NODULATION BACTERIA, CUCURBITACIN-CONTAINING PHYTONEMATICIDES, DOSAGE MODEL AND NUTRITIONAL WATER PRODUCTIVITY OF *SUTHERLANDIA FRUTESCENS* IN THE CONTEXT OF CLIMATE-SMART AGRICULTURE

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THESIS SUBMITTED IN FULFILMENT FOR THE DEGREE DOCTOR OF PHILOSOPHY IN AGRICULTURE (PLANT PRODUCTION), DEPARTMENT OF PLANT PRODUCTION, SOIL SCIENCE AND AGRICULTURAL ENGINEERING, SCHOOL OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF LIMPOPO, SOUTH AFRICA

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DECLARATION

I, Tsobedi Absalom Masenya, declare that the thesis hereby submitted to the University of Limpopo for the Doctor of Philosophy in Agriculture (Plant Production) has not been submitted previously by me or anybody for a degree at this or any other university. Also, this is my work in design and in execution, while related materials contained herein had been duly acknowledged.

Candidate: Tsobedi Absalom Masenya	Signature	Date
Supervisor: Professor K.M. Pofu	Signature	Date
Co-Supervisor: Professor P.W. Mashela	Signature	Date

DEDICATION

I dedicate this thesis to my late family members, my granny Ethel Masenya, uncles Dick and Peter Masenya, who passed on during the execution of this study. May their souls rest in eternal peace. Along with my lovely wife Tshedza, my daughter Angel and my son Junior Masenya.

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ABSTRACT

The unique phytochemical composition of the medicinal plant cancer bush (*Sutherlandia frutescens*) have made its foliage to gain much attention in South Africa due to its health benefits. In situ harvesting of the plant parts of this important species serve as one potential strategy to avert its extinction through whole plant harvesting, a common practice by rural communities. However, such a strategy is limited by lack of information on the agronomic requirements of the plant species and its susceptibility to root-knot (*Meloidogyne* species) nematodes. The objectives of the study were four-fold, namely, to: (1) identify nodulation bacteria associated with wild *S. frutescens* using morphological and biochemical techniques, (2) assess the efficacy of the nodulation isolates from different centres of biodiversity of *S. frutescens* in Limpopo Province, South Africa (3) test the compatibility of cucurbitacin-containing phytonematicides on *S. frutescens* for managing population densities of *Meloidogyne* species and (4) determine the nutritional water productivity (NWP) of *S. frutescens* in association with water scarcity of the region where the plant species originated. In achieving Objective 1, nodules from *S. frutescens* roots were washed in distilled water and healthy, undamaged, firm and pink nodules were sterilised. Aseptic nodules from *S. frutescens* roots and commercial strains were transferred into a smasher biomerieux polythene bag containing 10 ml distilled water and crashed to produce a milky suspension the milky suspension was streaked on Yeast extract mannitol agar (YEMA). After gram reaction, colony characterisation includes the investigation of shape, colour, configuration, elevation and margin of bacterial colony as observed in colonies on nutrient agar plates of overnight grown microorganisms using a microscope. The medium for biochemical test was prepared, inoculated with 5 µl purified

bacterial cultures and incubated at 37˚C for 48 h. Identification of the bacterial isolates was performed using VITEK 2 Systems (bioMérieux, Inc., North Carolina, USA). Using morphological and biochemical techniques, the bacterial species associated with roots of *S. frutescens* in the wild were assayed primarily those in the genera *Raoutella ornithinolytica* and *Enterobacter cloacae* species d*issolvens*. The VITEK 2 Systems confirmed the identification of the bacterial species from 80 to 96% of the samples. Three species were confirmed from another sampling area, *Sphingomonas paucimobills*, *Raoutella ornithinolytica* and *Enterobacter cloacae* species *dissolvens* from by 86 to 96% of the samples. In achieving Objective 2, the five treatments, namely, *Bradyrhizobium* spp. (Arachis) strain, *Rhizobium leguminosarum* strain, Tubatse strain, Sebayeng strain and untreated control, were laid-out in a randomised complete block design, with seven replications during the first season (Experiment 1) and with eight replications during the second season (Experiment 2). The seasonal interactions (Experiment 1 × Experiment 2) on plant and nutrient elements were not significant ($P \leq 0.05$) and data for the two seasons were pooled ($n = 75$). Relative to untreated control, commercial (*Bradyrhizobium* and *Rhizobium* strain) and native strains (Tubatse and Sebayeng strain) significantly increased plant height by 31, 33, 44 and 40%, respectively, root length by 30, 41, 40 and 42%, respectively and dry shoot mass by 48,195 and 17%, respectively. Similarly, rhizobia strains significantly contributed to the increase in nitrogen assimilation by 7, 25 and 80%, respectively, protein synthesis by 13, 10, 24, 69%, respectively, and symbiotic efficiency by 31, 133, 292 and 82%, respectively. However, rhizobia inoculants had no significant effects on potassium and phosphorus in leaf tissues. In achieving Objective 3, in Mean Concentration Stimulation Point (MCSP) experiments, seven treatments,

namely, 0, 2, 4, 8, 16, 32 and 64% for each phytonematicide, were arranged in a randomised complete block design (RCBD), with 8 replicates. In application interval experiments, treatments, based on “weeks-per-month-of-30 days” for *M. javanica*, which translated to 1, 2, 3 and 4 weeks, were arranged in a RCBD, with 10 replicates. Nemarioc-AL and Nemafric-BL phytonematicides had MCSP values of 3.43 and 4.03%, respectively, with the plant having high tolerance level to the products. The respective application interval of the two products for managing population densities of *Meloidogyne* species were 29 and 17 days. The dosage models for Nemarioc-AL and Nemafric-BL phytonematicides were 6.62 and 13.26%, respectively. In achieving Objective 5, the study used nine treatments designated as T1, T2, T3, T4, T5, T6, T7, T8 and T9, respectively, consisting of 1, 2, 3, 4, 5, 6, 7, 8 and 9 seedlings/hole of drip irrigation transplanted using a 3S planter under field conditions, arranged in randomised complete block design (RCBD) with 9 replications (n = 81) in two seasons. The NWP of total flavonoids, total tannin and total phenol exhibited positive quadratic relations in varied planting density suggesting that this cultural practices could be manipulated to improve NWP of cancer bush. In conclusion, the wild bacterial isolates, sampled from *S. frutescens* plant grown in the field, outperformed the commercial bacterial strains in enhancing the productivity of the test plants. The empirically established dosage model for Nemarioc-AL and Nemafric-BL phytonematicides could be used to control *Meloidogyne* species in cancer bush production. There is a need to further investigate the responses of the identified strains to the test phytonematicides. Findings of the study openend new frontiers in the development and commercialisation of the observed native bacterial strains for the

cultivation of *S. frute*scens, which has excellent medicinal importance as a cure or management for cancer.

JOURNAL ARTICLES GENERATED FROM THE THESIS

1. **Masenya, T.A.**, Pofu, K.M. and P.W. Mashela. 2020. Responses of cancer bush (*Sutherlandia frutescens*) and *Meloidogyne javanica* to increasing concentration of Nemafric-BL phytonematicide. *Research on Crops.* 21 (3): 615-620.

2. **Masenya, T.A.**, Pofu, K.M. and P.W. Mashela. 2022. Efficacy of rhizobia strains on growth and chemical composition of cancer bush (*Sutherlandia frutescens*). *Acta Agriculturae Scandinavica, Section B-Soil & Plant Science.* 72 (1): 358-363. https://doi.org/10.1080/09064710.2021.2003852

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of the study

Cancer bush (*Sutherlandia frutescens* [(L.R.) Br.)], which is indigenous to South Africa, is a leguminous plant with pharmacological and ethnomedicinal applications (Faleschini *et al.*, 2013; Van Wyk and Wink, 2004). Due to its association with mitigation of the symptoms of Covid-19 (Dwarka *et al.*, 2020), which have since increased the destructive harvesting of *S. frutescens,* the plant faces the risk of becoming extinct. The use of *S. frutescens* is no longer confined to traditional healers but has entered both the informal and formal entrepreneurial sectors, with a large number of gatherers and traders (Raselabe, 2017; Wiersum *et al.*, 2006). The entire above-ground part of the plant is harvestable (Dunn, 2017; Tanga *et al.*, 2018), with repeated harvesting not providing opportunities for re-establishing the normal root/shoot ratio, which is critical in the sustainable survival of plants.

Global demand for medicinal plant products has been estimated at US$60 million per annum (Rasethe *et al.*, 2002), with current demand being even higher as reflected by the success of various medicinal plants in curbing various devastating global pandemics (Avdeenko *et al.*, 2020; Jang *et al.*, 2019). Overexploitation of *S. frutescens* is a major threat to biodiversity and emboldens a shortage in supply for pharmacological and ethnomedicinal uses, which in turn affect health care, income and employment (Wiersum *et al.*, 2006). Among the remedial actions to avoid extinction, large scale cultivation of the

plant using best practices could be viewed as a sustainable solution in the cultivation of *S. frutescens* as an underutilised crop (Dunn, 2017; Makgato *et al*., 2020; Tanga *et al*., 2018). However, limited empirically-based agronomic management information could derail attempts to successfully cultivate the plant, although adequate attempts are being made to promote the plant as one of the underutilised crops (Makgato *et al*., 2020). Multiple other challenges in cultivation of *S. frutescens* add to the lack of knowledge and resources on the successful cultivation of this plant. *Sutherlandia frutescens*, as a leguminous crop, requires suitable and sustainable nodulation bacteria to ensure successful cultivation (Gyogluu *et al*., 2018). Although commercial nodulation bacteria are mainly in the genera *Rhizobium* and *Bradyrhizobium*, there are many other genera with high nodulation efficiencies in wild leguminous plant species, which have evolved with the plant species over extended periods of time (Gyogluu *et al*., 2018). Additionally, *S. frutescens* is highly susceptible to root-knot (*Meloidogyne* species) nematodes, with certain reports suggesting that some species in the genus causes complete crop failure (Raselabe, 2017).

Due to global warming, there is growing concern for pest management due to the threat of the emergence of new pests, including nematodes with shorter life cycles, which make management increasingly difficult (Jones *et al*., 2013). In the midst of limited management options due to the withdrawal of most synthetic chemical nematicides from the agrochemical markets (Mashela *et al*., 2017a), options for managing nematodes are increasingly limited. Recently, it was shown that in addition to traditional *Meloidogyne* species such as *M. incognita* and *M. javanica*, with life cycles of over 30 days, *M.*

enterolobii with the life cycle of 15 days is becoming increasingly common in various parts of South Africa, particularly in Limpopo Province (Collett, 2020). Global warming also significantly impacts the cultivation of various plant species due to increased evapotranspiration, which increase water demand, especially for herbaceous plant species such as *S. frutescens* (Carr *et al.*, 2016).

1.1.1 Description of the research problem

The unique phytochemical composition of medicinal plants like *S. frutescens* has made its shoots to gain much attention to the already over-harvested plant species with limited restrictions and conservation measures such as cultivation and conservative harvesting (Dunn, 2017; Dwarka *et al.*, 2020). Knowledge on cultivation protocols of *S. frutescens* in water-scarce regions and management of *Meloidogyne* species would enhance the successful production of this crop. The development of sustainable and environment-friendly methods as best practice strategy under semi-arid regions with heavy nematode infestations could improve production and agricultural productivity of semi-arid originated plants as underutilised crops under the impeding climate change inland South Africa, where predictions suggest that extremes related to drought and high temperatures would be common place (Zuluaga *et al.*, 2020).

Among available low-input strategies to increase the productivity of crop husbandry, the use of nodulation bacteria, cucurbitacin phytonematicides and plant selection for high water-use efficiency, have had great potential in achieving the goal of sustainability and improved productivity (Zuluaga *et al.*, 2020). Commercial nodulation bacteria, for

example, can directly contribute to plant nutrition and also alleviate the detrimental effects caused by biotic and abiotic stress to which plants are regularly subjected (Zuluaga *et al*., 2020). Most indigenous legume plant species have evolved with their unique nodulation bacteria, which could be different from the usual commercial genera of *Rhizobium* and *Bradyrhizobium*. Such native strains could have great potential for being developed to provide cheap and efficient inoculants for indigenous leguminous crops intended for cultivation as underutilised crops (Koskey *et al*., 2017; Zuluaga *et al*., 2020). Native nodulation bacteria have the added advantage of being more adapted to the soils than the introduced commercial inoculant strains (Koskey *et al*., 2017; Zuluaga *et al*., 2020).

