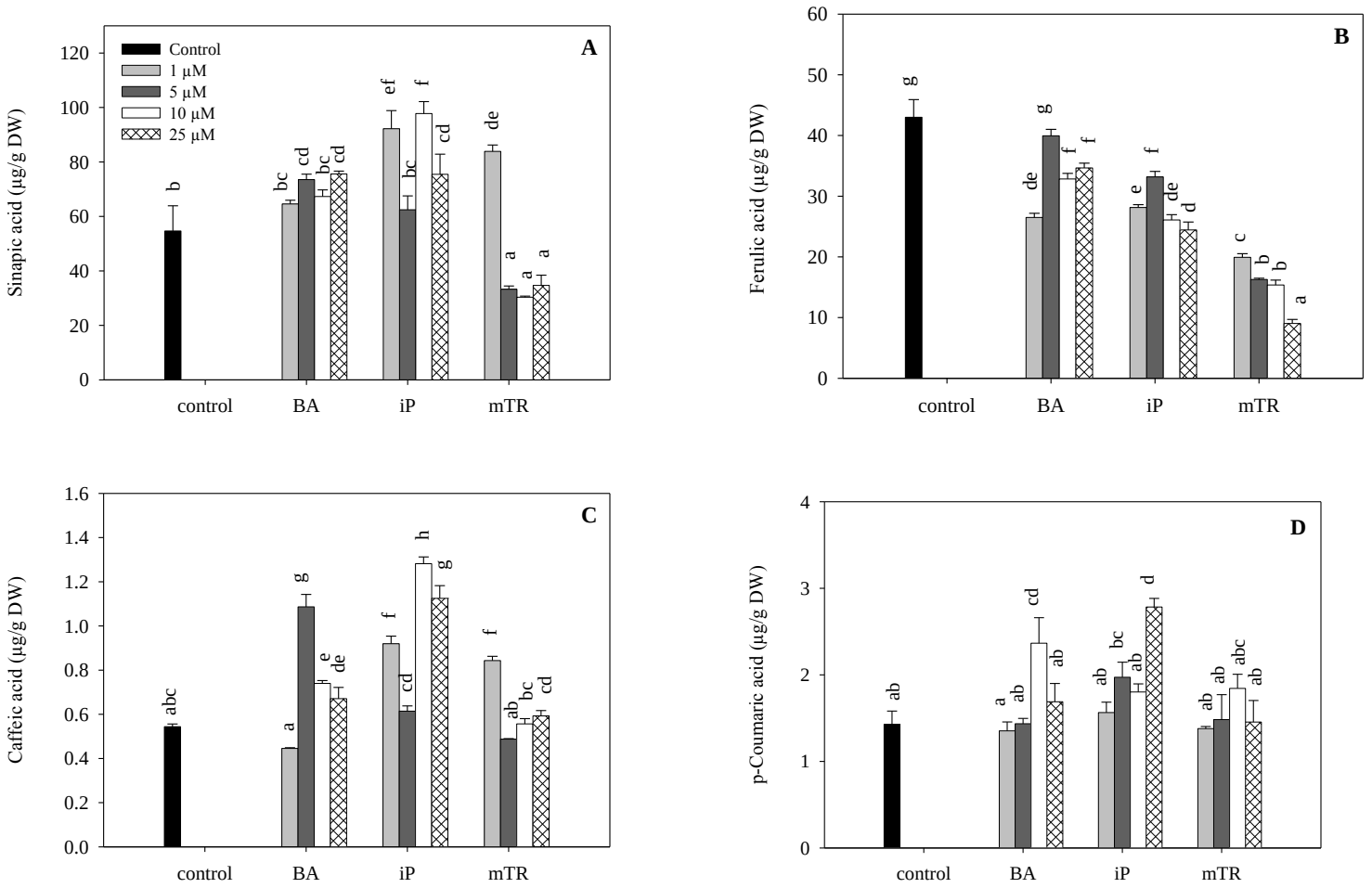

Figure 4.5: Effect of cytokinins on cinnamic acid derivatives, in *Brachystelma pygmaeum*. In each graph, bars represent means with standard error. Different letter(s) indicate significant differences ($p \leq 0.05$) among concentrations and cytokinin types. BA= N^6-benzyladenine, iP= isopentenyladenine and *m*TR= *meta*-topolin riboside.

