THE EFFECT OF GEOGRAPHY, CULTIVATION AND HARVEST TECHNIQUE ON THE UMCKALIN CONCENTRATION AND GROWTH OF *PELARGONIUM SIDOIDES* (GERANIACEAE)

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Abstract

Pelargonium sidoides DC. (Geraniaceae) root extracts are used in the Eastern Cape Province of South Africa as a traditional medicine for the treatment of respiratory tract and gastro-intestinal infections. Ethanolic extracts are used globally as herbal treatments for bronchitis, asthma and as an immune system booster. Despite documented exploitation of wild populations by illegal harvesters, this species has not been awarded a protected status. The high level of harvest in the years preceding this study prompted this investigation of the prospects for sustainable root harvest through wild harvest and greenhouse cultivation. A novel method was developed for the purification of umckalin, a bioactive constituent in root extracts, such that the root umckalin concentrations of wild and cultivated plants could be quantified by HPLC. As part of the cultivation experiments, the concentration of umckalin in roots was measured for plants across part of the species' distribution range in the Eastern Cape Province. This survey revealed that root umckalin concentrations were inversely related to the average annual rainfall of the collection site ($r^2 = 0.94$, $p = 0.007$) and directly related to soil pH ($r^2 = 0.97$, $p = 0.002$). Thus, the possibility of inducing high umckalin concentrations in greenhouse-cultivated plants was investigated by subjecting plants to rapid and prolonged water stress treatments. Two leaf applied hormone treatments (cytokinin and gibberellin) and a root competition treatment with a fast growing annual (*Conyza albida*) were also investigated based on the potential function of umckalin in *P. sidoides* plants. These five treatments did not significantly affect root umckalin concentrations compared to well-watered controls. The results of further experiments suggested that umckalin production may have been influenced by the geographical origin and genetics of plants rather than environmental variation. Following wild harvest experiments, the regrowth of replanted shoots from which a standard proportion of the root was harvested showed that water availability affected shoot survival but not root regrowth rate. Regrowth rates were low, questioning the viability of wild harvest. In contrast, greenhouse cultivated plants showed *ca.* six times greater growth rates, supporting the cultivation of roots to supply future market demand.

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The Sovereign Lord is my strength; He makes my feet like the feet of a deer, He enables me to go on the heights (Hab. 3:19). Thanks Dad.

Chapter 1

Introduction

Many South African medicinal plants, including *Harpagophytum procumbens* and *H. zerheri* (Devil's claw), *Aloe ferox* (Cape aloes) and *Agathosma betulina* (buchu), are used in the production of internationally marketed herbal remedies for the treatment of various ailments (Van Wyk *et al.,* 2000). The wild resources of these species, and others like *Pelargonium sidoides,* may become over-exploited should excessive sourcing of wild plant material take place to meet foreign and local demand.

Therefore, the intention of the research presented here was to investigate the wild harvest and cultivation of *Pelargonium sidoides* and gain knowledge that would aid informed decision-making on the future conservation of the species. Additionally, experiments were designed to investigate the function of a known *P. sidoides* bioactive metabolite, umckalin, in plants of this species. The implementation and assessment of novel harvest techniques formed the focus of wild harvest experiments, while cultivation experiments focused on optimising the concentration of umckalin in the roots of greenhouse-grown plants. Assessing the latter was achieved through the development of novel methods for the isolation of umckalin and for the quantification of this compound in root extracts.

1.1 Botanical description

P. sidoides DC. (*Geraniaceae,* section *Reniformia*) is commonly known as Kalverbossie or Rabassam and would be recognized in the field as an erect, woody to herbaceous subshrub. Its leaves are clustered, velutinous, rosulate, fairly aromatic, long-stalked and silver-green. The leaf shape is described as ovate-cordate and the base as cordate. The inflorescences are formed by flowering branches more or less throughout the year, peaking in December, with several pseudo-umbels, each with 4-12 flowers (Dreyer and Marais, 2000; and Van der Walt and Vorster, 1988).

Plants of this species are very easily confused with those of *P. reniforme* Curt. (Rooirabas or Kidney-leaved pelargonium; Van der Walt, 1977). Both species are very similar in appearance and the form of each varies between locations (White, 2004-5, *personal observations*). The diagnostic features that can be used to distinguish the two are sepal and petal colour (Figure 1.1). Unlike the pink to purple petals of *P. reniforme*, those of *P. sidoides* are maroon to black. The sepals of *P. sidoides* are green with white margins with those of *P. reniforme* being red with pink margins (Dreyer and Marais, 2000).

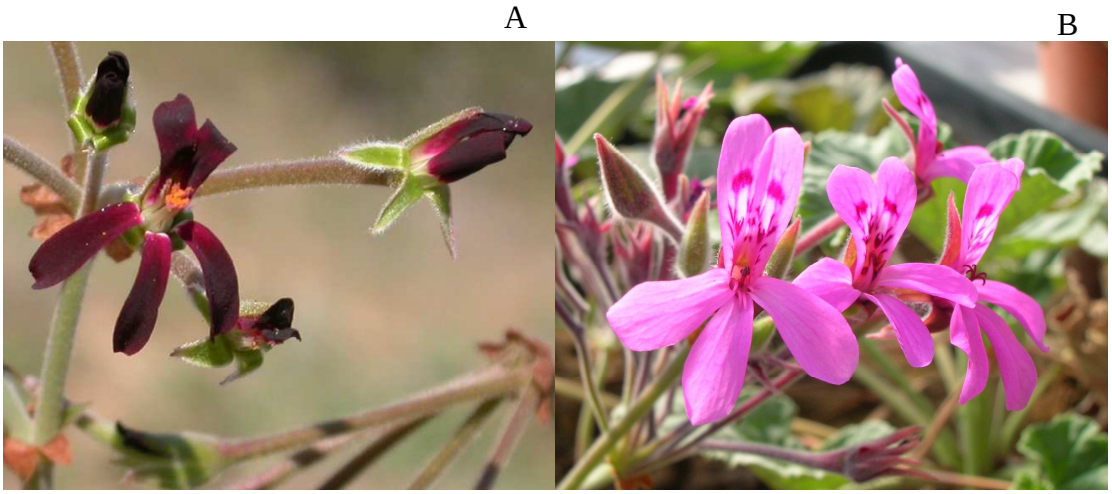


Figure 1.1: The diagnostically coloured flowers of *P. sidoides* (A) and *P. reniforme* (B).

P. sidoides plants are evergreen in cultivation (Van der Walt and Vorster, 1988) but wild plants die back in winter at some localities (White, 2004-5, *pers. obs.*). The distribution of this species is very widespread across the central to eastern parts of Southern Africa; occurring in Lesotho as well as the Eastern Cape, Free State, Northwest, Gauteng and Mpumalanga Provinces of South Africa. *P. sidoides* plants are said to thrive in direct sunlight and are found predominantly on sand or loamy soils from near sea level to 2300 m (Figure 1.2). The rainfall in the majority of the distribution range, falling mostly in summer, is between 200 and 800 mm per year (Dreyer and Marais, 2000; and Van der Walt and Vorster, 1988).

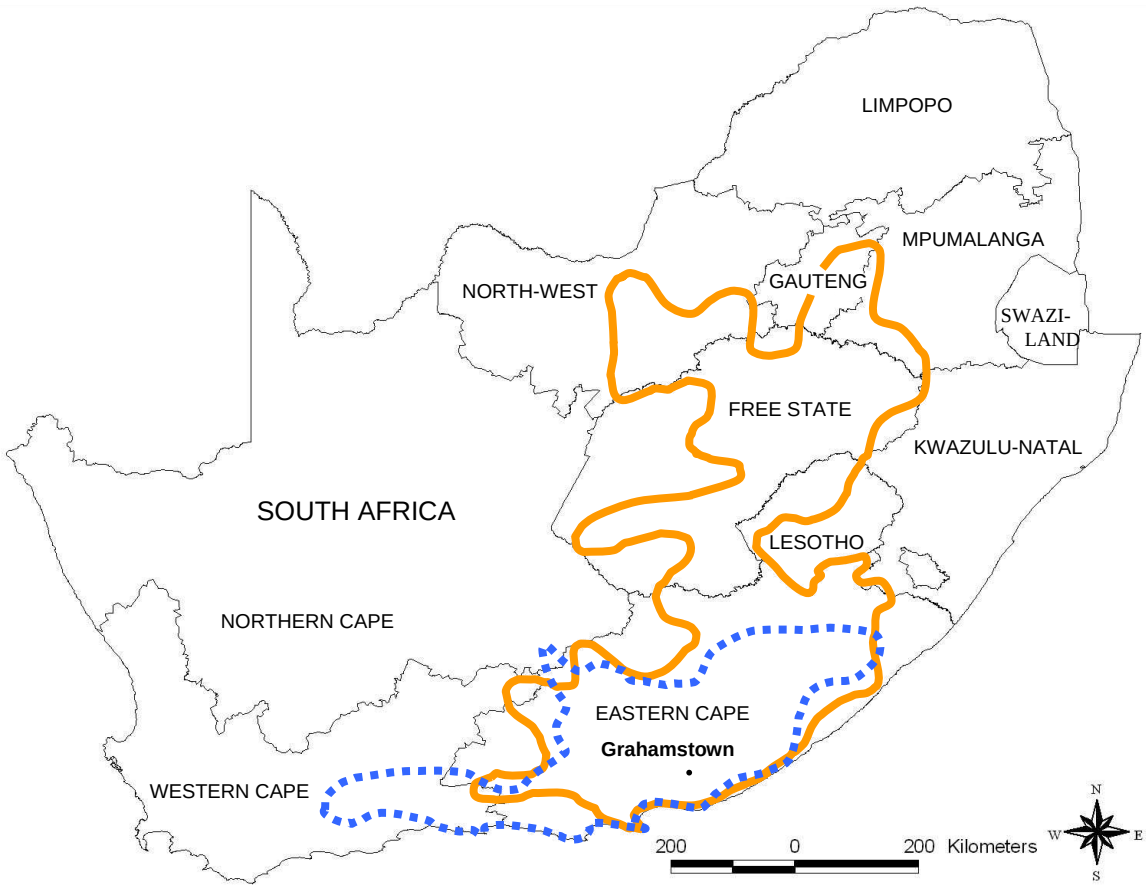


Figure 1.2: Map showing the distribution of *P. reniforme* (blue dashed line) and *P. sidoides* (orange solid line) in South Africa and Lesotho. Adapted from Dreyer and Marais (2000).

1.2 Medicinal use

1.2.1 Introduction

The tuberous, woody roots of *P. reniforme* and *P. sidoides* are traditionally used in the Eastern Cape Province of South Africa for their medicinal properties (Gerardy, 2002; and Van der Walt and Vorster, 1988), particularly amongst the Xhosa people who use infusions of the roots for the treatment for diarrhoea, dysentery, colds and lung infections including tuberculosis (Bladt, 1977; and Van Wyk and Gericke, 2000).

Recently, both *P. reniforme* and *P. sidoides* populations in the Eastern Cape, particularly in rural areas, have also been targeted by local and international pharmaceutical companies for commercial products. These products (e.g. Pelargonium, Medicherb UK; Pelargonium Syrup, Bioharmony Africa and Umckaloabo®, Spitzner) are effectively used to treat upper respiratory tract infections and are available in many regions including Germany, Mexico, Baltic states, in the Commonwealth of Independent States (CIS), Turkey, Brazil (e.g. Matthys *et al.*, 2003; and Chuchalin *et al.*, 2005) and South Africa (White, 2004-5, *pers. obs.*).

1.2.2 Clinical trials

In their randomized, double-blind, placebo controlled trial of 468 adults Matthys *et al.* (2003) clearly showed that a *P. sidoides* extract (EPs 7630) was effective in the treatment of acute bronchitis and had only mild and no serious adverse events. Specifically, treatment with the extract reduced the time-off from work by two days, an important aspect to consider when assessing the efficacy of a treatment (Matthys *et al.*, 2003).

Like Matthys *et al.* (2003), Chuchalin *et al.* (2005) found in a similar clinical trial that EPs 7630 was more effective than a placebo in the treatment of acute bronchitis amongst adults. Their trial included 124 patients and was based at six outpatient clinics in Germany. Treatment with EPs 7630 was well tolerated by patients and no serious side effects were reported during the trial.

The rational basis for the effective treatment of acute bronchitis (*vide supra*) with *P. sidoides* extracts is provided by chemical, immunological and antibacterial assays performed using similar extracts and some of the compounds they contain (Kayser and Kolodziej, 1997; Kolodziej *et al.*, 2003; Kolodziej *et al.*, 2005; Seidel and Taylor, 2004 and Trun *et al.*, 2006).

1.2.3 Antibacterial and antifungal activity

Extracts made from the roots of *P. sidoides* contain the following bioactive constituents: the coumarins umckalin and 6,8-dihydroxy-5,7-dimethoxycoumarin (Kayser and Kolodziej, 1997), gallic acid and its methyl ester (Kayser and Kolodziej, 1997), (+)-catechin (Kolodziej *et al.*, 2003), certain fatty acids (Seidel and Taylor, 2004) and tannins (Kolodziej *et al.*, 2005).

Kayser and Kolodziej (1997) investigated the antibacterial activity of crude root and leaf extracts and their individual constituents against a range of pathogenic bacteria known to cause respiratory tract infections. Minimum inhibitory concentrations (MIC) were between 5-10 $mg.mL^{-1}$ for crude extracts and 0.62-2.5 $mg.mL^{-1}$ for fractionated root extracts (water, ethyl acetate and butanol). Mativandlela *et al.* (2006) found that acetone and ethanolic *P. sidoides* root extracts were active at 5 $mg.mL^{-1}$ against three bacteria associated with respiratory tract infections (*Haemophilus influenzae* and *Moraxella catarrhalis*, both gram-negative, and *Streptococcus pneumoniae*, gram positive). Kayser and Kolodziej (1997) identified umckalin (MIC between 200-500 $\mu g.mL^{-1}$) and 6,8-dihydroxy-5,7-dimethoxycoumarin (MIC between 220-500 $\mu g.mL^{-1}$) to be the most active single compounds in their root extracts.

Subsequently, Kolodziej *et al*. (2003) reported that extracts of *P. sidoides* showed significant activity against multi-resistant *Staphylococcus aureus* strains. They also presented evidence to suggest that *P. sidoides* extracts possess antimycobacterial activity as claimed in the traditional use for the treatment of tuberculosis. Mativandlela *et al.* (2006) observed inhibition of *Mycobacterium tuberculosis* by *P. reniforme* root extracts (acetone, ethanol and chloroform) at 5 $mg.mL^{-1}$, but not by extracts of *P. sidoides* roots. In their bioassay-guided fractionation of *P. reniforme* and *P. sidoides* extracts for antimycobacterial activity, Seidel and Taylor (2004) identified active fractions containing complex mixtures of saturated and mono- and di-unsaturated fatty acids.

The authors found oleic and linoleic acid, two of the three major components in active fractions, to be active against *M. aurum* at 4 and 2 μg.mL^{-1}, respectively. Streptomycin, used as an antibiotic control, had an MIC of 0.5 μg.mL^{-1}.

Additionally, Mativandlela *et al.* (2006) found that *P. sidoides* root extracts (acetone and ethanolic) inhibited the growth of several fungal species associated with respiratory tract infections (*Aspergillus niger*, *Fusarium oxysporum* and *Rhizopus stolonifer*)

1.2.4 Immunomodulating activity

In addition to the antibacterial and antifungal activity described above, there is functional and molecular evidence to suggest that the constituents of *P. sidoides* extracts have significant immunomodulating capabilities. These help the immune system combat the viruses that frequently cause respiratory tract infections. Dorfmüller *et al.* (2005) found that a *P. sidoides* extract (EPs 7630) significantly increased opsonophagocytosis by leukocytes in whole blood from healthy individuals, infected with *Candida albicans*, *in vitro*. Furthermore, Kolodziej *et al.* (2003) showed that three constituents of *P. sidoides* extracts (umckalin, gallic acid and 6,8-dihydroxy-5,7-dimethoxycoumarin) stimulated the non-specific immune system. This stimulation was the result of the induced expression of the genes coding for tumour necrosis factor (TNF).

Gallic acid, (+)-catechin and umckalin were identified as having the highest interferon (INF)-like cytoprotective activity of the compounds isolated from a *P. sidoides* extract. This extract also included phenols and coumarins, besides umckalin, that accounted for less that 10% of the activity compared to the control (recombinant murine IFN-γ). In the above experiment, the combined effect of gallic acid, (+)-catechin and umckalin was far less than the effect of the crude *P. sidoides* extract. Gallic acid showed complete cytoprotection at 100 μg.mL^{-1}, with no cytotoxic effects, and was shown to elicit the expression of the genes for nitric oxides (NO) in addition to TNF. This result provided further evidence for the stimulation of the non-specific immune system by *P. sidoides* extracts (Kolodziej *et al.*, 2003).

Trun *et al.* (2006) further investigated the basis for the immunomodulatory activity of the EPs 7630 at the molecular level. For the first time, they showed convincing evidence for the immunomodulating action of EPs 7630 at the molecular level of macrophage-like (RAW 264.7) cells in response to infection by *Leishmania major* parasites. They found that a methanol insoluble fraction of EPs 7630 contained constituents that were responsible for remarkable expression of nitric oxide synthase (iNOS), cytokine and interferon (INF) mRNA in only infectious conditions. Their results suggested that EPs 7630 only mediates an immunological effect when an infectious agent is present. This is highly appropriate as that is when additional help may be needed by the immune system to combat the given infection. The authors also found that umckalin, a chemical marker for *P. sidoides* (Latté *et al.*, 2000, in Trun *et al.*, 2006), was not responsible for the observed effects on iNOS and cytokine expression. In contrast to umckalin, gallic acid showed the same positive expression results as a methanol insoluble root fraction. Comparison of the relatively low concentration of gallic acid in extracts and that needed to induce gene expression suggested, however, that the potent action of the extract was the result of a then unknown metabolite (Trun *et al.*, 2006).

In order to determine the likely identity of the then unknown compound(s) responsible for the immunological effects seen on the molecular level (Trun *et al.*, 2006; and Kolodziej *et al.*, 2003), Kolodziej *et al.* (2005) used reverse-transcriptase (RT)-PCR to analyse the effects of polyphenols on gene expression. As in Trun *et al.* (2006), the authors used macrophage-like RAW 264.7 cells infected or not infected with *Leishmania* parasites. Their tested extracts and compounds included *P. sidoides* and *Phyllanthus amarus* extracts containing polyphenols; and representative standards of simple phenols, proanthrocyanidins, flavan-3-ols and hydrolysable tannins. Their results showed that all of the tested compounds and extracts were capable of enhancing iNOS and cytokine mRNA levels in parasitized compared to non-infected cells.

Based on their results and subsequent work on the pharmacological activities of extracts and their constituents, Kolodziej *et al.* (2003) reasoned that there were two possibilities for the efficacy of *P. sidoides* extracts: either the constituents synergistically contributed

to the powerful overall action of the root extracts, or then unknown compounds were responsible for the activity.

Collectively, these research findings clearly show that constituents of *P. sidoides* extracts are effective against bacteria and also stimulate the non-specific immune system, thus helping the body combat infectious agents, specifically viruses, and their harmful effects on cells. It can be concluded that umckalin, the compound quantified in this study, is an antibacterial agent with INF-like cytoprotective activity that induces the expression of TNF *in vitro.*

1.2.5 Further investigations

In addition to the immunomodulating, antifungal and antibacterial effects, preliminary evidence suggests that topical treatment of the nasal passages with EPs 7630 may aid the clearance of mucous from the nose. Neugebauer *et al.* (2005) showed *in vitro* that treatment with EPs 7630 resulted in a significant increase in the ciliary beat frequency of ciliated cells in human nasal epithelium cultures. The isolation of the active constituent(s) is the subject of further research by the authors. The activity of EPs 7630 *in vivo* and the contribution of its effects to the mucociliatory system as a whole are also part of ongoing experimentation.

1.3 Conservation

1.3.1 Introduction

In an article in the East London based Daily Dispatch newspaper, Gerardy (2002) quoted local Environmental Affairs district manager Quintus Hahndiek in saying that the quantity of roots removed from the wild was several tons per week. At the time, plants thought to be *P. reniforme* were being collected illegally.

P. reniforme and *P. sidoides* often grow together and as they are difficult to distinguish from one another when not in flower it is likely that both species were collected together. Nine harvesters were arrested as a result.

Up until that time no research had been published on the environmental impacts of harvesting, the size of the wild resource or the recovery of populations after harvest. Additionally, the current volume and export value of dried *P. sidoides* roots to the international and national markets is not known.

1.3.2 South African legislation

In a positive development, progressive changes to South African and Eastern Cape legislation are leading towards the legal protection of these two species and the justified intellectual property rights of indigenous communities.

The Eastern Cape Environmental Conservation Bill (the Bill, Department of Economic Affairs, Environment and Tourism, Eastern Cape Province, 2003) makes the provision for *P. reniforme* to be added to the list of protected species (schedule 5) in the Eastern Cape Province of South Africa (the Province), whereas it was not previously listed. *P. sidoides*, however, was not listed in this most recent Bill. This may have been an oversight or due to the once popular literature citing that *P. reniforme* is the species of dominant medicinal value. Conversely, the current volume of literature focuses predominantly on *P. sidoides*, suggesting that a protected species listing should be considered. When the Bill is enacted the provisions of sections 115-118 and 120 will apply specifically to *P. reniforme* as a protected species. These include the requirement for an individual to hold a permit in order to sell or grow *P. reniforme* and that buyers may only purchase from licensed sellers. *P. reniforme* may not be imported into or exported from the Province unless the carrier holds a permit to do. Persons in possession of material bought from a licensed grower or seller will be exempt from this provision. Potential growers and sellers will have to apply for a permit.

The issuing of these permits will be at the discretion of the Head of the Department of Economic Affairs, Environment and Tourism of the Eastern Cape Province (Head of Department). The sale of *P. reniforme* for charitable or other similar purposes will be possible after the issue of an appropriate permit at the discretion of the Head of Department. Although *P. sidoides* is not listed as a protected species in the Bill, it is afforded some protection as an indigenous Eastern Cape plant. As such, it may not be picked, uprooted or destroyed 90 m from the middle of a public road without a permit.

Flowers may be picked provided the plants are not uprooted, picked or destroyed in the process. The most important difference between the provisions for protected and unprotected flora concerns the collection of plants on private land. Unprotected flora may be collected on private land without a permit if the harvester has the written permission, including the specific details of harvest, from the land owner or delegated authority. No provision is made in the Bill specifically for the regulation of cultivating or selling unprotected flora. Fortunately, as it is an indigenous species, the export of *P. sidoides* from the Province will require a permit. Until the Bill is enacted, *P. sidoides* will be protected as an indigenous plant under the Cape Nature Conservation Ordinance (No. 19 of 1974; in Egan and Cavvadas, 1977). The Cape Nature Conservation Ordinance provides near identical legislation for the protection of indigenous unprotected flora, like *P. sidoides,* in comparison to the above mentioned Bill. The only differences are found in the regulations concerning flower picking.

The South African Parliament has recently enacted a bill by the Department of Environmental Affairs and Tourism (DEAT) largely restructuring the management of biodiversity and bioprospecting in South Africa. In this regard, the National Environmental Management: Biodiversity Act (the Act; DEAT, 2004) protects South African plant species and the interests of affected people through legislation concerning bioprospecting, and the interlinked benefit-sharing and export of indigenous resources.

Under the Act, interested parties must be holders of a permit before they may engage in bioprospecting. The export of indigenous resources from South Africa for bioprospecting or other kinds of research also requires a permit. Additionally if a stakeholder, such as an indigenous community or researcher, has an interest in the proposed action, all information concerning the proposed bioprospecting must be disclosed to him/her/them. Frameworks for benefit-sharing and subsequent indigenous resource management are also provided by the Act.

1.3.3 Conservation options

There are two interlinked options that would promote the conservation of wild *P. sidoides* populations for future use, to the benefit of people and the biodiversity of the natural environment. The first option would be to develop and implement sustainable harvesting practices for wild populations. The second would be to effectively cultivate *P. sidoides* plants in order to supply local and international demand, thus reducing the harvest of roots from the wild (World Health Organization *et al.*, 1993). A combination of good cultivation and wild harvest practices may provide for the sustainable harvest of *P. sidoides* in the future.

In this study we made significant steps towards realizing the above options, firstly by assessing the regrowth and survival of field harvested plants and secondly by assessing the levels of a bioactive metabolite, umckalin, in the roots of greenhouse cultivated plants.

1.3.4 Determining a sustainable harvest

Ticktin (2004) reviewed the ecological implications of harvesting non-timber forest products (NTFP) and identified a number of variables to be considered when studying the effects of harvest on individual plants and populations. These included documenting the plant part used, the life history of plants, variation in environmental conditions, management practices, harvest method and land use context.

Ticktin (2004) recommended that, in order to manage and conserve NTFP populations effectively, researchers must identify what the ecological effects of harvesting are, what the mechanisms are that cause them and identify any management practices that may reduce the negative effects of harvesting. To complete the investigation, socio-economic and political issues must also be addressed. The following case studies, of important natural resources, shed light on some of the variables that may need to be considered concerning the conservation of *P. sidoides* for sustainable harvest.

Pterocarpus angolensis is an important hardwood tree with many traditional and commercial uses found in the African miombo woodland vegetation type (Swartz *et al.*, 2002). In their study the authors highlighted the importance of gaining knowledge concerning the effects of harvest on population dynamics. The authors estimated population size structures, tree growth rates and seed production; and interviewed harvesters to learn about their harvesting patterns. They used this information to develop a simple population projection model and showed that the then harvesting was unsustainable.

Findings on the commercial harvest of devil's claw, an important southern African medicinal plant, may be very useful to the development of conservation and harvest strategies for *P. sidoides*. Devil's claw is the collective common name for *Harpagophytum procumbens* and *H. zerheri* and has recently been the subject of a comprehensive and valuable review by Stewart and Cole (2005). Evidence was presented to suggest that improving benefit-sharing for harvesters results in improved resource management and conservation at the local level. The case in point was the successful Sustainably Harvested Devil's Claw (SHDC) project initiated in Namibia. Some of the activities of the SHDC project were to ensure good market prices and strengthen harvesters' buying position, make information available, create options, and provide general support. The project saw an increase in the responsibility taken by harvesters for the management of the resource and in supplying a high quality product to the marketplace. These positive results were not generally the case in areas where the resource was extensively harvested.

The SHDC project also showed the importance of traditional knowledge about sustainable harvest. The authors recommended that traditional knowledge be recognized, legitimized and used as the base for a "best practices" message to harvesters (Stewart and Cole, 2005). As stated by the authors, the study was performed on a limited and local scale and would benefit from including a larger study area.

Like Stewart and Cole (2005), Shinwari and Gilani (2003) also considered increased coordination and cooperation between communities and traders regarding plant use and regeneration to be important. Other factors were identified as valuable to promoting the sustainable use of plants in northern Pakistan and included: protecting areas where herbs were over-exploited, conservation training, securing the protection of intellectual property rights and the cultivation of nutritionally and medicinally used plants in nurseries. The authors noted that, in the region of Pakistan they studied, a lack of knowledge of sustainable use and conservation had contributed to an increased shortage of medicinal plants.

For *P. sidoides*, much information is currently lacking concerning the plant characteristics required by industry. It is known, however, that harvesters are instructed by middlemen to harvest large roots and those with darker red internal tissue (Dold, 2004, *personal communication*). The lack of published information concerning the quantity of the resource harvested, harvesting methods applied and management systems on the local scale for *P. sidoides* is similar to the situation described by Ghimire *et al.* (2005) for many Himalayan medicinal plants. In a study on the ecology and rhizome harvesting patterns of two threatened species, *Nardostachys grandiflora* and *Neopicrorhiza scrophulariiflora*, Ghimire *el al.* (2005) found that there was great variation in the harvesting practices and knowledge of resource users. Their study was based in a protected area where traditional doctors, *amchi*, collected rhizomes for home use; and a non-protected area where commercial harvesters collected for trade. The harvesting approaches of the two major user groups were studied and experimental trials were then implemented to analyze the effects on population growth in response to the different harvesting practices.

The *amchi* were selective in their approach to plant harvest and collected less on a seasonal basis for homecare use. In contrast, the commercial harvesters were not selective, collected intensely and did not hold to seasonal collection, only stopping in the three months of annual snowfall. The authors' findings on the response of population growth to harvest showed that differences in growth strategy between species affected the ability of each species to react to different harvesting intensities. In addition, growth form was found to affect the ability to selectively harvest plants. Collectively, differences in growth strategy, growth form, intensity and frequency of harvest - in addition to the survival and regrowth of replanted ramets - influenced the potential for each species to be harvested sustainably. Replanting of younger plants and selectively harvesting mature plants was recommended for *Neopicrorhiza*. Due to differences in harvest response and growth form, sustainable harvest of *Nardostachys* was not considered possible unless a long rotation between successive harvests was established. Ghimire *et al.* (2005) concluded that it is important that management plans be established for threatened Himalayan medicinal plants based on applied harvesting practices, growth patterns in different habitats, life forms and the plant part used.

In our study prominent steps were taken towards assessing the potential for the sustainable harvest of *P. sidoides* roots. The regrowth and survival of harvested plants under two management practices in two climatically different habitats was monitored over one year. In addition to field harvest experiments, plants were grown in cultivation so that regrowth and bioactive metabolite concentrations could be compared to those of wild plants. Fundamental to the assessment of *P. sidoides* cultivation as a viable part of sustainable harvest was the isolation of a bioactive standard from roots and the development of an effective assay to assess its concentration in cultivated and wild roots.

1.4 Aims

The aims of this study were:

1) To isolate a standard of umckalin, a bioactive coumarin, from dried *P. sidoides* roots and develop an effective High Performance Liquid Chromatography (HPLC) assay for its detection in root extracts.

An umckalin standard was isolated via a novel method incorporating acetone extraction and chromatography using HP-20 polystyrene resin, silica and HPLC guided by diagnostic Thin-Layer Chromatography (TLC) and Nuclear Magnetic Resonance (NMR) spectra. The standard was then used in various analytical HPLC systems, adapted from the literature, to develop an effective and robust method for the quantification of umckalin in ethanolic *P. sidoides* root extracts.

2) To determine the effects of different management practices, habitat and season of harvest on the regrowth of replanted *P. sidoides* plant parts after harvest.

Large sets of plants from two distinct annual rainfall areas were harvested and subjected to three treatments (management practices). Two treatments included the removal of 80% of the defoliated plant mass and replanting with or without a colloid to gradually release water into the soil surrounding the plants. A third treatment involved the replanting of defoliated plants without the removal of root. The emergence of leaves and the annual production of root regrowth were measured.

3) To assess the relationship between *P. sidoides* root umckalin concentration and local climate.

Plants and soil samples were collected from three locations of distinct annual rainfall and the average umckalin concentration of dried roots compared.

4) To determine the effects of cultivation and various applied treatments on the root umckalin concentration of non-clonal *P. sidoides* plants.