Globally, the management of *Meloidogyne* species remains the most challenging and tedious in crop production systems (Mashela *et al*., 2017a). In South Africa, it has been shown that *S. frutescens* was host to different thermophilic root-knot (*Meloidogyne* species) nematodes (Raselabe, 2017). The withdrawal of environment-unfriendly synthetic fumigant chemical nematicides such as methyl bromide (Sikora *et al.,* 2005), led to the development of various nematode management strategies, including the use of systemic chemical nematicides, which were nematostatic with a wide range of acropetal (root-shoot) and basipetal (shoot-root) movement-related challenges (Morris, 2015), organic manure (Mashela *et al*., 2020a), nematode resistance (Mashela *et al*., 2017a; Pofu, 2012) and cucurbitacin phytonematicides (Mashela *et al*., 2017a). All the listed strategies, just like the synthetic chemical nematicides, had their associated challenges. For example, the cucurbitacin-containing phytonematicides had challenges of phytotoxicity to crops being protected against nematode damage (Mashela *et al*., 2015).

The latter was resolved through the establishment of the dosage model, which is a four-step model, each having its empirical basis (Mashela *et al*., 2017a).

Simulations related to the outcomes of climate change suggested the advent of more pressure on water resources, with water scarcity being predicted to increase by more than 20% in 2050 relative to the current status (Carr *et al*., 2016). Such precautionary outcomes demand the need of developing new technologies and strategies for improving water use efficiency (Carr *et al*., 2016). Strategies for efficient management of water for agricultural use involves conservation of water, integrated water use, optimal allocation of water and selecting crops that would enhance water use efficiency in a given region (Das *et al*., 2019). Nutritional water productivity (NWP) is a concept established in agriculture to address improved water use efficiency, as well as being a strategy to address nutrition and health issues (Nyathi *et al*., 2016). The concept has several descriptors, which include nutrient mineral elements and organic secondary metabolites a prescribed in medicinal plants (Li *et al*., 2021; Mabhaudhi *et al*., 2017). Concurrently, this strategy can be used to look at the need for transdisciplinary dialogue and studies to develop more appropriate metrics that can be useful to operationalise transdisciplinary efforts to link water, agriculture and nutrition (Modi and Mabhaudhi, 2017). The concept allows for studies that explore water regimes and related effects such as management practices (fertiliser levels, planting densities), climate, edaphic and biotic factors such as plant-parasitic nematodes (Modi and Mabhaudhi, 2017; Ramputla, 2019). The concept of NWP can assist in improving cultural practices for cultivation of crops in water scare areas

and in the development of water use efficiency in crop selection for a given region (Hadebe *et al*., 2021).

In summary, the research problem for the study involves the need to establish suitable nodulation bacteria for *S. frutescens* with the focus being on establishing sustainable associated nodulation bacteria for the plant, along with establishing the compatibility of cucurbitacin phytonematicides and *S. frutescens* for managing population densities of *Meloidogyne* species*,* thereby ascertaining whether the test crop would be worth introducing as an underutilised crop in semi-arid regions using the concept of NWP.

1.1.2 Impact of the research problem

Cultivation of plants in high demand such as *S. frutescens* that are facing extinction as a result of poor wild harvesting and poor conversation measures, with limited agronomic practices, are emerging challenges in context of climate-smart agriculture. The Department of Science and Innovation, South Africa, has introduced a bio-economy strategy, which includes promoting the cultivation of commercially important indigenous medicinal plants as an adaptation strategy to global warming (Mofokeng *et al*., 2020). Constant supply of medicinal plant material from plants such as *S. frutescens* is essential because they play a vital role in the health care needs of three quarter of the world's population, most living in developing countries (Peabody *et al*., 2006). However, cultivation is hindered by lack of reliable best agronomic practices.

Large parts of South Africa have been reported to be infested with plant-parasitic nematodes (Bango, 2019; Rashidifard *et al*., 2019). The withdrawal of synthetic chemical nematicides from the agro-chemical markets has made the development of alternatives pivotal, effective cucurbitacin phytonematicides such as Nemarioc-AL and Nemafric-BL phytonematicides from fruits of indigenous plants being in the forefront (Mashela *et al*., 2015). Originally, reports related to phytotoxicity were viewed as being challenges that would limit the adoption of cucurbitacin phytonematicides (EPPO, 2010). Generally, the registration authorities have zero-tolerance to products with phytotoxicity (EPPO, 2010), which resulted in an effort to resolve the challenge using a computer-based algorithm model, the Curve-fitting Allelochemical Response Dose (CARD) model (Mashela *et al*., 2017b). The CARD model could also be applied in the development of appropriate concentration for managing nematode population densities in *S. frutescens* and thereby enhancing the availability of its raw material for product development. Generally, evidence exists that the extinction of this plant would threaten certain pharmaceutical and food industries, including the traditional practitioners, cosmetic and flavour industries and many other users (Nirmal *et al*., 2013). Additionally, the extinction would not auger well since it would threaten the biodiversity that the Department of Science and innovation is currently attempting to promote through the cultivation of such important indigenous plants, with potential economic benefits.

1.1.3 Possible causes of the research problem

Globally, there is currently a higher demand for *S. frutescens* and *Sutherlandia*-based products (Dunn, 2017; Raselabe, 2017). Unsustainable harvesting results an

unprecedented decline in the population densities of medicinal plants such as *S. frutescens* and therefore, such plants are becoming a high priority for conservation, which include a strong case to cultivate the species (Dunn, 2017; Mofokeng *et al*., 2020). Successful cultivation requires empirically-based propagation and cultivation protocols, which include water and nutrient element requirements under the ever-changing climatic conditions. Such information is limited in the cultivation of *S. frutescens* in semi-arid regions where the plant originated (Raselabe, 2017).

Sutherlandia frutescens as a legume plant species should be associated with wild nodulation bacterial isolates, which could be dissimilar from the available commercial genera of *Rhizobium* and *Bradyrhizobium* as observed in other indigenous leguminous plants (Gerding *et al*., 2012; Koskey *et al*., 2017; Zuluaga *et al*., 2020). Host range and cross-infectivity studies are important for identifying rhizobial strains with the potential for use as inoculants to improve the productivity of underutilised indigenous leguminous plants (Gyogluu *et al*., 2018). Isolation and characterisation of *S. frutescens* nodulation bacteria that originated in Limpopo Province, as well as comparing the compatibility of indigenous rhizobia with commercial genera inoculants on the test plant, remains undocumented.

Nemarioc-AL and Nemafric-BL phytonematicides have been shown to be highly efficient in managing population densities of root-knot nematodes in various crops (Chokoe, 2017; Mashela *et al*., 2015; Sebothoma, 2019; Sithole, 2016). However, the use of these products has been hindered by the tendency of phytotoxicity in the test crops (Mashela

et al., 2015; Mashela *et al*., 2017b). The incidence of phytotoxicity in various phytonematicides has had negative impact in the adoption of such products in various production systems. Phytotoxicity challenges have been resolved through the establishment of the dosage model concept using the CARD algorithm computer model. The dosage model is a four-step model, namely, (a) establishment of non-phytotoxic concentration, referred to as Mean Concentration Stimulation Point (MCSP), (b) the use of the derived MCSP to establish the application interval, (c) the use of the derived application interval to establish the application frequency and then (d) the use of the application frequency and MCSP to establish the dosage model (Mashela *et al*., 2015; Mashela *et al*., 2017b). The four steps, since they are based on empirical information, have been referred as the first, second, third and fourth laws of phytonematicides (Mashela *et al*., 2017b)

The concept of NWP allows crops to be explored for nutrition, adaptability and suitability to a particular environment (Hadebe *et al*., 2021). Studies have been conducted on NWP using macronutrient elements, micronutrient elements and vitamins in leguminous, fruit and vegetable crops as the descriptors of NWP, whereas phytochemicals as the descriptor were hardly used (Chibarabada *et al*., 2017; Hadebe *et al*., 2021; Li *et al*., 2021; Nyathi *et al*., 2016; Renault and Wallender, 2000). The latter could be due to the fact that the test crops used by the listed workers were not medicinal in nature. Additionally, in most NWP studies the focus was on assessing factors that would enhance crop choice for a specific region with respect to irrigation interval, with the focus being dependent upon the interest of the research team (Nyathi *et al*., 2016). Currently, information on how

management practices such as planting densities and irrigation interval could influence NWP, where phytochemicals are used as the descriptor, in medicinal plants, is limited (Modi and Mabhaudhi, 2017). Development of such information would promote decision-making in the successful cultivation of *S. frutescens* in semi-arid regions.

1.1.4 Proposed solutions

The concept of NWP is an important criterion for selecting a crop within a broad range of crops with different productive potential in relation to crop selection for improving consumer health. Generally, the descriptor is used with the focus being on the harvestable produce. The NWP concept links up water and agricultural production strategies, thereby providing the possibility to ensure that crops that accumulate nutritive elements and chemical compounds in edible produce are prioritised in production systems (Chibarabada *et al*., 2017; Li *et al*., 2021). The NWP in crops with different planting densities and under different irrigation intervals are critical since the listed cultural practices can be easily manipulated.

Cultivation using best agricultural practices has been the most reliable option for the conservation of plant species that are increasingly under threat of becoming extinct (Makgato *et al*., 2020; Mofokeng *et al*., 2020). The latter achieves sustainable development and biodiversity protection, thereby creating jobs, generating wealth, income and eventually a better life for the participating stakeholders (Raselabe, 2017). Generally, in the event there is no empirical information on best agricultural practices for an indigenous plant that is being introduced, those for any crop within the family could be

used, with traditional methods serving as an alternate during the initial stages of developing such methods (Mofokeng *et al*., 2020). Cultivation protocols of *S. frutescens* in semi-arid regions such as those in Limpopo Province, South Africa have not been documented.

The investigation of suitable nodulation bacteria for *S. frutescens* with the focus being on establishing its compatibility with nodulation bacteria is essential for improving the successful cultivation of this medicinal plant. The identification of indigenous rhizobia with a wider host range that are tolerant to abiotic stresses is important for inoculant development, especially for soils with unidentified effective indigenous rhizobia species. The introduction of suitable rhizobia species as inoculants can enhance N nutrition in cropping systems that include *S. frutescens* (Gyogluu *et al*., 2018).

The sustainable production of *S. frutescens* on commercial basis would also depend on the successful management of nematode population densities, especially *Meloidogyne* species (Mashela *et al*., 2017a). Currently, *M. enterolobii*, with a life cycle of 15 days (Collett, 2020), is increasingly becoming a serious threat to most crops, even those with Mi resistance genes. The withdrawal of synthetic nematicides from the agrochemical markets due to their environment-unfriendliness had since increased the use of alternative management strategies such as biocontrol agents and phytonematicides. However, each innovative management strategy has both advantages and disadvantages (Mashela *et al*., 2015). Inconsistent results in suppression of root-knot nematodes was originally a major issue facing the introduction of phytonematicides,

which was resolved using the concept of density-dependent growth (DDG) patterns (Mashela *et al.*, 2015). Briefly, the DDG patterns states that three phases exist, namely, stimulation, neutral and inhibition, which are specific to concentration ranges. In other words, if the concentration is within the stimulation, neutral and inhibition phase, the plant response to the product would emulate the phase in which the concentration occurs. Similarly, when the point is within the inhibition phase, phytotoxicity would occur. Generally, phytotoxicity has been the major challenge in the successful use of cucurbitacin Nemarioc-AL and Nemafric-BL phytonematicides. Originally, phytotoxicity was viewed as a major setback in the potential registration of the cucurbitacin phytonematicides (Bango, 2019; Mashela *et al.*, 2015; Pelinganga, 2013). Mashela *et al.* (2015) developed the dosage model concept, through the adoption of the CARD computer-based model (Liu *et al.*, 2003). The CARD model generates biological indices, where D_{50} on the inhibition phase was used to find its counterpart on the stimulation phase, which was technically referred to as MCSP (Mashela *et al.*, 2017a). Additionally, the CARD model provides the sensitivity index (k), which provides the level of sensitivity of the measured variable to the test product and the overall sensitivity ($\sum k$). Since the CARD is a statistical tool, the model also provides the coefficient of determination (R^2), which is important in indicating whether the output of the model were free from human bias. The MCSP = $D_m + (R_h/2)$ (Mashela *et al.*, 2017a), is described as the concentration at which a given phytonematicide concentration would not be phytotoxic to the test crop being protected against nematode damage, whereas nematode population densities would be suppressed consistently. The MCSP had been fondly referred to as the first law of phytonematicides. The first law is further used to derive the application interval (T_a),

where the concept of 'weeks-per-month-of-30 days' was multiplied by the life cycle of the test nematode in days. For example, for most thermophilic *Meloidogyne* species, the life cycle is 30 days, whereas for the citrus nematode (*Tylenchulus semipenetrans* Cobb 1913), the life cycle is 42 days (Van Gundy, 1958). In this manner, the application of the MCSP would disrupt the life cycle of the test nematode, at most twice within a given life cycle without inducing phytotoxicity to the protected crop (Mashela *et al*., 2015). Generally, the application interval constitutes the Second law of phytonematicides. Thereafter, the application frequency (T_f), which is the proportion of the crop cycle to the application interval, [(T_f = crop cycle (days)/application interval (days)] is computed as the third law of phytonematicides (Mashela *et al*., 2015). Once the application frequency is established the dosage model (D), as the fourth law of phytonematicides is computed (Mashela *et al*., 2017a), D (%) = C (%) × T_f.