4.3.2 Antioxidant activity

4.3.2.1 2,2 Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity

As depicted in **Figure 4.6**, extracts (50% MeOH) from leaves and tubers of *B. ngomense*, *B. pulchellum* and *B. pygmaeum* gave dose-dependent radical scavenging activity (RSA %). Leaf extracts had higher activity at lower concentrations compared to tuber extracts, with the exception of the *B. ngomense* tuber extracts. A similar observation was documented for *Brachystelma edulis*, where leaf material was reported to possess higher antioxidant activity (DESHMUKH AND JADHAV, 2014). Based on the EC_{50} values, the extracts had noteworthy radical scavenging activity in comparison to the standard antioxidant agent, ascorbic acid (**Table 4.1**). There was no significant difference between EC_{50} values of the ascorbic acid and the plant extracts, namely *B. ngomense* leaf and tuber extracts, *B. pulchellum* tuber extracts and *B. pygmaeum* leaf extracts. *B. ngomense* leaf extract (13.5 µg/ml) was more potent than ascorbic acid (14.5 µg/ml). Leaf extracts of wild plant species are generally found to have a higher antioxidant activity compared to other plant parts (CHAVAN et al., 2013; DESHMUKH AND JADHAV, 2014), for example, in some Apocynaceae members such as *Ceropegia – C. bulbosa*, *C. juncea*, *C. panchganiensis* and *C. santapaui* (CHAVAN et al., 2014a; DHIR AND SHEKHAWAT, 2014; SHARMA et al., 2011). From a conservation perspective, obtaining a higher antioxidant activity in leaf extracts of *Brachystelma* species will be beneficial mainly because they are perennial herbs and their subsequent reproduction is dependent on the tubers. The results obtained suggest the potential of *Brachystelma* species as a source of natural antioxidants.

Table 4.1: 2,2 Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity (EC_{50}) obtained using 50% methanol extracts from three *Brachystelma* species.

Plant	Part used	EC_{50} (µg/ml)
B. ngomense	Leaf	13.5 ± 1.80^{a}
	Tuber	20.8 ± 4.08^{bc}
B. pulchellum	Leaf	27.8 ± 0.35^{c}
	Tuber	20.9 ± 1.26^{bc}
B. pygmaeum	Leaf	15.7 ± 0.55^{ab}
	Tuber	24.1 ± 3.04^{c}
Ascorbic acid	-	14.5 ± 1.60^{ab}

Values are means ± standard error with the different letter(s) indicating significant differences ($p \leq 0.05$).

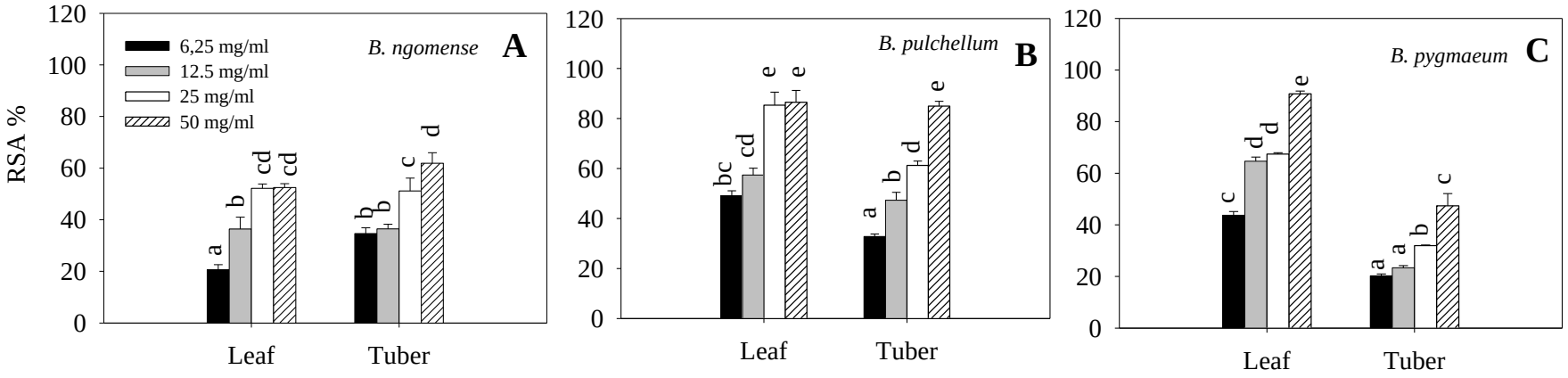

Figure 4.6: 2,2 Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity (RSA) of three *Brachystelma* species collected from the wild. In each graph, bars represent means with standard error. Different letter(s) indicate significant differences among concentrations and plant organs ($p \leq 0.05$). Ascorbic acid RSA value = 95.84.