Wild harvested plants were grown under greenhouse conditions for between 16 and 18 months before the application of one of five treatments. These treatments included rapid and prolonged water stress, two hormone treatments (cytokinin and gibberellin), and a root competition treatment with a fast growing annual (*Conyza albida*). Each treatment was hypothesized to increase root umckalin concentrations relative to well-watered controls.

5) To determine the variation of root umckalin concentrations amongst clonal *P. sidoides* plants.

Cuttings were made from the roots of individual wild parent plants and grown under greenhouse conditions. The variation of root umckalin concentrations between cultivated clonal plants was hypothesized to be lower than that between non-clonal wild plants.

Chapter 2

Umckalin Standard Purification and Attempted Verification of Biological Activity

This chapter describes the development of a method to isolate, and the subsequent isolation of, bioactive umckalin (6-hydroxy-5,7-dimethoxycoumarin) from the roots of *P. sidoides* plants.

In their isolation of umckalin, Kayser and Kolodziej (1995) used a method incorporating an initial aqueous-acetone extraction, subsequent ethyl acetate extraction and chromatography using Sephadex LH-20, silica gel, RP-18 and Medium Pressure Liquid Chromatography (HPLC). The method developed here included acetone extraction and chromatography using HP-20 polystyrene resin, silica gel and High Performance Liquid Chromatography (HPLC); guided by diagnostic Thin-Layer Chromatography (TLC) and Nuclear Magnetic Resonance (NMR) spectra.

2.1 Plant material

The roots of replicate plants, from two distinct geographical locations, were used in this study. The two sites are situated in the Greater Fish River Nature Reserve (GFRR) (33° 07’ 13” S, 26° 43’ 29” E) and on Killaloe Farm near Kei Road (KR, 32° 43’ 03” S, 27° 39’ 30” E). Both sites are in the Eastern Cape Province of South Africa and were specifically chosen as they represent areas of vastly different annual rainfall, 397 and 760 mm for the GFRR and KR sites, respectively. Voucher specimens were collected from each site and deposited in the Selmar Schönland Herbarium (GRA), Grahamstown. From here on “GFRR” will designate extracts and fractions originating from Greater Fish River Nature Reserve plants and “KR” will designate those from Killaloe Farm.

2.2 Development of an umckalin isolation method

Fresh roots were collected from each site, sliced using a stainless steel knife, oven dried at approximately 40 °C (Gallenkamp), crushed using a pestle and mortar and powdered to a 1 mm particle size using a hammer mill.

500 g of powdered dry root from each site was soaked in 2 L of redistilled acetone for four days in separate 5 L conical flasks. The solvent root mixture was agitated occasionally by swirling until all the settled powder was suspended in the supernatant. 50 mL of the burgundy coloured solutions yielded 18.1 and 15.5 $mg.mL^{-1}$ of dried acetone soluble extract for the GFRR and KR samples, respectively. These dried extracts were further fractionated as follows:

The above dried GFRR (903.2 mg) and KR (772.7 mg) extracts were re-dissolved in acetone (*circa* 60 mL) with the addition of a minimal volume (*ca.* 7 drops) of deionised water, to improve solubility, before they were loaded onto columns for separation using HP-20 (Diaion®, Supelco, Sigma-Aldrich) chromatography. The HP-20 chromatography employed three steps: (1) loading of increasingly aqueous diluted fractions, originating from an individual extract, onto an HP-20 column (cyclic loading); (2) the elution of fractions off a cyclically loaded column using a range of solvent combinations of varying polarity and (3) the loading and elution of individual fractions from (2) onto and off smaller HP-20 columns (backloading). This method was adapted from that used by Keysers (2003).

HP-20 columns were prepared as follows. 500 mL glass columns were rinsed with acetone and stoppered with cotton wool before being filled with 1 mL of HP-20 polystyrene resin beads per 25 mg of dried extract to be chromatographed. The beads were primed with three bed volumes of methanol followed by three bed volumes of acetone.

In loading the columns, the re-dissolved acetone extracts were poured onto the top of each column and the eluted fraction collected (step 1). The eluted fraction contained compounds that did not bind to the HP-20 stationary phase at the mobile phase solvent concentration. Fractions collected from each column were transferred to separate measuring cylinders and diluted by 25-33% with deionised water or until the solutions became cloudy. The cloudy nature of the fraction indicated that dissolved compounds were beginning to come out of solution before precipitating. Diluted fractions were then reloaded onto the original respective columns. This cyclic loading was continued with each column until the concentration of the diluted fractions to be loaded was 5% acetone. Cyclic loading results in a high proportion of the compounds in original extracts being absorbed onto the beads in the column.

Fractions were then separated off the columns (step 2), in elutions beginning with three bed volumes of water changing to three bed volumes of 100% acetone in 20% increments. Fractions eluting at each acetone concentration were collected in separate conical flasks (Figure 2.1).

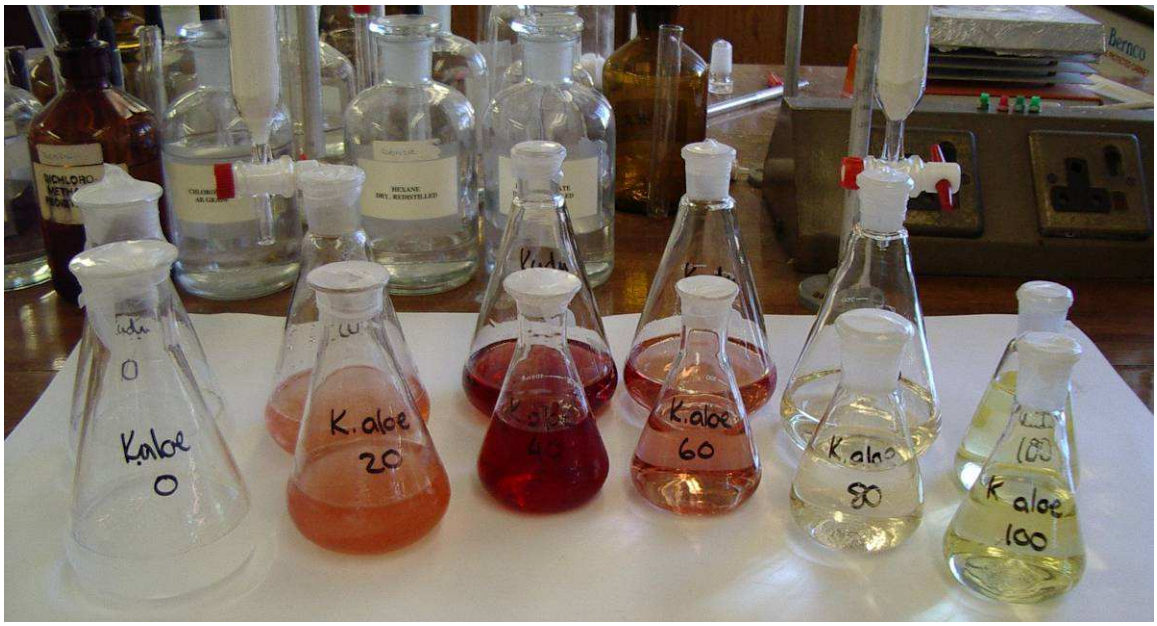


Figure 2.1: Photograph showing the fractions collected after the HP-20 chromatographic separation described above. Kei Road samples (K. aloe) are in the foreground with the Greater Fish River Reserve (Kudu) samples behind.

Backloading was performed to remove water from each of the fractions that contained acetone and water. Two columns, one for GFRR fractions and one for KR fractions, were filled with 15 mL of HP-20 beads and rinsed with three bed volumes of acetone. Each of the above 20 to 80% acetone fractions were loaded onto, and eluted off the columns individually. Before loading, each fraction was diluted with water, while swirling, until the solution started to become cloudy. The appropriate column was then equilibrated to the solvent concentration of the diluted fraction. Once loaded, the columns were air dried to remove water and eluted with 50 mL of acetone, followed by 50 mL of methanol. Methanol and acetone fractions were collected in the same round bottom flask and dried under reduced pressure at 35 °C on a rotary evaporator (Bűchi Rotavapor R-200, heating bath B490). The columns were rinsed with three bed volumes of acetone between backloadings.

100% water fractions were frozen using liquid nitrogen and dried using a freeze drier over three days (LyoLab 3000, Heto, with an Edwards high vacuum pump). 100% acetone fractions were dried under reduced pressure at 35 °C on a rotary evaporator.

A small dried sub-sample (*ca.* 10 mg) of every fraction was dissolved in deuterated methanol (Merck) and analysed using a Bruker 400 MHz "Avance" DPX 2 Channel NMR Spectrometer. The GFRR 60% acetone fraction (27.3 mg and named Fraction Alpha from here on) provided a proton NMR spectrum for umckalin, comparable with published data (Kayser and Kolodziej, 1995), and was therefore chosen for further purification.

At this stage TLC was used as rapid means of assessing the effectiveness of the possible purification options to follow.

A dissolved sample of Fraction Alpha was spotted onto normal phase (silica gel 60 F_{254s}, Merck) and reversed phase (C-18, RP-18 F_{254s}) TLC plates and allowed to dry before being placed in a solvent bath containing one of the solvent combinations shown in Table 2.1.

Once the solvent had migrated to within 5 mm of the top of a given plate, it was removed from the solvent and solvent bath. All TLC plates were first observed under 254 and 365 nm wavelength light to reveal UV absorbing compounds, then sprayed with 10% sulphuric acid in methanol and heated to reveal the presence of non UV absorbing organic molecules, and finally they were dusted with iodine to reveal any further compounds undetected by the two other plate development techniques. The sulphuric acid with heat and iodine treatments did not frequently reveal any compounds that had not already been observed under 365 or 254 nm wavelength light.

Table 2.1: RF values of a compound, likely to be umckalin (as seen in NMR spectra), determined using various solvent combinations on normal and reversed phase TLC plates in an experiment to find the combination that resulted in the separation of the compound closest to 30% from the baseline relative to the solvent front. Compounds were observed under 365 or 254 nm wavelength light.

Stationary phase	Solvent combination	RF value
Normal	Ethyl acetate	0.61
Normal	Ethyl acetate: methanol (1:1)	0.37
Normal	Ethyl acetate: methanol (9:1)	0.82
Normal	Ethyl acetate: chloroform (1:1)	0.55
Reversed	Acetonitrile:methanol (1:1)	0.85
Reversed	Methanol	0.78
Reversed	Acetonitrile	0.82
Normal	Ethyl acetate: hexane (4:1)	0.73
Normal	Ethyl acetate: hexane (3:1)	0.66
Normal	Ethyl acetate: hexane (2:1)	0.6
Normal	Hexane	0 (no separation)
Normal	Ethyl acetate: hexane (1:2)	0.15
Normal	Ethyl acetate: hexane (1:1)	0.32

Normal phase separation using ethyl acetate: hexane (1:1) produced the best RF value for further HPLC purification. This result led to the choice of a column based silica stationary phase with ethyl acetate:hexane (1:1) as the mobile phase in the next separation step.

22 mg of Fraction Alpha was dissolved in 2 mL of ethyl acetate and 1 mL of methanol and loaded onto a column filled with 50 g of silica gel 60 (0.040-0.063 mm, 230-400 mesh ASTM, Merck) stoppered with cotton wool below acid washed sand. The column was eluted with two bed volumes of ethyl acetate:hexane (1:1) and test tubes were used to collect fractions of about 10 mL in volume. The first 19 fractions were spotted on normal phase TLC plates and run in ethyl acetate. Separated compounds from fractions five and six (RF values of 0.78 and 0.81, respectively) were observed under 365 nm wavelength light as blue spots. The contents of these fractions was combined and dried to yield a 4.6 mg product that was pale yellow when dissolved in methanol. This fraction is named Fraction Beta from here on.

^{1}H NMR (Figure 2.2) and TLC (ethyl acetate:hexane, 1:1) showed that Fraction Beta contained a single major compound (> 90% purity). A combination of 1D and 2D NMR (Figures 2.2-2.6) were used to unequivocally establish the structure of this major compound as umckalin. Further confirmation of the structure of umckalin was provided by High Resolution Fast Atom Bombardment Mass Spectroscopy (HRFABMS) and UV spectroscopy as described below in Section 2.3.

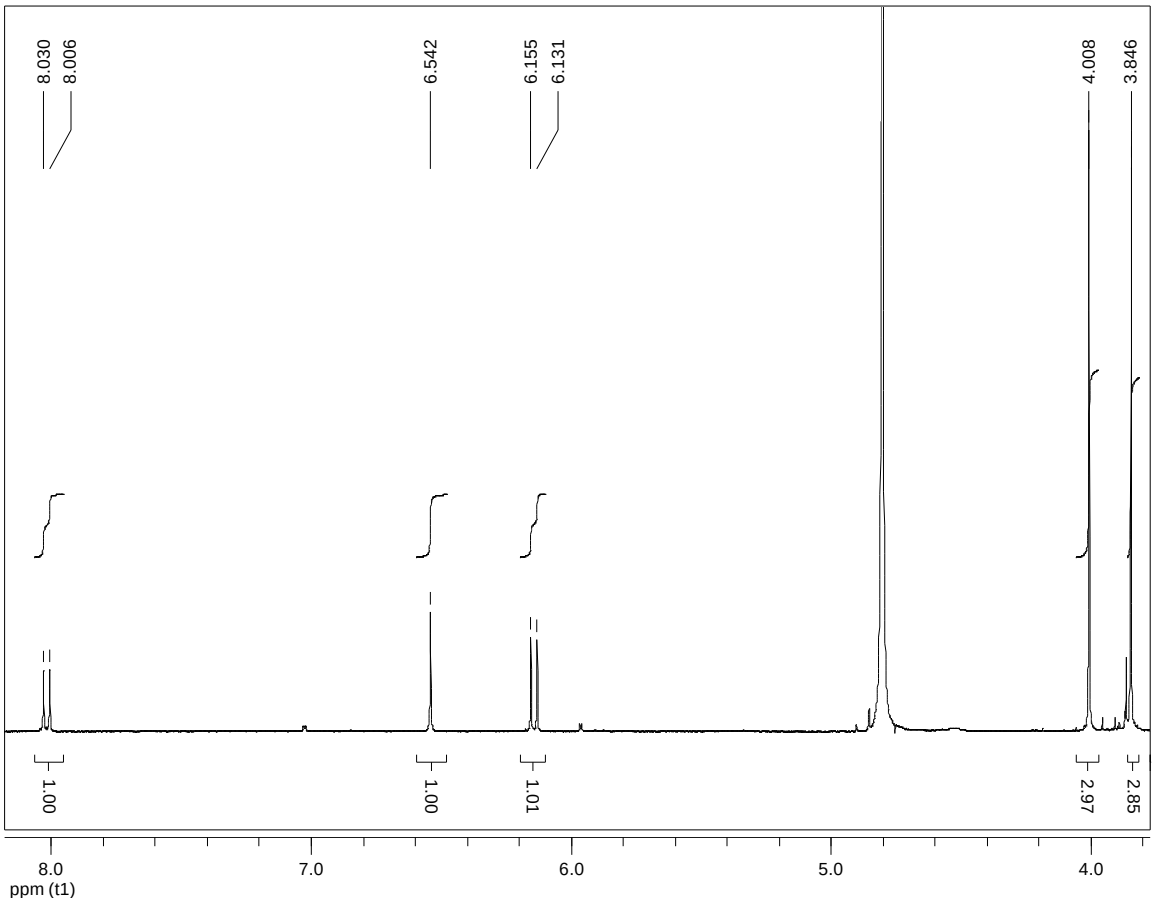


Figure 2.2: Proton NMR spectrum (400 MHz) of Fraction Beta, umckalin isolation method development (spectrum acquired in methanol- d_4).

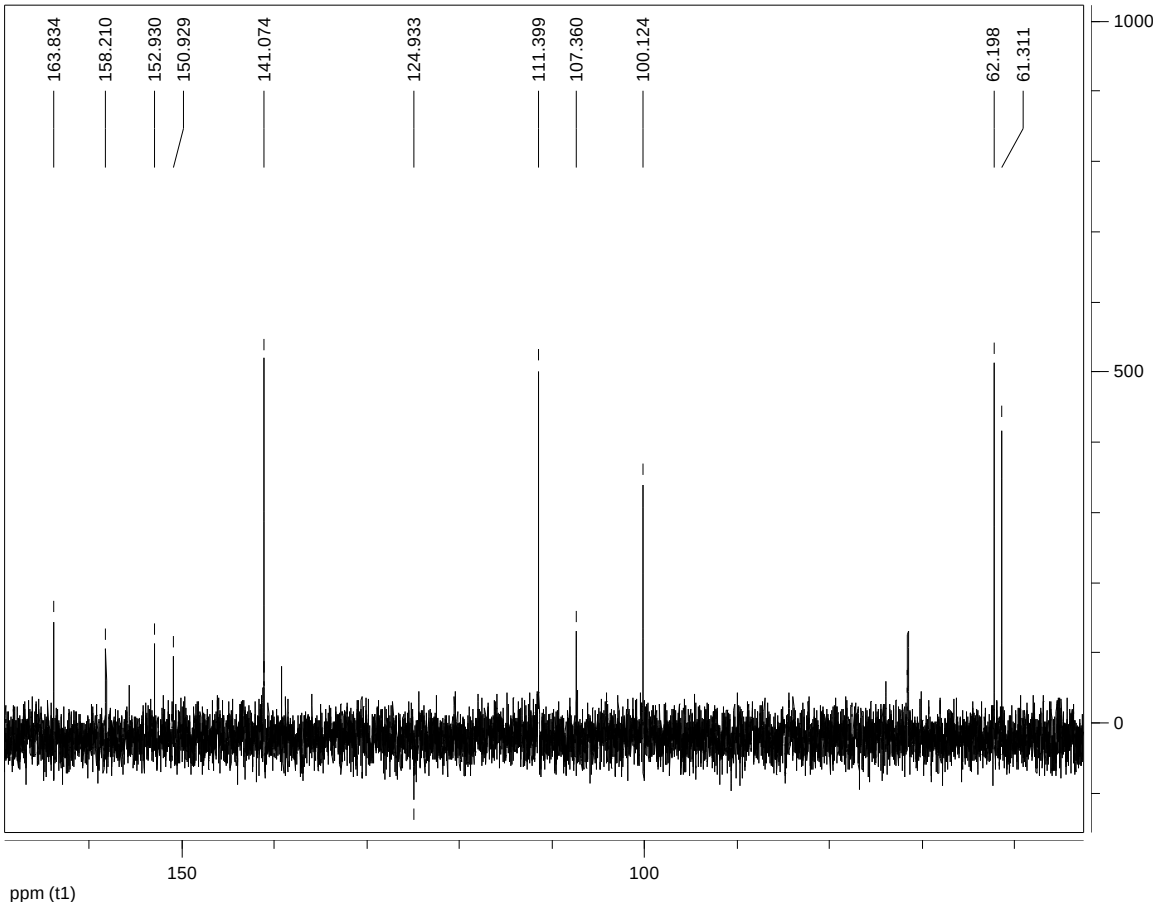


Figure 2.3: ^{13}C NMR spectrum (100 MHz) of Fraction Beta, umckalin isolation method development (spectrum acquired in methanol- d_4).

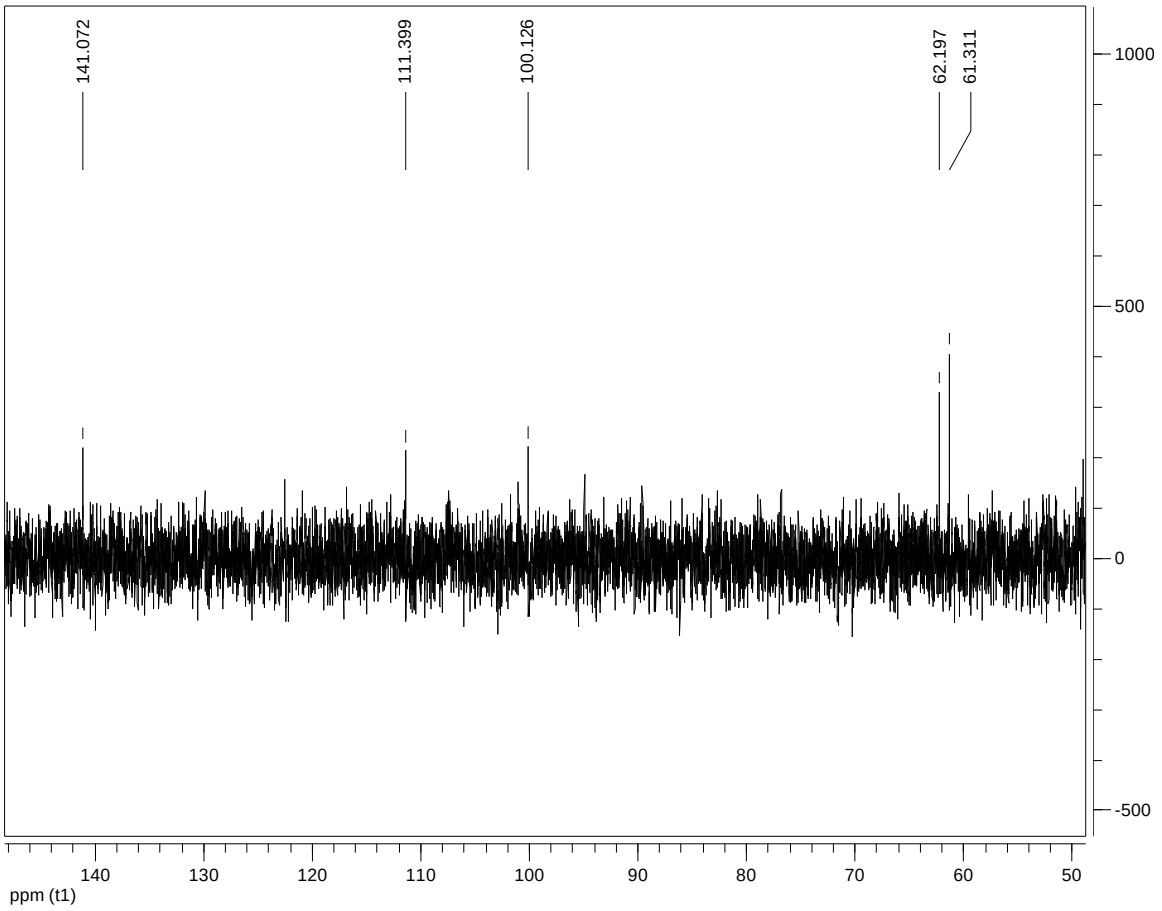


Figure 2.4: Distortionless enhancement by polarization transfer (DEPT)-135 NMR spectrum (100 MHz) of Fraction Beta, umckalin isolation method development (spectrum acquired in methanol- d_4). Only CH and CH_3 carbon signals appear in this spectrum.

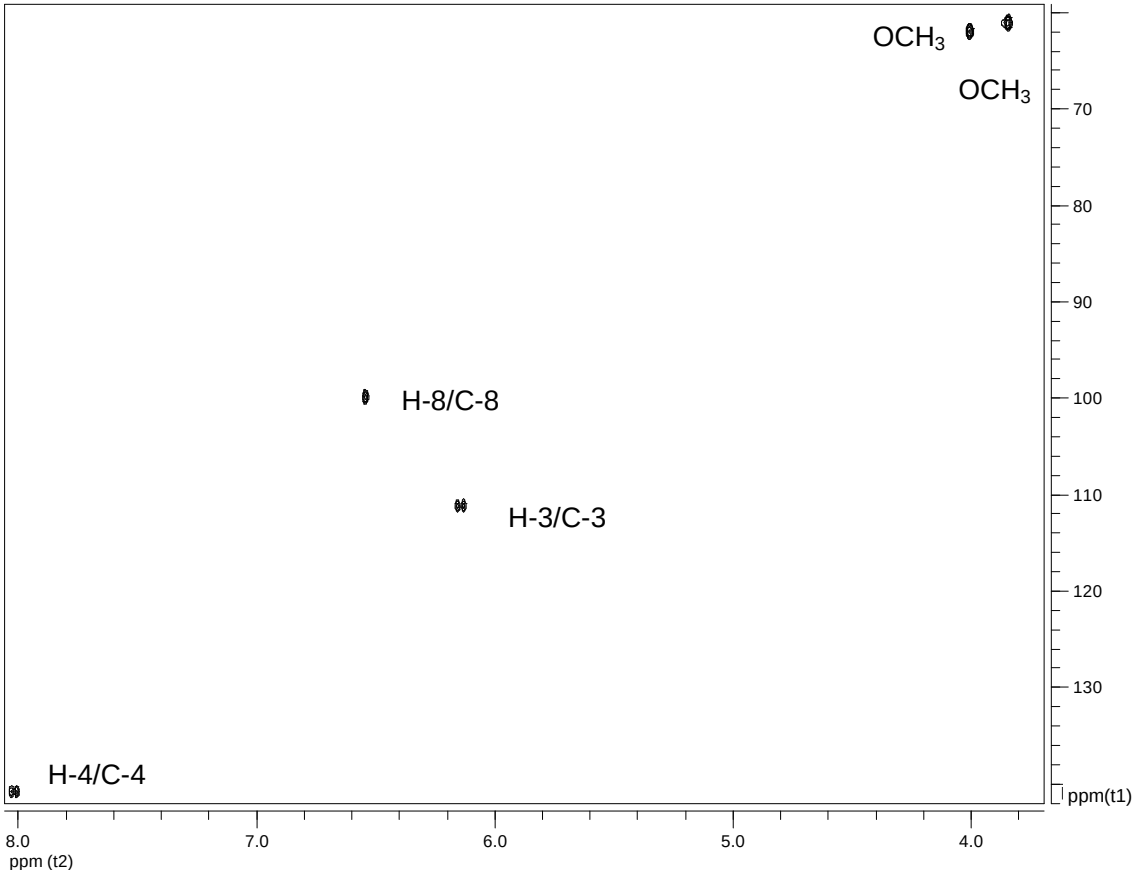


Figure 2.5: Heteronuclear single quantum correlation (HSQC) NMR spectrum (400 MHz) of Fraction Beta, umckalin isolation method development (spectrum acquired in methanol- d_4). The HSQC spectrum shows correlations between hydrogen (x-axis) and carbon atoms (y-axis) separated by one bond.

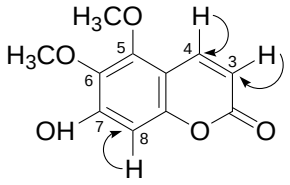


Figure 2.6: The structure of umckalin with a diagrammatic representation of the correlations between hydrogen and carbon atoms shown in Figure 2.5.

Table 2.2: Structural elucidation - ^{1}H and ^{13}C NMR spectral data for Fraction Beta (400 Hz, methanol-d_4) and umckalin (75 Hz, acetone-d_6, Kayser and Kolodziej, 1995) (d = doublet, s = singlet). Coupling constant, J in Hz, in parentheses. Note: there will be some small differences in chemical shifts (δ ppm) in different NMR solvents.

H	^{1}H δ (ppm) for Fraction Beta	^{1}H δ (ppm) for umckalin
3	6.14 d (9.6)	6.13 d (9.7)
4	8.02 d (9.6)	7.97 d (9.7)
8	6.54 s	6.59 s
OMe	3.85, 4.01 (each s)	3.86, 4.01 (each s)

Table 2.3: Structural elucidation - ^{13}C NMR spectral data for Fraction Beta (100 Hz, methanol-d_4) and umckalin (75 Hz, acetone-d_6, Kayser and Kolodziej, 1995).

C	^{13}C δ (ppm) for Fraction Beta	^{13}C δ (ppm) for umckalin
2	163.8	162.2
3	111.4	112.5
4	141.1	139.6
4a	107.4	107.3
5	158.2	155.9
6	124.9	138.1
7	152.9	152.5
8	100.1	99.6
8a	150.9	150.4
OMe	61.3, 62.2	61.3, 62.0

Comparison of Fraction beta proton and carbon NMR data (Tables 2.2 and 2.3) with umckalin data in the literature (Kayser and Kolodziej, 1995) confirmed that umckalin was the dominant compound in the fraction and that further purification would be needed to isolate a standard.

At this point it was decided to pursue the purification of umckalin from a larger mass of crude acetone extract with the inclusion of a separation step on semi-prep HPLC after silica column chromatography.

2.3 Umckalin isolation

The successful development of the above method to isolate a > 90% pure sample of umckalin prompted the use of a similar method to isolate a larger quantity of the compound with greater purity.

The method in Section 2.2 employed acetone extraction and two purification steps: HP-20 column chromatography followed by silica column chromatography. In the method described here (Section 2.3), an umckalin standard (14.1 mg) was isolated from dried GFRR acetone root extract (8.62 g) using a similar method to that above but with the addition of a semi-prep HPLC separation step after silica column chromatography. This additional step improved the purity of the isolated umckalin to *ca.* 99%.

HPLC was performed on a normal-phase Whatman Magnum 9 Partisil 10, 10 x 150 mm column, equilibrated with ethyl acetate:hexane (1:2), connected to a Spectraphysics series P100 pump and a Water R401 detector. Appropriately combined silica column fractions were loaded onto the equilibrated column in two separate injections and eluted with ethyl acetate:hexane (1:2, 4 $ml.min^{-1}$). Eluting peaks were combined according to their respective retention times (RT). The fraction containing the largest peak from each separation, 12.7 and 12.8 min RT respectively, was dried under reduced pressure at 35 °C on a rotary evaporator, yielding a pale yellow crystalline solid. This fraction is named Fraction Gamma from here on. A sample of Fraction Gamma was dissolved in deuterated acetone (Merck) and analyzed using NMR. The choice of deuterated acetone as the NMR solvent was to enable the data obtained for this compound to be compared directly with published data for umckalin.