1.1.5 General focus of the study

The general focus of the study was to develop protocols that would enhance cultivation of *S. frutescens* plants that would produce optimum concentration of phytochemicals. The latter would entail isolation and characterisation of nitrogen-fixing bacteria collections associated from cancer bush, morphological and biochemical techniques and pathogenicity tests, optimisation of cucurbitacin containing phytonematicides on cancer bush production to control root-knot nematode (*Meloidogyne* species) and its also about use of varied planting density to improve NWP of cancer bush.

1.2 Problem statement

Sutherlandia frutescens is an economically important legume with extensive medicinal properties of economic value. For example, the plant has been used in ailments such as HIV/AIDS-related illness, cancer and diabetes due to its unique phytochemical properties (Makgato *et al*., 2020; Raselabe, 2017). In South Africa, products of *S. frutescens* are available in liquid and capsule formulations for blood cleansing as “Lerumo la madi”[R], translated as the “Spear of blood”. The use of *S. frutescens* is increasingly being viewed as important in various health systems due to its extensive pharmacological and ethnomedicinal attributes, which prompted high harvesting pressure and potential with concomitant extinction of the crop (Chen *et al*., 2005). Due to global warming and the emergent of a wide range of disease-inducing pathogens, there is increased need for extensive evaluation of medicinal plants with phytochemicals that can be harnessed to ameliorate some of the vagaries induced by pathogens (Avdeenko *et al*., 2020; Herbst, 2020). Attempts to cultivate *S. frutescens* are limited by lack of sufficient knowledge on best agricultural practices that include appropriate nodulation bacteria, planting density, irrigation interval and suitable nematode management options, all with a focus on increasing the desired phytochemicals in the foliage of the plant. The concept of NWP, using the target phytochemicals, would enhance the availability of the materials from the plant, thereby enhancing its conservation from becoming extinct.

Successful cultivation of leguminous crops requires efficient nodulation bacteria that would be compatible with its host in order to ensure successful harvesting of inert nitrogen from the atmosphere, which is being viewed as best practice in husbandry of low input

leguminous crops (Gyogluu *et al*., 2018). Inoculation of medicinal plant species with *Rhizobium* and the association formed with various microorganisms can be formulated as biofertiliser and biocontrol tools to improve sustainable agriculture (Makgato *et al*., 2020). The nodulation bacterium in *S. frutescens*, along with their efficacy as compared to other commercial bacterium species, remains undocumented. In the wild, it was observed that roots of *S. frutescens* had prominent nodules. The first step in assessing the suitability of the association is comparing the efficacy of the wild strains with the commercial strains on plant growth variables and biosynthesis of nitrogen and proteins. Isolation and characterisation techniques of nodulation bacteria in *S. frutescens* and comparative evaluation of the nodulation bacteria with commercial strains would enhance the development of cheap and efficient inoculant for *S. frutescens*, as done in other indigenous plant species (Koskey *et al*., 2017).

Cucurbitacin Nemarioc-AL and Nemafric-BL phytonematicides have been shown to be highly effective and consistent in managing population densities of *Meloidogyne* species in several crops (Mashela *et al*., 2015; Mashela *et al*., 2017a). The MCSP, intended to ameliorate phytotoxicity was shown to be plant-specific (Mashela *et al*., 2017a). Additionally, it was shown that non-phytotoxicity was a function of plant type, application interval, application frequency and finally, the dosage that would have been applied at harvest.

In semi-arid regions, the availability of irrigation water becomes an increasingly limiting factor during the introduction of new crops (Chibarabada *et al*., 2017; Das *et al*., 2019).

The concept NWP was introduced to enhance the selection of crops by integrating the relationship among nutritional content, crop yield and water used to assimilate nutrient elements and chemical compounds of interest (Chibarabada, 2018). In this regard, agriculture could simultaneously address the challenges of increasing productivity and improving nutrition in areas with limited irrigation water.

1.3 Rationale of the study

Strategies to mitigate extinction of *S. frutescens*, such as large-scale cultivation, could be limited by lack of information on best agricultural practices, particularly in context of climate-smart agriculture. The phytochemical properties of *S. frutescens* have made its products to gain unprecedented attention in health circles current demand for the plant is even higher due to the devastation of Covid-19 pandemic, along with its related complications (Herbst, 2020). The development of agronomic practices in cultivating *S. frutescens* in water scarce and nematode infested areas, would enhance the successful cultivation of the test plant species as observed in other introduced plant species (Makgato *et al*., 2020). *Sutherlandia frutescens* as a legume requires compatible rhizobia inoculum to maximise production of this medicinal plant. The evaluation of natural nodulation bacteria in *S. frutescens* as potential inoculum and related capabilities to outperform the commercially available highly effective nodulation bacteria strains is yet to be documented. Inoculum using native rhizobia strains would provide cheap and efficient inoculum that even smaller scale farmers could afford as shown in other introduced leguminous plant species (Koskey *et al*., 2017; Zuluaga *et al*., 2020). Sustainable production of *S. frutescens* would certainly also depend on the management

of root-knot nematode population densities using the existing cucurbitacin phytonematicides.

1.4 Purpose of the study

1.4.1 Aim

Establishment of protocols that would enhance the successful cultivation of *S. frutescens* in context of climate-smart agriculture.

1.4.2 Objectives

1. To determine whether nodulation bacteria in roots of *S. frutescens* could be identified using morphological and biochemical techniques.
2. To investigate whether the efficacies of natural *S. frutescens* nodulation bacteria would be similar to those of commercial nodulation bacteria on growth and chemical composition of *S. frutescens.*
3. To investigate whether the dosage models of Nemarioc-AL and Nemafric-BL phytonematicides on *S. frutescens* would be similar under microplot conditions.
4. To determine whether planting density of *S. frutescens* would affect NWP of phytochemicals to enhance the cultivation of this medicinal plant under field conditions in semi-arid regions.

1.5 Null hypotheses

1. Nodulation bacteria in roots of *S. frutescens* could not be identified using morphological and biochemical techniques.
2. The efficacies of natural *S. frutescens* nodulation bacteria would not be similar to those of commercial nodulation bacteria on growth and chemical composition of *S. frutescens.*
3. The dosage models of Nemarioc-AL and Nemafric-BL phytonematicides on *S. frutescens* would not be similar under microplot conditions.
4. Planting density of *S. frutescens* would not affect NWP of phytochemicals to enhance the cultivation of this medicinal plant under field conditions in semi-arid regions.

1.6 Reliability, validity and objectivity

Reliability is being described as the extent to which a measuring instrument yields consistent results when the variable being measured repeatedly has not changed (Leedy and Ormrod, 2005). Statistical analyses provide various reliability checks on the data. In this study, reliability in various experiments was ensured by using appropriate levels of statistical significance for mean separation and when evaluating the variance explained by models as measured by coefficients of determination (R^2). Validity is described as an extent to which the instrument measures what was actually intended to be measured (Leedy and Ormrod, 2005). In empirical research, experiments are either replicated in time or space in order to increase the range of validity of conclusions drawn from it (Little

and Hills, 1981). Validity was ensured by conducting the experiment at the same location over time (Little and Hills, 1981). Objectivity is described as striving, as far as is practicable, to eliminate biases, prejudices or subjective evaluations by relying on verifiable data (Leedy and Ormrod, 2005). Objectivity was achieved by discussing the findings on the basis of empirical evidence as shown by statistical analyses, with findings compared and contrasted with findings in other studies (Little and Hills, 1981).

1.7 Bias

Bias is described as any influence, conditions or set of conditions that singularly or collectively distort the data (Leedy and Ormrod, 2005). In this study, bias was minimised by ensuring that the experimental error in each experiment was reduced through increased replications and randomisation of the treatments (Little and Hills, 1981).

1.8 Scientific significance of the study

The study would develop best agricultural practices for producing *S. frutescens* in the semi-arid regions. Such practices would include suitable nodulation bacteria, planting density, irrigation interval using the NWP with the descriptor being the phytochemicals, and the alternative nematode management strategy for the crop.

1.9 Structure of the thesis

Following the detailed outlining of the research problem (Chapter 1), the work done and not yet done on the problem statement was then reviewed (Chapter 2). Subsequent research chapters (Chapters 3, 4, 5 and 6) each addressed an objective. In order to

improve the flow of the thesis from one chapter to the other, at the end of the research chapters it was spelt out whether, on the basis of the findings, the null hypothesis was accepted or rejected. Also, a sentence was included to link up the current chapter to the next chapter by paraphrasing what would be done in subsequent chapter. In the final chapter (Chapter 7), findings were summarised, with emphasis being on the related significance. Recommendations with respect to the identified gaps were provided, followed by an overall conclusion which tied the entire study into a coherent unit. In the entire thesis, citation and reference styles followed the Harvard format of the author-alphabet as spelt out by the University of Limpopo Senate.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Cancer bush (*Sutherlandia frutescens* (L.R) Br.), a legume plant species indigenous to South Africa, contributes substantially to modern and traditional medicines (Gonyela, 2016; Van Wyk and Wink, 2004). The genus *Sutherlandia* belongs to the order Galegeae and is closely related to the genera *Astragalus* L. and *Lessertia* DC, within Fabaceae Family (Gonyela, 2016; Van Wyk and Wink, 2004). The medicinal uses of *S. frutescens* originated from the Kohi and Nama people for treating internal cancers and sterilising external wounds (Chen *et al*., 2005). *Sutherlandia frutescens* has long been known and respected as a medicinal plant in southern Africa and is regarded as the African adaptogens par excellence, which are unidentified chemicals with health benefits (Faleschini, 2013). In South Africa, the plant is often referred to as cancer bush, with other more than at least 25 local names (Gonyela, 2016). The stems, leaves, flowers, pods and roots are together or separately used to make infusions and decoctions (Aboyade *et al*., 2014). A decoction of *Sutherlandia* is used to wash wounds and eyes and one can drink it to reduce fevers, whereas infusions from leaves and stems are used to treat cancers, fever, diabetes, kidney/liver problems, rheumatism and stomach ailments (Aboyade *et al*., 2014).

In Southern Africa, *S. frutescens* is widely spread in the Western, Eastern and Northern Cape Provinces and some areas of KwaZulu-Natal, including the southern parts of Namibia and Botswana, along with the western, central and eastern parts of South Africa

and most of regions of Lesotho as shown in Figure 2.1 (Aboyade *et al.*, 2014; Gonyela, 2016; Raselabe, 2017; SANBI, 2014).