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4.3.2.2 Oxygen radical absorbance capacity (ORAC)

Figure 4.7 presents the oxygen radical absorbance capacity (ORAC) of extracts from *B. pulchellum* and *B. pygmaeum in vitro*-derived shoots treated with different concentrations of BA, iP and *m*TR. The different cytokinin treatments had a significant effect on antioxidant capacity. For *B. pulchellum* in particular, all cytokinin treatments, from lowest to highest concentration (1 – 25 µM), showed an increased antioxidant capacity compared to the control (35.0 µmol TE /g). The highest antioxidant capacity (72.8 µmol TE /g) was observed in shoots treated with 25 µM of iP. For *B. pygmaeum*, *m*TR, from lowest to highest concentration, significantly increased antioxidant capacity compared to the control (45.1 µmol TE /g). On the other hand, only some concentrations of the BA and iP treatments showed an increased antioxidant capacity. The highest antioxidant capacity (75.8 µmol TE /g) was observed with 25 µM *m*TR. An increase in cytokinin concentration did not necessarily result in a gradual increase or decrease in antioxidant capacity for both species, with the exception of iP on *B. pulchellum*. Further studies are necessary to establish a possible correlation between the treatments and the ORAC. Overall, the use of cytokinins significantly increased the level of ORAC. BASKARAN et al (2014) also reported significant increase in antioxidant activity in response to *in vitro* cytokinin treatments among which was BA.

The stimulatory effect of cytokinin treatments on *in vitro* propagated plants is likely to have a direct influence on secondary metabolite production as well as biological activity. Studies have reported on the influence of cytokinins on secondary metabolite production in *in vitro* cultures of various plant species (AREMU et al., 2013; BASKARAN et al., 2012; DEIKMAN AND HAMMER, 1995). Cytokinins such as BA, iP and *m*TR have been reported to increase the accumulation of important specific phenolic acids under *in vitro* conditions (AREMU et al., 2013; BASKARAN et al., 2014; COSTE et al., 2011). Phenolics have a direct association to a range of biological activities such as anti-microbial and antioxidant activity (BASKARAN et al., 2014; CHIRINOS et al., 2008; GÜLÇIN, 2012; JABERIAN et al., 2013). The observation of increased antioxidant capacity, in this current study, may be attributed to the stimulatory effect of the cytokinin treatments on phenolic acids seeing as untreated shoots (i.e. the control) had a lower antioxidant capacity.