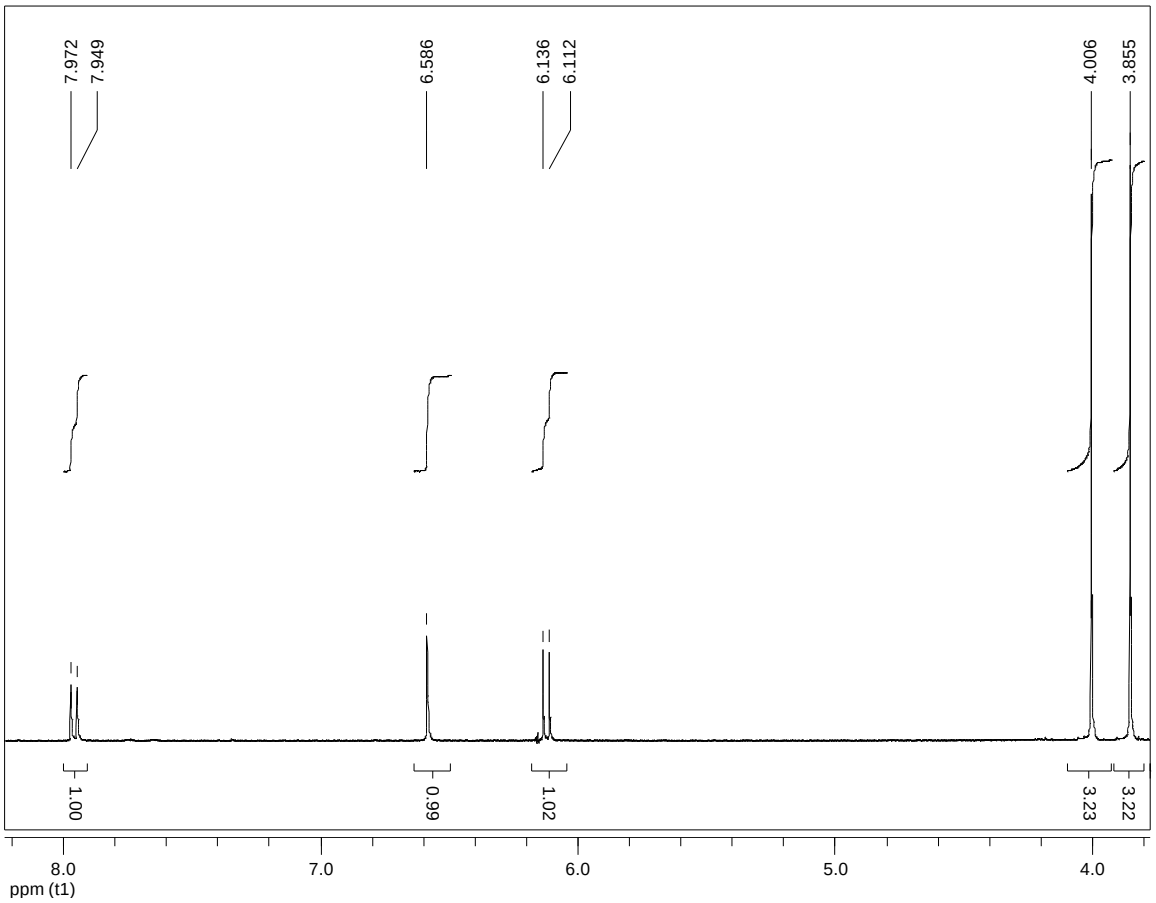


Figure 2.7: ^{1}H NMR spectrum (400 MHz) of Fraction Gamma, umckalin isolation (spectrum acquired in acetone-d_6).

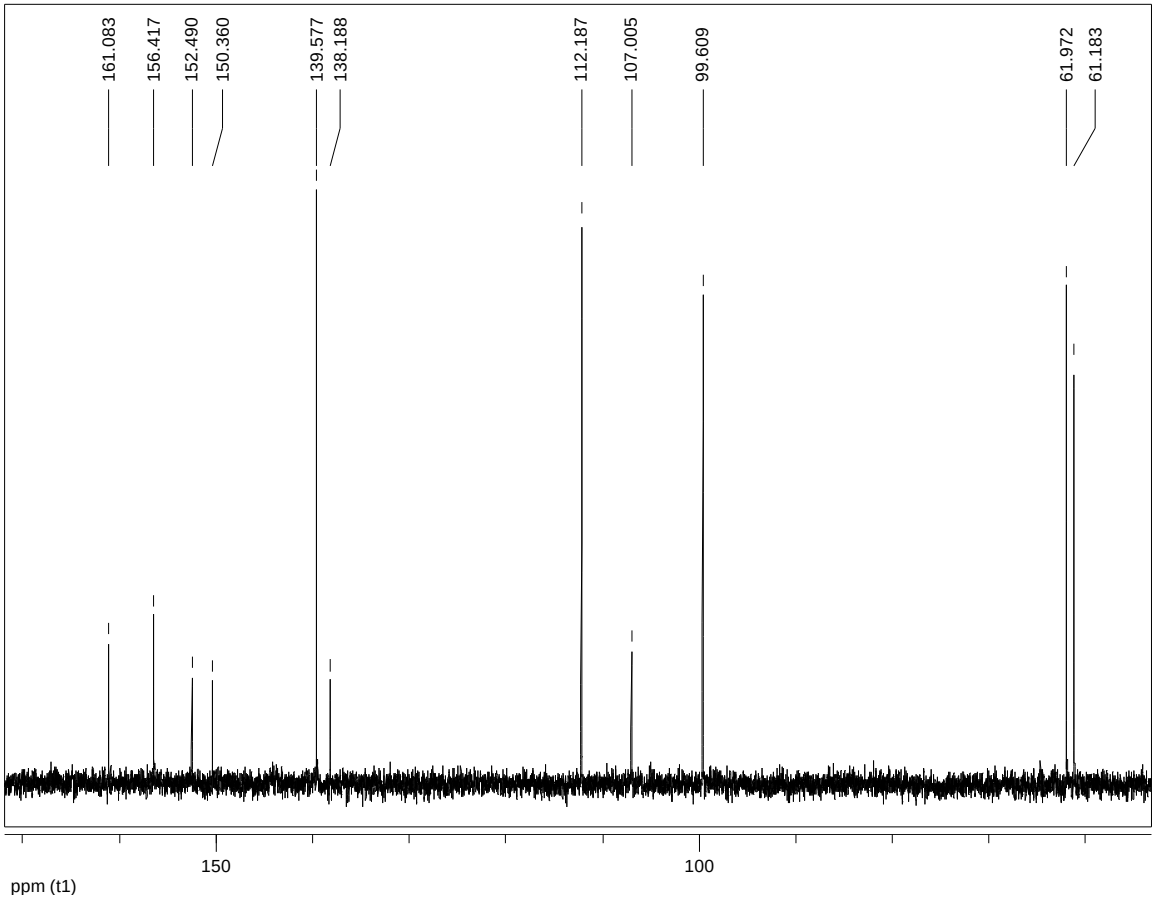


Figure 2.8: ^{13}C NMR spectrum (100 MHz) of Fraction Gamma, umckalin isolation (spectrum acquired in acetone-d_6).

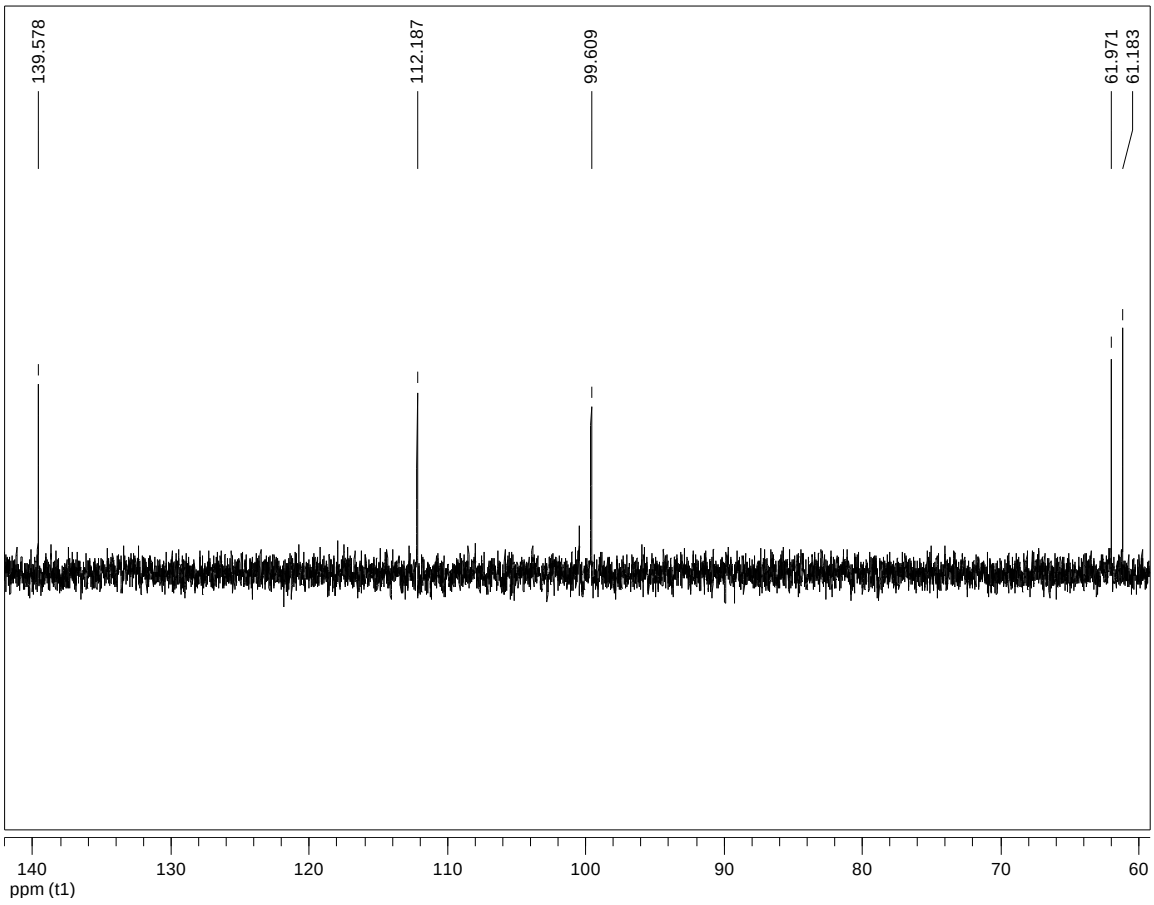


Figure 2.9: DEPT-135 NMR spectrum (100 MHz) of Fraction Gamma, umckalin isolation (spectrum acquired in acetone-d_6).

Table 2.4: Structural elucidation - ^{1}H spectral data for Fraction Gamma (400 Hz, acetone-d_6) and umckalin (75 Hz, acetone-d_6, Kayser and Kolodziej, 1995) (d = doublet, s = singlet). Coupling constant, J in Hz, in parentheses.

Position	^{1}H δ (ppm) for Fraction gamma	^{1}H δ (ppm) for umckalin
3	6.12 d (9.6)	6.13 d (9.7)
4	7.96 d (9.6)	7.97 d (9.7)
8	6.59 s	6.59 s
OMe	3.86, 4.01 (each s)	3.86, 4.01 (each s)

Table 2.5: Structural elucidation - ^{13}C NMR spectral data for Fraction Gamma (100 Hz, acetone-d_6) and umckalin (75Hz, acetone-d_6, Kayser and Kolodziej, 1995).

Position	^{13}C δ (ppm) for Fraction gamma	^{13}C δ (ppm) for umckalin
2	161.1	162.2
3	112.2	112.5
4	139.6	139.6
4a	107.0	107.3
5	156.4	155.9
6	138.2	138.1
7	152.3	152.5
8	99.6	99.6
8a	150.4	150.4
OMe	61.2, 62.0	61.3, 62.0

The proton and carbon NMR data in Tables 2.4 and 2.5 confirmed that a pure sample of umckalin had been isolated (as first described by Wagner *et al.*, 1974).

In order to determine the UV absorbance spectrum of the isolated compound (Fraction Gamma), 3 µL of a 0.01 $mg.\mu L^{-1}$ HPLC grade methanol solution was analysed using a Varian Cary 500 UV-Vis-NIR spectrophotometer (Figure 2.10). These results are compared below with those in the literature (Kayser and Kolodziej, 1995).

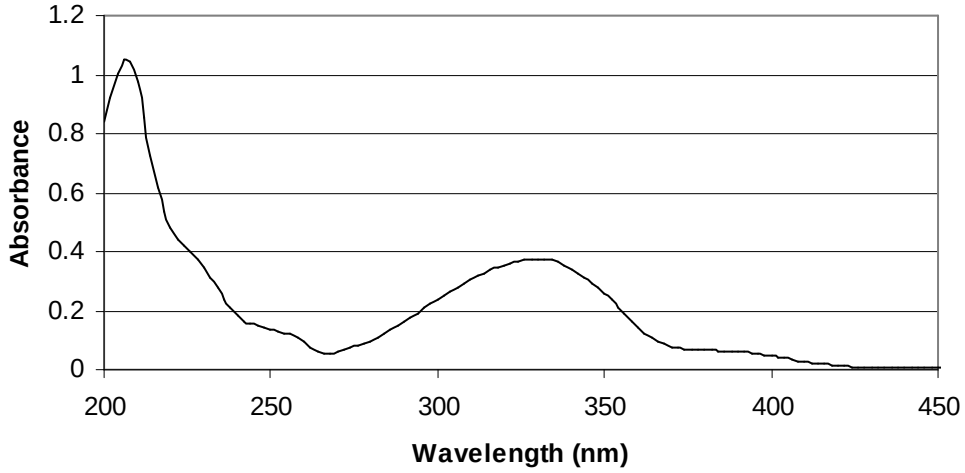


Figure 2.10: The UV absorbance spectrum of Fraction Gamma (spectrum acquired in HPLC grade methanol).

In a further verification of the chemical structure of the compound isolated here, a High Resolution Fast Atom Bombardment Mass Spectrum (HRFABMS, Micromass 70-70E spectrometer) was obtained by Professor L. Fourie of the Mass Spectrometry Unit, North-West University, Potchefstroom, South Africa. The molecular formula of umckalin ($C_{11}H_{10}O_5$) requires m/z 222.05282 and we found m/z 222.05281, indicating unequivocally that these are the same compound.

NMR analyses, combined with UV absorbance and mass spectrometer data, confirmed that the following compound had been isolated:

6-Hydroxy-5,7-dimethoxy-2H-benzopyran-2-one (umckalin). 14.1 mg.
Molecular mass: 222.0528 $g.mol^{-1}$ (222 $g.mol^{-1}$, Kayser and Kolodziej, 1995 and Wagner *et al.*, 1974). UV^{MeOH}_{max} 206, 330 nm: (210, 328 nm, Kayser and Kolodziej, 1995; and 221, inflection at 255, 330 nm Wagner *et al.*, 1974). C^{13} and H^1 NMR data (see Tables 2.4 and 2.5).

2.4 Attempted verification of biological activity

An assay was conducted in order to confirm the documented biological activity of umckalin.

2.4.1 Quick microplate method

The quick microplate method (Eloff, 1998) was used to test for the bacteriostatic activity of the umckalin isolated above. The method employed the use of sterile 96 well microplates (0.5 mL volume) and the dye p-iodonitrotetrazolium violet (INT) as an indicator of metabolic activity. Ampicillin (Roche) was used as an antibiotic control and dissolved in sterile triple distilled water (SW). Umckalin was dissolved in a minimal amount of 96% ethanol (rectified, Saarchem UNIVAR, Merck) with SW so that the final ethanol concentration in wells was less than 0.35%. Solvent controls were run at the same concentrations as those in the umckalin wells. INT was dissolved in SW (0.2 $mg.mL^{-1}$) with sonication (UMC5, Ultrasonic Manufacturing Company). Liquid bacterial cultures were prepared by first inoculating Müller-Hinton (M-H) broth (23 $g.L^{-1}$, Fluka, Merck) with a loop of *E. coli* (ATCC 29181) under sterile conditions. The inoculated broth was incubated overnight at 37 °C with 200 rpm rotation (Labson incubator). The following morning fresh sterile M-H broth was inoculated with the overnight culture and incubated at 37 °C with 200 rpm rotation for approximately five hours so that cultures were in their log phase of growth at the time of use.

At the beginning of each microplate experiment 50 µL of sterile M-H broth (23 $g.L^{-1}$) was added to each of twelve wells in a single row. 50 µL of test solution (10 and 11 $mg.mL^{-1}$ for ampicillin and umckalin, respectively) was then added to the first well of each row and thoroughly mixed by sucking it into an autopipette tip and releasing it back into the well three times. 50 µL from the first well was then transferred to the next well, mixed and the process repeated until the final well where 50 µL was discarded after mixing. 25 µL of the above log phase culture was added to each well (except for zero culture controls).

The microplates were covered with a sterile lid and incubated overnight at 37°C in a controlled environment (CE) room. Three replicates were used for each test and control solution.

After overnight incubation, 40 µL of INT (0.2 $mg.ml^{-1}$) was added to each well before the plates were returned to the 37 °C CE room. Digital photographs were taken of each microplate before adding INT and at 30, 60, 120 and 270 minutes afterwards to create a permanent record of colour and rate of colour development. The contents of selected wells was triple streaked on M-H agar plates (23 $g.L^{-1}$ M-H broth and 18 $g.L^{-1}$ Agar Bacteriological, Merck) which were inverted and incubated at 37 °C overnight. This verification was used to determine the accuracy of the results found in the visual assessment of the microplate wells and also to identify possible contamination.

Like Eloff (1998), we found that using broth instead of water for the serial dilutions of ampicillin resulted in MIC closer to those found in the literature. The MIC of ampicillin diluted with water was 23.24 $\mu g.mL^{-1}$ and 8.64 $\mu g.mL^{-1}$ with broth. The literature value for ampicillin against *E. coli* (ATCC 27853) was 2-8 $\mu g.mL^{-1}$ (National Committee for Clinical Laboratory Standards (1990), in Eloff (1998). We also observed that after one hour INT was reduced to red in some of the wells where the ampicillin concentration was above the MIC reported here, indicating false positives. This observation was confirmed by triple streak verification. The presence of false positives was not found to be the effect of using broth in the serial dilutions.

Colour developed in all the wells containing umckalin test solutions, indicating possibly a lack of bacteriocidal activity. However, the development of colour over time did not appear to correlate to umckalin concentration. As such the published bacteriostatic activity of umckalin could not be clearly reproduced in our hands using Eloff's method. Kayser and Kolodziej (1997) used an agar dilution method to describe the antibacterial activity of umckalin against a range of bacteria. The quantity of umckalin required to reproduce the assay, however, was more than that available for this study.

2.5 Discussion

Umckalin is a reported antibacterial agent (Kayser and Kolodziej, 1997), has INF-like cytoprotective activity and has been shown to induce the expression of TNF (Kolodziej *et al.*, 2003). The latter activity accounts for umckalin's role in stimulating the non-specific immune system. These findings and the conclusive evidence for the isolation of umckalin here, offered rational justification for its use as a bioactive metabolite marker in this study.

Chapter 3

HPLC Method Development

3.1 Introduction

Following the successful isolation of umckalin (Chapter 2), a High Performance Liquid Chromatography (HPLC) based assay was developed for the quantification of umckalin concentrations in ethanolic *P. sidoides* root extracts. The developed assay was then used in subsequent experiments to assess the variation of root umckalin concentrations between wild harvested plants from a range of geographical locations and of greenhouse cultivated plants subject to various treatments.

The published assay of Kayser and Kolodziej (1997) was used as the starting point for the development of a more efficient method. The authors' analytical HPLC was performed on a C-18 column (250 x 4 mm; RP-18, 7 μm). The mobile phase was a linear gradient system of water:methanol (H_2O:MeOH, 9:1 to 3:7 over 40 min, at a flow rate of 1.5 $ml.min^{-1}$) yielding an umckalin retention time (RT) of 32.94 minutes. The linear gradient system was misprinted in the original paper as being MeOH:H_2O instead of H_2O:MeOH, this was confirmed by Professor H. Kolodziej (2005, *pers. comm.*) as the convention is to have the most polar solvent (H_2O in this case) mentioned first. The use of MeOH:H_2O could have a significant effect on the resolution and separation of compounds on the column. The guidelines of Snyder *et al.* (1997) were followed here in order to improve the assay to one that would be accurate, rapid and reproducible.

3.2 Method development on a 250 x 4 mm reversed phase column

Our HPLC method development began using a similar method to that of Kayser and Kolodziej (1997) on a C-18 column (Nucleosil, 250 x 4 mm; C-18, 5 μm, Macherey-Nagel) connected to a Spectra-Physics SP8800 ternary HPLC pump. The absorbance of compounds from 20 μL injections eluting from the column was measured by a Linear UVIS 200 detector at a wavelength of 330 nm. The resultant peaks in absorbance, their areas and RT, were recorded on chromatograms created by a Spectra-Physics SP4290 integrator.

Linear gradient and isocratic systems of HPLC grade H_2O:MeOH (Academic A10 MilliQ, Millipore and Sigma-Aldrich, respectively) were used as eluting solvents delivered at a flow rate of 1 $mL.min^{-1}$. Separation was first assessed using injections of the umckalin standard dissolved in ethanol; these were followed by injections of ethanolic root extracts. The value of umckalin standard separations for the subsequent analytical work was assessed according to the RT, width, clarity and definition of the umckalin peak. The value of the separation for ethanolic extracts was assessed according to the separation of the umckalin peak from other peaks on the chromatogram in addition to the characteristics listed above. A 20 minute post-analysis column clean-up elution using MeOH was included after each separation to prevent RT drift and a loss of peak resolution. The success of this stage was exemplified by standard curves A and B in Figure 3.2.

The long umckalin RT using the protocol of Kayser and Kolodziej (1997) was not deemed suitable for the assay of the anticipated 100 ethanolic extracts or for the routine assessment of wild harvested or cultivated plants. It was therefore decided to develop a modified method with a shorter RT for umckalin. The results of our separations using altered solvent protocols on a 250 x 4 mm column are shown as Methods 1 to 4 in Table 3.1. Reducing the RT was the focus of early method development.

RT was reduced to 15.2 min using the above column and a 7:3 to 54:46 (H_2O:MeOH) linear gradient system over 16 min. The RT could not be reduced further and it was therefore decided to change to a shorter column.

3.3 Method development on a 125 x 4 mm reversed phase column

Method development on the second column (Nucleosil 100-5 C-18, 125 x 4 mm, particle size 125 μm with a CC 8/4 Nucleosil 100-5 C18 guard column, Macherey-Nagel) began using similar linear gradient systems of H_2O:MeOH to those used with the 250 x 4 mm column. We quickly discovered a method giving a very good umckalin RT (8.0 min, Table 3.1, Method 6). The RT using this system, however, was slightly longer than the gradient time and as a result the advance of the umckalin peak fell into the column clean-up stage which used MeOH as the elutant. This had the potential to cause inaccurate results when assaying plant extracts should other compounds have eluted in the MeOH clean-up stage. Methods 7 to 16 (Table 3.1) represent our subsequent attempts at reducing the RT to within the gradient time, while still retaining the good separation and peak width of Method 6. None of the Methods 7 to 16 (gradient or isocratic) produced the desired result.

The desired result was however attained when 0.05% trifluoroacetic (TFA) was added to solvents and Method 6 (Table 3.1) used. TFA acts as a proton acceptor, deprotonating the molecules in solution and therefore making them more polar and quicker-moving on the non-polar C-18 stationary phase. The method using TFA resulted in a reduction of the umckalin RT to well within the gradient time and increased the distance between the umckalin peak and the peaks of other compounds in the ethanolic root extracts (Figure 3.1).

Table 3.1: Retention times (RT) of umckalin standard absorption peaks of known concentrations, in ethanol, using reversed phase HPLC, on 250 and 125 x 4 mm C-18 columns and linear and gradient systems of H_2O:MeOH. Duration of isocratic elution and gradient time given in parentheses. Scanned chromatograms are included to show the shape of each resultant umckalin peak, shape was used as one of the credentials in judging the merit of separations. Solvent peaks are not shown.

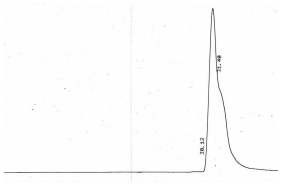
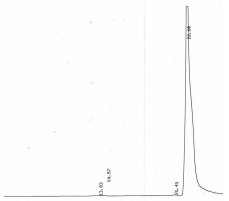
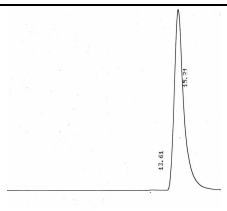
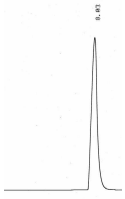
Method number	Column length L=250 mm S=125 mm	Gradient or isocratic (G/I)	Solvent regimes (H_2O:MeOH) over time (min)	RT (min)	Scanned chromatogram, showing the shape of the umckalin peak
1	L	G	1:9 → 7:3 (50)	36.0	
2	L	G	9:1 → 3:7 (50)	31.4	
3	L	G	9:1 → 3:7 (30)	22.6	
4	L	G	7:3 → 54:46 (16)	15.2	
5	S	G	3:7 → MeOH (8)	2.0	
6	S	G	7:3 → 3:7 (8)	8.0	

Table 3.1: Continued…

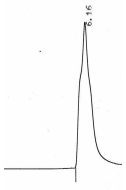
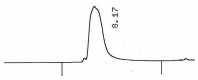
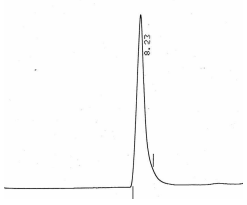
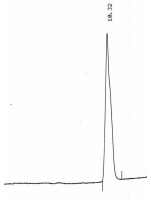
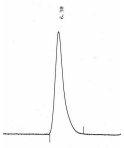
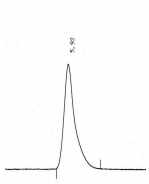
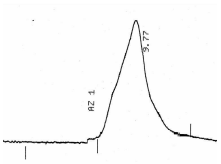
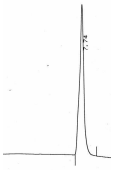
Method number	Column length L=250 mm S=125 mm	Gradient or isocratic (G/I)	Solvent regimes (H_2O:MeOH) over time (min)	RT (min)	Scanned chromatogram, showing the shape of the umckalin peak
7	S	G	1:1 → 3:7 (8)	4.1	
8	S	G	65:35 → 3:7 (6)	6.2	
9	S	G	7:3 → 3:7 (8)	7.0	
10	S	G	7:3 → 3:7 (11)	8.2	
11	S	G	7:3 → 3:7 (12)	8.2	
12	S	G	9:1 → 3:7 (11)	10.3	

Table 3.1: Continued…

Method number	Column length L=250 mm S=125 mm	Gradient or isocratic (G/I)	Solvent regimes (H_2O:MeOH) over time (min)	RT (min)	Scanned chromatogram, showing the shape of the umckalin peak
13	S	G	MeOH → 1:9 → H_2O (2 and 18, respectively)	1.2	
14	S	I	55:45 ⟶ 55:45 (19)	6.3	
15	S	I	55:45 ⟶ 55:45 (9)	5.9	
16	S	I	3:2 ⟶ 3:2 (12)	9.7	
17	S	G	7:3 ⟶ 3:7 (8)	9.3	
18	S	G	7:3 ⟶ 3:7 (8) with 0.05% TFA	7.7	

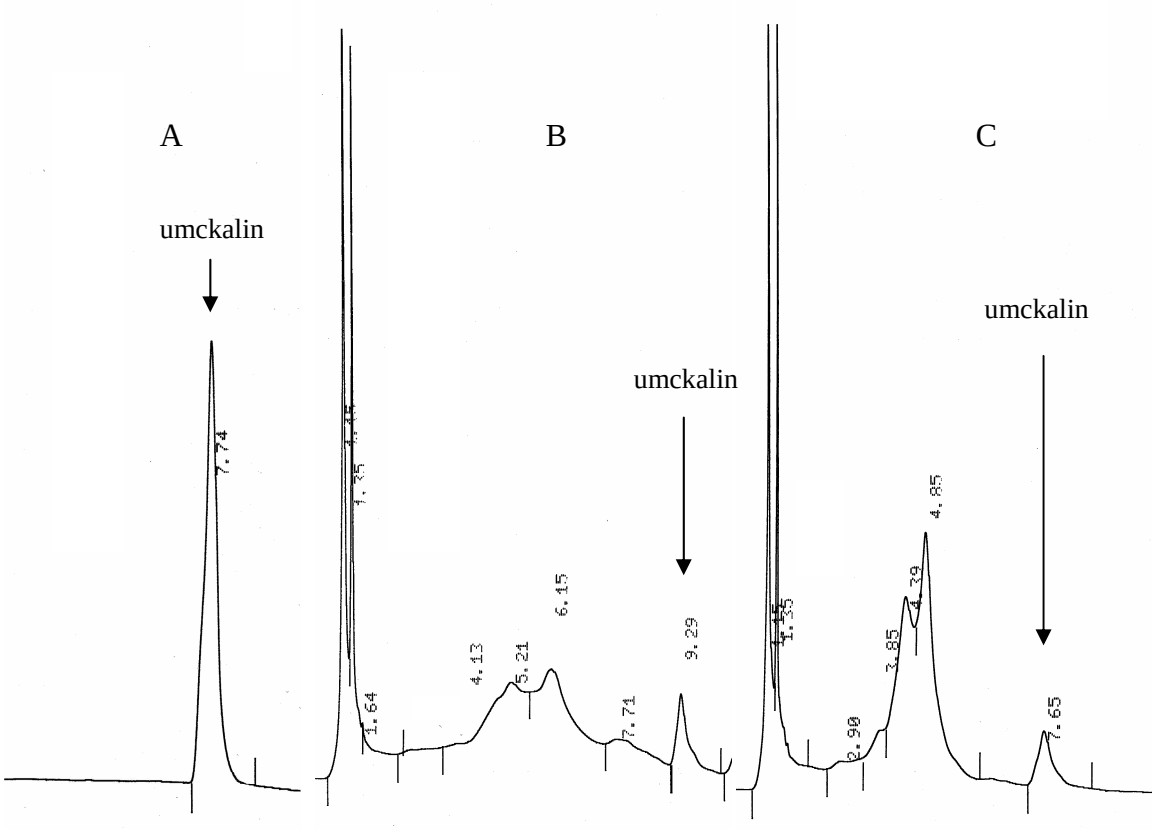


Figure 3.1: HPLC chromatograms for an umckalin standard with 0.05% TFA in eluting solvents (A) and an ethanolic extract of powered dry *P. sidoides* root eluted without and with 0.05% TFA in eluting solvents, (B) and (C), respectively. Results were obtained using a linear gradient of 7:3 to 3:7 (H_2O:MeOH) over eight minutes on a 125 x 4 mm C-18 column.

Method 18 (Table 3.1) was chosen as the best method developed in this study based on umckalin peak width, separation and retention time. Additionally, the rapid separation made Method 18 better suited than that of Kolodziej and Kayser (1997) for use in the experiments to follow (see Chapters 4 and 5).

The analyses of root umckalin concentrations were performed at two experimental times separated by three months. As such, two standard curves were created for umckalin dissolved in ethanol and used for analyses corresponding to these experiment times.

For the first set of experiments, umckalin concentrations ranging from 0.1388 to 15 µg.mL^{-1} were analysed using Method 18 and the resultant absorption peak areas used to create a standard curve of peak area versus the mass of umckalin injected (Figure 3.2A). The concentrations used for the second standard curve (Figure 3.2B), ranged from 2.5 to 50 µg.mL^{-1}. A standard curve of umckalin dissolved in acetone (50 to 200 µg.mL^{-1}) was also created so that the effects of using acetone as an extracting solvent could be assessed. The linearity of the standard curves confirmed that the developed method could be used to accurately determine the concentration of umckalin in ethanolic and acetone root extracts.