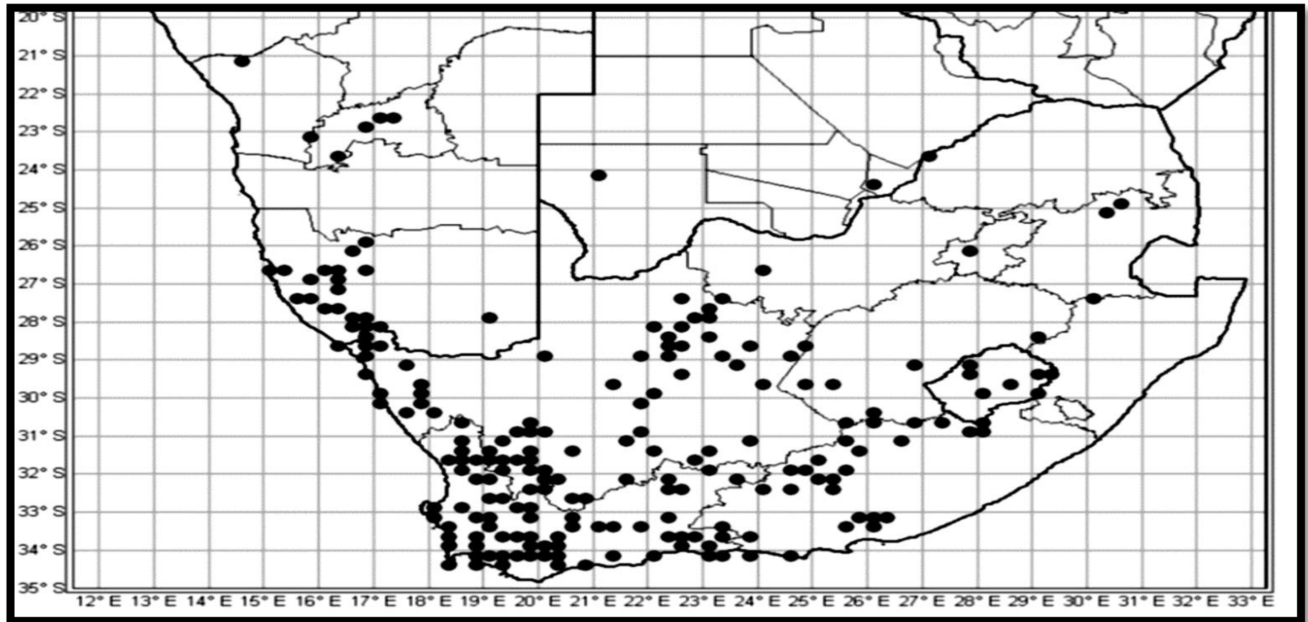


Figure 2.1 Distribution map of *Sutherlandia frutescens* in Southern Africa. Source: South African National Biodiversity Institute – (SANBI, 2014; Raselabe, 2017).

Sutherlandia frutescens is a drought-tolerant perennial short-lived shrub with the plant height being as high as 2 m. Leaves are divided into numerous small leaflets, which are slightly to densely hairy, often giving the plant a silvery appearance (Ojewole, 2004; Raselabe, 2017). The stems are globous or sparsely pubescent, with numerous pinnately compound leaves borne mainly towards the tip of shoots (Raselabe, 2017). The large, red flowers of the plant develop into characteristic balloon-like seed pods (Ojewole, 2004). The attractive, butterfly-like red flowers are pollinated mainly by sunbirds. The lightweight, papery, inflated pods enable the seed to be easily dispersed by wind (Raselabe, 2017).

Due to the health attributes of *S. frutescens*, there has been a marked increase in both scientific and commercial interests in the herb (Street and Prinsloo, 2013). The harvesting of medicinal plants is increasing, with recent surveys suggesting that at least 500 000 tons of dried medicinal plants were annually traded internationally, with substantial quantities being traded in South Africa's national and local markets (Loundou, 2008). More than 50% of the plants were harvested from the wild, and the demand for these materials kept increasing (Tanga *et al*., 2018). Global demand for medicinal plant products had been estimated at US$60 million per year (Rasethe *et al*., 2002), with current demand being even much higher with recent devastating global pandemics such as Covid-19.

Lately, there has been higher demand for *S. frutescens* and *Sutherlandia*-based products. Therefore, a better understanding of its optimal cultivation and processing practices have been identified as being in urgent need to assist in the successful commercialisation of the herb (Raselabe, 2017; Shaik *et al*., 2010). Currently there is improvement in development of strategies for enhancing successful cultivation of *S. frutescens* (Makgato *et al*., 2020; Raselabe, 2017). Such strategies include the biotechnological strategy (Shaik *et al*., 2010), which firstly involves micro propagation of *S. frutescens*, that aims to resolving the problems related to wild harvesting of material by supplementing *ex situ* cultivation or providing an *ex-situ* resource of acclimated plants. Secondly, through *Agrobacterium-mediated* transformation, a system which produces interesting biochemical changes would inform on the impact of transgenesis in *S. frutescens* (Shaik *et al*., 2010). Raselabe (2017) investigated the effects of pruning and fertiliser on growth,

phytochemistry and biological activity of *S. frutescens*. Makgato *et al.*, (2020) recently evaluated the effects of *Rhizobium* inoculation on N_2 fixation, phytochemical profiles and rhizosphere soil microbes of *S. frutescens*. Although there is some knowledge on cultivation protocols, information on isolation and characterisation of nodulation bacteria and its efficacy compared to commercial bacteria strain, pest management strategies and the value of crops in terms of nutritional water productivity (NWP) concept on *S. frutescens* in semi-arid Limpopo Province are yet documented.

2.2 Work done on the problem statement

2.2.1 Nodulation bacteria in cancer bush

The isolation and characterisation of nitrogen-fixing bacteria associated with the rhizosphere of a given leguminous plant species provide indispensable information on the development of an effective local strain for maximising the productivity of the test plant (Majeed *et al.*, 2015). Bacteria commonly found in leguminous crops belong to the Rhizobiaceae family, consisting of 61 species that belong to 13 different genera, which include *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Methylobacterium, Burkholdera, Cupriavidus, Devosia, Herbaspirillum, Ochrobactrum* and *Phyllobacterium* (Ndusha, 2011). Currently, the genera *Agrobacterium, Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Allorhizobium*, are recognised, with a rising number of species in the genera of rhizobia, with 53 species (Ndusha, 2011). Characterisation studies of bacterial strain suggested that *Rhizobium* and *Bradyrhizobium* strains are the commonly identified in different legumes (Gyogluu *et al.*, 2018; Zahran, 1999). *Rhizobium* species associated

with legumes have been described as being from very fast, moderate or to slow growers, depending on their growth rate on Yeast Extract Mannitol Agar (YEMA) (Odee *et al*., 1997; Simon *et al*., 2014). The *Bradyrhizobium* strains are predominantly slow growing bacteria and host-specific, whereas *Rhizobium* species have a wide host range and are fast growing genus. The growth of *Rhizobium* on YEMA occurs within 3-5 days, whereas *Bradyrhizobium* takes 6-8 days (Simon *et al*., 2014). However, there are many other nodulation bacterial genera associated with the rhizosphere of leguminous plants, which might or might not play any role in nitrogen-fixation (Rivas *et al*., 2009; Stepkowski *et al*., 2012).

Studies of rhizobia diversity have been conducted using several phenotypic and biochemical approaches. Such studies have shown that more than one *Rhizobium* type strain have been found from a single nodule, which is technically referred to as dual nodule occupancy (Ndusha, 2011). Initial isolation and characterisation of *S. frutescens* was rhizobia on different *Sutherlandia* species in Australia collected from Northern, Eastern and Western Cape Provinces of South Africa (Gerding *et al*., 2012). The aerobic gram negative *Burkholderia cepacia* was also found in nodules of root samples (Gerding *et al*., 2012). However, Rhizobia strains can also differ from one environment to another (Ndusha, 2011). *Enterobacter cloacae* MSR1 was identified from roots of non-nodulating alfalfa (*Medicago sativa L.*) using API20E biochemical identification system (Biomerieux, France) (Khalifa *et al*., 2016).

The rhizobia isolate of Brown hemp (*Crotolaria junceae* L.), grown in Assam State, India, were investigated for seven different biochemical characteristics that included catalase, oxidase, nitrate reduction, starch hydrolysis, urease, citrate utilisation and gelatin liquefaction tests. Findings based on morphological and biochemical characteristics suggested that most of the isolates were closely related to *Rhizobium leguminosarum* MTCC-99 and *Mesorhizobium thiogangeticum* MTCC-7001 (Singha *et al*., 2013). Similarly, Shahzad *et al*. (2012) confirmed the presences of *Rhizobium meliloti* from root nodules of alfalfa (*Medicago sativa* L.) using the seven morphological and biochemical characteristic tests. Additionally, the tests demonstrated the presence of *R. japonicum* and *B. japonicum* on root nodules of soybean (*Glycine max* L. Merr) (Gachande and Khansole, 2011). However, characterisation of nodulating bacteria on *S. frutescens* using similar tests had not been documented.

2.2.2 Capabilities of nodulation bacteria

Biological nitrogen fixation (BNF) has been widely used as a replacement of nitrogen fertilisers in legume production because of its economic efficiency in the provision of sustainable agroecosystem services (Koskey *et al*., 2017; Ouma *et al*., 2016). Fertilisation of a crop with nitrogen fertilisers represents a significant reduction in input cost both economically and environmentally (Arafa *et al*., 2018). Production of nitrogen fertilisers requires high amounts of non-renewable fossil energy, which is one of the reasons for the release of greenhouse gases (Arafa *et al*., 2018). Increased cultivation of legumes is essential as a mitigation strategy of global warming and the regeneration of nutrient-deficient soils, thereby providing needed nutrients to humans and animals (Laranjo *et al*.,

2014). The nitrogen fixing bacteria are the essential feature of leguminous plants due to their ability to colonise the rhizosphere soon after seed germination and then converting atmospheric N_2 into nitrate forms available to plants through the nitrogen fixation process (Laranjo *et al*., 2014).

There is increased preference of indigenous rhizobia strains for BNF due to their ability to interact positively with the resident soil microbiota and their adaptability to the local agroecological climatic conditions which often explain their superior performance over the exotic commercial strains (Koskey *et al*., 2017). The utilisation of native rhizobia as inoculants could promote ecologically sustainable management of agricultural ecosystems and enhance legume production due to their growth promoting traits and adaptability to soil and environmental stress (Koskey *et al*., 2017). Rhizobia can vary widely in their ability to nodulate various legume species, with some strains showing nodulation specificity and therefore nodulating only a limited number of hosts, whereas others are highly promiscuous and can nodulate a wide range of host plants (Gyogluu *et al*., 2018). The use of host-specific native rhizobia isolates is recommended because they adapt better to the local environmental conditions (Koskey *et al*., 2017; Ouma *et al*., 2016). In addition, native rhizobia isolates are persistent and have better survival rate and this could increase the chances of successful nodulation and nitrogen fixation in host plants (Koskey *et al*., 2017; Ouma *et al*., 2016). On the other hand, the inability of introduced commercial inoculants to compete well with native rhizobia population due to negative microbial interactions impedes their commercial use (Koskey *et al*., 2017; Ouma *et al*., 2016). Authentication of rhizobia to determine their symbiotic efficiency is required

to screen out effective native rhizobia isolates (Koskey *et al*., 2017).The use of the most probable number (MPN) technique to estimating efficacy of microbial populations in soils and agricultural products by the fact of its ability to estimate a microbial population size based on a process-related attribute has been proved efficient in rhizobia diversity studies and evaluating efficacy of rhizobia isolates (Ndusha, 2011). This method is a four-step process, first, inoculation of viable rhizobia on its specific host results in development of nodules. Second, nodulation on inoculated plants becomes a proof of the presence of infective rhizobia. Third, the absence of nodules is a proof of the absence of infective rhizobia (Ndusha, 2011). Finally, uninoculated plants are used as control, with the absence of nodules serving as negative control (Ndusha, 2011).

Various studies have compared the efficacy of commercial and wild isolates, without contradictions. Kawaka *et al*. (2014) observed that two natural nodulating bacterial strains had higher efficacy on growth of beans (*Phaseolus vulgaris* L.) when compared to two commercial strains, CIAT 899 and Strain 446. Similar evaluations on soybean when inoculated with indigenous rhizobia NAC46 and NAC17, resulted in greater growth and productivity when compared with soybean inoculated with commercial strains (Ndusha, 2011). Also, Gicharu *et al*. (2013) observed that nodulation was improved among three climbing bean cultivars when inoculated with native strains when compared with the commercial strains (Gicharu *et al*., 2013). Also, higher symbiotic efficiencies of four native rhizobia isolates ELM3, ELM4, ELM5 and ELM8, when compared to the commercial inoculant Biofix in climbing bean were observed.

2.2.3 Dosage model of cucurbitacin-containing phytonematicides

Sutherlandia frutescens is generally highly susceptible to root-knot (*Meloidogyne* species) nematodes and in most cases could not withstand the infection (Raselabe, 2017). Under field conditions, most plant nematodes were associated with the rhizosphere of *S. frutescens* suggesting that the plant is a good host (Raselabe, 2017). Nematodes were extracted from roots of a healthy living, a wilted and a dead *S. frutescens* plant, as well as from the soil. Examination of the root of an infected plants showed the presence of *M. javanica* in large numbers (Raselabe, 2017). A small number of *Scutellonema, Pratylenchus, Helicotylenchus* and *Tylenchorhynchus* species were also identified.