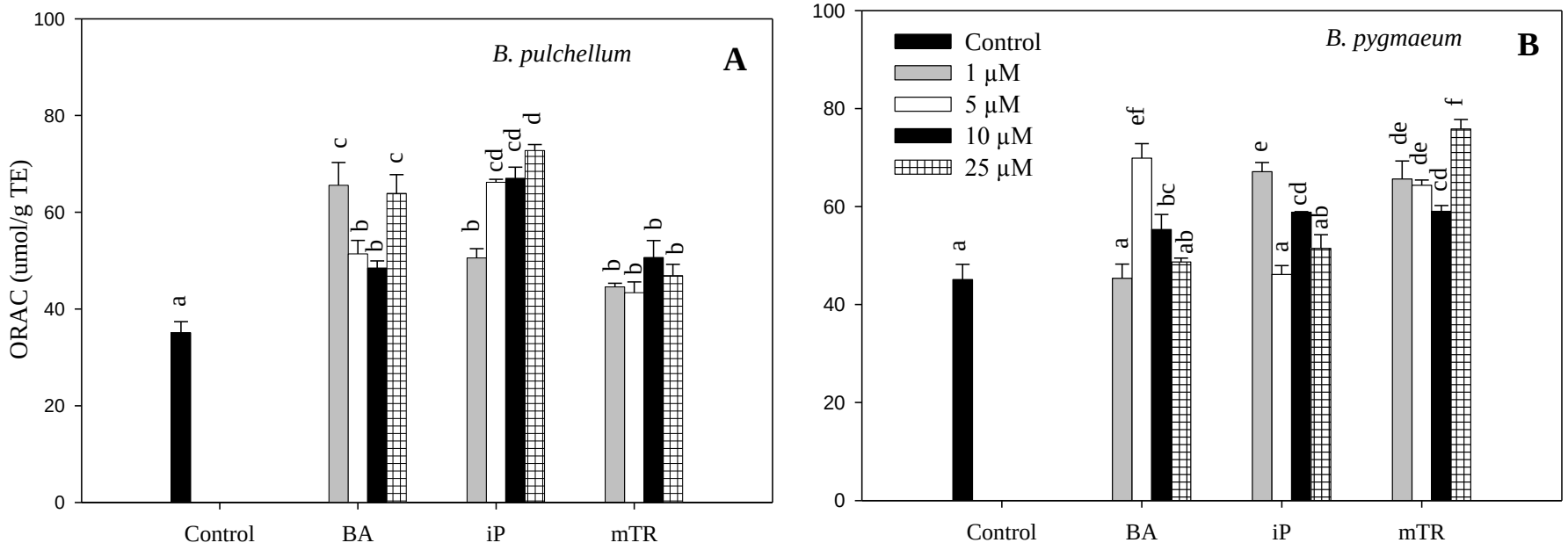

Figure 4.7: Effect of cytokinins on oxygen radical absorbance capacity (ORAC) of *in vitro*-derived shoots of two *Brachystelma* species. In each graph, bars represent means with standard error. Different letter(s) indicate significant differences among the tested concentrations and cytokinin type ($p \leq 0.05$). TE-trolox equivalents, BA= N^6-benzyladenine, iP= isopentenyladenine and *m*TR= *meta*-topolin riboside.

4.4. Conclusions

All the extracts obtained from the three *Brachystelma* species possessed phenolics, flavonoids and demonstrated antioxidant activity. Leaf extracts had higher phenolics, flavonoids and antioxidant activity compared to tuber extracts. Based on the results, *B. pulchellum* and *B. pygmaeum* contained a number of phenolic acids and demonstrated antioxidant activity which was influenced by the application of cytokinin treatments. Ferulic and sinapic acids were the most abundant phenolic acids found in *in vitro* shoots and increased in response to cytokinin treatments. Significant antioxidant levels were found in the leaves and tubers of wild *Brachystelma* species used in this study, thus indicating their potential in protecting against the damaging effects of reactive oxygen species and free radicals. The observations in this study serve, to a certain extent, as support for the reported use of *Brachystelma* species by communities in Africa and India for various ailments. The use of the tubers of certain *Brachystelma* species for medicinal purposes such as wound healing (*B. buchananii*) and chest pains (*B. johnsonii*) can be evaluated as there is likelihood that the leaves of these species have a higher potency compared to their tubers which would also benefit conservation of the species. A thorough investigation of secondary metabolites present in *Brachystelma* plant material and their bioactivity can be achieved by using extraction solvents of varying polarities. Thus further investigations are encouraged to continue exploring the potential medicinal and nutritional use of the *Brachystelma* genus.

Chapter 5: Overall conclusions and recommendations

Plant tissue culture has been acknowledged by commercial and research facilities as a fundamental tool for achieving improved plant regeneration rates and phytochemical production. Micropropagation is among the most used of the tissue culture techniques. The current study evaluated the role of plant growth regulators during the micropropagation of three *Brachystelma* species, two of which are of conservation concern. The highest shoot production (4.44 ± 0.41, 2.04 ± 0.20 and 2.57 ± 0.26) was obtained from medium supplemented with 25 µM *m*TR, 25 µM iP and 25 µM BA for *B. ngomense*, *B. pulchellum* and *B. pygmaeum*, respectively. An increase in concentration of all the cytokinin treatments was found to typically result in significantly higher shoot growth parameters, mainly fresh weight and shoot proliferation compared to the control, for *B. ngomense* and *B. pygmaeum* especially. During the shoot proliferation stage, 1 µM and 5µM BA displayed the least root inhibiting effect. In the rooting investigations of *B. pygmaeum*, the highest number and length of roots was obtained from medium supplemented with the lower concentrations of 2,4-D (0.5 µM and 5 µM, respectively). The observed shoot and root growth parameters show the potential of growth regulators in improving the growth of *Brachystelma* species. However, the study has not achieved an efficient micropropagation protocol for any of the *Brachystelma* species due to the limit in acclimatization of shoots *ex vitro* under tested green house conditions. Inadequate rooting was one of the main reasons for the failure to achieve a successful micropropagation protocol. *Ex vitro* rooting of regenerated shoots after exposure to 492.1 µM IBA pulse treatment was also found to yield poor results and short lived green house survival. Only a very small percentage (0.5%) of plantlets was successfully acclimatized. The use of a combination of various cytokinin and auxin treatments during *in vitro* culture stages could potentially assist in improving acclimatization. Likewise, pulse treatment using different concentrations of IBA could possibly yield better rooting. Subsequent investigations towards the optimization of a propagation protocol for *Brachystelma* species can also include testing different *ex vitro* growth environments (acclimatization stage) considering mainly factors such as humidity, temperature and soil substrate such that some of the environments are similar to the natural habitat in which *Brachystelma* thrive.