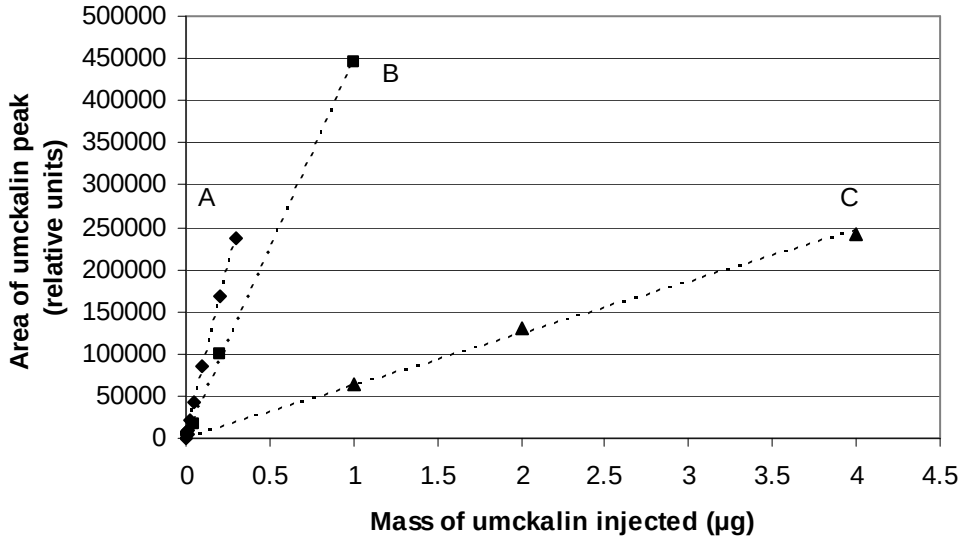


Figure 3.2: Standard curves of peak areas corresponding to masses of umckalin in absolute ethanol (A and B) and acetone (C) injections as determined by HPLC using a linear gradient of 7:3 to 3:7 (H_2O:MeOH) over eight minutes on a 125 x 4 mm C-18 column. The y-axis intercept of the trendlines were set to zero. For A: y = 807430x (r^2 = 0.99, N = 10), B: y = 446737x (r^2 = 0.99, N = 3) and C: y = 61637x (r^2 = 0.99, N = 3). In testing method robustness, the standard error of four umckalin peak areas corresponding to 0.025 µg injected mass on curve A equaled 542/20932.

The above standard curves (A and B) allowed for the concentration of umckalin in ethanolic root extracts from both wild and cultivated *P. sidoides* plants to be assessed.

As seen in Figure 3.2, the gradient of curve B is not identical to that of curve A and may have been due to differences in ambient temperature during the two sets of elutions. Alternatively, the change in gradient may have been due to changes in the condition of the HPLC column during the three months between the sets of analyses. The reduced detection of umckalin dissolved in acetone (Figure 3.2C), in comparison to that in absolute ethanol (Figures 3.2A and B), could not be readily explained but may have been due to differences in the solubility of umckalin in the two solvents.

3.4 Root extraction method development

Following the successful development of a robust analytical HPLC method and initial standard curve (Figure 3.2A), a series of experiments were run to determine an efficient and practical method of preparing ethanolic extracts for HPLC analysis. The dry powdered root used in the following experiments was taken from the same GFRR stock that was prepared for and used in the isolation of umckalin (Chapter 2).

For the extractions, 1 g of powdered dry root was combined with 100 mL of absolute ethanol in conical flasks, stoppered with rubber bungs and extracted for one, two and six days at 25 °C in a shaking water bath (N = 3). The ethanol portion of each mixture was poured into plastic centrifuge tubes and centrifuged (RC-5 Superspeed Refrigerated Centrifuge; Du Pont Instruments, Sorvall) at 12 000 rpm for 20 minutes between zero and 20 °C. Supernatants were poured into a glass syringe and filtered (0.45 μm nylon filters, Cameo) into 100 mL volumetric flasks. The internal wall of the neck of each flask was rinsed and the volume made up to 100 mL with absolute ethanol. After mixing (Whirlimixer, Fisons Scientific Apparatus), a volume from each flask was transferred into a clean 10 mL glass vial, capped, and was then ready for HPLC analysis. The re-dried powder from the one and six day extracts was extracted in a second 100 mL of absolute ethanol for a further one and six days, respectively, to determine if any umckalin remained subsequent to the first extraction.

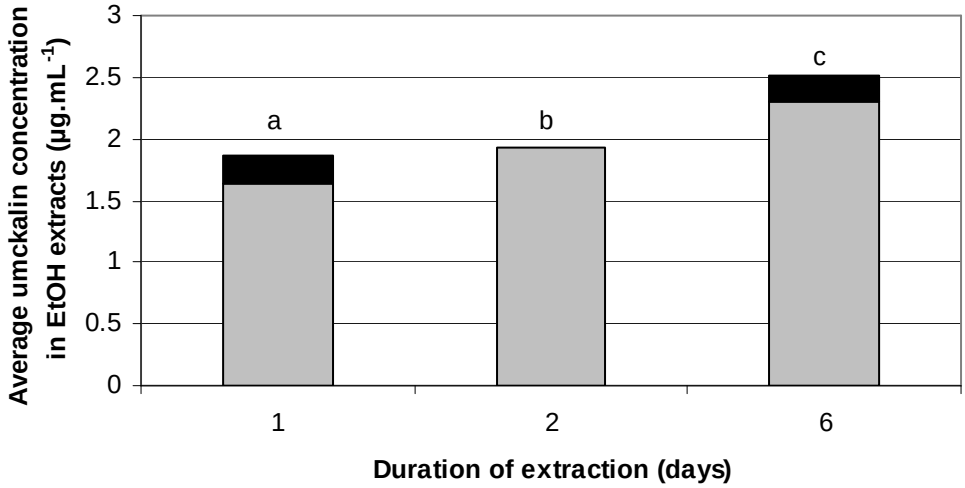


Figure 3.3: Yield of umckalin in absolute ethanol extracted over time, the grey shading shows the average umckalin concentration of the first extractions. N = 3. a, b and c represent statistically homogeneous groups at the 95% confidence level, $p < 0.0002$, $F = 53.76$. The black shading shows the average umckalin concentration of extracts made of the powder remaining after the first one and six day extractions.

Less umckalin was extracted from the re-dried powder of the six day extractions than from the one day extractions (grey shading in Figure 3.3, $p = 0.897$). Following this observation and for the purposes of this study, the combined umckalin concentration of the first and second six day extractions was taken to represent a 100% yield. The average umckalin concentration of the initial one day extracts represented 65.06% of this total. For practical reasons, a single day extraction of 1 g of powdered dry root in 100 mL of absolute ethanol was chosen for the preparation of samples for HPLC analysis in the experiments to follow. Based on the information in Figure 3.3 the umckalin concentration detected in one day extractions was adjusted by 34.94% to represent a 100% yield.

3.4.1 Root extraction with acetone versus ethanol

Ethanol extraction is used in the manufacture of commercially available *P. sidoides* products (e.g. Pelargonium, Medicherb UK; Pelargonium Syrup, Bioharmony Africa and Umckaloabo®, Spitzner). However, acetone and aqueous-acetone extraction was used here and in other studies (Kayser and Kolodziej, 1995; Kayser and Kolodziej, 1997; Kolodziej *et al.*, 2003 and Latté *et al.*, 2000), respectively, for the isolation and testing of active constituents. Therefore, the rationale of using ethanol as the commercial extracting solvent was investigated by comparing the concentration of umckalin in ethanol and acetone extracts of dried *P. sidoides* root.

As shown in Figure 3.4 the umckalin concentration in acetone extracts was *ca.* five times greater than that in ethanol extracts. Therefore, the use of ethanol in the manufacture of commercial products may be due to tradition as opposed to it being a more effective extracting solvent. If the yields of other active constituents, like gallic acid and 6,8-dihydroxy-5,7-dimethoxycoumarin, are also higher in acetone extracts it would make sense for the industry to change solvents and use dried acetone extracts in the manufacture of *P. sidoides* products. Acetone is toxic to people and would need to be removed from extracts before they were administered to patients.

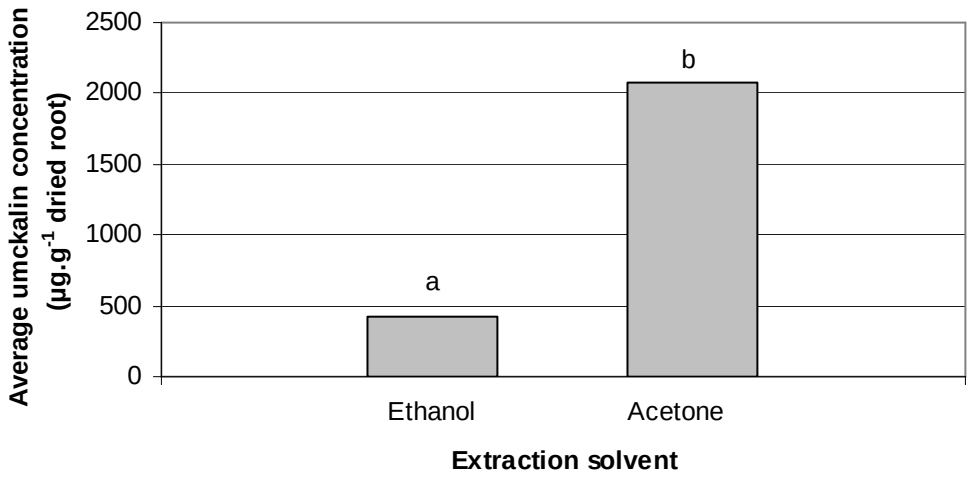


Figure 3.4: Concentration of umckalin per gram dried root following extraction with absolute ethanol and acetone. N = 3. a and b represent statistically homogeneous groups at the 95% confidence level, $p < 0.0008$, $F = 83.85$.

3.5 Summary of the developed method

The following is a summary of the preparation procedure followed for all subsequent root samples: whole roots were washed under running water and dried with paper towel before being sliced with a stainless steel knife and dried at 35-40 °C, dried root was either crushed between the fingers or with a pestle and mortar before being ground to a 1 mm particle size using a hammer mill, 1 g of root powder was combined with 100 mL absolute ethanol and extracted with shaking at 25 °C for one day, the ethanolic extract was than centrifuged at 12 000 rpm and filtered through a 0.45 µm nylon filter. Such samples were then analysed using a linear gradient of 7:3 to 3:7 (H_2O:MeOH) over eight minutes at a flow of 1 $mL.min^{-1}$ through a 125 x 4 mm C-18 HPLC column. Detected concentrations were corrected by 35.84%, as above.

Chapter 4

Wild Harvest

4.1 Introduction

Essentially, the research presented in this chapter investigated the effects of harvest on the survival and regrowth of individual replanted *P. sidoides* plant parts, post-harvest, at distinct field sites.

4.1.1 The recent history of *P. sidoides* harvest

Preceding 2003, *P. sidoides* roots were extensively harvested in the Eastern Cape to supply the demand generated by an international market. Despite the international interest, speculation still surrounds the quantity of *P. sidoides* roots harvested from the wild. The most recent reports date back to 2002 when the issue of unsustainable harvest first became apparent to Eastern Cape Nature Conservation officials and the public. Popular articles in two newspapers and one online publication reported on rural harvesting coordinated by two individuals. Conservatively, at least 20 tonnes of combined *P. reniforme* and *P. sidoides* roots were harvested from populations in the Eastern Cape in 2002 (Bisseker, 2002 and Limson, 2002). Some of the potential problems associated with the reported harvest included the removal of entire plants from the ground, not replanting any unwanted plant parts (like stems and leaves) and the rate at which roots were harvested for export. In about four villages middlemen would visit almost weekly (Dold, 2002, *pers. comm.*). This information was sufficient to identify the investigation of potential mitigation techniques as an area of high priority research.

4.1.2 Towards a sustainable harvest

Broadly, "sustainable harvest refers to the activities that involve the extraction of a natural resource in a manner that it is not depleted and can renew itself so that similar levels of exploitation can occur indefinitely" (Struhsaker, 1998). Many approaches, cited in the literature, are followed in assessing resource utilisation towards the goal of sustainable management and nature conservation. These recommendations include examples on ecological, public participatory and policy making levels. On the ecological level, it is recommended that the effects of harvest on post harvest recovery of target species, population dynamics and whole ecosystems be evaluated before permission is granted for extensive harvest (Peres *et al.*, 2003; Rock *et al.*, 2004 and Struhsaker, 1998; respectively). On the public participatory level, the promotion of cultivation and the gathering of potentially valuable information on resource management from traditional users are viewed in high regard (Stewart, 2003 and the World Health Organization, WHO, *et al.*, 2003). The use of legislation to monitor and regulate harvest is also suggested (Rock *et al.*, 2004; Stewart, 2003 and the WHO *et al.*, 2003). Additionally, Stewart (2003) recommended the protection of wild populations and the enrichment of harvested populations with cultivated plants. Gaining an understanding of the ecology of a given plant in its natural environment is also important, especially if researchers are to provide policy makers and conservationists with clear criteria for evaluating the above and other measures for biodiversity protection and maintaining a sustainable harvest (Egli *et al.*, 2006).

In 1993 the World Health Organization (WHO), The International Union for the Conservation of Nature and Natural Resources (IUCN) and the World Wide Fund for Nature (WWF) published the document "Guidelines on the conservation of medicinal plants". In this document the authors recommended that techniques be investigated for improving the harvest of medicinal plants and that governments take an active role in regulating medicinal plant trade and harvest. Specifically, they recommended that governments control the trade of plants and derived products.

The suggested control measures were a total ban on the collection of all plants or the establishment of a permit system. A permit system would help ensure that certain species or populations from certain locations were not over-harvested. For *P. sidoides* and *P. reniforme* populations in the Eastern Cape, middlemen are currently required to apply for, and obtain, a permit from Eastern Cape Nature Conservation (ECNC) before harvesting may proceed (Hahndiek, 2005, *pers. comm.*). Over-exploitation was highlighted by the WHO *et al.* (1993) as particularly important for threatened or rare species, for which they recommended collection for propagation purposes only.

The results of a study on Brazil nut (*Bertholletia excelsa*) harvest by Peres *et al.* (2003) and the review of African cherry (*Prunus africana*) bark harvest by Stewart (2003) provide valuable insight relevant to the future harvest of *P. sidoides*. Before extensive wild resource extraction is initiated, thorough research needs to be completed on post-harvest population dynamics and recovery following resource removal. Peres *et al.* (2003) studied the effects of Amazonian Brazil nut collection on *B. excelsa* population dynamics in relation to geographic location, forest type (reflecting the canopy openness), annual rainfall, degree of seasonality (number of dry months with $\leq$ 50 mm rainfall), the level of hunting pressure and soil nutrient availability, over a 20 year period. The authors showed that the juvenile plant numbers following the excessive Brazil nut harvest during the 20th century were insufficient to maintain populations over the long term. Some of their suggestions to promote sustainability were the spatial rotation of fruit harvest, so that areas would have a chance to undergo a degree of seedling recruitment, and the management of annual harvest quotas. Unlike *P. sidoides*, the immediate threats to *B. excelsa* populations are habitat destruction and degradation. The protection of this species is particularly important as it is only collected from wild populations and the Brazil nut industry forms the cornerstone of the Amazonian economy (Peres *et al.*, 2003).

Stewart (2003) cites the lack of adequate initial harvest experiments as negatively contributing to the fate of *P. africana* in the wild. The bark of *P. africana* (African cherry), an endemic African medicinal tree, is used internationally for the treatment of benign prostatic hyperplasia and other disorders.

Although *P. africana* has only a recent history of harvest (*ca.* 38 years), it was placed on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II list of endangered species in 1996 (Stewart, 2003; see CITES, 2005). Harvesting, which began in the 1970's and was originally well controlled by a single company in Cameroon, has since ceased in certain countries due to over-exploitation. This over-exploitation began with an increase in the number of harvest groups and the increasing neglect of traditional forest conservation practices. The effective conservation of *P. africana* in Cameroon was once performed by the *kwifon*, traditional forest managers. The depth of conservation knowledge they held may not be available for *P. sidoides* as it is not one of the most commonly used traditional medicinal plants in South Africa or the Eastern Cape. Therefore, traditional harvesting practices may not have been rigorously tested at the large scale necessary to supply international demand. To successfully conserve *P. africana* populations in the wild, Stewart (2003) proposed *ex situ* small and large scale farm cultivation, protection for wild plants and planting greenhouse-grown plants to enrich wild populations *in situ*. *Ex situ* and *in situ* cultivation of economically important medicinal plants may have the potential to supplement future wild harvest (e.g. Devil's Claw; Stewart and Cole, 2005; and *Paeonia lactiflora*, Kim *et al.*, 2006). The WHO *et al.* (1993) listed the potential increase in crop yield of plants under irrigation as a positive implication of cultivating medicinal plants commercially. Furthermore, Wang *et al.* (2006) found that increasing the frequency of drip irrigation for cultivated potatoes resulted in enhanced potato tuber growth and increased tuber yields per total plant dry weight. This provided a rationale for growing *P. sidoides* plants under greenhouse conditions with frequent watering to promote tuberous root growth.

In this study, a hydrophilic colloid made up of crosslinked potassium polyacrylate-polyacrylamide copolymers, Stockosorb™, was used to promote the survival and regrowth of replanted *P. sidoides* plant parts, post-harvest. The addition of Stockosorb™ to the soil surrounding replants increased water provision immediately after replanting and would also have increased the retention of water in the soil after rainfall.

Dehgan *et al.* (1994) investigated the response of *Photinia* x*fraseri* and *Podocarpus macrophyllus* to different percentages of Stockosorb™ added to growing medium under greenhouse conditions. The authors found that the interaction between frequency of irrigation and percentage of Stockosorb™ in the soil on root and shoot dry mass produced by *P.* x*fraseri* plants was significant. However, the effect on the growth of *P. macrophyllus* was not significant. Dehgan *et al.* (1994) concluded that the effectiveness of Stockosorb™ may vary between plant species and under different cultivation conditions.

It is important that experimental design takes into account traditional harvest practices, as in the work of Egli *et al.* (2006). The authors' experiments were well designed to use the available information about Swiss wild mushroom harvest and thus produced a relevant result. Performed in Swiss forests, the experiments included mushroom collections by cutting or picking compounded by a ground trampling treatment to accurately simulate the effects of harvest. In addition, the authors used exclusion plots in areas where harvesting by private harvesters was expected to occur during the course of their experiments. Although some information was gained through observation of the practices used by *P. sidoides* harvesters employed in the industry (Dold, 2003, *pers. comm.*), much traditional harvest information is lacking for this species. The novel harvest techniques used in this study were therefore developed independently using a reasoned approach and are described in the methods section to follow.

4.1.3 Field sites

As the survival of replanted harvested plant parts may depend on environmental factors like rainfall (Ticktin, 2004), potential sites were surveyed across a broad range of rainfall areas in the Grahamstown, Fort Brown/Fish River and Kei Road regions of the Eastern Cape. Two sites, one in the Greater Fish River Reserve (GFRR; 33° 07' 13" S, 26° 43' 29" E) and one on Killaloe farm, Kei Road district (KR; 32° 43' 03" S, 27° 39' 30" E) were chosen on the basis of their marked difference in annual rainfall, 397 ± 122 mm and 760 ± 168 mm (1984-2003, average ± standard deviation), respectively.

Figure 4.1 shows the location of each site used in the wild harvest experiments presented in this chapter, as well as those used in studies presented in Chapter 5. To observe the effects of harvest season, the survival and regrowth of *P. sidoides* replants was assessed following April/May (autumn) and November/December (summer) harvests. The regrowth of similarly treated plants under greenhouse conditions was also assessed.

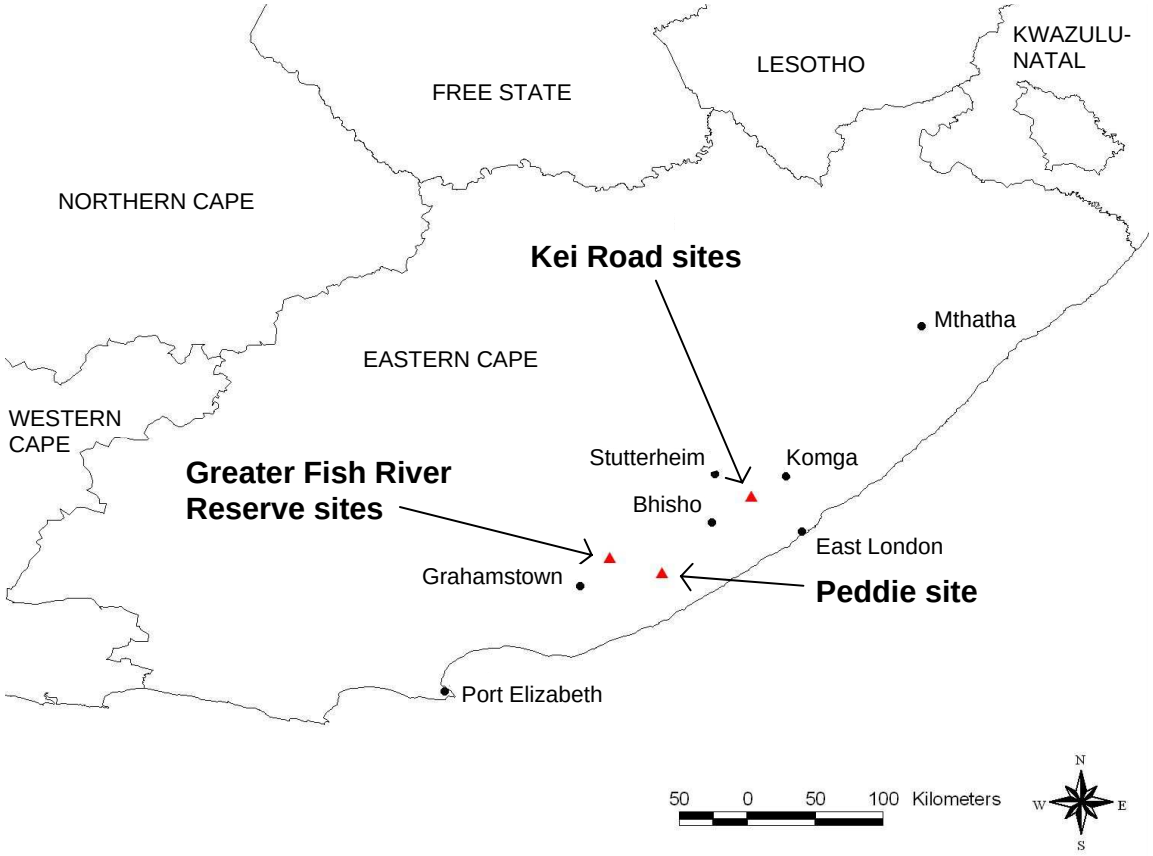


Figure 4.1: Map centered on the Eastern Cape Province of South Africa showing the locations of the Greater Fish River Reserve (GFRR), Kei Road (KR) and Peddie field sites (red triangles) in reference to key towns and cities (black circles).

Both wild harvest experiment sites were within protected areas and neither were exclusion plots. The KR site was used as pasture for sheep and cattle while non-domesticated large and small herbivores including red hartebeest, springbok, eland and warthog were present at the GFRR site.

The latter site was at an altitude of 456 m with a southeast aspect while the former site was at an altitude of 623 m with a north-northwest aspect. An interesting observation was that most *P. sidoides* plants not included in the experiment at the KR site lost their leaves in winter while the plants at the GFRR site were only partially defoliated in the same season. Xeric succulent thicket and eastern thorn bushveld on gentle slopes, 2 and 3°, characterized the vegetation at the GFRR and KR sites, respectively (Low and Rebelo, 1998). The chosen *P. sidoides* population at the GFRR site occupied an area of 250 m^2 with plants growing at a density of *ca.* 1 plant.m^{-2} separated mostly by open ground and small herbs. This contrasted with the lower plant density of *ca.* 0.3 plants.m^{-2} observed at the KR site for plants separated by dense grass cover within an area of 3000 m^2.

4.1.4 Aims, rationale and hypotheses

The aims of the research described in this chapter were:

1) To determine the effects of different management practices, habitat and season of harvest on the regrowth of replanted *P. sidoides* plant parts post-harvest.

Novel harvest techniques were designed and tested in order to address the lack of information concerning traditional harvest. These harvest techniques ("management strategies"; Ticktin, 2004) included the replanting of harvested plant parts and the addition of a water releasing colloid in two seasons at two climatically different field sites. The plant parts replanted in field experiments were 20% portions of leafless harvested plants, by mass (20% replant). The 80% portion was removed as root. This strategy was considered a small concession for harvesters to make in order to promote plant regrowth and hence potential sustainability. Additionally, an approximate 20% replant mass can be easily estimated by harvesters without the necessity of a sensitive balance and in instances comprises a 2 cm long section of the root with attached stem(s). It was hypothesized that the 20% replants would have a higher production of root than whole replants following harvest. This hypothesis was based on the anticipated allocation of assimilated carbon to root production, post-harvest, to replace harvested root.

Conversely, the survival rate amongst 20% replants was expected to be less than that for whole replants. The survival and growth of replanted 20% replants was ascertained and compared to results obtained for 20% replants planted with a water releasing colloid, Stockosorb™. The addition of Stockosorb™ to the soil surrounding 20% replants was hypothesized to increase the percentage survival and regrowth of the replants. Following the findings of Wang *et al.* (2006), it was hypothesized that the root growth of 20% replants under cultivation would be higher than the corresponding growth under field conditions. Additionally, it was hypothesized that harvest and replanting in the wet season, or in an area of higher rainfall, would increase the subsequent growth of root and leaf biomass.

2) To investigate the relationships between above and below ground plant biomass, and root colour and umckalin concentration, respectively.

The findings of this study may be useful in aiding harvesters and providing advice for the industry, researchers and local and international governments; however, it was not designed specifically to do so. The relationships between leaf and root mass, and root colour and umckalin concentration, were investigated based on the following information concerning the root characteristics sought by middlemen in the industry. Firstly, harvesters are requested to collect larger as opposed to smaller roots (Dold, 2004, *pers. comm.*). It would therefore be useful to harvesters if the biomass of the underground roots could be determined by an above ground observation. Potential relationships were investigated and a relationship between above ground biomass and root mass was hypothesized. Secondly, harvesters are instructed to preferentially collect roots with darker red internal tissue over lighter coloured ones (Dold, 2004, *pers. comm.*). To determine the relevance of this request, the relationship between the colour and umckalin concentration of powdered dry root was investigated.

4.2 Methods

4.2.1 Field harvest experiments

In order to test the proposed hypotheses, harvested plants were treated in one of three ways following the removal of their leaves. Initially all plants were weighed and had their leaves and petioles removed by cutting the petioles approximately 2 cm from the basal attachment to the stem. These leafless plants were weighed and control plants (CN) were returned to the ground as such (whole replants). Plants in the second two treatments had 80% of their leafless mass removed as root, to give 20% leafless plants (20% replants), and were either returned to the soil with the application of Stockosorb™ (SA - Stockosorb™ added) or without the application of Stockosorb™ (NS - no Stockosorb™ added). Stockosorb™ was hydrated 1 $g.L^{-1}$ and 500 mL was thoroughly mixed into the soil surrounding appropriate replants.

The plants used in harvests at the GFRR and KR sites represented 70 and 18.5% of the total number of plants in the selected populations, respectively. The largest populations at each site, 270 (GFRR) and 1000 (KR), were chosen for experimentation in order to minimize the local impact of harvest.

As survival and regrowth were likely to be affected by geographical location, climate and season, these experiments were repeated at two sites of significantly different rainfall in both autumn and summer. Harvests at the GFRR and KR sites were conducted at the beginning of the dry season (autumn) and in the wet season (summer; Figure 4.4). The autumn harvests represented the beginning of the low rainfall period for both sites; summer treatments represented the middle and beginning of the highest rainfall period for the GFRR and KR sites, repectively. For the autumn harvests at the GFRR and KR sites, 50 plants were randomly selected from within the chosen *P. sidoides* populations at each site for each of the three treatments. 10-15 randomly selected plants were used for the three treatments in the summer harvests at the same sites.

Fresh total plant, leaf and replanted mass were recorded for each plant at the time of harvest. Individual plants were marked with labeled stainless steel tags secured into the ground with six inch steel nails. The coordinates of each plant were recorded using perpendicular transects intersecting a primary transect that was marked with wooden stakes.

Sets of 50 plants were chosen for the autumn treatments so that subsets of ten replants from each treatment could be destructively harvested at four month intervals during the course of the experiments. This would have allowed for the assessment of replant survival and regrowth over time in relation to environmental factors and treatments. However, at the first proposed destructive harvest it was found that the percentage survival of replants was not high enough to support more than one destructive harvest. The method was therefore changed to include a single harvest in May 2005, one year after the autumn harvests and four months after the summer harvests. In order to compare regrowth under field conditions with that of cultivated plants, 127 20% replants from the KR site were grown under well-watered conditions in a greenhouse at the Rhodes University Botany Department. Surviving greenhouse cultivated plants were harvested after 18 months. Leaf, inflorescence, stem, and root masses were recorded at the ultimate harvest of surviving greenhouse and field grown replants. The stems included the above ground part of the plants that bore the leaves, buds and flowers (Allaby, 1998) without the diagnostic red coloured cortical tissue of the roots (White, 2004, *pers. obs.*). Leaves included the petioles and laminae (Allaby, 1998).

Both sites were visited approximately every four months during the study to note the presence and absence of leaves on each replant. The final visit corresponded to the ultimate harvest of the surviving replants in May 2005. Replants were said to have survived if they had green leaves at the time of observation. Plants that emerged independently of the replants subsequent to either harvest, observed within a 30 cm radius immediately around any replant, were considered to be growth resulting from a piece of root inadvertently left in the soil during harvest.

The number of such plants (called resprouts) and the distance of each resprout from the head of the nail positioning the appropriate replant label were recorded.

Due to a faulty field balance, the total root mass of leafless plants and the proportion of root to be excised for KR autumn harvest treatments were determined volumetrically. Once the leaves had been cut off, the root sections were individually submerged in a 5 L plastic measuring cylinder half filled with water. Total root volumes were recorded for the CN plants before they were returned to the ground. For the NS and SA treatments, total volume measurements were first made of the whole roots. Remaining in the water, each was then raised so that 20% of the total root volume was above the water level. The 80% portion of the root that remained below the surface was removed and resulting 20% replants were returned to the soil with or without Stockosorb™, as above.

The root portions that accounted for 80% of the total root volume were weighed in the laboratory and the value extrapolated to a 100% mass. The direct linear relationship between the extrapolated 100% root mass and the total root volume is shown in Figure 4.2, verifying the accuracy of using volume to determine root mass.