Global ban of synthetic nematicides from agrochemical markets due to their harmful effects on the environment, non-target organisms and chemical residues in produce, have led to nematode damage escalating to as high as 37% (Mashela *et al.*, 2017b). Following the suspension, focus to alternatives to managing nematode damage on crops resulted in the development of the alternative nematode management options (Mashela *et al.*, 2017a). The concept of nematode resistance in crops as a nematode management option was hampered by various challenges (Mashela *et al.*, 2015). The use of fermented plant extracts with nematicidal properties had been a success in managing plant nematodes in various crops and were primarily adopted as a mitigation strategy to the drawbacks of conventional organic amendments and as an alternative to the withdrawn synthetic chemical nematicides (Mashela *et al.*, 2020a; Mashela *et al.*, 2015).

Nemarioc-AG (G = granular formulation), Nemafric-BG Nemarioc-AL (L = liquid formulation) and Nemafric-BL phytonematicides, have cucurbitacin A and B active ingredients as shown before G or L. The A and B groups are being developed from dried fruits of wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* L.F.), respectively. Both the A and B groups had been highly effective in suppression of root-knot (*Meloidogyne* species) nematodes on various crops (Chokoe, 2017; Mafeo *et al*., 2011; Mashela *et al*., 2017a; Pelinganga, 2013; Sithole, 2016), in most cases with the efficacies being comparable to those of commercially available synthetic chemical nematicides (Mashela *et al*., 2015; Mashela *et al.,* 2017a).

Cucurbitacin B ($C_{32}H_{46}O_8$) is nonpolar and stable, whereas cucurbitacin A ($C_{32}H_{46}O_9$) is slightly polar and disintegrates rapidly to cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$) (Mashela *et al.,* 2017a). The tendency of the cucurbitacin phytonematicides to being phytotoxic to certain crops was resolved through the establishment of the dosage model (Mashela *et al*., 2015), which is a function of the Mean Concentration Stimulation Point (MCSP), the application interval and then the application frequency (Mashela *et al*., 2017a).

Mean concentration stimulation point (MCSP): The MCSP is a concept that was developed in an attempt to avoid phytotoxicity (Mashela *et al*., 2015) using the biological indices from the Curve-fitting Allelochemical Response Dose (CARD) algorithm model (Liu *et al*., 2003). The MCSP refers to the concentration of the phytonematicide which stimulates plant growth, while suppressing population densities of the target nematode

(Mashela *et al*., 2015; Mashela *et al*., 2017a). The MCSP is derived by subjecting the test plant to increasing concentration of the test phytonematicide which is applied on weekly basis for 56 days (Pelinganga, 2013). All the CARD-derived curves were skewed to the right (Liu *et al*., 2003, Mafeo, 2012), which suggested that the data were not having normal distribution. Thus, inference for the population mean could not be made from the means derived from such data. Consequently, the x-axis data were transformed in order to normalise the interval between any two points (Causton, 1977). The geometric series of the phytonematicide concentrations was further transformed using $\log_2 2^x$, where $\log_2 2 = 1$, with the x-axis resulting in 0, 1, 2, 3, 4, 5 and 6% phytonematicide (Tseke and Mashela, 2018).

The generated seven biological indices included:(1) threshold stimulation (D_m), (2) saturation point (R_h), (3) 0% inhibition (D_0), (4) 50% inhibition (D_{50}), (5) 100% inhibition (D_{100}), (6) degree of sensitivity (k) and (7) coefficient of determination $^{(R2)}$ (Liu *et al*., 2003). Opposite the biological index D_{50} on the stimulation phase was supposed to be the mid-stimulating point, however, the CARD model did not provide its value. After analysing CARD-generated curves from various experiments, Mashela *et al*. (2017a) derived the MCSP relation using the biological indices on the stimulation phase as:

$$MCSP = D_m + (R_h/2)$$

The D_m and R_h indices were as explained above. The relationship was validated in a wide range of crops (Chokoe, 2017, Malebe, 2019, Sithole, 2016), and demonstrated that at MCSP, the phytonematicide would not induce phytotoxicity on the protected plant, but would consistently suppress nematode population densities (Mashela *et al*., 2015;

Mashela *et al*., 2017a) as depicted in Figure 2.2. As depicted, it has been well-established that most allelochemicals affect biological systems through density-dependent growth (DDG) patterns (Liu *et al*., 2003), which comprise three phases, stimulation, neutral and inhibition phase (Salisbury and Ross, 1992) depending on the level of concentration and the degree of sensitivity of the test plant organs (Liu *et al*., 2003; Malebe, 2019; Mashela *et al*., 2015).

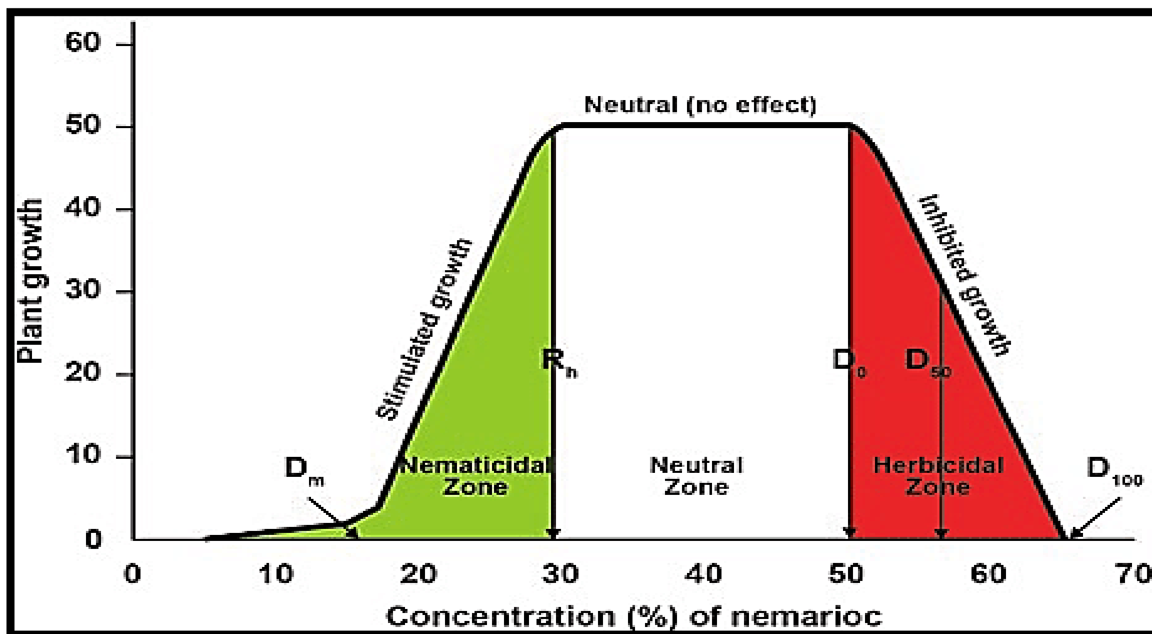


Figure 2.2 Indices of Curve-fitting response Dose model (Mashela *et al*., 2015).

The successful use of the cucurbitacin phytonematicides relied on the establishment of the MCSP, which was plant-specific (Mashela *et al.,* 2017). Importantly, in the empirical establishment of the MCSP, in addition to a geometric series of concentration that allowed the inclusion of the three phases, various plant organs are collected in order to base the MCSP on the whole plant rather than one organ. The MCSP values are interpreted alongside the k values for separate plant variables and the overall sensitivity of the plant

(Σk) to the phytonematicide (Liu *et al*., 2003; Mashela *et al*., 2015). Basically, Σ*k* is a function of the phytonematicide concentration, application rate, plant organs used and plant species (Mashela *et al*., 2015). The closer the (Σk) of the plant is to zero, the higher the sensitivity of the plant to the test phytonematicide, while the opposite is true (Liu *et al*., 2003).

Research findings on MCSP and overall sensitivity values of cucurbitacin phytonematicides to several plant species have been reported. Sithole *et al*. (2016) developed MCSP for Nemarioc-AL and Nemafric-BL phytonematicides for *Pelargonium sidoides* as 6.2 and 2.9% respectively, along with ∑k value of 3 units for both products. Similarly, Mathabatha *et al*. (2016) observed MCSP values of 8.6 and 6.3% with ∑k of 2 and 4 units of for Nemarioc-AL and Nemafric-BL phytonematicides on Citrus volkameriana seedling rootstock, respectively. On green bean (*Phaseolus vulgaris* L.) cv. 'Tahoe', the MCSP value for Nemarioc-AL phytonematicide was 2.67%, with the Σk = 20 units, whereas for Nemafric-BL phytonematicide the MCSP was 0.5% with the Σk = 6 units (Chokoe, 2017). The MCSP values for Nemarioc-AL and Nemafric-BL phytonematicides on squash (*Cucurbita pepo*) were 11.9 and 2.8%, with the ∑k) values of 0 and 3 units, respectively (Lebea, 2017). Generally, since the intention of developing MCSP values is to avoid phytotoxicity, when the value is excessively high, it could be adjusted to a lower value such as less than 3%, which were shown to consistently supress nematode population densities (Mashela *et al*., 2017a). As explained earlier (Chapter 1), since it is empirically-based, the MCSP had been referred to as the first law of phytonematicides.

Application interval: The application interval represents an interval between adjacent the successive applications of the phytonematicide that would not be phytotoxic, but would successfully suppress nematode population densities (Mashela *et al.,* 2015; Pelinganga, 2013). The MCSP for the test crop, along with the lifespan of the test nematode, are used in the empirical determination of the application interval (Mashela *et al.,* 2015). Mashela *et al.* (2017a) developed the concept 'weeks-per-month-of-30 days' for *Meloidogyne* species, where 30 days was the lifespan from egg to mature adult of *Meloidogyne* species. The concept, for *Meloidogyne* species could be expressed in daysas 0, 7.5, 15, 22.5 and 30 days or in weeks as 0, 1, 2, 3 and 4 weeks, which constitute the application interval for the previously derived MCSP value. The best would be in weeks since this would comply with inferences, referred to earlier in this chapter. Using MCSP 2.18% for Nemafric-BL phytonematicide on sweet potato 'Bophelo', the application interval was 19 days (Selomo, 2019). Using MCSP of 3% for Nemarioc-AL and Nemafric-BL phytonematicides the application intervals were 20 and 16 days, respectively (Pelinganga, 2013). The application interval constitutes the second law of phytonematicides.

Application frequency (Tf): The application interval allows the computation of the application frequency (Tf), which is defined as the proportion of the crop cycle to the application interval Tf = crop cycle (days)/application interval (days) (Mashela *et al*., 2017a), which is the third law of phytonematicides. The application frequency provides is primarily for planning purpose and it is also used in the establishment of the dosage model.

Dosage: Dosage (D) model is a product of MCSP (denoted as C (%) and the application frequency (Tf), summarised as D (%) = C (%) × Tf, and constitutes the fourth law of phytonematicides. Dosage is defined as the amount of the total active ingredient that would have been placed in a given environment by the end of the crop cycle (Mashela *et al.*, 2015). Generally, active ingredients of most pesticides are removed from the environment through bacteria, fungi and plants in a process referred to as bioremediation (Mashela *et al.*, 2021). Recently, Mashela *et al.* (2021) argued that bioaccumulation of cucurbitacins did not occur in produce since there were no cucurbitacin residues in fruit and leaves of various crops. Additionally, bacteria and fungi did not have enzymes that could biodegrade cucurbitacins. However, it was shown that another group of animals, the ecdyzoans, which include nematodes, were highly effective in bioremediation of cucurbitacins due to the unique properties that confer these group of chemical compounds the capabilities of isoprenylation (protein breakdown) and farneslation (protein biosynthesis). Further, Mashela *et al.* (2021) argued that due to the large number of ecdyzoans in the soil, their role in bioremediation could explain the relative shorter application intervals of cucurbitacin phytonematicides when compared with fumigant nematicides, which were renowned for their persistence in the soil, at times lasting for as long as 12 years (Van Gundy and McKenry, 1975).

2.2.4 Nutritional water productivity

Global climate change suggests extremes in climate, with high temperatures and droughts, which would impose unprecedented pressure on water resources (Carr *et al.*, 2016). The predictions have necessitated the development of new technologies and

adaptation strategies intended to help in improving water use efficiency in crop production (Carr *et al*., 2016). Adopting Water Productivity (WP) index in crop production is limiting since the concept aims at maximising the output by using less inputs without addressing the quality of the output (Kanda *et al*., 2020). Opportunities to improve WP of crops include deficit irrigation, water harvesting, soil fertility management, mulching, and installation of drip irrigation and selection of adapted crops for a particular region (Nyathi *et al*., 2018; Renault and Wallender, 2000). Linking WP to nutritional quality resulted in the concept nutritional water productivity (NWP) (Renault and Wallender, 2000). The NWP of a food crop focuses on energy (calories), vitamins, malnutrition mineral elements or phytochemicals (Li *et al*., 2021; Mabhaudhi *et al*., 2017). The concept NWP results in agricultural production being altered from focusing on quantity to focusing on improving quality, which is beneficial to ensuring nutrition security (Li *et al*., 2021). The concept allows for exploring cultural practices in a given environment that would improve the nutritional benefit of crops (Chibarabada *et al*., 2017; Nyathi *et al*., 2018).