The significance of plant growth regulators was also observed in the *in vitro* phytochemical investigation. Cytokinin treatments improved the concentration of secondary metabolites quantified in this study. Phenolics were generally higher in shoots treated with cytokinins. The most abundant phenolic acids found in *in vitro* shoots were ferulic and sinapic acids which were observed to increase in response to the cytokinin treatments. Generally, the phenolic acids reached their highest concentration at higher cytokinin levels. The observed *in vitro* phenolic acid accumulation is of potential benefit to their use as therapeutic agents. The current study supports the association of enhanced therapeutic activity with increased secondary metabolites. Better antioxidant activity was observed from *in vitro* shoots that had higher phenolic content as a result of the applied cytokinin treatments. Thus, it is necessary to optimize the tissue culture treatments as a means of enhancing secondary metabolites for commercial use. There is, however, a likelihood that other compounds are significant contributors to the observed biological activity. Phytochemical and pharmacological investigations conducted using extracts from wild *Brachystelma* species showed phenolic content to be higher in leaf extracts compared to tuber extracts. The observation of higher phenolic and flavonoid content in leaf extracts is significant with regards to conservation since the use of above ground parts is often less detrimental to the survival of field plants compared to using the bulbs. In addition, the leaves and tubers of wild *Brachystelma* species were found to have significant antioxidant levels which indicates their potential antioxidant activity against the damaging effects of free radicals and reactive oxygen species. Even though the quantity of plant material posed a major challenge, it is necessary to explore exhaustively other potential bioactivities, for example anti-microbial activity, of *Brachystelma* species. As plant material becomes available, a more thorough investigation of secondary metabolites present in the *Brachystelma* plant material as well as their bioactivity can be achieved by using extraction solvents of varying polarities. Thus further investigations are encouraged to establish the potential medicinal and nutritional use of the *Brachystelma* genus.

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Appendix

Appendix 1: In vitro propagation of some species belonging to the family Apocynaceae.

Plant name	**Type of explant**	**Most optimal media composition**	**Response**	**Survival *ex vitro* (%)**	**Reference**
***Boucerosia diffusa* Wight.**	Nodal segments	MS + (8.87 µM BAP+ 2.27 µM TDZ) + 2.69 µM NAA	Shoot multiplication (7.0 ± 0.19) + root induction (10.37 ± 0.03)	56	Ramadevi et al., (2012)
Cantharanthus roseus	Axillary bud, nodal segment, shoot tip	MS + (4.0 mg/l BA + 3.0 mg/l KN) + 4.0 mg/l IBA	Shoot multiplication (19.6 ± 0.28) + root induction (½MS + IBA)	80	Bakrudeen et al., (2011); Debnath et al., (2006); Kumar et al., (2013)
***Caralluma bhupenderiana* Sarkaria.**	Nodal segments	MS + 8.87 µM BA +2.69 µM NAA	Shoot multiplication (8.40 ± 0.50) + root induction (½MS +NAA)	80	Ugraiah et al., (2011)
***Carissa carandas* L.**	Nodal segments, shoot apex	MS + (1.5 mg/l BAP + 1.0 mg/l Kn + 1.0 mg/l TDZ + 15 mg/l Ads) + 2.0 mg/l IAA	Shoot multiplication + root induction (½ MS IAA)	NS	Imran et al., (2012)
***Ceropegia attenuata* Hook.**	Leaf, nodal segment	MS + 13.30 µM BA + 2.46 µM IBA	Shoot multiplication (12.9 ± 0.50) + root induction (9.80 ± 0.50)	85	Chavan et al., (2011); Palawat and Lodha, (2014)
Ceropegia bulosa Roxb.	Leaf, nodal segment, shoot tip	MS + 1.5 mg/l NAA + 100.0 mg/l IBA	Callus formation (80%) + *ex vitro* root induction (7.46 ± 0.48)	100	Palawat and Lodha, (2014); Phulwaria et al., (2013)