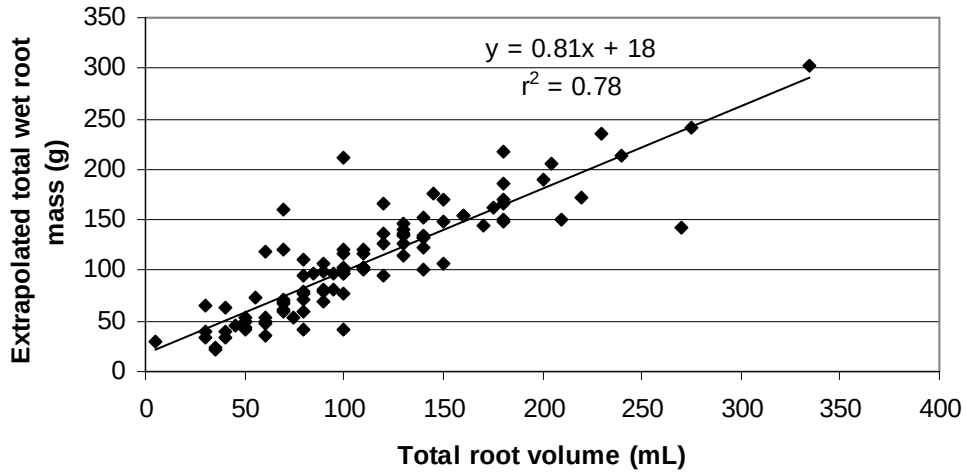


Figure 4.2: Description of total root mass by total root volume for KR autumn harvest NS and SA treated plants. N = 98. $p < 0.0001$.

In addition to the assessment of treated replant survival after harvest, the regrowth of root and leaf biomass was an important factor to consider. In April 2005, 12 months after the autumn harvests and four months after the summer harvests, all surviving plants were dug up so that the regrowth of root and leaf biomass could be measured. As all leaves were excised at the time of replanting, regrowth was represented by the subsequent leaf production. Old root growth was separated from the new root growth based on cortical tissue colour, red/brown as opposed to creamy white; and root bark appearance, dark brown and brittle as opposed to light brown and non-brittle. Dry leaf and root masses and not wet masses were recorded for treated plants re-harvested in April 2005. However, only wet replant masses could be measured and were measured at the beginning of each treatment. As such, dry leaf and root masses were converted to wet masses using the relationship between wet and dry mass found for greenhouse cultivated plants (Figure 4.3). Average leaf and root yields were calculated using the wet replant masses recorded at the beginning of the harvest experiments, giving wet yields. In the results to follow (Section 4.3), these wet yields were therefore plotted against the average wet root and leaf masses produced and not the average dry masses produced.

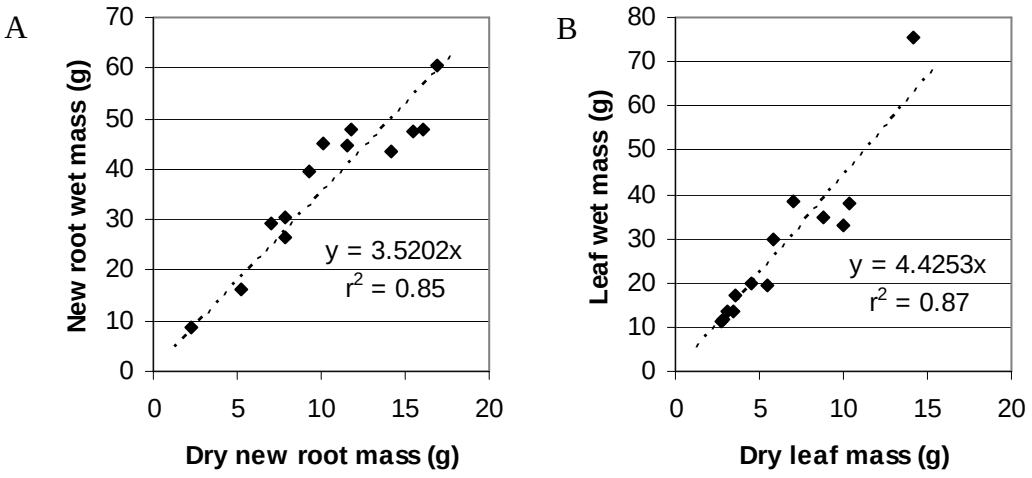


Figure 4.3: The relationship between wet and dry leaf (A) and new root mass (B) for leaves and roots harvested from greenhouse cultivated plants. The y-axis intercepts were set to zero for both straight lines.

Plant voucher specimens were collected from each site used in the above experiments and deposited in the Selmar Schönland Herbarium (GRA), Grahamstown.

4.2.2 Colour assessment of dry powered root

Eight plants were collected from the above KR harvest site in order to investigate the relationship between the colour and umckalin concentration of old roots. Colour and appearance of the root bark was used as a basis for distinguishing between the old and new root growth of plants (as above). Root samples were thinly sliced with a stainless steel knife, dried at approximately 40 °C for 3-4 days and powdered using a hammer mill. In order to determine the colour of each root sample, portions of powdered root were individually fixed onto approximately 1 cm^2 sections of black card with Alcolin glue. After air drying, the reflectance spectrum of each sample was measured using an Ocean Optics USB2000 spectrophotometer (Analytical Instrument Systems, Inc.) with an AIS model mini-DT light source. The integration time was set to 300 msec. Transmission data were plotted at 10 nm intervals and total reflectance, an indication of sample colour, was measured as the area under the curve between 400 and 700 nm wavelengths light (the spectrum visible to the human eye). Comparison of the shape of the reflectance curves with curves in the literature (Wyszecki and Stiles, 2000) was used to determine the colour of each sample (e.g. red, brown or red-brown). The area under the curve was indicative of the shade of the colour: the greater the area under the curve the lighter the colour of the root powder and *vice versa*. The umckalin concentration of each sample was determined using the method described in Chapter 3.

4.2.3 Estimation of root regeneration time

The time taken for 20% replants to regrow to the average pre-harvest mass under field conditions was estimated using a mathematical modeling technique. Firstly, replant mass was plotted against the mass of root produced subsequent to harvest for individual replants that survived the CN and 20% replant (NS and SA) treatments following the GFRR autumn harvest.

The exponential relationship between these variables gave an indication of how root regrowth may change with increasing accumulated root mass. The equation of the trendline fitted to the CN data was then used to extrapolate the growth of 20% replants from the average 20% replant mass to the average pre-treatment whole replant mass.

4.2.4 Statistics

Levene's test was used to test the normality of all the datasets generated and homogeneity of variances was tested using Shapiro-Wilk's test. Data that were not homogeneous or normally distributed were transformed to their natural logarithm or square root. One way analysis of variance (ANOVA) was used for testing variance within continuous data. Non-parametric analyses of variance were used for non-normal and non-homogenous continuous data that could not be corrected by transformation. Post-harvest survival data was analysed using observed versus expected Chi-square (X^2) tests or log-linear models. For the log-linear models, the significance of differences in the X^2 values of different models was assessed using a table of critical values of the X^2 distribution (Zar, 1999). Log-linear analyses were performed in order to assess the effects of treatment and time after harvest on the survival of replants following autumn harvest at the GFRR and KR field sites. In log-linear analyses, statistical models are created using various combinations of factors (e.g. treatment, site and time after harvest) to describe the frequency of observed data (e.g. survival). In this study best fit models were automatically created using Statistica 7 (StatSoft Inc., 2004). Maximum likelihood X^2 tests are used to test the fit of log-linear models and should not be significant ($p > 0.05$) if a model effectively fits the observed frequency data. Once an effective model had been created, including various two or three way interactions between the factors, different factor combinations are either added or removed (one change at a time) from the model and subsequent X^2 tests performed (hierarchical tests of alternative models; StatSoft Inc., 2004). Changes in X^2 values and degrees of freedom (ΔX^2 and Δdf, respectively) between the best fit and subsequent models are used to determine the significance of the interaction of the added or removed factor combination on the model fit. Statistica, version 7, statistical analysis software (StatSoft) was used to perform the above analyses.

4.3 Results

The total average rainfall in 2004 at each site (789 mm for KR and 377 mm for GFRR) was well within the variation of the respective 20 year average, although the harvests were conducted during a prolonged national drought. The distribution of the 2004 rainfall (solid lines on Figure 4.4) was unusual at both sites in comparison with the 20 year average (Figure 4.4).

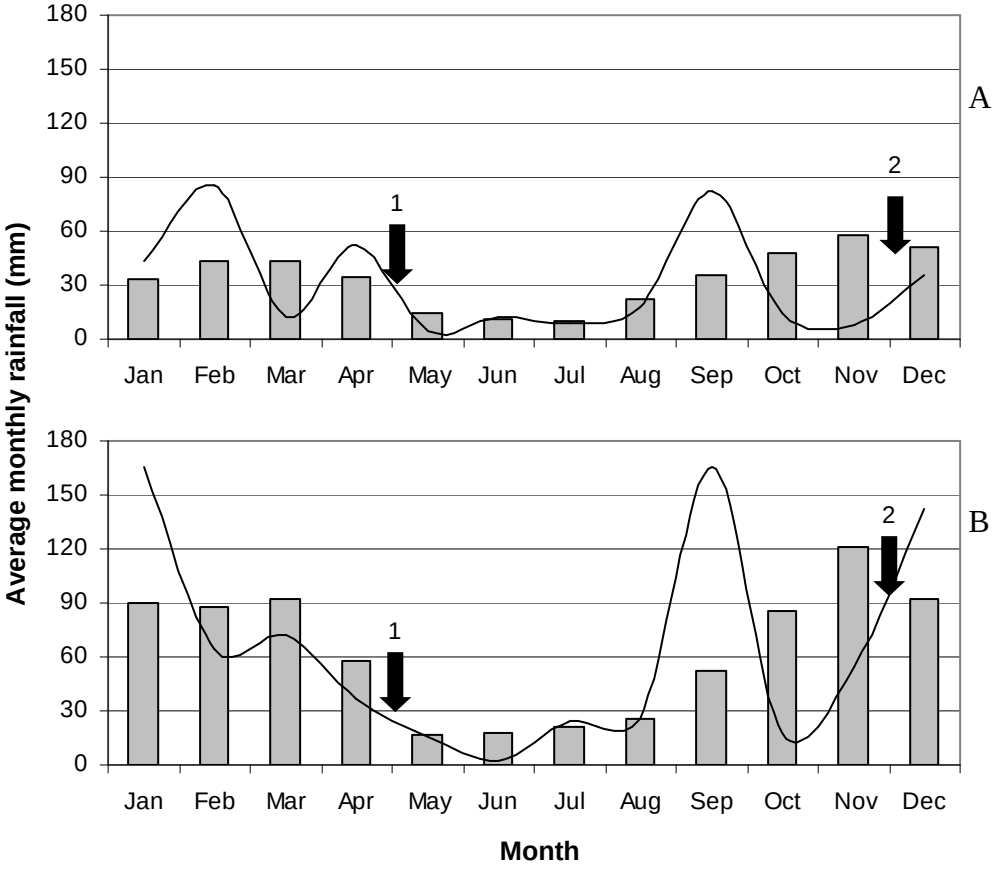


Figure 4.4: Average 1984-2003 monthly rainfall for the GFRR (A) and KR (B) sites recorded at the Main Office Complex in the Andries Vosloo Kudu Reserve (GFRR) and Rangerton Farm, Kei Road district (shaded bars), respectively. The solid lines show the total monthly precipitation for 2004. The arrows below 1 and 2 show the dates of the autumn and summer harvests, respectively.

4.3.1 Relationship between above and below ground plant biomass

A weak relationship was found between the total fresh replant mass (constituted mainly of root mass) and the fresh leaf mass of plants from both the GFRR and KR sites, as indicated by the low r^2 values of the straight lines fitted to the data of Figures 4.5A and 4.5B, respectively. This relationship may have been influenced by plants only bearing leaves seasonally (White, 2004-5, *pers. obs.*) while roots represent years of biomass accumulation. As water becomes more freely available following the spring and summer rains, the plants produce new leaves and inflorescences dependent on the availability of water. This idea was substantiated by observation of well watered greenhouse cultivated plants, most of which produced masses of foliage, were nearly continually in flower throughout the year and originated from a range of root masses. However, many cultivated plants lost their leaves when severely water stressed.

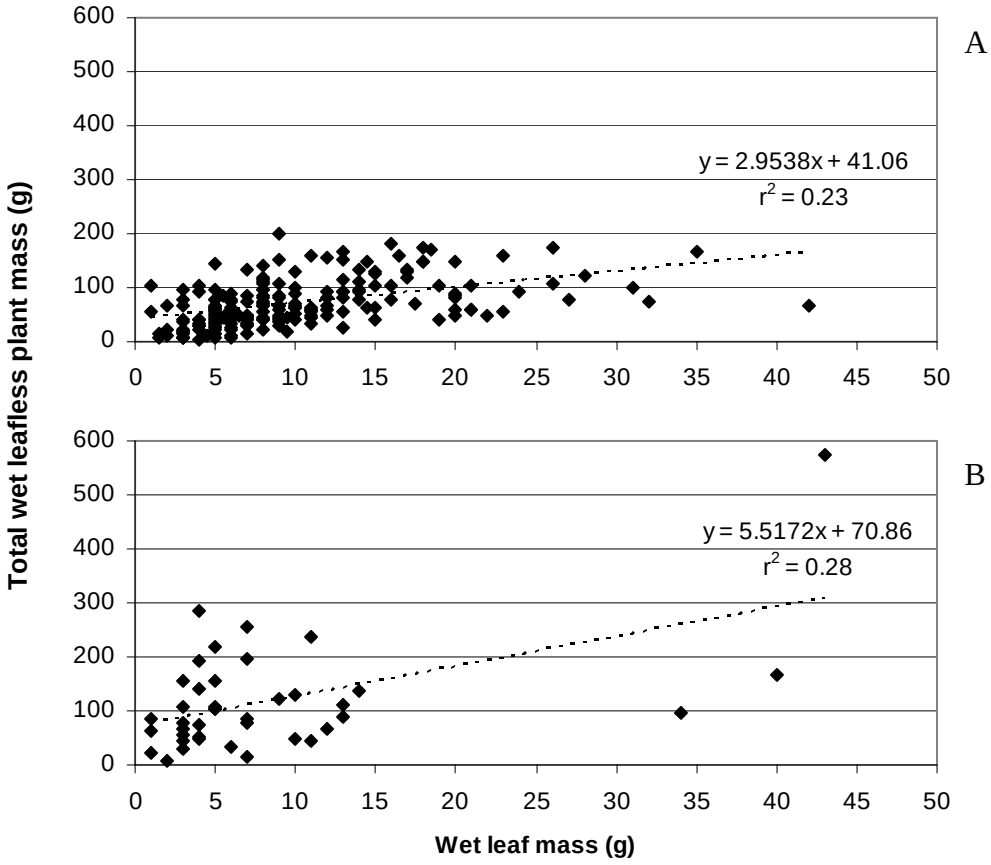


Figure 4.5: The relationship between wet leaf mass and wet replant mass for all the plants in the autumn and summer GFRR harvest (A, N = 194, $p < 0.0001$) and all the plants in the summer KR harvest (B, N = 40, $p = 0.0005$).

The implication of this finding is that harvesters may not be able to determine the size of the unseen roots merely by observation of above ground features like leaf and inflorescence biomass.

4.3.2 Relationship between root colour and umckalin concentration

This analysis was performed to determine the relationship between the colour and umckalin concentration of field harvested *P. sidoides* roots. The shape of all the reflectance curves generated from the *P. sidoides* root samples closely matched those of a red-brown brick published by Wyszecki and Stiles (2000). The area under the curves was inverted so that increasing values corresponded to an increase in the darkness of the samples (Figure 4.6). The lack of a direct relationship between the darkness of colour and umckalin concentration for dry *P. sidoides* roots can be seen clearly in Figure 4.6, questioning the harvesters' rationale for selecting dark red roots.

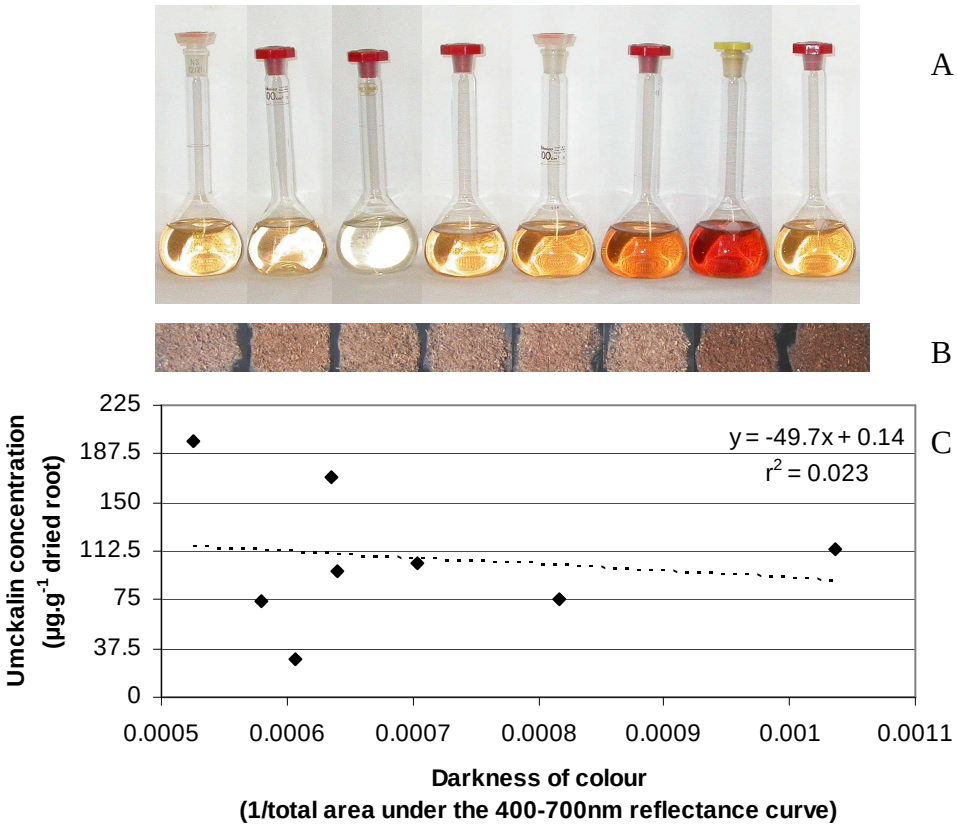


Figure 4.6: The relationship between the darkness of powdered root colour and umckalin concentration of old root collected from the KR harvest site (C). p = 0.722. A shows the colour of the ethanolic extracts produced from the powdered dried root (B), both observed under fluorescent lighting. Each data point on C corresponds to one of the ethanolic extracts and powdered root samples shown in A and B from left to right, respectively.

4.3.3 Replant survival and regrowth after harvest

This investigation was conducted in order to determine the effect of harvest season and local climate on the survival of treated plants post-harvest. The emergence of leaves after replanting was taken as an indication of replant survival.

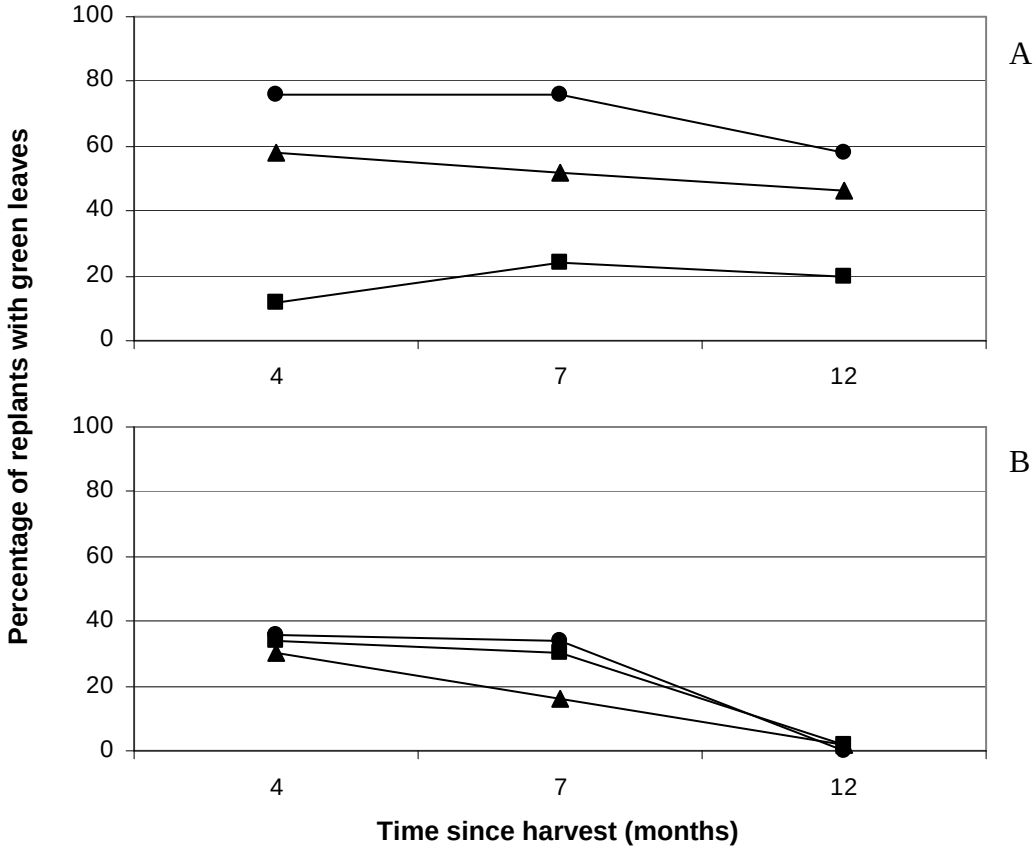


Figure 4.7: Presence of green leaves on replants observed three times following autumn harvest at the GFRR (A) and KR sites (B). Control (CN), 20% leafless plant without Stockosorb™ (NS) and 20% leafless plant with Stockosorb™ (SA) treatments are represented by circles, squares and triangles, respectively. The maximum likelihood X^2 value = 8.85, df = 16 and p = 0.919 for the log-linear model describing the combined frequency data of A and B. Four, seven and twelve months after harvest correspond to the months of August, December and May; and the seasons spring, summer and autumn, respectively.

The p-value of 0.919 for the combined autumn harvest survival data (Figure 4.7) confirmed that the specified model was sufficient to explain the frequencies observed in the harvest data. The relationships between the three treatments following the autumn harvest in the GFRR (Figure 4.7A) show a clear and consistent trend throughout the year following harvest. The observed percentage survival was consistently the highest in the CN treatment followed by the SA treatment and then the NS treatment. The percentage survival of GFRR replants was significantly affected by treatment ($\Delta X^2 = 86.49$, $\Delta df = 3$, $p < 0.001$), but did not vary significantly between observation times ($\Delta X^2 = 0$, $\Delta df = 6$, $p > 0.05$). In contrast to the GFRR site, replant survival at the KR site was significantly affected by time after harvest ($\Delta X^2 = 228.64$, $\Delta df = 6$, $p > 0.05$) and not treatment ($\Delta X^2 = 2.78$, $\Delta df = 4$, $p > 0.05$). Owing to the drier conditions at the GFRR site, the effect of adding Stockosorb™ (SA) on the survival of replants compared to treatment without Stockosorb™ (NS) was more pronounced than that seen at the higher rainfall KR site. However, empty replant holes and evidence of plant labels in cow dung suggested that the decrease in the percentage survival of replants was due to grazing by local cattle, compromising the experimental results. The autumn harvest results (combined GFRR and KR) also revealed that the interaction of treatment, site and replant survival was significant ($\Delta X^2 = 87.56$, $\Delta df = 8$, $p < 0.001$), as was the interaction of site, observation time and replant survival ($\Delta X^2 = 67.29$, $\Delta df = 8$, $p < 0.001$). The above two analyses suggest that harvest site was a significant factor, although the effect of site alone on replant survival could not be analysed. The survival of replants following harvest in summer, at both sites, is shown in Figure 4.8.

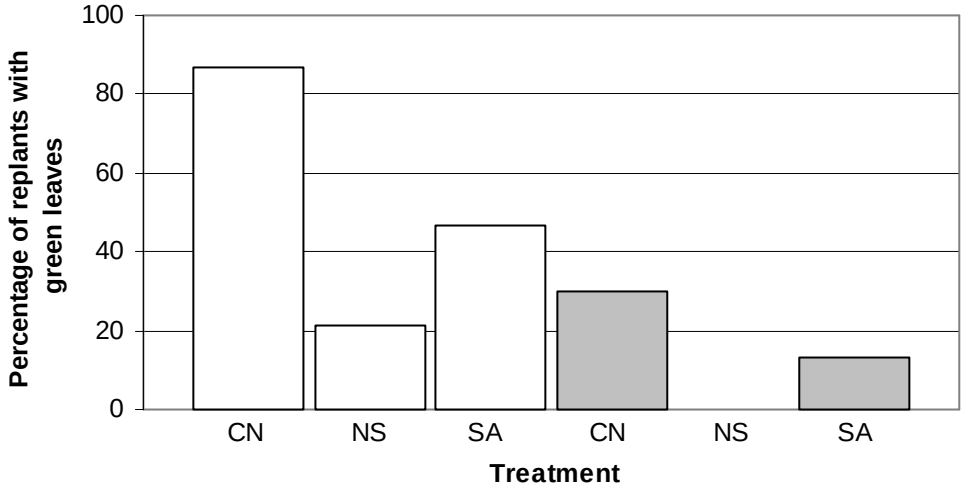


Figure 4.8: Presence of green leaves on replants observed four months after summer harvest at the GFRR (White) and KR sites (gray). CN, NS and SA represent the control, 20% leafless plant without Stockosorb™ and 20% leafless plant with Stockosorb™ treatments, respectively. The maximum likelihood X^2 value = 1.29, df = 4 and p = 0.862 for the log-linear model describing the combined frequency data of the two sites.

Unlike the analysis of the autumn harvest data, the effect of site alone on replant survival could be tested for the summer harvest data. Log-linear analysis of the combined GFRR and KR summer harvest data (Figure 4.8) revealed that site had a significant effect on the percent survival of treated plants four months after harvest (ΔX^2 = 14.49, Δdf = 2, $p < 0.001$). Additionally, the survival of replants in the CN treatments was the highest at both sites followed by the SA and then the NS treatments. These trends were the same as those observed following the autumn harvest in the GFRR (Figure 4.7A). The effect of treatment on the percentage survival of replants was found to be significant at both sites (X^2 = 6.35, df = 2, $p < 0.05$ and X^2 = 12.33, df = 2, $p < 0.003$ for the GFRR and KR treatments, respectively). Like the autumn harvest treatments, the survival of replants in all summer harvested GFRR treatments was higher than that of the KR treatments. This may have been influenced by livestock grazing at the KR site.

The average root and leaf production of replants that survived the autumn and summer harvests, at the GFRR site, are shown in Figures 4.9-4.12. Too few plants survived the harvests at the KR site for the same analyses to be performed.

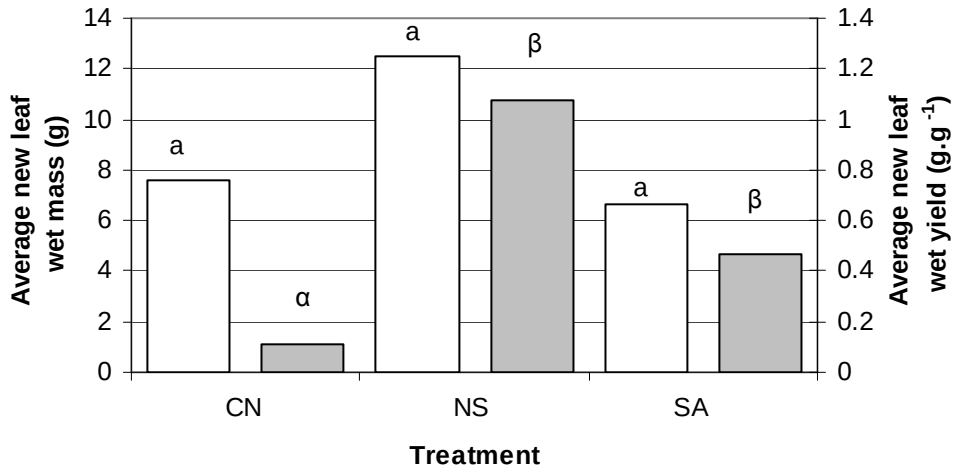


Figure 4.9: Average leaf production measured one year after the autumn harvest in the GFRR. Leaf production was expressed as the average wet mass produced (white bars, primary y-axis) and the average wet mass yield produced per original wet gram of the replant mass (shaded bars, secondary y-axis). a represents a statistically homogeneous group within the average mass data at the 95% confidence level, $p = 0.235$, $F = 1.50$. α and β represent statistically homogeneous groups within the average yield data at the 95% confidence level, $p < 0.0006$, $F = 9.24$. $N = 19$, 4 and 20 for CN, NS and SA treatments, respectively.

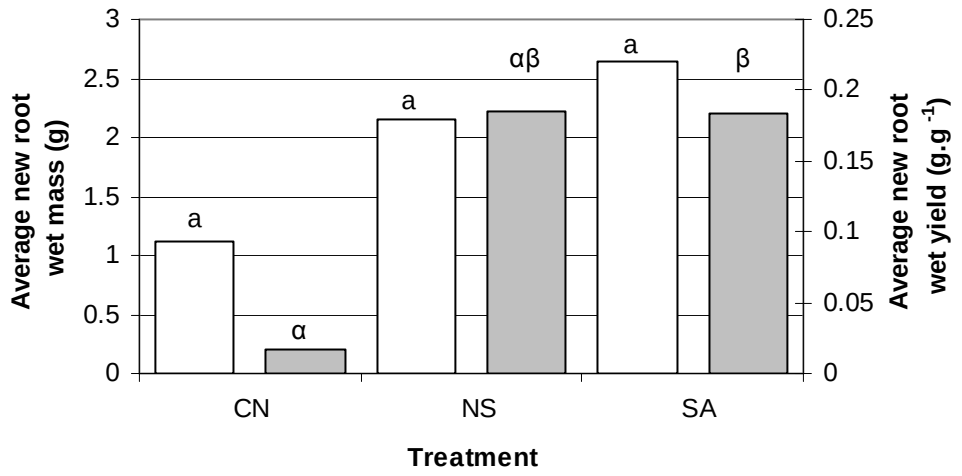


Figure 4.10: Average root production measured one year after the autumn harvest in the GFRR. Root production was expressed as the average wet mass produced (white bars, primary y-axis) and the average wet mass yield per original wet gram of the replant mass (shaded bars, secondary y-axis). a represents a statistically homogeneous group within the average mass data at the 95% confidence level, $p = 0.203$, $F = 1.66$. α and β represent statistically homogeneous groups within the average yield data at the 95% confidence level, $p = 0.003$, $F = 6.63$. N = 19, 4 and 20 for CN, NS and SA treatments, respectively.

Figures 4.9 and 4.10 show that both root and leaf yields were significantly higher for the SA plants than the CN plants and not significantly different between the SA and NS treatments. This was the result of higher leaf masses amongst the CN replants coupled with a lack of significant difference between the average root and leaf biomass produced between the three treatments. Relative to mass, the SA treated plants produced more root biomass than CN treated plants. The implication is that higher root mass (CN treatments) aided the survival of replants but did not promote regrowth. Additionally, these results show that new leaf production was dependent on photosynthate produced subsequent to replanting rather than re-allocation of carbon from the roots. The addition of Stockosorb™ (SA) did not significantly affect new biomass production over the treatment without Stockosorb™ (NS). The insignificant difference between the NS root yield and that of the CN treatment may be accounted for by the high variability of new biomass production and the low number of plants that survived the NS treatment.