The NWP concept is one of the integrated irrigation systems currently being advanced on different crops in context of climate-smart agriculture (Chibarabada *et al*., 2017; Das *et al*., 2019; Nyathi *et al*., 2018). For example, in comparison to other irrigation methods, the environment under drip irrigation was more beneficial to the accumulation of phytochemicals and the formation of total antioxidant activity in tomato fruits, which also significantly increased the NWP (Li *et al*., 2021). In groundnut (*Arachis hypogaea* L.), cowpea (*Vigna unguiculata* (L.) Walp), common bean (*Phaseolus vulgaris* L.) and Bambara groundnut (*Vigna subterranean* (L.) Verdc), different irrigation intervals across

different environments significantly affected NWP (Chibarabada *et al.*, 2017). In leafy vegetables amaranth (*Amaranthus cruentus* L.), cleome (*Cleome gynandra* L.) and Swiss chard (*Beta vulgaris* L.), the descriptors of NWP were fat, protein, Ca and Fe (Nyathi *et al.*, 2018). Although in most traditional studies of NWP the focus is on chemical compounds in edible produce as has been the case in leafy vegetables (Nyathi *et al.*, 2018), wheat (Moreira-Ascarrunz *et al.*, 2016) and sweet potato (Nyathi *et al.*, 2019), at times the descriptors include phytochemicals in the form of secondary metabolites, as had been the case in tomato (Li *et al.*, 2021). In general terms, NWP is described as the amount of water required to assimilate the descriptor of interest, say, for instance, Ca/m^3 water in fruit tissue of tomato. The NWP is suitable for selecting a crop that would be fit-for-purpose in a particular environment (Nyathi *et al.*, 2018; Renault and Wallender, 2000).

2.2.5 Effect of planting density on secondary metabolites

Plant secondary metabolites are a group of naturally occurring compounds biosynthesised through different biochemical pathways. The biosynthesis and resultant content of secondary metabolites are affected by various environmental factors that could be either abiotic or biotic factors (Pavarini *et al.*, 2012). Various genetic, ontogenic, morphogenetic and environmental factors have been among the factors with influence on the biosynthesis and accumulation of secondary metabolites (Yang *et al.*, 2016). Such metabolites are an important part of the plant defence system against attack by pests and gradual extremes imposed by environmental factors. Due to their remarkable biological activities, plant secondary metabolites are increasingly used as medicine ingredients and

food additives for therapeutic, aromatic and culinary purposes (Pavarini *et al*., 2012; Yang *et al*., 2016).

For centuries, humanity has exploited the physiological adjustments in medicinal plants as a source of improving biosynthesis of bioactive compounds. Considerable evidence of biotic and abiotic elicitors was shown to improve the biosynthesis of secondary metabolites in plant cells, particularly during the amelioration process of oxidative stress defence response (Selmar and Kleinwächter, 2013).

Secondary metabolites such as phenolic acids, flavonoids, terpenoids and alkaloids are crucial in plant adaptation to environmental stresses that include excessive light irradiation, temperature, soil water, soil fertility and salinity, which had been observed to enhance the production of certain amino acids, sugars, indoles and phenolic compounds (Kałużewicz *et al*., 2017; Yang *et al*., 2016). The effects of planting density on secondary compounds could emanate from competition for resources such as nutrients and water. Planting density had been adopted in several production systems as a physiological manipulation to increase the concentration of plant secondary metabolites (Kałużewicz *et al*., 2017; Wu *et al*., 2020). The manipulation was previously shown to influence the content of phenolic acids and flavonols in cauliflower curds (Kałużewicz *et al*., 2017), total phenolic content in globe artichokes (Lombardo *et al*., 2009), flavonoids, volatile oil, soluble sugar and soluble proteins in perilla sprouts (Wu *et al*., 2020), along with phenolic compounds in cabbage (Riad *et al*., 2009). Generally, high planting density modifies the photosynthetic rate and photosynthetic carbon assimilation capacity of different parts of

leaves (Wu *et al*., 2020). Although the planting density mainly affects the structure of plant population by increasing intraspecific competition for photosynthetically active radiation, water and nutrient minerals, the competition in turn impacts positively on assimilation of secondary metabolites (Wu *et al*., 2020).

2.3 Work not done on the problem statement

In Limpopo Province, *S. frutescens* has an economic potential of serving as an alternative crop in smallholder farming systems. Thus, isolation and characterisation of nodulation bacteria in *S. frutescens* along with assessing the efficacy of such bacteria in relation to existing commercial strains, would constitute an important component of cultivating the test crop under water-scarce environment. Hoverer, since the test plant is highly susceptible to infection by *Meloidogyne* species, it was imperative that existing alternative management strategies such as the use of cucurbitacin phytonematicides be investigated to ensure the successful introduction of the crop. Since the test plant is cultivated for its medicinal attributes, it was important that existing cultural practices such as planting densities, be included in order to assess if the practice could improve the descriptor, NWP, which was used to assess the suitability of the test plant under semi-arid conditions.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF NITROGEN-FIXING BACTERIA IN *SUTHERLANDIA FRUTESCENS*

3.1 Introduction

Leguminous plants such as *Sutherlandia frutescens* usually depend upon combined, or fixed, forms of nitrogen, such as ammonium and nitrate because it is unavailable in its most prevalent form as atmospheric nitrogen (Al-Mujahidy *et al*., 2013; Makgato *et al*., 2020). Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers (Al-Mujahidy *et al*., 2013). Use of these fertilizers has led to worldwide ecological problems as well as affects the human health and economic related issues for smaller holder farmers who struggle to afford this fertiliser (Al-Mujahidy *et al*., 2013). Biological nitrogen fixation (BNF) is the cheapest and environment-friendly procedure in which nitrogen-fixing micro-organisms, interacting with leguminous plants, fix atmospheric nitrogen into soil (Al-Mujahidy *et al*., 2013; Franche *et al*., 2009). Biological nitrogen fixation has been found to be 3 to 4 times more efficient than the N fertilisers, besides offering an environment-sound source of N to cropping systems (Ouma *et al*., 2016).

Rhizobium is the most well-known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen (Rasul *et al*., 2012; Zuluaga *et al*., 2020). The rhizosphere, which is defined as a thin soil layer close to the root system and is actively influenced by metabolic activity, has often been used as the preferential site for the isolation of plant

growth-promoting bacterial (PGPB) with potential applications as biofertilisers (Zuluaga *et al*., 2020). The legume-rhizobium interaction is the result of specific recognition of the host legume by *Rhizobium*, where various signal molecules that are produced by both *Rhizobia* and the legume confer the specificity (Al-Mujahidy *et al*., 2013).

The development of plant growth-promoting bacterial bio-inputs ultimately requires the collection of rhizo-competent strains and the elucidation of their role in plant growth and health, their resilience as a halobiont component and their ecological relationships within the plant rhizosphere, where important PGPB associations occur (Zuluaga *et al*., 2020). The isolation and characterisation of new *Rhizobium* isolates from different leguminous species helps to understand the diversity, evolution of rhizobia and for effective inoculation for effective nodulation using adapted rhizobial strain (Rasul *et al*., 2012; Valetti *et al*., 2016). Considering the potential value of *S. frutescens* in sustainable agriculture, in modern and traditional medicines, agroforestry, and the lack of studies on the diversity of rhizobia associated with these plants in the proposed study area, we aimed to collect and characterize rhizobia associated with this plant in the Limpopo province, South Africa. Therefore, the objective of the study was to determine whether nodulation bacteria in roots of *S. frutescens* could be identified using morphological and biochemical techniques. The null hypothesis was that nodulation bacteria in roots of *S. frutescens* could not be identified using morphological and biochemical techniques.

3.2 Materials and methods

3.2.1 Description of the study site

Cancer bush plants with nodulated roots were collected from two locations during spring 2018 and 2019 at Tubatse (24°63'52.5"S; 30°16'4.28"E) situated in the Sekhukhune District Municipality and Sebayeng (23°88'92.5"S; 29°17'8.38") in Capricorn District Municipality. The two sites with have mean annual rainfall of less than 600 mm and 500 mm, respectively, as centres of biodiversity for cancer bush in Limpopo Province. The two sites have minimum/maximum average temperatures of 7/28°C and 13/30°C, respectively. Plant roots (from 10 randomly selected plants) were collected using a spade, placed in cooler boxes and transported to Limpopo Agro-food Technology Station (LATS) University of Limpopo, South Africa (23°53'10"S, 29°44'15"E), for further processing.

3.2.2 Preparation of materials

Roots from each location were washed in distilled water and with healthy, undamaged, firm and pink nodules detached and sterilised in 0.1 mercuric chloride ($HgCl_2$) for 30 seconds to break the surface tension (Hamza and Alebejo, 2017). Nodules were then rinsed 10 times in pasteurised distilled water to remove traces of ($HgCl_2$) (Hamza and Alebejo, 2017) and transferred into a smasher biomerieux polythene bag containing 10 ml distilled water and crashed to produce a milky suspension. Components of rhizobium yeast extract mannitol agar medium (YEMA) (Table 3.1) were prepared as described previously (Hamza *et al.*, 2017). Yeast extract mannitol agar is the most widely used medium for the culture of rhizobia in leguminous crops (Niste *et al.*, 2013). The components of the medium were weighed and mixed in a 1 000 ml conical flask, with the

flask opening covered using aluminium foil (Table 3.1). The flask was half-filled with distilled water and mixed with the medium, with further adjusting to the mark and autoclaved at 121°C for 15 min. The cooled media was then poured into petri dishes.

Table 3.1 Materials for the preparation of rhizobium medium (Hamza *et al.*, 2017; Pervin *et al.*, 2017).

Compound	Formula	Concentration (g/l)
Distilled water	H_2O	1 000 ml
Mannitol	$C_6H_{14}O_6$	10.00
Anhydrous magnesium sulphate	$MgSO_47H_2O$	0.20
Sodium chloride	NaCl	0.10
Di-potassium hydrogen phosphate	K_2HPO_4	0.50
Anhydrous calcium chloride	$CaCl_22H_2O$	0.20
Anhydrous Iron chloride	$FeCl_36H_2O$	0.01
Yeast extract	-	1.00
Agar	$C_{14}H_{24}0_9$	20.00

3.2.3 Isolation of nodule bacteria from *Sutherlandia frutescens* roots

The milky suspension at 5 µl material was streaked on YEMA and incubated at 32°C for 48 h (Hamza and Alebejo, 2017). In order to enhance purity, repeated sub-culturing from a single colony streaking nutrient agar plates was done and the purified bacterial cultures were maintained by inoculating into the slant culture media in test tubes for future use (Hamza *et al*., 2017).

3.2.4 Morphological characterisation of nodule bacteria isolates from *Sutherlandia frutescens* roots

Bacterial colony characterisations were done including shape, colour, configuration, elevation and margin of bacterial colony were observed in colonies on nutrient agar plates of overnight grown microorganisms (Hamza and Alebejo, 2017). Typical rhizobia were recognised by their appearance and the growth rate. Usually, rhizobium colonies have milky colour, whereas bacterial colonies dark red (Figure 3.1) (CIAT, 1988). The cultures were examined microscopically by gram staining and colony count, with rhizobia being gram negative (CIAT, 1988).