Plant name	**Type of explant**	**Most optimal media composition**	**Response**	**Survival *ex vitro* (%)**	**Reference**
***Ceropegia candelabrum* L**	Axillary bud, nodal segments	MS + (8.87 µM BA + 2.46 µM IBA) + 0.49 IBA	Axillary bud proliferation (7.8 ± 0.0) + root induction (6.9 ± 0.0)	90	Beena et al., (2003)
***Ceropegia juncea* Roxb.**	Nodal segments	MS + 7.50 µM Kin + (1.0 µM 2,4-D+ 5.0 µM BA) + 7.50 µM IBA	Shoot multiplication (8.5 ± 3) + callus formation (0.55 ± 0.02) + root induction (½ MS - 9.4 ± 0.7)	100	Nikam and Savant, (2009)
***Ceropegia noorjahaniae* Ans.**	Nodal segments	MS + 2.0 mg/l BAP + 1.0 mg/l IBA	Shoot multiplication (18.3 ± 1.3) + root induction (8.1 ± 0.7)	85	Chavan et al., (2014b)
***Ceropegia thwaitesii* Hook**	Axillary bud	MS + (13.94 µM Kin + 28.54 µM IAA) + 2.46 µM IBA	Shoot multiplication (6.42 ± 0.26) + root induction (4.43 ± 0.52)	73.3	Muthukrishnan et al., (2012); Muthukrishnan et al., (2015)
***Chonemorpha grandiflora* Roth.**	Axillary bud, shoot tip	MS + 13.30 µM BA + 0.49 µM IBA	Shoot multiplication (3.1)+ root induction (½ MS- 3.9)	90	Nishitha et al., (2006)
Decalepis arayalpathra	Nodal segments	MS + (2.22µM BA + 0.24 µM 2-iP) + 1.07 µM NAA	Nodal production (9.8 ±0.3) + root induction (½ NAA)	86	Sudha et al., (2005)
***Holostemma annulare* Roxb.**	Shoot tip, basal node	MS + (4.43 µM BA + 0.54 µM NAA) + 1.48 µM IBA	Shoot multiplication (3.8 ± 0.2) + root induction (½MS IBA)	80	Sudha et al., (1998)
Hoya wightii* ssp. *palniensis	Shoot tip	MS + (4.65 µM KN+ 1.47 µM IBA) + 0.28 µM IAA	Shoot multiplication (3.80 ±0.50) + root induction (16 ± 0.6)	56	Lakshmi et al., (2010)
Mandevilla moricandiana	Nodal segments	MS + 1.0 mg/l BA	Shoot multiplication + callus induction	40	Cordeiro et al., (2012)
Nerium odorum	Leaf, stem	MS + 2.5 mg/l 2.4-D	Callus induction (80%)	NA	Rashmi and Trivedi, (2014)
Rauvolfia micrantha	Root	½ MS + (0.2 mg/l BA + 0.1 mg/l NAA)	Embryogenesis (63%)	80	Sudha and Seeni, (2006)

Plant name	Type of explant	Most optimal media composition	Response	Survival *ex vitro* (%)	Reference
Hook. f.					
***Rauvolfia serpentina* Benth.**	Nodal segments	MS + (5.0 µM BAP + 0.5 µM IAA) + 50 µM IBA + NOA	Shoot multiplication + *ex vitro* root induction (½ MS)	80	Kataria and Shekhawat, (2005)
Sarcostemma brevistigma	Nodal segments	MS + (4.0 µM BA + 1 µM NAA) + (10 µM BA+ 1 µM NAA) + 5 µM IBA	Shoot multiplication (10.9 ± 1.8) + callus formation (13.4 ± 1.9) + root induction (½MS -4.4 ± 0.2)	86	Dennis Thomas and Shankar, (2008)
Wattakaka volubilis	Seedling	MS + (4.40 µM BAP + 0.288 µM GA3)	Adventitious shoots (10.4)	60	Chakradhar and Pullaiah, (2014)

NA- Not available

Appendix 2: Pharmacological uses of some plants belonging to the family Apocynaceae.