The average wet leaf yield produced per gram wet replant mass by greenhouse cultivated plants was 2.02 g (± 2.16 g), approximately four times higher than plants in the SA treatment of the GFRR autumn harvest. The average wet root yield produced per gram wet replant mass by greenhouse cultivated plants (see Chapter 5) was 1.1 g (± 2.01 g), approximately six times higher than plants in the SA treatment of the GFRR autumn harvest.

The results following the GFRR autumn harvest contrasted with those following the summer harvest at the same site. No significant difference was found between the average wet leaf and new root yields of surviving plants in the CN and SA treatments following the GFRR summer harvest (Figures 4.11 and 4.12). This result implies that harvest time had an effect on subsequent root and leaf production, especially for CN treated plants. Insufficient data was available for comparison of the summer CN and SA treatments with the NS treatment at the GFRR site.

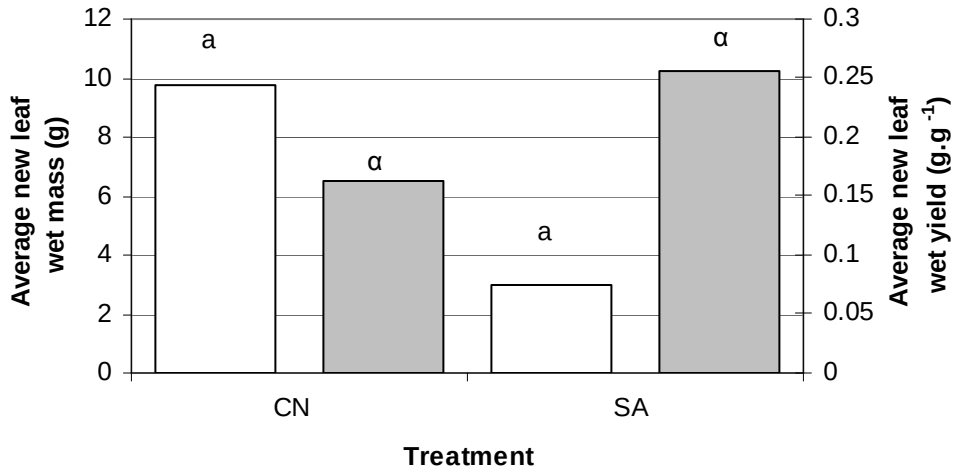


Figure 4.11: Average new leaf production measured four months after the summer harvest in the GFRR expressed as the average wet mass produced (white bars, primary y-axis) and average wet mass yield per wet gram replanted (shaded bars, secondary y-axis). a represents a statistically homogeneous group within the average mass data at the 95% confidence level, p = 0.346, F = 0.94. α represents a statistically homogeneous group within the average yield data at the 95% confidence level, p = 0.433, F = 0.65. N = 13 and 6 for CN and SA treatments, respectively.

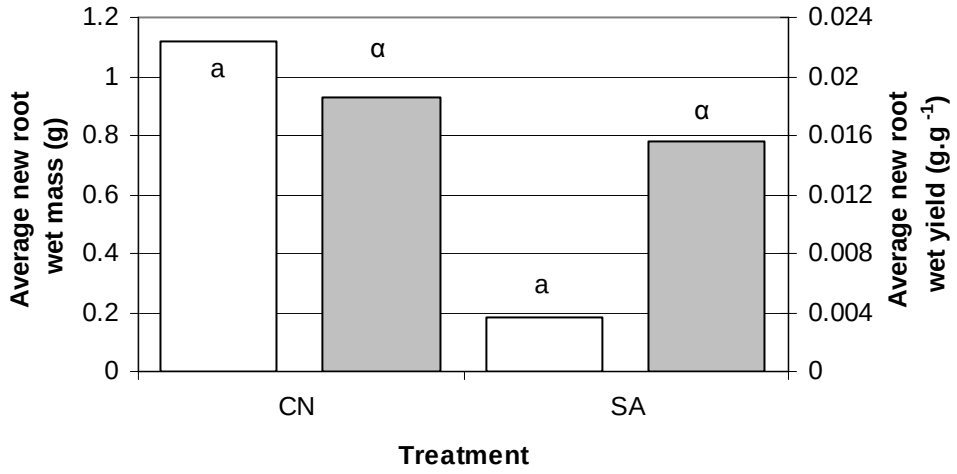


Figure 4.12: Average new root production four months after the summer harvest in the GFRR site expressed as the average wet mass produced (white bars, primary y-axis) and average wet mass yield per wet gram replanted (shaded bars, secondary y-axis). a represents a statistically homogeneous group within the average mass data at the 95% confidence level, $p = 0.826$, $U = 36.5$, $Z = 0.22$. α represents a statistically homogeneous group within the average yield data at the 95% confidence level, $p = 0.693$, $U = 34.5$, $Z = -0.40$. $N = 13$ and 6 for CN and SA treatments, respectively.

4.3.4 Root regeneration and respouting

Root regeneration was extrapolated beyond the measured annual growth using a mathematical modeling technique, as described above.

The first step in modeling the growth of replants was to assess the relationship between the mass planted and the root mass produced after one year. Figure 4.13 shows this relationship for the CN and combined NS and SA treatments (20% replants) following the autumn harvest in the GFRR and for 20% replants that were grown under greenhouse conditions. The CN data produced the strongest relationship ($r^2 = 0.65$) when compared to the field-grown 20% replants ($r^2 = 0.026$) and greenhouse-grown 20% replants ($r^2 = 0.046$). The root growth by field-grown 20% replants was higher than that of CN replants post-harvest (Figure 4.10). Hence, a potential limitation of using the trendline fitted to the CN data was that it may not accurately represent the regrowth of 20% replants. However, as the 20% replants showed no viable relationship between replant mass and subsequent annual root production, the trendline fitted to the CN data, together with 95% confidence limits, was used for all subsequent modeling purposes. The relationship between this trendline for control (CN) field replants and the 20% field and greenhouse replants was shown by superimposing it on the 20% replant data (Figure 4.13 B and C).

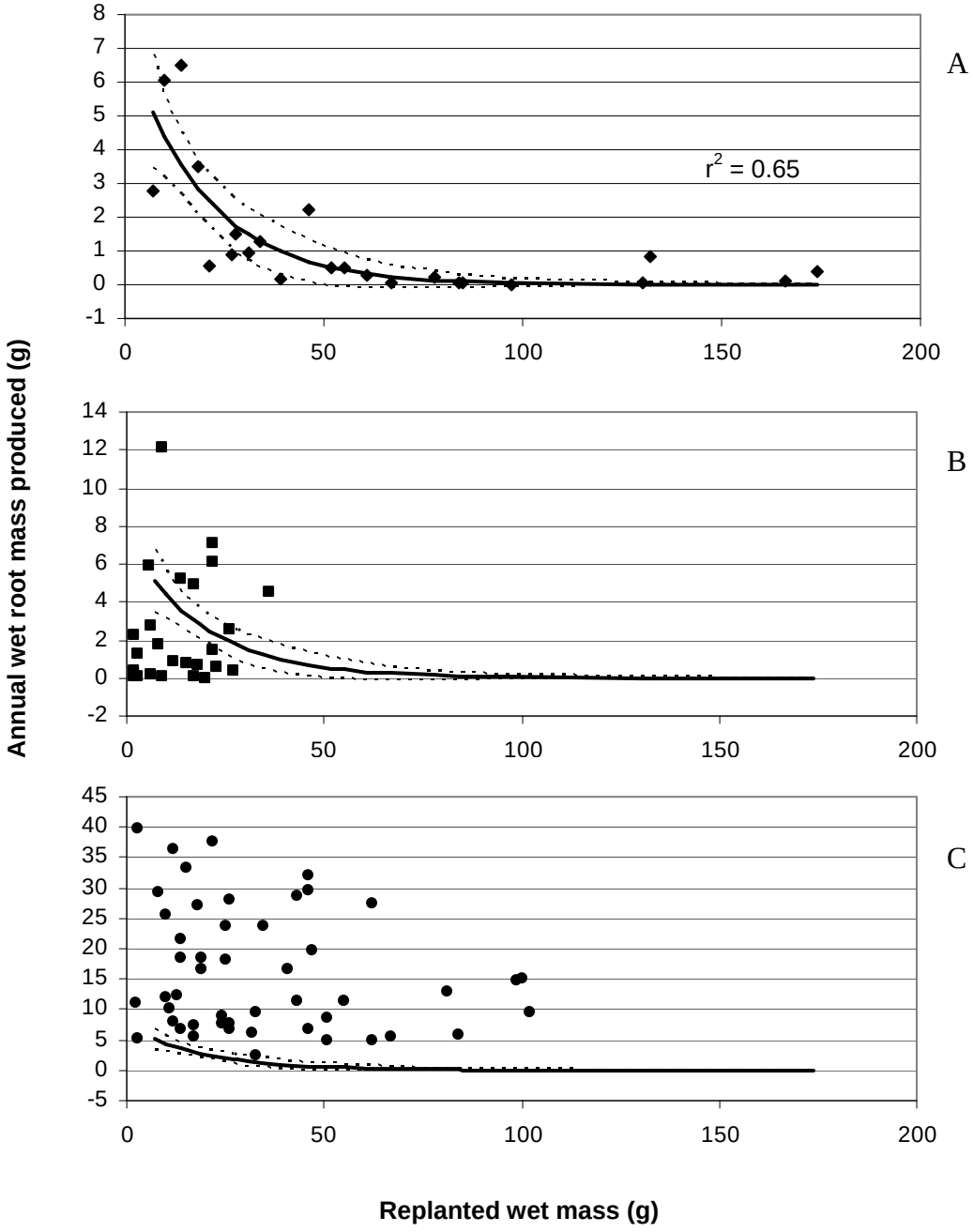


Figure 4.13: Relationship between replant mass and subsequent annual mass of root produced by plants in the CN (diamonds - A) and 20% replant (squares - B) treatments following the GFRR autumn harvest and 20% replants under greenhouse cultivation (circles - C). The line describing the relationship for the CN data (solid line) is fitted to each graph, $y = 7.37^{-0.0516x}$, with 95% confidence intervals (dashed lines).

The equation of the trendline fitted to the CN data (Equation 1) was chosen, based on its r^2 value, for use in modeling the regrowth rate of 20% replants over time (Figure 4.14). The relationship between the mass planted and the root mass produced for cultivated plants was not strong enough to warrant further investigation ($r^2 = 0.046$). Furthermore, the average wet root yield of greenhouse cultivated 20% replants was *ca.* 67 times greater than that of CN replants and *ca.* six times greater than field grown 20% replants. As such, Equation 1 was used to determine the annual mass increment on the average 20% replant mass under field conditions only. These increments were added to the respective average replant masses and the process repeated to produce the modeled growth curve for 20% replants under field conditions (line A) in Figure 4.14. The average mass of the NS and SA replants that produced root subsequent to harvest, 13.4 g, was used as the starting mass for the curve.

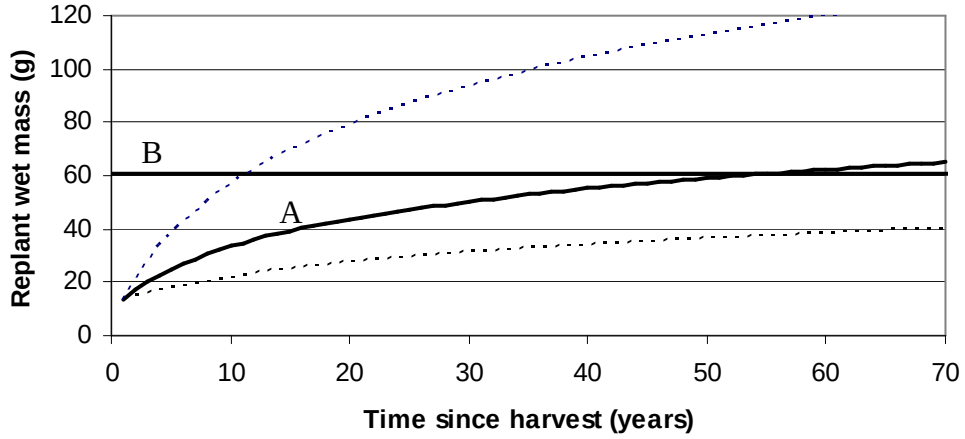


Figure 4.14: The estimated regrowth of 20% replants over time under field conditions (A) modeled using equation: $y = 7.37^{-0.0516x}$ (Equation 1). Line (B) marks the average pre-harvest mass (61.1 g) of the 20% replants that survived till one year after the GFRR autumn harvest. The upper and lower estimations (dashed lines) were created by substituting the upper and lower 95% confidence values of the fitted parameters a and b into the equation used to fit line A, where $y = a^{-bx}$.

The average time estimated for 20% replants to regrow to the average pre-harvest mass was *ca.* 56 years under field conditions (Figure 4.14). The upper and lower limits show that the estimated regeneration time could range from *ca.* 11.5 to an unrealistic *ca.* 410 years. The *ca*. six times greater wet root yield under cultivation lends support to the lower estimated regeneration time of 11.5 years. The average regeneration time and range were obtained by inspecting the curve and noting where the lines plotting the average regrowth and upper and lower 95% confidence limits crossed line B, the average pre-harvest 20% replant mass (Figure 4.14).

Between three and nine resprouts, from the 50 plants harvested, were noted during the three field site visits following the autumn harvest at the KR site. A similar result was found at the GFRR site where between three and five resprouts were observed during the same visits. Two resprouts were observed four months after the summer harvest of *ca.* 15 plants per treatment at the GFRR site and none were observed four months after the same harvest at the KR site. Hence, the regeneration of harvested *P. sidoides* populations by resprouts seems unlikely.

4.4 Discussion

The ultimate aim of the research described in this chapter was to investigate the effects of harvest season, different harvesting practices and habitat on the regrowth and survival of replanted *P. sidoides* plant parts after harvest. Novel harvest techniques were designed and tested in order to address the lack of information concerning traditional harvest.

The three treatments employed showed a clear and significant relationship to survival when performed on plants from the GFRR site (Figure 4.7). The CN treatment resulted in the most number of replants surviving the harvest; this was most likely due to the fact that these plants remained relatively undisturbed besides the removal of their leaves. The cause of death amongst the 20% replants was most likely due to the loss of root mass and the resultant loss of water reserves to supply transpiration and counteract dehydration. This hypothesis is supported by the higher percentage survival of the SA treated plants compared to NS treated plants at the GFRR site following both the autumn and summer harvests and the KR summer harvest. The seasonal loss of leaves by non-treated wild plants at the KR site (White, 2004, *pers. obs.*) may have had a compounding effect on the observed survival of replants as survival was judged on the presence and absence of leaves. However, the results from the GFRR treatments confirm that the addition of Stockosorb™ to the soil surrounding replants was effective in increasing their survival post-harvest in a xeric succulent thicket environment. The use of Stockosorb™ or a similar product should therefore be considered by commercial operators in the industry and included in potentially mitigating harvest techniques investigated in the future.

Fungal infection and livestock grazing may have contributed, as confounding factors, to the death of replants. Roots that were collected and returned to the Botany Department showed fungal growth if they were not dried a few days after harvest (White, 2004, *pers. obs.*). Additionally, the KR autumn harvest was conducted during wet weather; conditions the WHO *et al.* (1993) recommended avoiding.

Evidence for the interaction between replants and livestock, particularly cows, at the KR site may have been significant, thus compromising the results following both the autumn and summer harvest. This result, however, is of important consequence as *P. sidoides* is often harvested on communal rangelands where livestock grazing is common. Following confirmation of this observation, it may be necessary to account for the presence of livestock in the development of suitable conservation strategies. The thick grass cover and relatively high rainfall would be expected to have minimized plant water loss and thereby promoted the survival of replants, had the KR experiment not been compromised.

The relative leaf and root yields per replanted mass were significantly different between the three treatments (Figure 4.9 and Figure 4.10; for leaf and root yields, respectively). In contrast, the average absolute mass of leaves and root produced by replants was not significantly different (Figures 4.9 and 4.10; for leaf and root masses, respectively). The SA treated replants produced a significantly higher root yield compared to the CN plants, and replants in both 20% replant treatments produced significantly higher leaf yields than CN replants. These results show that the 20% replants produced significantly more root and leaf mass than CN plants relative to replant mass. This allocation of carbon by 20% replants agrees with the balanced-growth strategy of carbon allocation (Shipley and Mezaine, 2002). Balanced-growth simply describes the preferential allocation of biomass, by plants, to plant organs that collect growth-limiting resources. Under balanced-growth, a water-limited plant, like the replants that had root removed, would be expected to produce more root, the plant organ responsible for collecting water. The only exception was the average root yield of the NS treated plants which was not significantly different to the control. This can be attributed to the low number of replants that survived the NS treatment and higher variability of the yields between the individual plants.

The yield of root and leaf biomass was much higher for 20% replants grown under greenhouse as opposed to field conditions (four and six times greater for leaf and root yields per replant mass, respectively).

These results, coupled with the low survival rates of field grown 20% replants, long root regeneration time (*ca.* 11.5 to 410 years) and low regeneration from resprouts raise questions about the sustainability of wild harvest. Additionally, they highlight the need to know the size of the available resource in the wild. Determining the wild resource size and the effects of these and other harvest treatments on the survival of plants returned after harvest would aid decision-makers in judging whether or not this species can be harvested sustainably from the wild. A similar rationale was presented by Rock *et al.* (2004) following their study on the sustainability of harvesting whole wild *Allium tricoccum* plants for their bulbs. The authors assessed the effects of removing between 25 and 100% of the total number of *A. tricoccum* plants from different sites within protected areas in the southernmost parts of the species' distribution range (Appalachian region, USA). They used a population growth model and found that population recovery could be affected by harvests as low as 5%. As a result, the authors recommended further research in other areas of the species' distribution range, improved monitoring of private party harvests and greater enforcement of harvest limits by way of a permit system.

The widespread occurrence of *P. sidoides* across a variety of climate and vegetation types (Dreyer and Marais, 2000; see Chapter 1, Figure 1.2) necessitates the gathering of harvest response data as well as population dynamics information for each proposed commercial harvest site. The distribution of *P. sidoides* populations within several South African provinces and Lesotho will also require streamlining provincial legislation concerning indigenous plant conservation and harvest so that over-exploitation is stopped completely and not only moved from one province to another. *P. sidoides* has been harvested for international export from populations in the Eastern Cape Province of South Africa and in Lesotho (Smith, 2006, *pers. comm.*). Once appropriate legislation is implemented for the protection of *P. sidoides* in the Eastern Cape, it will therefore be necessary for the other provinces and Lesotho to follow suit with similar legislation. This will help facilitate the protection of the wild *P. sidoides* resource as a whole and prevent the shift of over-exploitation from one province or country to another in the future.

The importance of this rationale was highlighted by Mayer *et al.* (2006) in their study of tree harvest, for timber, in the boreal forests of south-eastern Finland and just over the border in north-western Russia. The authors suggested that the resources of both regions would be best conserved if a large scale, co-ordinated conservation strategy was implemented. The authors stated that the strategy should be constructed to address long-term conservation goals and wood consumption, forest industries, logging practices and trade. These principles will be useful in creating a strategy for the sustainable use and conservation of *P. sidoides*.

The relationships between root colour and umckalin concentration, and leaf and root mass were investigated based on recommendations given to harvesters by middlemen in the industry. Leafless plant mass (made up mostly of root) was not found to be related to leaf mass (Figure 4.5). Two alternative strategies of biomass allocation are balanced-growth or allometric growth (Shipley and Mezaine, 2002). Under balanced-growth, the proportion of roots to shoots (root:shoot ratio) is constant for a given species under certain conditions. Under allometric growth this ratio is hypothesized to change as a given plant ages. The relationship between leafless plant mass and leaf mass shown in Figure 4.5 is therefore best explained by the allometric growth hypothesis. However, the relationship between these two variables may be influenced by the seasonal loss of leaves by plants (White, 2004-2005, *pers. obs.*). The leaf growth on the plants at any one time only represents the growth of a particular season while the root growth represents the growth accumulated over many seasons, complicating the assessment of root biomass by leaf mass. Additionally, the production of leaves may vary from season to season depending on the rainfall received. The influence of water provision on root growth can be inferred from the relationship between replant mass and annual root mass produced by 20% replants grown under field (lower water provision) and greenhouse (higher water provision) conditions (Figures 4.13B and C). These figures show that greenhouse cultivated replants produced more root mass, relative to replant mass, than field grown plants. Additionally, much higher variation in root mass production was shown amongst the greenhouse cultivated replants (Figure 4.13C).

Moreover, the following observation of plant leaf masses reveals a potential upper limit of leaf production despite the range of root masses (Figure 4.5). Only three of the 40 plants from the KR summer harvest had fresh leaf masses above 15 g. Similarly, only one summer harvested GFRR plant had a fresh leaf mass above 20 g. The majority of plants in the autumn GFRR harvest had fresh leaf masses of 20 g and below (138 out of 150). Above ground biomass is therefore not a good indicator of below ground root biomass. As a result, it may be necessary for harvesters to adopt a different method of selecting plants. One example would be to harvest only every second or third plant across a large area, as opposed to harvesting all the plants from a limited area and leaving other areas unharvested.

Root colour and umckalin concentration produced a very weak relationship (Figure 4.6), thus challenging the instruction by middlemen for harvesters to harvest darker as opposed to lighter coloured roots. The observed brown to red colour is most probably due to the presence of tannins and would potentially mask the pale yellow colour of umckalin. The presence of equivalent levels of umckalin in very light brown new roots compared to the dark red older root growth confirmed the lack of relationship observed (see Chapter 5). It would be valuable to compare the colour of roots and the biological activity of the extracts made from different coloured samples as umckalin does not account for the entire biological activity of root extracts (Kayser and Kolodziej, 1997; Kolodziej *et al.*, 2003; and Seidel and Taylor, 2004). The relationship between root colour and biological activity therefore requires further investigation before the plausibility of the middlemen's instruction can be ruled out completely. On the socio-economic level, it may be important for a governmental or non-governmental organisation to investigate the fairness of the trade between harvesters and middlemen.

The results of the study presented in this chapter and future studies will help develop an increased understanding of the ecological and environmental impacts of *P. sidoides* harvest and the management structures that are needed to promote sustainable wild harvest. The wild plant recovery data presented here for *P. sidoides* coupled with the low number of resprouts and the higher root yield by cultivated replants indicate that greenhouse cultivation may provide a more sustainable source of roots than wild harvest to supply local and international markets in the future. The potential for producing a viable *P. sidoides* root harvest from cultivated plants forms the subject of Chapter 5 to follow.

Chapter 5

Greenhouse Cultivation

5.1 Introduction

5.1.1 Guidelines for medicinal plant cultivation

The WHO *et al.* (1993) recommended that medicinal plants be cultivated, wherever possible, as the source of supply for the market. The most prominent advantage of cultivation over wild harvest is the potential for reducing the quantity of material extracted from the natural environment. The use of plant breeding techniques can also provide plants that are more genetically uniform, potentially resulting in less chemical variation in final products. The growth of plants under controlled conditions, most notably under irrigation, can increase the biomass yield and decrease chemical variability that may otherwise have resulted from environmental variation. Additionally, there would be less chance of misidentifying plants under cultivation; this would be especially useful given the similarity of *P. sidoides* and *P. reniforme*. The guidelines presented by the WHO *et al.* (1993) include sourcing the most suitable genetic material for propagation, the genetic improvement of the stock, finding optimal cultivation conditions and the best form of propagation; and finding ways to protect plants from pests and diseases and control weeds. The WHO *et al.* (1993) also recommended identifying the best time of harvest, considering the possibility of mechanization and developing an optimal system for post-harvest storage.

This study focused on the prospects of *P. sidoides* wild harvest and cultivation. As such, the following investigations of root cultivation under greenhouse conditions encompassed some of the above guidelines recommended by the WHO *et al.* (1993).

These included the selection of suitable plants from the wild, irrigated cultivation, investigating the effects of various treatments on root umckalin concentration and measuring the variation of root umckalin concentration between plants cloned from the same parent material.

5.1.2 Variation in plant secondary metabolite production

The yield and composition of secondary metabolites within a species may vary between plants from different geographical locations and may be influenced by environmental and genetic differences. A number of studies have investigated this phytochemical variation and revealed differences within or between populations, as in the following examples for *Melaleuca alternifolia* (Australian tea tree), *Echinacea* spp. (purple coneflower), *Aloe ferox* and *Paeonia lactiflora* (paeony), all important medicinal plants (Homer *et al.*, 2000; Binns *et al.*, 2002; and Wills and Stuart, 1999; Van Wyk *et al.*, 1995; and Kim *et al.*, 2006; respectively).

Homer *et al.* (2000) used the chemical variation of three bioactive markers, terpinolene, 1,8-cineole and terpinen-4-ol, in leaf oil extracts to classify *M. alternifolia* plants into three geographically distinct regions. Additionally, the authors' study of 615 individual plants at 41 sites identified six chemotypes within the distribution range of *M. alternifolia*. In their paper, Homer *et al.* (2000) used the term chemotype to classify plants of distinct chemical composition. Most of the six chemotypes were found throughout the distribution range in south-eastern Australia. However, the authors found a definite correspondence between location and oil chemotype. This observed variation was accredited to genetic rather than environmental differences between the locations (Penfold *et al.*, 1948 in Homer *et al.*, 2000; and Butcher *et al.*, 1992 and 1994). Although the above authors ascribe the differences in chemical composition to genetic variation, comparison with the works of Binns *et al.* (2002) and Wills and Stuart (1999) suggests that genetic and environmental effects may depend on the plant species and compounds under investigation.

Binns *et al.* (2002) surveyed the variation of alkylamide and cichoric acid concentrations in *Echinacea angustifolia* root extracts of plants collected from wild populations in North America, the indigenous habitat of *E. angustifolia*. The authors' findings were based on the qualitative phytochemical analysis of populations grown from seed sourced from nine different regions. These findings provided evidence supporting the existence of chemotypes within the species. Interestingly, Binns *et al.* (2002) found a positive relationship between increasing phytochemical quantity and increasing latitude of wild parent populations. This relationship held for certain compounds while the inverse relationship was shown for certain others. Similarly, Wills and Stuart (1999) found that alkylamide levels in root and aerial samples of *E. purpurea* were significantly higher in samples from populations cultivated north of the 32 °S latitude than those cultivated south of this division in Australia. The authors noted that differences in daylight hours and annual distribution of rainfall may have accounted for the variation. However, the sample size was too small to fully determine the effects of region on alkylamide concentration. Additionally, while alkylamide concentration changed with latitude, the composition of alkylamides in extracts was not affected in the same way. No distinction was observed in the cichoric acid levels north and south of 32 °S. Similarly, Van Wyk *et al.* (1995) found that the composition of the four major compounds in *A. ferox* leaf exudate was invariable across the natural distribution range of the species in South Africa. The concentration of the main purgative principle, aloin, was clearly related to location, although no trends with other geographical variables were identified.

A similar study to that reported here for *P. sidoides* was conducted by Kim *et al.* (2006) for *Paeonia lactiflora*, a traditionally used medicinal plant indigenous to Korea. The authors used a gradient High Performance Liquid Chromatography (HPLC) method to quantify the variation of active compound concentrations in root extracts. Large variation was identified within a set of 12 samples collected from regional markets across Korea. Additionally, the concentrations of the two known active ingredients were observed to change independently of one another in response to changes in season.

The root concentration of one compound, paeoniflorin, showed little variation during the year while the concentration of a second, albiflorin, was highest in spring and decreased dramatically to a lower level after the onset of summer. The concentration of both compounds in cultivated plants was higher in root extracts compared to extracts made of leaf and branch material, thus supporting the traditional use of roots.

5.1.3 Coumarin production by plants

Cultivation provides the potential to grow plants under environmental or physiological conditions that may promote the production of desired bioactive secondary metabolites. The concentration of umckalin in the roots of cultivated *P. sidoides* plants was therefore expected to increase following the application of treatments based on knowledge presented in the literature of the role of coumarins in plants, of which umckalin is an example.

Coumarins, the class of compounds to which umckalin belongs, have a range of functions within plants. For example, the production of coumarin phytoalexins, plant produced antifungal agents (Bailey, 1999), has been shown in an allelopathic function following fungal infection (Urdangarín *et al.*, 2004 and Uritani, 1999). Here, allelopathy involves "the release into the environment by an organism of a chemical substance that acts as a germination or growth inhibitor to another organism" (Allaby, 1998). Godoy *et al.* (2005) tested a range of eight coumarins and found five to inhibit the symbiotic fungus of leaf-cutting ants. The typical coumarin (1,2-benzopyrone), scopoletin (6-methoxy-7-hydroxycoumarin) and other coumarin derivatives have also been shown to have varying inhibitory effects on the growth of root cells (Avers and Goodwin, 1956; Goodwin and Taves, 1950; Kupidlowska *et al.*, 1994; and Pollock *et al.*, 1954). Additionally, Abenavoli *et al.* (2004) showed that 1,2-benzopyrone had a varying effect on the carbon allocation between three different root types in maize plants, while not affecting biomass accumulation.

The authors' findings on the selective effect of 1,2-benzopyrone led to the suggestion that it may favour tap-rooted over fibrous-rooted species and therefore influence plant composition within communities (Abenavoli *et al.*, 2004).

Water stress and wilting (Ojala, 2001), in addition to mechanical damage, are possible conditions that result in the production of coumarins. It must be noted here that Ojala only hypothesized that water stress and mechanical damage enhance coumarin production and did not provide any experimental evidence in support of this hypothesis. No other scientific evidence for water-stress-induced coumarin production has subsequently appeared in the literature. As such, the effects of two water stress treatments on root umckalin concentrations were investigated here. The first, a rapid water stress (RWS) treatment, was designed to provide extraordinary water stress and mimic a potential agricultural practice. The practice involves farmers removing a *P. sidoides* crop from the ground and leaving them on the soil surface in the sun to transpire for two days to stimulate water stress. The second was a prolonged water stress (PWS) treatment designed to represent plants subjected to a natural one month drought. Leaf water potentials (Ψ_{leaf}) were assessed throughout the above experiments using a pressure bomb apparatus (Scholander *et al.*, 1965) to indicate the water stress experienced by plants. The same technique was followed by Alcocer-Ruthling *et al.* (1989) and Pegoraro *et al.* (2004) where a decrease in Ψ_{leaf} was used as an indicator of increasing water stress. Ψ_{leaf} is a "measure of the energy available in an aqueous solution to cause the migration of water molecules across a selectively permeable membrane during osmosis" (Bailey, 1999). As such, the smaller (more negative) the Ψ_{leaf}, the greater the available energy to drive the transfer of water between cells. All plants in the RWS experiment were rehydrated each evening so that water potential gradients between the leaves and the medium surrounding the roots could reach equilibrium before measurements were made the following morning (Wei *et al.*, 2000). Having the water potential gradients in equilibrium is a necessity when using the pressure bomb apparatus (Scholander *et al.*, 1965).