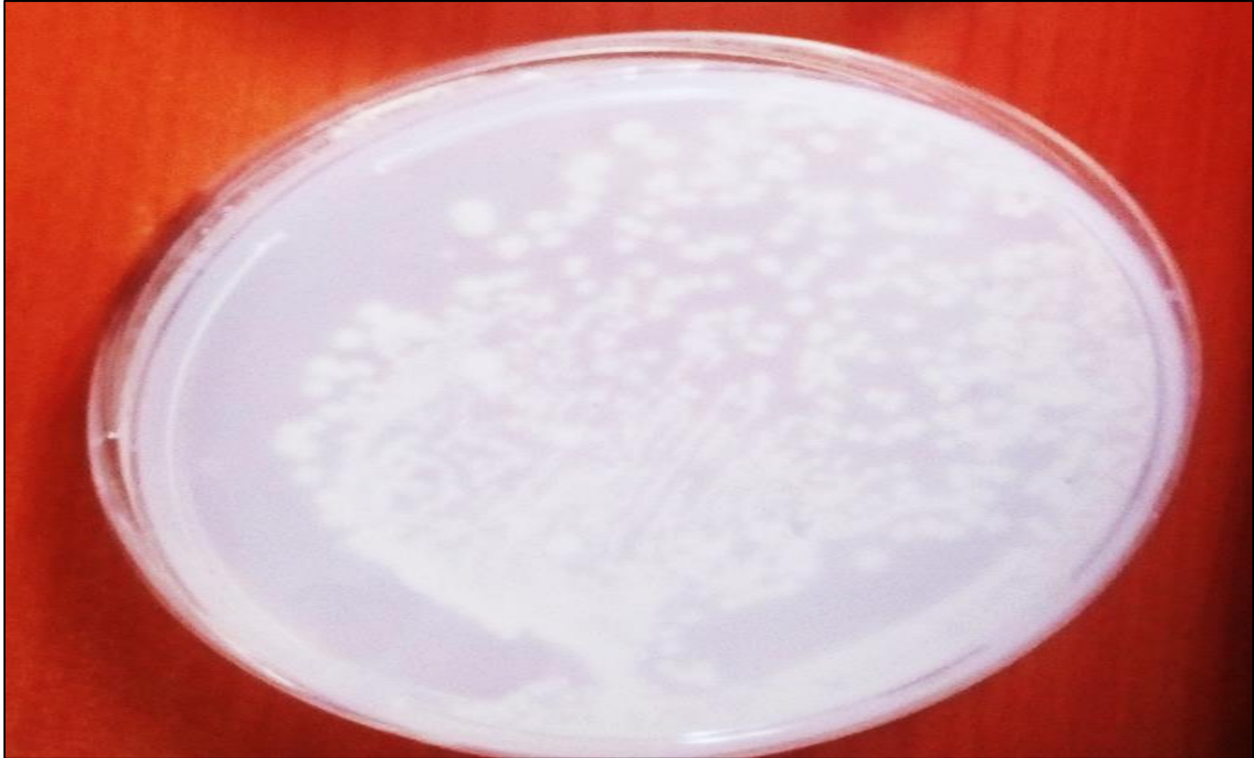


Figure 3.1 Growth of Rhizobium on yeast extract mannitol agar (YEMA) medium.

3.2.5 Reaction of nodule bacteria isolates from *Sutherlandia frutescens* roots to chemical stains

A loopful of normal saline was dropped at the centre of a slide and then a wire loop used to collect specimens from plates. Specimens were smeared in a circular manner at the centre of the slide, with the slide gently passed over the Bunsen burner flame for heat fixing (Hamza *et al.*, 2017). Crystal violet was put on the smear for 30 s and poured off. A drop of aqueous iodine was poured on the smear and left for 60 s, and thereafter washed off in tapwater (Hamza *et al.*, 2017). Ethanol was added for decolonisation and subsequently poured off, with counterstaining using Safranin done with water within a

minute. Finally, the slide surface was dried, with immersion oil added and isolates viewed under × 100 oil immersion objective lens (Hamza *et al*., 2017).

3.2.6 Biochemical characterisation of nodule bacteria isolates from *Sutherlandia frutescens* roots

The medium for biochemical test was prepared, inoculated with 5 µl purified bacterial cultures and incubated at 37°C for 48 h. Identification of the bacterial isolates was performed using VITEK 2 Systems (bioMérieux, Inc., North Carolina, USA) (Figure 3.2) (Titah *et al*., 2014). The Vitek 2 compact is an automated microbiology system utilising the methodology based on characteristics of data and knowledge about the organism and the reactions being analysed (Titah *et al*., 2014). Sufficient data were collected from known strains to estimate the typical reactions of the claimed species to a set of discriminating biochemicals (Pincus, 2006).

Generally, when a unique identification pattern was not recognised, a list of possible organisms was given, or the strain was determined to be outside the scope of the database (Pincus, 2006). The printed laboratory report was obtained whenever contained suggestions for any supplemental tests were necessary to complete the identification (Pincus, 2006). Bacterial isolates that were Gram negative rods were analysed using Vitex gram negative (GN) cards (Joyanes *et al*., 2001).

Figure 3.2 Biochemical identification machine, VITEK 2 Systems (bioMérieux, Inc., North Carolina, USA).

3.3 Results

3.3.1 Morphological characterisation of Tubatse isolates from *Sutherlandia frutescens* roots

A total of five isolates were detected as nitrogen-fixing bacteria through their growth on YEMA from which further characterisation was undertaken (Table 3.2). The isolated bacteria were able to grow on YEMA, turning the medium into a moderately cream white to yellowish colour (Al-Mujahidy *et al.*, 2013). Nitrogen-fixing bacteria isolates from *S. frutescens* grew rapidly, with maximum growth happening in 1 to 2 days after inoculation. Morphological characterisation of the isolates suggested that they were gram negative

with a circular configuration, cream white, flat, regular margins and rod-shaped colonies (Table 3.2).

Table 3.2 Morphological description of nodule bacteria isolates from *Sutherlandia frutescens* roots from Tubatse site.

Medium used	Isolates Code	Growth period (days)	Colon colour	Colony configuration	Colony margin	Elevation	Gram staining	Cell shape
YEMA	Aa-1	1−2	Creamy white	Circular	Regular	Flat	Negative	Rod
YEMA	Ac-1	1−2	Creamy white	Circular	Regular	Flat	Negative	Rod
YEMA	Ad-1	1−2	Creamy white	Circular	Regular	Flat	Negative	Rod
YEMA	Af-1	1−2	Creamy white	Circular	Regular	Flat	Negative	Rod
YEMA	AH-1	1−2	Creamy white to yellowish	Circular	Regular	Flat	Negative	Rod

3.3.2 Morphological characterisation of Sebayeng isolates from *Sutherlandia frutescens* roots

The collected 10 plants resulted in five isolates forming transparent creamy white to yellow colonies with growth rate duration from 2 to 4 days after incubation on Petri YEMA plates (Table 3.3) (Al-Mujahidy *et al*., 2013; Hamza *et al*., 2017). Similarly, the media turned moderately cream white to yellowish colour indicating the ability of isolates bacteria to grow on YEMA (Al-Mujahidy *et al*., 2013). Morphological characterisation of the isolates suggested that they were gram negative with a circular configuration, regular margins and rod-shaped colonies (Table 3.3).

Table 3.3 Morphological description of nodule bacteria isolates from *Sutherlandia frutescens* roots from Sebayeng site.

Medium used	Isolates Code	Growth period (days)	Colon colour	Colony configuration	Colony margin	Elevation	Gram staining	Cell shape
YEMA	Ma-1	2−4	Creamy white to yellowish	Circular	Regular	Flat	Negative	Rod
YEMA	Ma-2	2−4	Creamy white to yellowish	Circular	Regular	Flat	Negative	Rod
YEMA	Ma-3	2−3	Creamy white	Circular	Regular	Flat	Negative	Rod
YEMA	Ma-4	2−3	Creamy white	Circular	Regular	Flat	Negative	Rod
YEMA	Ma-6	2−4	Creamy white to yellowish	Circular	Regular	Flat	Negative	Rod

3.3.3 Biochemical characterisation of Tubatse isolates from *Sutherlandia frutescens* roots

Biochemical identification of isolates done using the VITEK 2 Systems bioMérieux, the genera *Raoutella ornithinolytica* and *Enterobacter cloacae spp. dissolvens* were identified in root nodules of *S. frutescens* (Table 3.4). The Aa-1, Af-1 and Ac-1 isolates were identified as *E. cloacae spp. Dissolvens*, whereas bacterial endophytes Ad-1 and AH-1 isolates are *R. ornithinolytica* (Table 3.4). The examined isolates varied slightly in utilisation of some carbon sources, Aa-1 and AH-1 tested positive to Alpha-Glucosidase, while the other isolates tested negative (Table 3.4). All isolates tested negative to Glu-Gly-Arg-Arylamidase, lipase and D-Tagatose (Table 3.4). Carbon source L-Pyrrolydonyl-Arylamidase was not used by Aa-1, Ac-1 and AH-1 isolates, whereas Ad-1 and Af-1 isolates used the source, also EllMan was not utilised Aa-1 and Af-1 isolates, but tested positively for Ac-1, Ad-1 and AH-1 isolates (Table 3.4). The identified isolates were regarded as fast-growing bacteria by positively utilising carbon sources such as urease, D-Mannitol, D-glucose and D-Mannose (Table 3.4). *Raoutella ornithinolytica* and *E. cloacae spp. dissolvens* consumed certain carbohydrates such as D-glucose, D-maltose and glucose as a sole carbon source and certain amino acids such as L-Proline Arylamidase as nitrogen source.

Table 3.4 Biochemical characterisation of nodule bacteria isolates from *Sutherlandia frutescens* roots from Tubatse site.

	Selected Isolates codes				
Biochemical test	Aa-1	Ac-1	Ad-1	Af-1	AH-1
Ala-Phe-Pro-Arylamidase (APPA)	−	−	−	−	−
H_2S production (H_2S)	−	−	−	−	−
Beta galactosidase (BGLU)	+	+	+	+	+
L-Proline Arylamidase (ProA)	+	+	+	+	+
Saccharose/Sucrose (SAC)	+	+	+	+	+
L-Lactate alkalinization (ILATK)	+	+	+	+	+
Glycine Arylamidase (GLYA)	+	+	+	+	+
0/129 Resistance (0129 Resistance)	+	+	+	+	+
Adonitol (ADO)	−	−	−	−	−
Beta-N-Acetyl-Glucosaminidase (BNAG)	+	+	+	+	+
D-Maltose (dMAL)	+	+	+	+	+
Lipase (LIP)	−	−	−	−	−
D-Tagatose (dTAG)	−	−	−	−	−
Alpha-Glucosidase (AGLU)	+	(−)	−	−	+
Ornithine Decarboxylase (ODC)	+	+	+	+	+
Glu-Gly-Arg-Arylamidase (GGAA)	−	−	−	−	+
L-Pyrrolydonyl-Arylamidase (PyrA)	−	−	+	+	−

Glutamyl Arylamidase (AGLTP)	−	−	−	−	−
D-Mannitol (dMan)	+	+	+	+	+
Palatinose (PLE)	+	+	+	+	+
D-Trehalose (dTRE)	+	+	+	+	+
Succinate alkalinization (SUCT)	+	+	+	+	+
Lysine Decarboxylase (LDC)	−	−	−	−	−
L-Malate assimilation (IMLTa)	−	+	−	−	−
L-Arabitol (IARL)	−	−	−	−	−
D-Glucose (dGLU)	+	+	+	+	+
D-Mannose (dMNE)	+	+	+	+	+
Tyrosine Arylamidase (TyrA)	+	+	+	+	+
Citrate (CIT)	+	+	+	+	+
Beta-N-Acetyle-Galactosaminidase (NAGA)	+	+	+	+	+
L-Histidine assimilation (IHISa)	−	−	−	−	−
ELLMAN (ELLM)	−	(+)	+	−	+
D-Cellobiose (dCell)	+	+	+	+	+
Gamma-Glutamyl-Tranferase (GGT)	+	+	+	+	+
Beta-Xylosidase (BXYL)	+	+	+	+	+

Urease (URE)	+	+	+	+	+
Malonate (MNT)	−	−	−	−	−
Alpha-Galactosidase (AGAL)	+	+	+	+	+
Coumarate (CMT)	−	−	−	−	−
L-Lactate assimilation (lLATa)	−	−	−	−	−
Beta-Galactosidase (BGAL)	+	+	+	+	+
Fermentation/Glucose (OFF)	+	+	+	+	+
Beta-Alanine Arylamidase (BALap)	−	−	−	−	−
D-Sorbitol (dSOR)	+	+	+	+	+
5-Keto-D-Gluconate (5KG)	−	−	−	−	−
Phosphatase (PHOS)	+	+	+	+	+
Beta-Glucoronidase (BGUR)	−	−	−	−	−
Identified organism (VTEK 2 compact system) Confirmed test 80 to 96%	*Enterobacter cloacae spp. dissolvens*	*Enterobacter cloacae spp. dissolvens*	*Raoutella ornithinolytica*	*Enterobacter cloacae spp. dissolvens*	*Raoutella ornithinolytica*

$^{+}$ Compatible, $^{-}$Incompatible.