Plant	Common name	Plant part used	Therapeutic potential (Research studies)	Ethnobotanical use	Reference
Allamanda	NS	Leaf, stem, root	Anti-proliferative	Cancer, malaria	Chan et al., (2016)
***Alstonia macrophylla* Wall. ex G. Don**	Hard alstonia	Bark	Anti-cancer, anti-microbial	Anticholeric, antidysentery	Khyade et al., (2014)
***Alstonia scholaris* (L.) R. Br.**	Devil's tree	Leaf, stem bark	Anti-inflammatory, anti-cancer, anti-microbial	Chronic respiratory disease, cancer, jaundice, malaria	Adotey et al., (2012); Chan et al., (2016); Khyade et al., (2014); Shang et al., (2010)

Plant	Common name	Plant part used	Therapeutic potential (Research studies)	Ethnobotanical use	Reference
Aspidosperma ramiflorum	Yellow peroba	Leaf, stem bark	Anti-inflammatory, anti-malarial, anti-oxidant	Leishmaniasis	Aguiar et al., (2015)
***Brachystelma edulis* Coll.**	Galya	Leaf, tuber	Anti-oxidant	Cough, dysentery, stomachache	Deshmukh and Jadhav, (2014)
***Calotropis gigantiea* (L.) R.Br.**	Crown flower	Leaf, stem, root, root bark	Anti-microbial, anti-oxidant, anti-diabetic, antidiarrheal	Central nervous system, skin disease, digestive and respiratory system	Kadiyala et al., (2013); Chan et al., (2016)
Cantharanthus roseus	Mada-gascar peri-winkle	Leaf	Anti-cancer, anti-microbial, anti-oxidant, anti-plasmodial	Cancer, malaria	Chan et al., (2016); Almagro et al., (2015)
***Caralluma* R.Br.**	NS	Whole plant	Anti-diabetic, anti-obesity, anti-microbial, anti-oxidant, anti-plasmodial	Diabetes, inflammation, malaria, obesity	Adnan et al., (2014); Dutt et al., (2012)
Cerbera	NS	Seed, Leaf, root	Anti-proliferative	Cancer, malaria	Chan et al., (2016)
***Ceropegia santapaui* Wadhwa and Ansari**	NS	Whole plant	Anti-oxidant	Diarrhea, dysentary	Chavan et al., (2013); Deshmukh and Jadhav, (2014); Dhir & Shekhawat, (2014); Sharma et al., (2011)
Kopsia	NS	Leaf, stem bark	Anti-plasmodial, anti-proliferative	Cancer, malaria	Chan et al., (2016)
Leptadenia	Yadiya	Stem	Anti-microbial	Labour and stomach pains,	Doughari and Odibah, (2008)

Plant	Common name	Plant part used	Therapeutic potential (Research studies)	Ethnobotanical use	Reference
***lancifolia* (Per) Decne**				topical application	
Mandevilla velutina	Rocktrum pet	Leaf, stem, rhizome	Anti-venom, anti-toxin	Inflammation, snakebites	Biondo et al., (2003)
Nerium	NS	Leaf, stem, flower	Anti-proliferative	Cancer, malaria	Chan et al., (2016)
Picralima nitida	NS	Whole plant	Anti-diarrheal, anti-inflammatory, anti-microbial, anti-plasmodial	Malaria, dysmenorrhoea, gastrointestinal disoders	Kouitcheu Mabeku et al., (2008)
***Picralima nitida* Durand and Hook**	Pile plant	Whole plant	Anti-inflammatory, antimicrobial, anti-plasmodial, anti-ulcer	Jaundice, malaria, gastrointestinal disorders	Erharuyi et al., (2014)
Plumeria	NS	Leaf, bark, flower	Anti-plasmodial, anti-proliferative	Cancer, malaria	Chan et al., (2016)
Thevetia peruviana	NS	Leaf and bark	Piscicidal	Poisonous	Singh et al., (2010)
Vallaris glabra	Bread flower	leaf	Anti-plasmodial, anti-proliferative	Cancer, malaria	Chan et al., (2016)
***Wrightia tinctoria* R. Br.**	Dyer's oleander	Leaf, whole plant	Anti-diabetic, anti-microbial	Herpes, jaundice, malaria, toothache	Khyade and Vaikos, (2014); Srivastava, (2014)

NS-Not specified

Appendix 3: Antioxidant activity of some Ceropegieae (tribe) species.