In addition to environmental factors, plant hormones may increase the production of coumarins *in vivo* as shown by Pastírová *et al.* (2004) and Kai *et al.* (2006) with salicylic and 2,4-dichlorophenoxyacetic acid, respectively. Additionally, preliminary studies presented here (Figure 5.14A) showed a relationship between dry inflorescence mass and the concentration of umckalin in cultivated roots. As plant flowering is closely regulated by hormones, including gibberellic acids (GAs), indoleacetic acid (IAA), abscisic acid (ABA) and cytokinins (e.g. de Melo Ferreira *et al.*, 2005; Koshita and Takahara, 2004; Koshita *et al.*, 1999; and Sirikul and Luukkanen, 1987), the effects of cytokinin and gibberellic acid treatments on root umckalin concentrations were investigated here.

5.1.4 Field sites

In order to identify the most suitable plant material for propagation, the geographical variation of *P. sidoides* root umckalin concentration was measured for plants from five sites representing three different regions of the Eastern Cape Province, South Africa. Two sites each were chosen in the vicinity of the Greater Fish River Reserve (GFRR) and Kei Road (KR) sites described in Chapter 4 and one site was chosen in the Peddie district (33° 13’ 17.7” S, 27° 03’ 55.5” E). The three regions differed widely according to average annual rainfall. The highest average annual rainfall was received at the KR sites (760 $mm.yr^{-1}$), followed by the Peddie site (513 $mm.yr^{-1}$, 1878-1991 average) and the GFRR sites (397 $mm.yr^{-1}$). Rainfall data for the Peddie site was recorded by the South African Weather Bureau (SAWB) and data for the GFRR and KR sites was recorded as described in Chapter 4, Figure 4.4. Similar to the GFRR sites, the vegetation at the Peddie site consisted of xeric succulent thicket (Low and Rebelo, 1998); however, the latter site was not in a protected area and was open to domestic livestock grazing. In addition to the above collections, plants were collected from the KR and GFRR sites for use in the greenhouse cultivation experiments described below. The location of each of the above sites is shown in Figure 4.1 with reference to key Eastern Cape towns and cities (see Chapter 4).

5.1.5 Aims, rationale and hypotheses

The aims of the research described in this chapter were:

1) To assess the relationship between *P. sidoides* root umckalin concentration and local climate.

Plant and soil samples were collected from three locations of distinct average annual rainfall and the average umckalin concentration of dried roots compared. It was hypothesized that the root umckalin concentration of plants from higher rainfall areas would be lower than that of plants from lower rainfall areas. Root mass was also measured, as the umckalin yield per plant, the product of root mass and root umckalin concentration, may influence the economic value of plants from various areas. Following the findings of Wang *et al.* (2006; see Section 4.1.2, Chapter 4), it was hypothesized that the roots of plants from higher rainfall areas would weigh more than those from lower rainfall areas.

2) To determine the effects of cultivation and various applied treatments on the root umckalin concentration of non-clonal *P. sidoides* plants.

Wild harvested plants were grown under greenhouse conditions for between 16 and 18 months before the application of one of five treatments. These treatments included rapid and prolonged water stress, two hormone treatments, and a root competition treatment with a fast growing annual (*Conyza albida*). Each treatment was hypothesized to increase root umckalin concentrations relative to well-watered controls.

3) To assess the effect of genetic uniformity on the variation of *P. sidoides* root umckalin concentrations.

Cuttings were made from the roots of individual wild parent plants and grown under greenhouse conditions. The variation of root umckalin concentrations amongst cultivated clonal plants was hypothesized to be lower than that amongst non-clonal wild plants.

5.2 Methods

5.2.1 Geographical variation of root umckalin concentration

At the end of April 2005, two sets of ten plants were collected from each of the GFRR and KR sites and one set of ten plants was collected from the Peddie site. Old root growth and new root growth were distinguished from one another on the basis of cortical tissue colour and root bark appearance (as in Chapter 4) and kept separate. Leaves, stems and root growth were dried and weighted. The umckalin concentration in new and old roots was analysed using the method described in Chapter 3. Soil samples were taken in triplicate from each site and their characteristics analysed by Matrocast Laboratories (Pty.) Ltd. (Brackenfell, South Africa).

5.2.2 Variation of root umckalin concentration under cultivation

Individual plants to be cultivated and used in the non-clonal treatments were collected from one of the KR sites. Plants to be used in clone experiments were collected from the vicinity of one of the GFRR sites. The non-clonal experiments will be discussed first (Section 5.2.2.1) followed by the clone experiments (Section 5.2.2.2).

5.2.2.1 Variation of root umckalin concentration amongst non-clonal plants

As the choice of collection site was made before the geographical variation in root umckalin concentration was known, plants for these experiments were harvested from the KR site. This followed the assumption that KR plants would have lower root umckalin concentrations compared to the other sites owing to the more temperate climate. Hence, it was anticipated that the KR plants would respond most positively to the imposed treatments, particularly those involving water stress. Plant collections were made from the KR site in April and August 2004 taking care to excavate as much of the intact roots as possible. Leaves were removed before plants were transported back to the Rhodes University Botany Department, Grahamstown. Each leafless plant was weighted before 80% of this leafless plant mass was removed as root. These 20% replants were then planted in locally sourced topsoil in 10 L black plastic nursery bags and grown in a plastic greenhouse tunnel (Figure 5.1).

Figure 5.1: Cultivated *P. sidoides* 20% replants following three months of growth under well-watered conditions in a greenhouse.

The arrangement of plants in the greenhouse was randomly changed occasionally to reduce the effects of spatial differences in light intensity and temperature. Soil samples from six bags were sent to Matrocast Laboratories (Pty.) Ltd. for analysis five months after planting. The results of these analyses are presented in Table 5.1.

Table 5.1: Average characteristics of the soil used in the non-clonal greenhouse cultivation experiments. N= 6.

pH	Carbon ($g.kg^{-1}$)	Nitrogen ($mg.kg^{-1}$)	Potassium (meq %)	Phosphorus ($mg.kg^{-1}$, citric)	Colour
8.26	29.8	2135.7	1.72	100.7	Dark brown
% course sand	% medium sand	% fine sand	% silt	% clay	Texture
12.8	14.6	59.4	13.3	0	Loamy sand

Cultivated plants were transplanted from the black bags to hard 8 L plastic pots in December 2004. In January 2005 these plants were moved to a second, cooler, tunnel as the temperature in the first tunnel reached 50 °C on some days causing many of the plants to wilt. Once in the second tunnel all plants produced new leaves, did not wilt again and were nearly continuously in flower till the end of the study. All plants were watered once every three or four days, and grew until mid September 2005 when the root umckalin concentration of three randomly selected plants was assessed. All three of these plants had been grown under cultivation for the same amount of time. This exploratory assessment showed that the umckalin concentration of new roots was not significantly different to that of old roots ($p = 0.623$, $F = 0.28$; Figure 5.2).

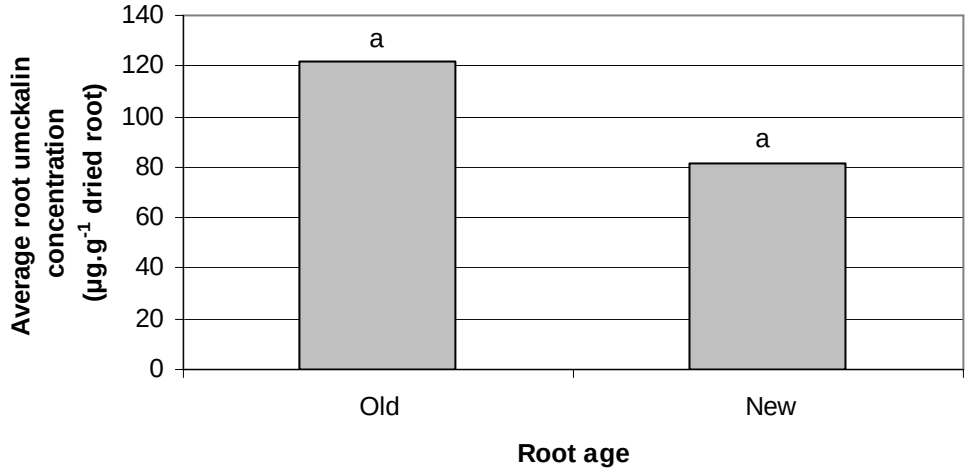


Figure 5.2: Average root umckalin concentration of new and old root growth from greenhouse cultivated plants. a represents a statistically homogenous group at the 95% confidence level. N = 3. p = 0.623, F = 0.28.

As such, the umckalin concentration of new root growth only was assessed after the following treatments, with the exception of the rapid water stress treatment.

5.2.2.1.1 Water stress treatments

Rapid water stress

This treatment was conducted using greenhouse cultivated plants exposed to high intensity lights (400 W, *ca.* 1140 µmol.m^{-2}.s^{-1}) and low humidity conditions generated by a Humex Turbo heater (*ca.* 28 °C and 36% relative humidity, Roberts Electrical Company).

Firstly, ten plants (five treated and five controls) were randomly selected from plants that had grown in the greenhouse for 18 months. Five intact plants had the soil carefully removed from their roots, were gently washed, dried and placed under the high light, low humidity conditions (rapidly water stressed-RWS plants). The control plants were subjected to the above conditions while remaining potted in the soil.

Air flow was deflected away from the roots of the RWS plants while control plants were arranged so that only their leaves were above the air flow level. A pressure bomb apparatus (Scholander *et al.*, 1965) was used to measure pre-dawn Ψ_{leaf} (Alcocer-Ruthling *et al.*, 1989; Pegoraro *et al.*, 2004 and Wei *et al.*, 2000) of single leaves from each plant before the treatment began. To avoid positional effects, plants were rotated clockwise through each position under the lights at intervals during the *ca.* 12 hours of light per day. Three sets of pre-dawn Ψ_{leaf} measurements were made over the two day duration of the treatments. Owing to limited space, the control plants were subjected to the same ambient conditions as those in the RWS treatment on the two days immediately following the RWS treatment. Ambient air humidity and temperature measurements were taken during both the control and RWS treatments and did not vary significantly between the two treatments. Plants from both treatments were rehydrated each evening by spraying them liberally with water, covering them with plastic bags and keeping them in the dark until pre-dawn the following morning. In addition to the water spraying, the roots of the RWS plants were carefully wrapped in water soaked cotton wool and paper towel.

Following the final pre-dawn Ψ_{leaf} measurement, plants were separated into inflorescence, leaf, stem, old and new root growth; weighed, dried and weighed again. New and old root growth was prepared for HPLC analysis as described in Chapter 3.

Prolonged water stress

Ten plants, five control and five treated, were randomly selected from the plants that had grown in the greenhouse for 18 months. Water was withheld from stressed plants (PWS treatment) for the duration of the one month greenhouse based experiment while controls were well watered every three or four days. Ψ_{leaf} and percent soil moisture content measurements were made for each potted plant at intervals during the course of the experiments using the pressure bomb apparatus (as above) and a Theta probe (type ML2x with a HH2 moisture meter, Delta-T Devices), respectively.

At the end of the experiment plants were separated into inflorescence, leaf, stem, old and new root portions; dried and weighed. New root was prepared for HPLC analysis as described in Chapter 3.

5.2.2.1.2 Hormone treatments

Two sets of eight plants each were randomly selected from the plants that had grown in the greenhouse for 16 months. 250 mg of benzyladenine (cytokinin) and gibberellic acid (both Sigma-Aldrich) were dissolved in small amounts of 5% aqueous NaOH and each hormone solution made up to 100 mL. 20 mL of each of these solutions was diluted with water to yield a final concentration of 25 $mg.L^{-1}$. These concentrations equated to 0.11 mM for cytokinin and 0.072 mM for gibberellic acid, agreeing with those used in the literature (25 $mg.L^{-1}$ for both hormones, Ranwala *et al.*, 2003; and 0.1 mM for cytokinin, Liu *et al.*, 2001). These solutions were applied to plants using individual spray-bottles for each hormone. 500 mL of solution was applied to isolated plants by spraying all leaves, both axial and abaxial surfaces, evenly until all solution was used up. During hormone application, plants from each treatment were isolated from those of the other treatments in order to prevent contamination through the air. Additionally, non-hormone treatments were raised off the ground to prevent runoff contamination. Initial applications were made in December 2005 and followed by two others, separated by *ca.* two weeks. Eight untreated well-watered plants were randomly selected from the same set of plants as above to serve as controls. All plants were harvested approximately one month after the final hormone application. At this time plants were separated into inflorescence, leaf, stem, old and new root portions; dried and weighed. The concentration of umckalin in new root growth was then determined as per the method described in Chapter 3.

5.2.2.1.3 Root competition treatment

Two or three individuals of an annual common to disturbed fields (*Conyza albida* - fleabane) were planted in the pots of eight *P. sidoides* plants randomly selected from those that had grown under cultivation for 16 months. All other treatments were maintained so that no weeds developed beyond the seedling stage. The treatment began in December 2005 and continued for one month. Treated plants were then harvested. These plants were separated into inflorescence, leaf, stem, old and new root portions; dried and weighed. The concentration of umckalin in new root growth then was determined as per the method described in Chapter 3. The same eight untreated well-watered control plants described for the hormone treatments served as controls here.

5.2.2.2 Variation of root umckalin concentration amongst cloned plants

In May 2005, eight intact *P. sidoides* plants were collected from the vicinity of the GFRR site described in Chapter 4 (Section 4.1.3), and returned to the Rhodes University Botany Department. The tuberous roots of these plants were cut into sections weighing between 2.9 and 22.1 g with an average mass of 7.3 g, dipped in a rooting hormone (Seradix® B No. 3, Bayer), planted in prepared seed trays and covered with soil. The trays were labeled and the position of each root cutting was recorded. Dried growing medium comprised of two parts of locally sourced topsoil for each part of washed beach sand, by mass. Additionally, the dry topsoil was sieved through *ca.* 4 mm square mesh before it was combined with the sand. Soil samples were collected from three of the six trays and sent to Matrocast (Pty.) Ltd. for analysis. Thereafter, the removed soil was replaced with the same growing medium as above. These trays were well-watered every two or three days and surviving clones were harvested after five months. At this time, inflorescence, leaf, stem, old and new root portions were dried separately and weighed. The concentration of umckalin in both new and old root growth, from three plants originating from a single parent plant, was determined as per the method described in Chapter 3. These concentrations were then compared with those of wild *P. sidoides* roots collected from the GFRR site (see Section 5.2.1, above, for collection details).

All dried plant material described in this chapter was stored under laboratory conditions in the dark. Dried roots remained under these conditions until just before chemical analysis. Table 5.2 lists the average soil characteristics of the growing medium used for the cultivation of the cloned plants.

Table 5.2: Average characteristics of the soil that the cloned plants were grown in. N = 3.

pH	Carbon ($g.kg^{-1}$)	Nitrogen ($mg.kg^{-1}$)	Potassium (meq %)	Phosphorus ($mg.kg^{-1}$, citric)
5.5	76	3.5	649	2
% course sand	% medium sand	% fine sand	% silt	% clay
14.2	32.7	49.3	3.8	0

5.2.3 Statistics

Statistical analyses were performed as described in Chapter 4, Section 4.2.4.

5.3 Results

5.3.1 Geographical variation of root umckalin concentration

The WHO *et al.* (1993) included selecting of the most suitable genetic material in their guidelines for the conservation of medicinal plants through cultivation. Towards this goal, this study investigated the variation of root umckalin concentrations across part of the geographical distribution of *P. sidoides* in the Eastern Cape Province. As the yield of umckalin per individual plant (Figure 5.7) is dependent on both root umckalin concentration and the mass of the roots, both of these parameters were assessed for each site (Figures 5.4 and 5.6, respectively). Firstly, the root umckalin concentration in the new and old roots of the plants from the KR sites was measured. The KR sites were the only sites to have plants with both root age classes and, as shown in Figure 5.3, the concentration of umckalin did not vary significantly between the two.

Therefore, the umckalin concentration of new and old roots was combined in proportion to the respective new and old root masses for use in Figures 5.6 to 5.9.

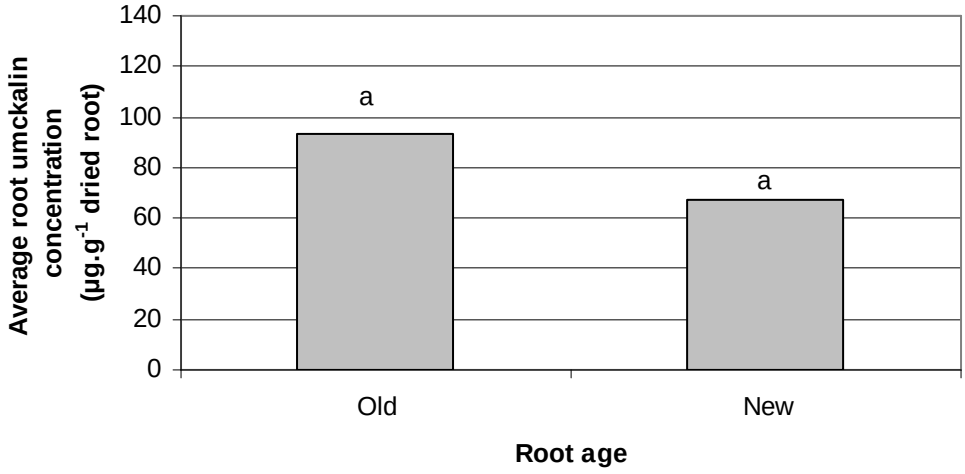


Figure 5.3: Average root umckalin concentration of new and old root growth from wild plants in the Kei Road region. a represents a statistically homogenous group at the 95% confidence level. N = 20 for old root and 5 for new root. p = 0.354, F = 0.89.

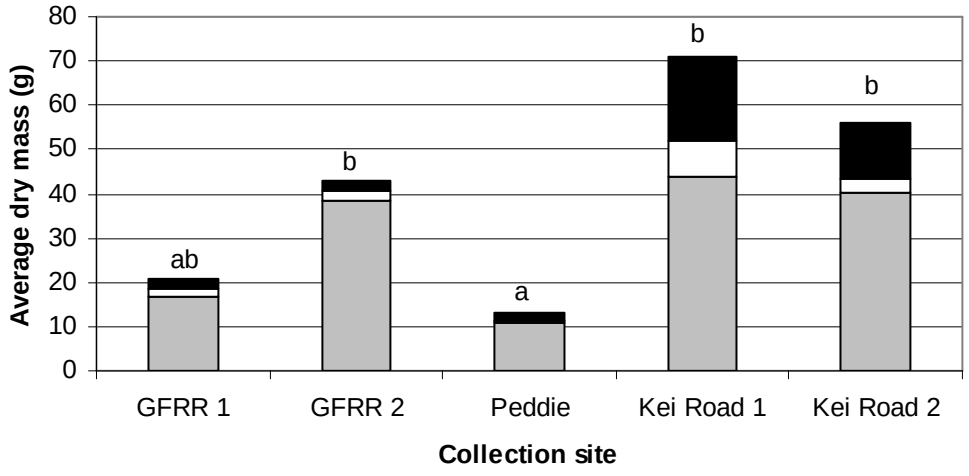


Figure 5.4: Contribution of total dry root (gray), stem (white) and combined leaf and inflorescence mass (black) to the total dry plant mass for plants from the five collection sites. N = 10. a and b represent statistically homogenous groups at the 95% confidence level for the average total dry root mass of plants from each site. p < 0.0005, H = 20.54.

Figure 5.4 shows that the average root biomass per plant from the highest rainfall area was higher that that for roots from lower rainfall areas. However, this trend was not significant ($p < 0.0005$) and root mass was not strongly related to average annual rainfall ($r^2 = 0.34$, $p = 0.304$; Figure 5.5).

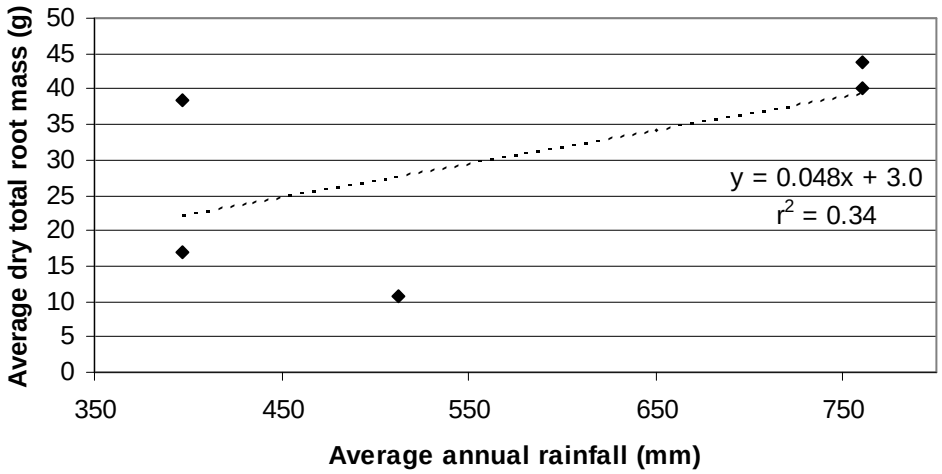


Figure 5.5: The relationship between average dry total root mass and average annual rainfall for plants from the five collection sites. N = 10 for each of the five average mass data points. $p = 0.304$.

Contrasting root mass variation, significant variation in the root umckalin concentration and in the yield (concentration multiplied by root mass) of umckalin per plant from each site was observed ($p < 0.0001$, Figure 5.6 and $p < 0.0001$, Figure 5.7; for umckalin concentrations and yields, respectively). Additionally, the average root umckalin concentrations at the five sites were found to be strongly related to annual rainfall ($r^2 = 0.94$, $p = 0.007$, Figure 5.8). The highest average umckalin concentrations were found in roots from the area of lowest rainfall and *vice versa*. Furthermore, the average root umckalin concentration and yield did not vary significantly between populations from the same region (Figures 5.6 and 5.7).

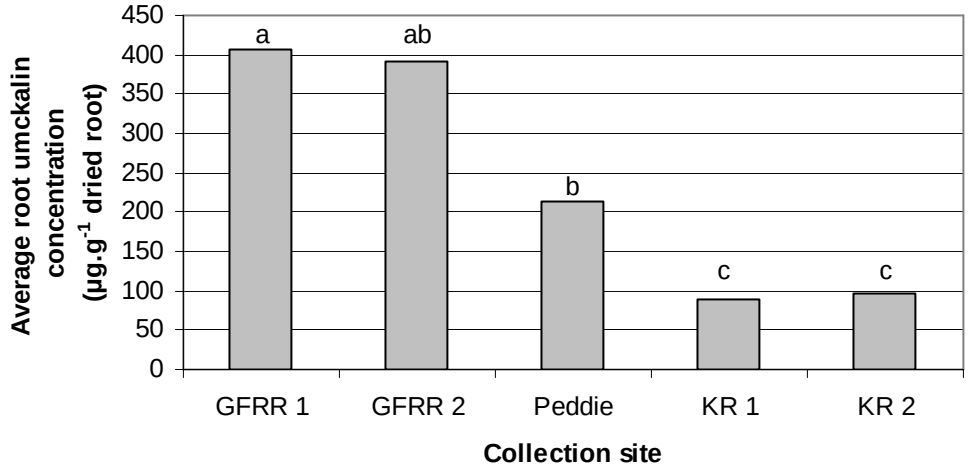


Figure 5.6: Average root umckalin concentration for plants from each of the five collection sites. a, b and c represent statistically homogenous groups at the 95% confidence level. N = 10 for the average root umckalin concentration at each of the five collection sites. $p < 0.0001$, $F = 19.85$.

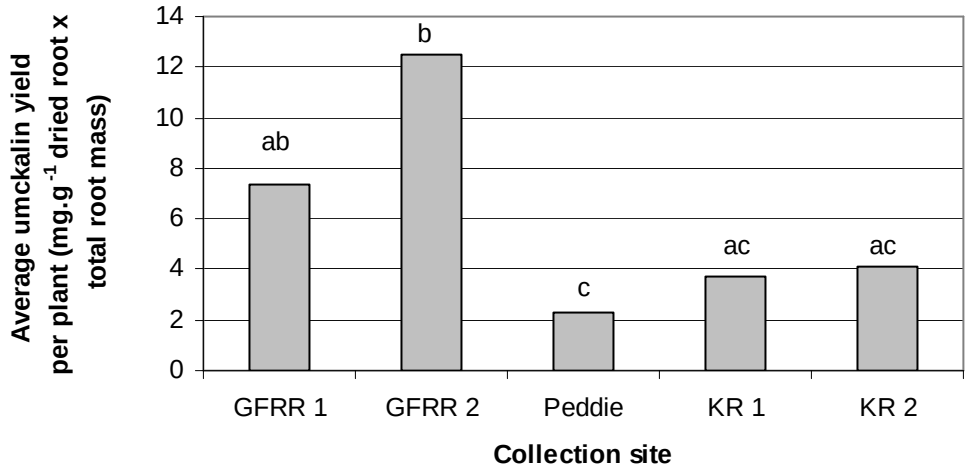


Figure 5.7: Average umckalin yield per plant from each of the five collection sites. a, b and c represent statistically homogenous groups at the 95% confidence level. N = 10 for the average umckalin yields at each of the five collection sites. $p < 0.0001$, $F = 9.17$.

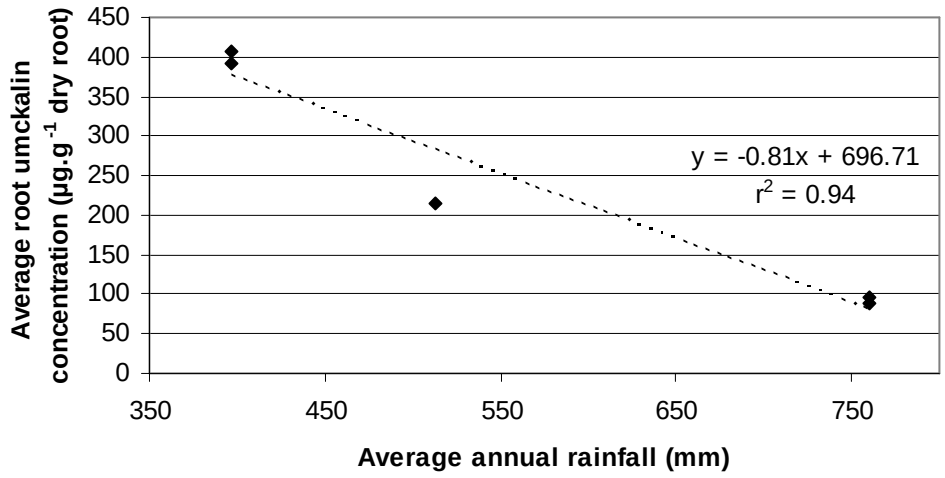


Figure 5.8: The relationship between average root umckalin concentration and average annual rainfall. N = 10 for each of the five average root umckalin concentration data points. p = 0.007.

The variation within the characteristics of the soil from each site (Table 5.3) was analysed in order to investigate the possible influence of biophysical factors on the root umckalin concentration of plants from these sites. Many of these characteristics varied significantly between the sites, including carbon, nitrogen, pH and percent course, medium and fine sand content (Table 5.3). Of these, soil pH was found to be strongly related to root umckalin concentration ($r^2 = 0.97$, $p = 0.002$, Figure 5.9) and was the only character to show a strong relationship.

Table 5.3: Average soil characteristics for each of the five collection sites. N = 3. a, b and c represent statistically homogeneous groups at the 95% confidence level. In the last column F is the value for parametric analyses of variance and H (in brackets) is for non-parametric analyses.

	GFRR 1	GFRR 2	Peddie	KR 1	KR 2	*P value*	*F statistic* (*H statistic*)
pH	a	a	b	b	B	< 0.001	17.43
	5.7	5.7	5.3	5.2	5.1		
Carbon ($g.kg^{-1}$)	bc	c	bc	ab	Ab	0.024	4.55
	12.9	11.76	14.3	16.1	16.2		
Nitrogen ($mg.kg^{-1}$)	abd	ab	bc	d	C	< 0.001	12.82
	1323.3	1270	978	1899.7	677		
Potassium (meq %)	a	a	a	a	A	0.629	0.67
	275.7	286	228.3	248.7	289		
Phosphorus ($mg.kg^{-1}$, citric)	a	a	a	a	A	0.689	(2.26)
	2.3	2.3	1.7	2.7	1.7		
% course sand	bc	ac	bc	ac	A	0.007	7.08
	15.1	18.7	8.4	16.2	28.4		
% medium sand	bc	ab	c	bc	A	0.046	3.59
	8.9	12.2	8	8.4	13.1		
% fine sand	a	a	a	a	B	0.048	3.53
	66.3	59.3	73.1	67.6	50.7		
% silt	a	a	a	a	A	0.769	1.82
	6.7	6.7	7.3	5.3	6		
% clay	a	a	a	a	A	0.63	0.67
	3.1	3.1	3.1	2.4	1.9		

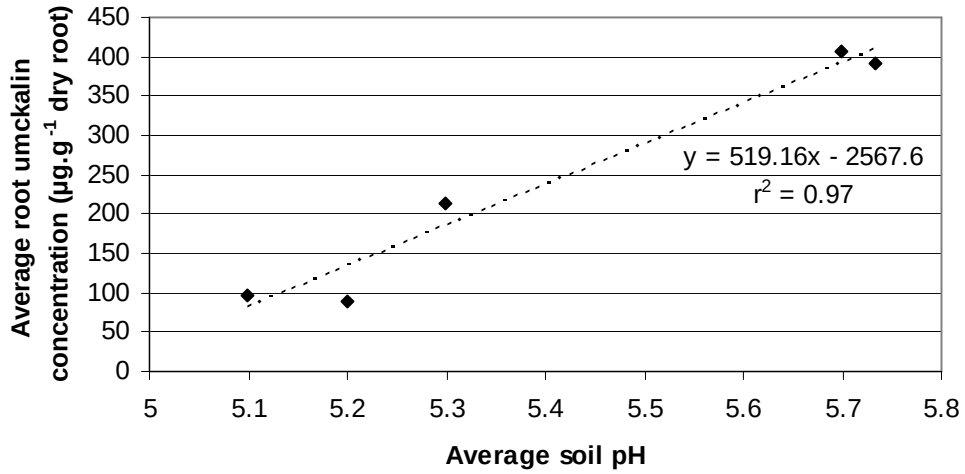


Figure 5.9: The relationship between average root umckalin concentration and average soil pH. N = 10 for each of the five average root umckalin concentrations and three for average soil pH measurements. p = 0.002.

The strong relationship between annual rainfall and root umckalin concentration (Figure 5.8) provided a rational basis for the production of umckalin under water limited conditions.

5.3.2 Variation of root umckalin concentration under cultivation

5.3.2.1 Variation of root umckalin concentration amongst non-clonal plants

The rapid water stress (RWS) treatment was designed to provide plants with extraordinary water stress conditions in order to promote the production of possible stress response compounds which may have included umckalin. The leaves of the RWS treated plants wilted by midmorning on the first day and did not recover during the remainder of the treatment. By the end of the treatment the leaves of the RWS treated plants were discoloured and showed signs of chlorosis. The leaves of control treated plants did not wilt or appear to be chlorotic at any time during the treatment.

Figure 5.10 shows the decrease of average leaf water potential (Ψ_{leaf}) amongst RWS treated plants compared to the controls, culminating in a significant difference by the end of the experiment ($p < 0.001$).

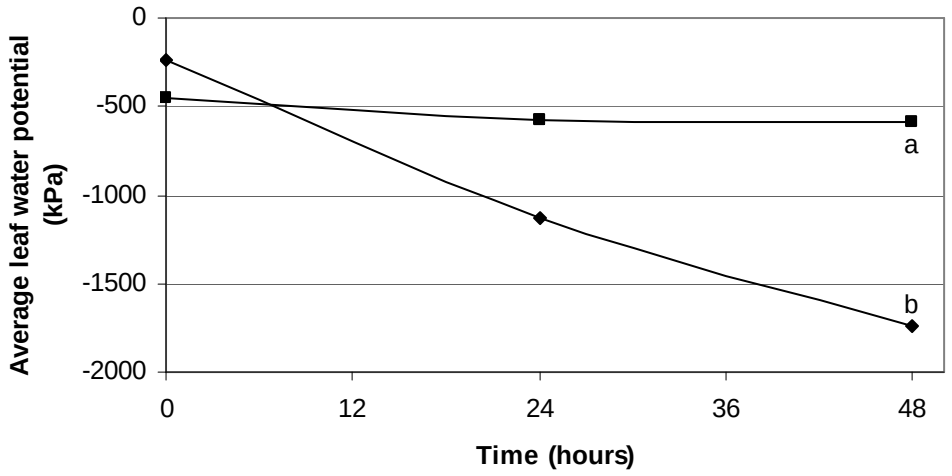


Figure 5.10: Change in average leaf water potential (Ψleaf) over time for rapidly water stressed (diamonds) and control plants (squares). The more negative the Ψleaf the more stressed the plant. N = 5 for each average leaf water potential data point. a and b represent statistically homogenous groups at the 95% confidence level for the average leaf water potentials at 48 hours. $p < 0.001$, $U = 0.00$, $Z = -2.61$.

The prolonged water stress (PWS) treatment was designed to mimic the effect of a natural one month drought on the root umckalin concentration of wild *P. sidoides* plants. The Ψ_{leaf} of plants in this treatment showed the same decreasing trend as those in the RWS treatment ($p < 0.001$, Figure 5.11A) and followed the decrease in soil moisture content during the treatment ($p < 0.001$, Figure 5.11B).

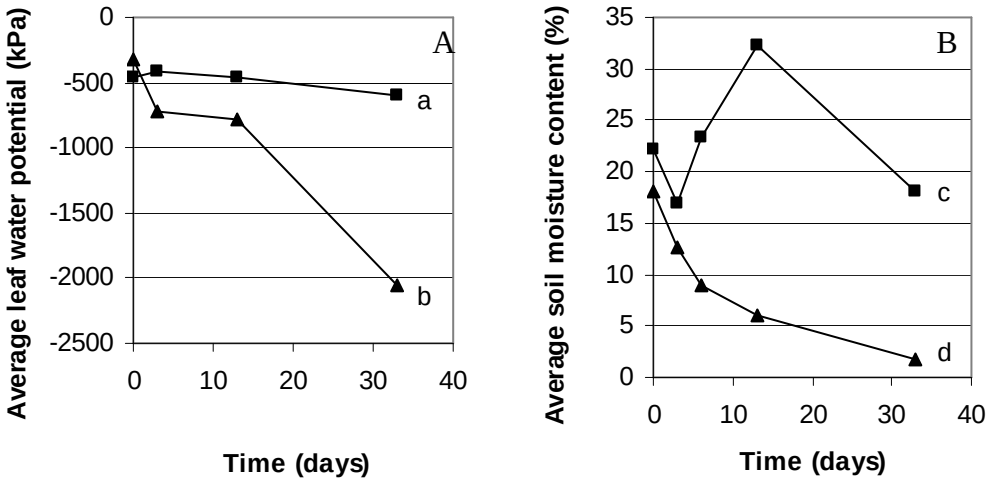


Figure 5.11: Change in average leaf water potential (A) and average percent soil moisture content (B) over time for well watered controls (squares) and plants from which water was withheld (triangles). N = 5. a and b represent statistically homogenous groups at the 95% confidence level for the average leaf water potentials on day 33. $p < 0.001$, $U = 0.00$, $Z = -2.61$. c and d represent statistically homogenous groups at the 95% confidence level for the average percent soil moisture content on day 33. $p < 0.001$, $U = 0.00$, $Z = -2.61$.

The RWS and PWS treatments significantly deceased the Ψ_{leaf} of treated plants, however, they did not result in significant changes in the root umckalin concentrations of treated plants ($p = 0.488$ and $p = 0.703$ for PWS and RWS treatments, respectively, Figure 5.12).

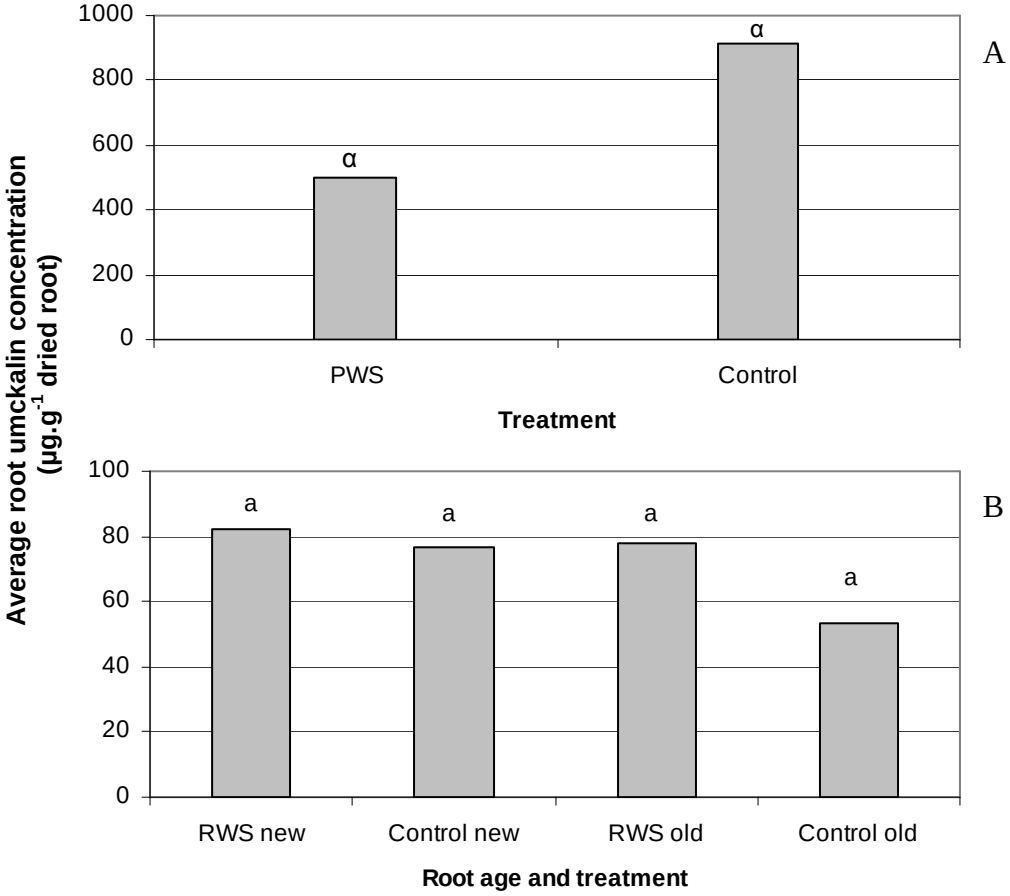


Figure 5.12: Average root umckalin concentration of old root from plants in the prolonged water stress treatment (PWS - A) and old and new root from those in the rapid water stress treatment (RWS - B), with their respective controls. α represents a statistically homogenous group at the 95% confidence level within the PWS data, $p = 0.488$, $F = 0.53$. a represents a statistically homogenous group at the 95% confidence level within the RWS data, $p = 0.703$, $F = 0.48$. $N = 5$.

Following the RWS experiment, the total inflorescence, leaf and combined leaf and inflorescence mass of each plant was plotted against the root umckalin concentration of each respective plant (Figure 5.13). Dry inflorescence mass showed the strongest relationship to root umckalin concentration ($r^2 = 0.53$, $p = 0.016$) and initiated postulation about the effects of applying hormones involved in flowering on the root umckalin concentration of greenhouse cultivated plants.

Additionally, the effect of the PWS treatment on the mass of the above plant parts is shown in Table 5.4. The average leaf mass of PWS treated plants was significantly lower than that of the controls (p = 0.0481, Table 5.4) and was the only plant part to show a significant difference.

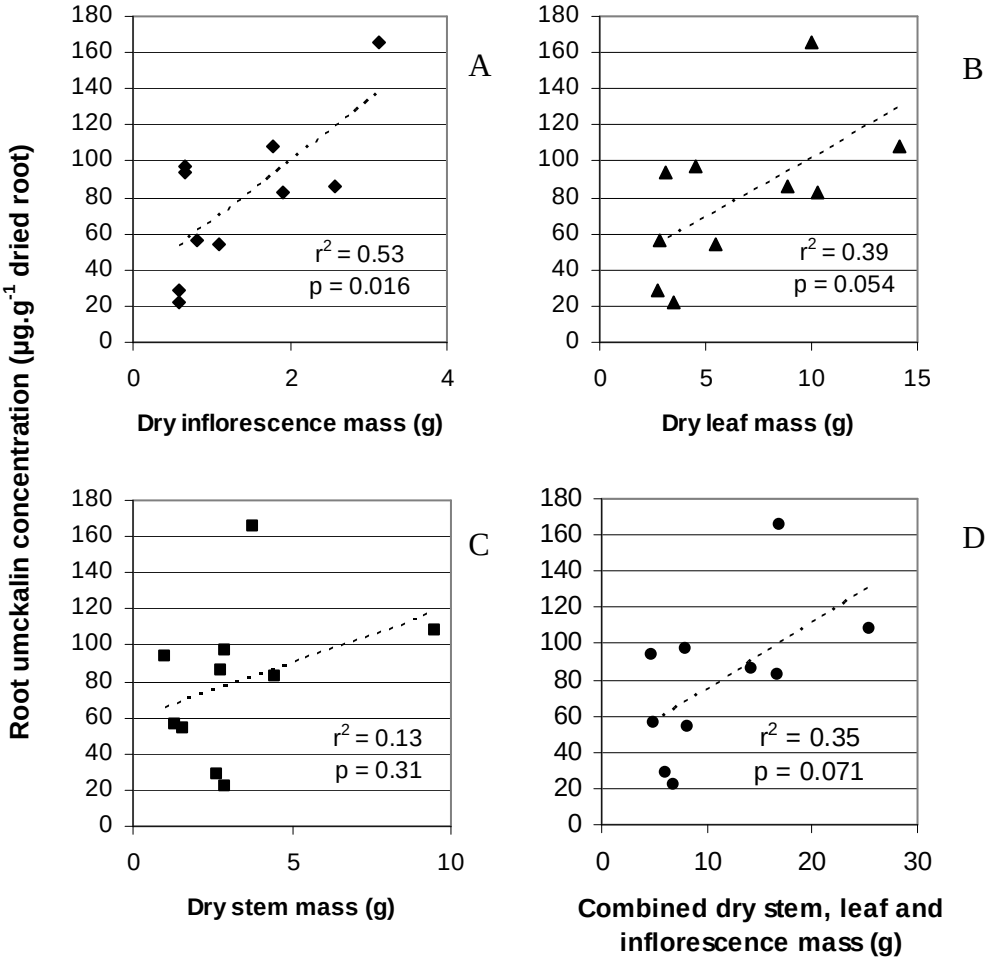


Figure 5.13: The relationship between the umckalin concentration in new roots and dry inflorescence (A - diamonds), leaf (B - triangles), stem (C - squares) and combined above ground mass (D - circles), for all plants in the RWS experiment. N = 10.

Importantly, the average umckalin concentration in the new roots of cultivated plants originating from the KR region (79.4 $\mu g.g^{-1}$) was not significantly different to the concentration of umckalin in the old roots of plants collected from the same region (92.6 $\mu g.g^{-1}$, p = 0.483, F = 0.51). This was a crucial discovery as it indicated that greenhouse cultivated plants may have the same medicinal value as wild harvested plants.

Table 5.4: Average dry inflorescence, leaf, stem, old root and new root mass for control and treated plants in the prolonged water stress (PWS) experiment. N = 5. a and b represent statistically homogeneous groups at the 95% confidence level.

	Control (g)	PWS (g)	*p value*	*F statistic*
	a	a	0.599	0.3
Inflorescence	1	0.3		
	a	b	0.0481	5.43
Leaf	8.7	4.9		
	a	a	0.0985	3.49
Stem	3	2.4		
	a	a	0.202	1.94
Old root	5.5	12.7		
	a	a	0.199	3.03
New root	8.2	15		

Figure 5.14 shows the effect of the cytokinin and gibberellin hormone treatments and the root competition treatment on average new root umckalin concentration compared to well-watered control plants. As seen for the two water stress treatments, no significant effects were observed (p = 0.76, Figure 5.14). Similarly, the above treatments did not have a significant effect on the dry mass of the various plant parts (Table 5.5).

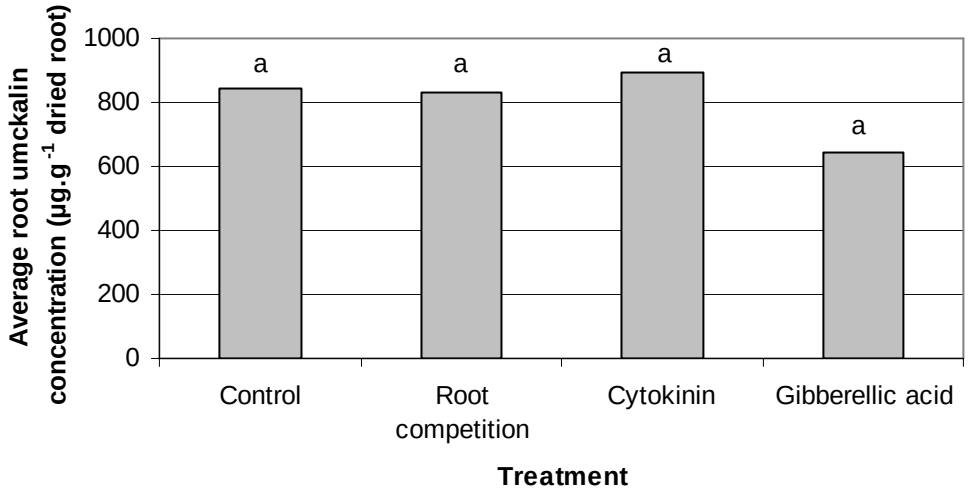


Figure 5.14: Average root umckalin concentration in the new roots of plants in the cytokinin, gibberellin, root competition and control treatments. N = 8. p = 0.76, F = 0.39.

Table 5.5: Average dry inflorescence, leaf, stem, old root and new root mass for control and treated plants in the cytokinin, gibberellin, root competition and control treatments. N = 8. a represents a statistically homogeneous group at the 95% confidence level.

	Control (g)	Cytokinin (g)	Gibberellin (g)	Root competition (g)	*p value*	*F statistic*
	a	a	a	a	0.488	0.83
Inflorescence	1.1	1.3	1	0.8		
	a	a	a	a	0.631	0.58
Leaf	12.1	10.2	8.5	10.1		
	a	a	a	a	0.216	1.52
Stem	3.4	2.3	1.8	1.5		
	a	a	a	a	0.471	0.86
Old root	14.9	16.8	18.1	11.1		
	a	a	a	a	0.537	0.74
New root	6.5	6.4	4.3	5.1		

5.3.2.1 Variation of root umckalin concentration amongst cloned plants

This analysis was performed to investigate the effects of genetic uniformity on root umckalin concentration. As such, Figure 5.15 shows the average root umckalin concentration of greenhouse cultivated cloned plants, originating from the same parent plant, compared to that of non-clonal wild grown plants. For wild plants from the GFRR site, the variation of the old root umckalin concentration accounted for 55.7 % of the average concentration. Likewise, the old root of greenhouse cultivated cloned plants showed a 49.5% variation about the average. However, the new root of the cultivated clones showed a much higher variation of 99.4%. Additionally, the average root umckalin concentration of the wild plants was significantly higher than that of both the old and new root of the cultivated clones ($p < 0.0001$; Figure 5.15), although all the plant material originated from the same area.

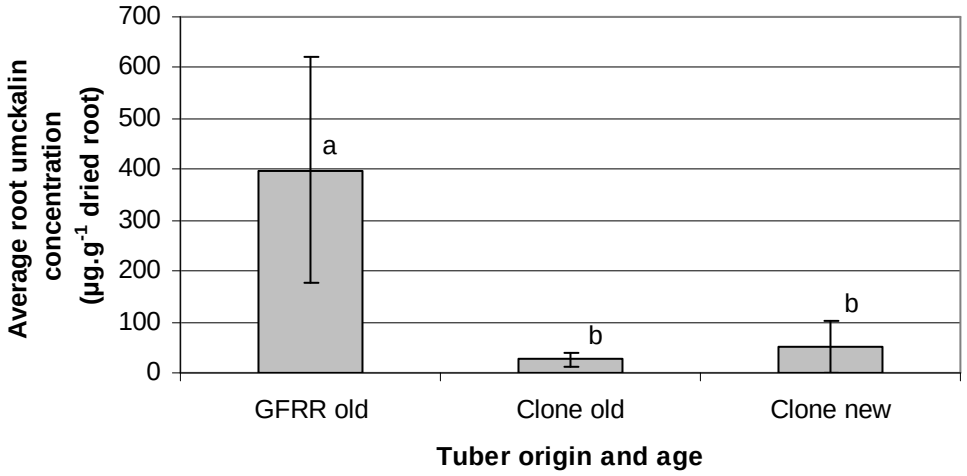


Figure 5.15: Average umckalin concentration in the old and new roots of greenhouse-cultivated cloned plants and the old roots of wild harvested GFRR plants. All plant material originated from the same area. Vertical bars show standard deviations. N = 20 for GFRR wild plant data and three for old and new clone data. a and b represent statistically homogenous groups at the 95% confidence level. $p < 0.0001$, $F = 19.47$.

5.4 Discussion

The aims of the research presented in this chapter were to assess the effect of climatic factors on the concentration of umckalin in the roots of wild *P. sidoides* plants, and to investigate the effect of greenhouse cultivation on root umckalin concentration. Additionally, the knowledge gained from the chemical survey of the wild plants was used, together with the relevant literature, to design cultivation treatments that would promote the synthesis of umckalin in the roots of greenhouse grown plants.

Identifying and sourcing the most suitable propagation material for medicinal plant cultivation is recommended by the WHO *et al.* (1993). Accordingly, the phytochemical survey of *P. sidoides* roots across three regions of the Eastern Cape was used here. The analysis of root extracts from these regions revealed that average root umckalin concentrations were strongly related to both the average soil pH and annual rainfall of the collection sites. Root umckalin concentrations were found to increase with decreasing average annual rainfall. Conversely, root umckalin concentrations were shown to increase with increasing soil pH. Precipitation influences soil pH as high rainfall tends to leach elements such as calcium, resulting in more acidic soils (Waugh, 1995). In contrast, soils are more alkaline in areas with low rainfall, such as deserts. This relationship describes the results presented here. As such, the relationship between root umckalin concentration and soil pH may be a result of the rainfall received at each site. Despite the relationship between rainfall and root umckalin concentration, the imposed rapid and prolonged water stress treatments did not significantly alter the root umckalin concentrations of cultivated plants relative to well-watered controls. As such, although the effect of pH on root umckalin concentration was not investigated here, pH may be a key factor and worth investigating in future studies.

Additionally, average root umckalin concentration did not vary significantly between plant populations from the same region. These results suggest that identifying the most suitable *P. sidoides* stock from within the entire distribution range could possibly be based on an analysis of environmental variables. The collection of additional data from other sites across the distribution range would help confirm this relationship. The yield of umckalin per plant was the highest in the lowest rainfall area and resulted from the combination of high umckalin concentrations and average root masses. However, the umckalin yields of both sites in this region were not consistently higher than the yield of umckalin per plant at the KR sites (highest rainfall area). The Peddie site had the lowest average umckalin concentration, root mass and thus yield of the three regions. The lower root mass may have been due to the selective removal of larger plants by harvesters as it was outside of a protected area and therefore the only site vulnerable to harvest. The GFRR plants are potentially the most economical for harvest owing to their high umckalin yield per plant compared to the other sites. However, as shown in Chapter 4, the regrowth rate of replanted parts of harvested GFRR plants was low, questioning the sustainability of long term harvest from these sites.

As discussed above, variation of bioactive metabolite composition and concentration in medicinal plants may be influenced by genetic as well as environmental variables (e.g. Wills and Stuart, 1999; Binns *et al.*, 2002 and Homer *et al.*, 2000). The analysis of cloned *P. sidoides* plants propagated from the GFRR stock revealed that the concentration of umckalin in their roots was significantly lower than that of wild GFRR plants. In contrast, no significant difference was found between the average root umckalin concentration of cultivated non-clonal KR plants compared to that of wild KR plants. This differential synthesis of umckalin by plants cultivated under the same conditions but originating from different areas suggests a genetic rather than an environmental control of production. Furthermore, the genetic control of umckalin production may be influenced by plant adaptation to the climate of the site of origin.

Accordingly, when plants adapted to survive in an arid climate, e.g. the GFRR site, are exposed to much more favourable greenhouse conditions umckalin may be produced at lower concentrations. For plants originating from a more moderate climate, e.g. the KR site, the change to greenhouse conditions may not have been pronounced enough to significantly alter the production of umckalin. The effect of cultivating plants from different regions under the same propagation technique needs to be investigated, however, before this explanation can be confirmed. As the significant differences in soil characteristics between the wild collection sites were not strongly related to root umckalin concentration, the differences seen under cultivation (Tables 5.1 and 5.2) were not regarded as having a significant effect.

In addition to the wild equivalent root umckalin concentrations (KR plants), the annual growth of new roots from cultivated replants was significantly higher than that of GFRR replants following the autumn harvest (*ca.* six times, see Chapter 4). The favourable greenhouse conditions preferred by *P. sidoides*, sunny positions and well drained sand to loamy soils (Dreyer and Marais, 2000), together with high water provision (Wang *et al.*, 2006), account for the higher root yield. The commercial cultivation of the species is also favoured by the ease of *P. sidoides* propagation from basal cuttings, seed and transplanting (Van der Walt and Vorster, 1988).

The five treatments imposed on the non-clonal greenhouse plants did not result in significant changes in root umckalin concentrations. However, other interesting findings were reported. These include the relationship between dry inflorescence mass and root umckalin concentration, and the significant decrease of dry leaf mass amongst PWS treated plants. The relationship between umckalin concentration and dry inflorescence mass suggests a relationship between the accumulation of umckalin in new roots and the reproductive stage of *P. sidoides* plants. Research on the accumulation of umckalin over time in cultivated *P. sidoides* roots would be useful in future investigations of the *in vivo* function of umckalin. Monitoring gradual changes would also help assess the effects of rapid changes in cultivation conditions that are difficult to control. An example would be the high greenhouse temperatures that caused leaf wilting here in December 2004.

As discussed in Chapter 3, the umckalin concentrations shown above were gained during two sets of HPLC analyses separated by three months. The roots of plants in the geographical collection and RWS and clone experiments were analysed during the first set of analyses. The roots of plants in the PWS, hormone and root competition experiments were analysed during the second set of analyses. When comparing Figures 5.6, 5.12 and 5.14 it is evident that the average concentration of umckalin measured in the second set of experiments were much higher than that of the first set of analyses, *ca.* 10 times. As the HPLC apparatus was calibrated before each set of experiments and separate standard curves were used for each set of analyses, this result was not due to an error in the calibration or operation of the apparatus. Therefore, the differences in root umckalin concentrations may have been due to seasonal variation; however, this is difficult to postulate as the function and seasonal variation of umckalin in plants has not been described.

The potential for supplying the market with cultivated roots is complemented by the concept of plant part substitution for conserving endangered medicinal plants (Zschocke *et al*., 2000). Recently, Lewu *el al.* (2006) compared the antibacterial activity of *P. sidoides* leaf and root extracts. The authors found that the activity of leaf extracts against a range of bacteria was not significantly different to that of root extracts. This result provides evidence for the potential plant part substitution of *P. sidoides* roots by their leaves. However, further investigations and clinical trials need to be performed before this substitute can replace root extracts in the market. Zschocke *et al*. (2000) conveyed a convincing message for plant part substitution. This was accomplished using a study comparing the phytochemical content and biological activity of various parts of four important and threatened South African medicinal plants. The results of this research on *Eucomis autumnalis*, *Siphonocilius aethiopicus*, *Ocotea bullata* and *Warburgia salutaris* showed that the potential for plant part substitution for medicinal use is very much species specific. For example, the bulbs and roots of *E. autumnalis* exhibited higher inhibition of cyclooxygenase-1 activity than the leaves, whereas for *S. aethiopicus*, the leaf and stem extracts showed higher activity than the underground parts.

The result for *S. aethiopicus* was particularly interesting as the roots and rhizomes are traditionally used and not the leaves or stems (Van Wyk *et al.*, 1997). To promote the conservation of a medicinal plant species, the authors recommended that all studies of medicinal plants include the investigation of leaves and other aerial parts, bark, stems and underground parts. Although not all parts may have a traditional use, the result will be beneficial in terms of finding potential substitutes for parts that are less easy to harvest sustainably.

The results presented here offer promising prospects for the commercial cultivation of *P. sidoides* roots to supply the international and local markets. Firstly, the growth of plants under irrigated greenhouse conditions resulted in yields *ca.* six times higher than wild grown replants. And secondly, the concentration of umckalin in the roots of cultivated plants was not significantly different to that of wild plants originating from the same high rainfall site, suggesting equal medicinal value.

Chapter 6

Conclusions and Further Research

Umckalin was successfully isolated from a crude acetone extract of *P. sidoides* roots using HP-20 resin, silica column and High Performance Liquid Chromatography (HPLC) guided by diagnostic Thin-Layer Chromatography (TLC) and Nuclear Magnetic Resonance (NMR) spectra. Thereafter, an effective and efficient analytical HPLC method was developed for the separation of umckalin from other compounds in ethanolic and acetone root extracts such that its concentration could be quantified. The method included a linear gradient of 7:3 to 3:7 (H_2O:MeOH) over eight minutes at a flow rate of 1 $mL.min^{-1}$ through a 125 x 4 mm C-18 HPLC column. An analytical HPLC method for the detection of umckalin in ethanolic root extracts has not been published previously. As such, the above method will be valuable for future academic and industrial research.

The effects of cultivation and climatic variation on the root umckalin concentrations of cultivated and wild *P. sidoides* plants, respectively, were then determined using the developed HPLC method. The survey of root umckalin concentrations across three distinct rainfall areas revealed that both soil pH and annual rainfall were strongly related to root umckalin concentration. Increasing annual rainfall was inversely proportional while increasing soil pH was directly proportional to the average root umckalin concentration of plants from each site. This discovery suggests that it may be possible to use climatic data in identifying *P. sidoides* populations with high root umckalin concentrations, and hence bioactivity, across the distribution range. This may prove valuable in sourcing the best propagation material for future cultivation studies and commercial operations. Additionally, the average root umckalin concentration of plants from the highest rainfall area did not differ significantly from that of greenhouse cultivated plants originating from the same area. This result suggests that, for plants from this area, cultivation may result in extracts that are as medicinally active as those of wild plants.

In contrast, the average umckalin concentration in the roots of greenhouse cultivated cloned plants was significantly lower than that of wild plants from that same low rainfall area. Collectively, the above results suggest that the umckalin production of the plants grown under cultivation may have been influenced by their origin and genetics rather than environmental variation. Before these findings can be confirmed, research should be conducted into the effects of cultivation on the concentration of bioactive metabolites in the roots of plants from different areas. Additionally, the effects of harvest season and various post-harvest storage options could be investigated.

The five imposed treatments - rapid and prolonged water stress, cytokinin and gibberellin hormone treatments, and the root competition treatment - did not significantly alter the average root umckalin concentration of cultivated plants relative to well-watered controls. The production of umckalin during inflorescence development, as an allelopathic agent or as a mitigation factor under conditions of water stress is therefore not supported by these cultivation results. Based on the findings of this study, the effects of other treatments including growth in soils of different pH and involving fungal infection could be investigated in future research. Root umckalin concentrations were not found to be related to root or ethanolic root extract colour. Further research to assess the relationship between total extract bioactivity against appropriate microorganisms and the concentration of known bioactive metabolites - specifically umckalin, 6,8-dihydroxy-5,7-dimethoxycoumarin, and gallic acid and its methyl ester - would be beneficial. Determining the relationships between extract bioactivity and colour, and between bioactive metabolite variation and colour, could also prove valuable. Additionally, DNA fingerprinting analyses could be used to assess the genetic control of bioactive metabolite biosynthesis of plants from across the distribution range and amongst cultivated plants, including those grown from tissue cultures. These analyses could be used concurrently to assess the genetic diversity of individual populations and of the species as a whole.

In terms of wild harvest, the yield of umckalin per root mass was the highest in the lowest rainfall area, making the plants from this area the most economically valuable. However, replant survival at this site was low. The three treatments in the wild harvest experiments, particularly the Stockosorb™ treatment, showed a clear and significant effect on the survival of replants at the GFRR site post-harvest. Yet, the very long estimated root regeneration time, low percentage survival of replants and the low regeneration from resprouts raised questions about the long-term sustainability of wild harvest. The rooting pattern of the observed *P. sidoides* plants consisted mostly of taproots in contrast to the more adventitious rooting pattern of *P. reniforme* plants. The difference in rooting pattern between the two species may favour the regrowth of *P. reniforme* plants post-harvest as the adventitious rooting pattern of this species is more likely to result in root pieces remaining in the soil during harvest. These results highlighted the need for additional wild harvest experiments to be investigated across the distribution range of the species, especially where commercial harvest is intended.

Additionally, the total size of the wild *P. sidoides* resource needs to be determined before sensible decisions can be made about the sustainability of wild harvest. A legislated monitoring system would help researchers determine the harvest and export volumes of *P. sidoides* and other medicinal plants and thus comment on the urgency to protect these species. Reviewing the compliance of commercial operations to current legislation and carefully reviewing and enforcing the current permit system are recommended. Until further research has been conducted, it is highly recommended that *P. sidoides* and *P. reniforme* be awarded a protected status in all South African provinces and in Lesotho.

In contrast to regrowth under field conditions, the annual growth of root biomass by replants under cultivation was *ca.* six times greater than that of similar plants under field conditions at the low rainfall harvest site. The higher growth rates amongst cultivated plants, and the potential to grow plants of optimal medicinal value, favour sustainable harvest through cultivation. Hence, in the best interests of the conservation of the species and the sustainable supply of roots for the local and international markets, the results of this study strongly recommend that cultivation be pursued as a promising alternative to wild harvest.

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