Species	Plant part used	Extract	Test/method	Reference
Brachystelma edulis	Leaf	Buffer	Titrimetric analysis	Deshmukh and Jadhav (2014)
***Ceropegia bulbosa* Roxb.**	Leaf	Methanol	Spectrophotometry	Dhir & Shekhawat (2014); Deshmukh and Jadhav (2014)
Ceropegia evansii	Leaf(FRAP), tuber	Methanol	DPPH, FRAP assay	Chavan et al., (2013)
Ceropegia hirsuta	Tuber	Buffer	Titrimetric analysis	Deshmukh and Jadhav, (2014)
Ceropegia juncea	Leaf	Ethanol	Malondialdehyde estimation	Sharma et al., (2011)
Ceropegia panchganiensis	Leaf	Methanol	DPPH	Chavan et al., (2013)
Ceropegia santapaui	Leaf	Methanol	DPPH, FRAP assay	Chavan et al., (2014a)
Ceropegia spiralis	Leaf, tuber (FRAP)	Ethanol, methanol(FRAP)	DPPH, FRAP assay	Chavan et al., (2013)

DPPH- Diphenyl-1-picrylhydrazyl, FRAP- Ferric reducing antioxidant power

Appendix 4: Details of methodology for specific phenolic acid quantification (including instrumentation and conditions) – Taken from Gruz et al. (2008)

UPLC –MS/MS instrumentation and conditions

UPLC-MS/MS analyses were carried out using an ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) linked simultaneously to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro *micro*™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with Z-spray electrospray ionisation (ESI) source operating in negative mode. MassLynx™ software (version 4.0, Waters, Milford, MA, USA) was used to control the instruments, and for data acquasition and processing.

Sample solutions were injected into a reverses phase column (BEH $C_{8,}$ 1.7 µm, 2.1 x 150 mm, Waters, Milford, MA), which was maintained at 30 ^{o}C. The mobile phase consisted of the following 9.5-min sequence of linear gradients and isocratic flows of the solvent B (acetonitrile) balanced with aqueous 7.5 mM HCOOH (solvent A) at a flow rate of 250 µL min^{-1}: 5% B for 0.8 min, 5-10% B over 0.4 min, isocratic 10% B for 0.7 min, 10-15% B over 0.5 min, isocratic 15% B for 1.3 min, 15-21% over 0.3 min, isocratic 21% B for 1.2 min, 21-27% B over 0.5 min, 27-50% B over 2.3 min, 50-100% B over 1min, and lastly 100-5% B 0ver 0.5 min. At the end of this sequence the column was equilibrated under initial conditionsfr 2.5 min. The pressure ranged from 4000 to 8000 psi duringthe chromatographic run. The effluent was introduced into a PDA detector (scanning range 210-600 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 100 ^{o}C, desolvation temperature 350 ^{o}C , capillary voltage 2.5 kV, cone voltage 25V). Argon was used as collision gas (collision energy 16 eV) and nitrogen as desolation gas (500 L h^{-1}). The retention windows used for quantification were as follows: 0.00-3.00 min; 3.00-4.25 min; 4.25-4.70 min; 4.70-5.15 min; 5.15-5.80 min;5.80-6.30 min; 6.30-7.30 min; 7.30-7.80 min; 7.80-8.90 min; 8.90-10.00 min

Identification and quantification

Phenolic acids (PHAs) and deuterium-labelled internal standards were detected in multiple reaction monitoring (MRM) mode using mass-to-charge (*m*/*z*) transitions of precursor and product ions. PHAs were identified by matching retention times and mass spectral data with those of the calibration standards. Analyte concentrations in the samples were derived from analyte:average internal standard peak area ratios, using the following equation:

$$(\text{analyte concentration}) = IS_c \times \text{slope} \times A \times 2(IS_1 + IS_2)^{-1} + c \quad (1).$$

where IS_c is the concentration of IS in the sample; A is the peak area of the analyte; IS_1 is the peak area of 4-hydroxybenzoic acid (2,3,5,6-D4); IS_2 is the peak area of salicylic acid (3,4,5,6-D4); slope and *c* are linear regression parameters.

Appendix 5: Phylogenetic relatedness of *Ceropegia* and *Brachystelma* (Apocynaceae) genera. Taken directly from Bruyns et al. (2015)