3.3.4 Biochemical characterisation of Sebayeng isolates from *Sutherlandia frutescens* roots

The genera *Sphingomonas*, *Raoutella ornithinolytica* and *Enterobacter cloacae spp. dissolvens* were identified in root nodules of *S. frutescens* (Table 3.5). The Ma1 and Ma2 bacterial isolates were identified as *S. paucimobills*, whereas bacterial endophytes Ma3 and Ma4 are *E. cloacae spp. Dissolvens,* whereas bacterial endophyte Ma6 was *R. ornithinolytica* (Table 3.5). The tested isolates varied in utilisation of carbon sources, Ma3, Ma4 and Ma6 tested positive in utilization of sucrose, L-Lactate alkalinisation (lLATK), 0/129 Resistance (0129), Beta-N-Acetyl-Glucosaminidase (BNAG) and Citrate (CIT) while the other isolates tested negative (Table 3.5). The identified isolates Ma3, Ma4 and Ma6 were fast growers by positively utilising carbon sources urease and D-Glucose (dGLU), but negatively utilizing D-Mannitol and D-Mannose unlike those from Tubatse (Table 3.5).

Table 3.5 Biochemical characterisation of nodule bacteria isolates from *Sutherlandia frutescens* roots from Sebayeng site.

	Selected Isolates codes				
Biochemical test	Ma1	Ma2	Ma3	Ma4	Ma6
Ala-Phe-Pro-Arylamidase (APPA)	−	−	−	−	+
H_2S production (H_2S)	−	−	−	−	−
Beta galactosidase (BGLU)	−	−	+	+	+
L-Proline Arylamidase (ProA)	+	+	−	−	+
Saccharose/Sucrose (SAC)	+	+	+	+	+
L-Lactate alkalinisation (lLATK)	−	−	+	+	+
Glycine Arylamidase (GLYA)	−	−	+	+	+
0/129 Resistance (0129 Resistance)	−	−	+	+	+
Adonitol (ADO)	−	−	−	−	−
Beta-N-Acetyl-Glucosaminidase (BNAG)	−	−	+	+	+
D-Maltose (dMAL)	−	+	+	+	+
Lipase (LIP)	−	−	−	−	+
D-Tagatose (dTAG)	−	−	−	−	−
Alpha-Glucosidase (AGLU)	−	−	+	+	+
Ornithine Decarboxylase (ODC)	−	−	+	+	+
Glu-Gly-Arg-Arylamidase (GGAA)	−	−	−	−	−
L-Pyrrolydonyl-Arylamidase (PyrA)	−	−	−	−	−
Glutamyl Arylamidase (AGLTP)	−	−	−	−	−
D-Mannitol (dMan)	+	−	+	+	+

Palatinose (PLE)	−	−	+	+	+
D-Trehalose (dTRE)	+	+	+	+	+
Succinate alkalinization (SUCT)	+	−	+	+	+
Lysine Decarboxylase (LDC)	−	−	−	−	+
L-Malate assimilation (IMLTa)	−	−	−	−	−
L-Arabitol (IARL)	−	−	−	−	−
D-Glucose (dGLU)	+	+	+	+	+
D-Mannose (dMNE)	−	−	+	+	+
Tyrosine Arylamidase (TyrA)	+	−	+	+	+
Citrate (CIT)	−	−	+	+	+
Beta-N-Acetyle-Galactosaminidase (NAGA)	−	−	−	−	−
L-Histidine assimilation (IHISa)	−	−	−	−	−
ELLMAN (ELLM)	−	−	−	−	+
D-Cellobiose (dCell)	−	+	+	+	+
Gamma-Glutamyl-Tranferase (GGT)	+	+	−	+	+
Beta-Xylosidase (BXYL)	−	−	+	+	+
Urease (URE)	+	+	+	+	+
Malonate (MNT)	−	−	+	+	+
Alpha-Galactosidase (AGAL)	−	−	+	+	+

L-Lactate assimilation (ILATa)	−	−	−	−	−
Fermentation/Glucose (OFF)	−	−	+	+	+
Beta-Alanine Arylamidase (BALap)	−	−	−	−	−
D-Sorbitol (dSOR)	−	−	+	+	+
5-Keto-D-Gluconate (5KG)	−	−	−	−	−
Phosphatase (PHOS)	+	−	+	+	+
Beta-Glucoronidase (BGUR)	−	−	−	−	−
Identified organism (VTEK 2 compact system) Confirmed test 86 to 96%	*Sphingomonas paucimobills*	*Sphingomonas paucimobills*	*Enterobact-er cloacae spp. dissolvens*	*Enterobact-er cloacae spp. dissolvens*	*Raoutella ornithinolytica*

$^{+}$ Compatible,
$^{-}$Incompatible
.

3.4 Discussion

3.4.1. Morphological characterisation of Tubatse isolates from *Sutherlandia frutescens* roots

The isolates observed in the current study from *S. frutescens* were fast growing bacteria. Fast growing bacterial strains grow in three days or less, whereas relatively slow-growing bacteria should take at least six days (Amin, 2014). Isolates displayed the typical morphological characteristics of members of the genus *Enterobacter cloacae spp. dissolvens.* Khalifa *et al*. (2016) observed similar morphological characteristics when *E. cloacae spp.* was isolated from roots of alfalfa (*Medicago sativa* L.) plants. The observed isolates in the current study on YEMA turned the media to moderately yellow, supporting the observed fast generation period. Kawaka *et al*. (2018) had similar observation where isolates from common bean (*Phaseolus vulgaris* L.) were considered to be acid producers and fast growers and turned the media moderately yellow. All isolates under inspection were gram negative, cream white, a circular configuration, flat, regular margins and rod-shaped colonies. Results in the current study had similar characteristics as isolates in another study Ramesh *et al*. (2014), where *E. cloacae spp. dissolvens* was isolated from roots of soybean (*Glycine max (*L.) Merr.).

3.4.2. Morphological characterisation of Sebayeng isolates from *Sutherlandia frutescens* roots

The isolates from Sebayeng were also fast growing as those from Tubatse. Singha *et al*. (2013) studied isolates with a growth duration of 1-3 days on YEMA plates, with rhizobia producing white or creamy white colonies on YEMA media. Sadowsky *et al*. (1983) noted

that the fast-growing rhizobia had mean generation time ranging from 2 to 4 h, whereas the rhizobia referred to as slow growing had mean generation time for at least 6 h, and did not lower medium pH (Amin, 2014). All isolates from *S. frutescens* were gram negative, circular configuration, flat, regular margins and rod-shaped colonies. Isolates in the study displayed typical morphological characteristics of members in the genera *Enterobacter cloacae spp. Dissolvens* and *Sphingomonas paucimobills.* Ramesh *et al*. (2014) recorded similar observation on gram-stain-negative, rod-shaped bacterial strain in the genus *Sphingomonas paucimobills*. Gram negative, cream white, a circular configuration, regular margins and rod-shaped colonies of *E. cloacae spp. Dissolvens* are present in roots of soybean (Ramesh *et al*., 2014). The genus *Sphingomonas* is an aerobic and yellow pigment-producing bacterium which belongs to the aproteobacteria class (Kim *et al*., 1998).

3.4.3. Biochemical characterisation of Tubatse isolates from *Sutherlandia frutescens* roots

The genera *R. ornithinolytica* and *E. cloacae* were previously isolated from root nodules of *S. frutescens.* Khalifa *et al*. (2016) were among the first to use biochemical identification system (Biomerieux, France) in identification of root rhizobia in plants and identified *Enterobacter cloacae* in alfalfa plants. Gerding *et al*. (2012) outlined dissimilar results when isolating rhizobia of different *Sutherlandia* spp. in Australia collected from the Northern, Eastern and Western Cape Provinces of South Africa. The different results of the two studies were primarily due to location, time and environment from which the root nodules were collected. The utilisation of the mentioned carbon sources by the two

effective microorganisms suggested that *E. cloaca* spp. and *R. Ornithinolytica* were the fast growers. Similar results were observed when nitrogen-fixing bacteria were isolated from roots of pea (*Pisum sativum* L.) plants, with the isolates growing faster when using similar carbon sources (Deshwal and Chaubey, 2014). The utilisation of carbohydrates such as D-glucose, D-maltose and glucose as a sole carbon source and certain amino acids such as L-Proline Arylamidase as nitrogen source by *Raoutella ornithinolytica* and *E. cloacae spp. dissolvens* were as observed in another study (Khalifa *et al*., 2016). Basically, Khalifa *et al*. (2016) demonstrated that *E. cloacae* primarily consumed glycerol, D-xylose, D-maltose and esculin melibiose as a sole carbon source and arginine and tryptophan ornithine as nitrogen source. *Raoutella ornithinolytica* is a gram-negative bacterium of the *Enterobacteriaceae* family and is occasionally reported to have genes that induce plant growth promoting abilities (Thijs *et al*., 2014).

Enterobacter cloacae are gram negative bacteria, the Proteobacterium which belong to the family *Enterobacteriaceae* (Liu *et al*., 2013). The viability of the results for the identified organism *R. ornithinolytica and E. cloacae spp. dissolvens* was verified by their ability to use a wide range of carbon sources (Liu *et al*., 2013). Similar results were observed when genetic comparative analysis of *E. cloacae* was conducted, where a wide range of carbon sources with variation in biochemical content were used to separate isolates (Gonzalez *et al.,* 2018). *Enterobacter cloacae spp. dissolvens* and *R. ornithinolytica* ordinarily have the ability to colonise, adapt to diverse environments and benefit plant growth as observed in soybean, cucumber, corn, rice and ginger (Liu *et al*., 2013). However, although the efficacy of *E. cloacae* spp. dissolvens and *R. ornithinolytica* to fix

atmospheric nitrogen had being reported in certain leguminous crops, its efficacy on *S. frutescens* was not documented.

3.4.4. Biochemical characterisation of Sebayeng isolates from *Sutherlandia frutescens* roots

The genera *Sphingomonas paucimobills*, *Raoutella ornithinolytica* and *Enterobacter cloacae spp. dissolvens* were identified in root nodules from *S. frutescens*. Luo *et al*. (2019) demonstrated that *S. paucimobills* played a role in promoting plant growth of the thale cress (*Arabidopsis thaliana* (L.) Heynh). *Sphingomonas paucimobilis* is a gram-negative bacterium that had seldom been studied as a plant-associated bacterium, and to the present time, it has been found to exist in only a few plants, such as *Petunia hybrida. Sphingomonas paucimobilis* has been shown as having the potential of inducing plant growth in the medicinal plant *Dendrobium officinale* (Feng *et al*., 2014). The genus *Sphingomonas* spp. is viewed as a soil-dweller with attributes of plant-associated bacterium (Kim *et al*., 1998).

Luo *et al*. (2019) observed that *Sphingomonas* spp. promote growth of *Arabidopsis thaliana* by driving developmental plasticity in roots, thus stimulating the growth of lateral roots and root hairs. The *Sphingomonas* spp*.* bacterial isolates varied in using carbon sources, Ma3, Ma4 and Ma6 tested positive in using carbon sucrose and Citrate (CIT), whereas the other isolates tested negative. The identified isolates Ma3, Ma4 and Ma6 were fast growers by positively using carbon sources urease and D-Glucose (dGLU), but negatively exploiting D-Mannitol and D-Mannose unlike the Tubatse isolates. The results

of the current study were in line with assimilation of D-glucose and sucrose observed in bacterial isolation of *S. paucimobills* (Yang *et al*., 2016). However, Yang *et al*. (2016) had contradicting results on urease, since all strains of *S. paucimobills* were negative for that carbon resources, which was attributable to differences in locations from where the bacterial isolates were collected.

3.5 Synthesis and conclusion

The interest in the use of bio-fertilisers in context of climate-smart agriculture is unprecedented. In the current study, it was shown that the first step was to identify the effective microorganism responsible for ensuring that inert nitrogen was converted into forms available to plants. Two rhizobia, *Sphingomonas paucimobills*, *R. ornithinolytica* and *E. cloacae* were identified using morphological and biochemical techniques as being present in root nodules of *S. frutescens* at two different locations in Limpopo Province. Consequently, the null hypothesis which suggested that nodulation bacteria in roots from *S. frutescens* could not be identified using morphological and biochemical techniques was rejected. In the next research chapter, the researcher investigated the comparative effects of the identified nodulation bacterial isolates and the commercial strains on growth and chemical composition of *S. frutescens* plants cultivated under microplot conditions.

CHAPTER 4

EFFECTS OF COMMERCIAL AND NON-COMMERCIAL STRAINS OF NODULATION BACTERIA ON GROWTH AND CHEMICAL COMPOSITION OF *SUTHERLANDIA FRUTESCENS*

4.1 Introduction

Sutherlandia frutescens is a legume, with unknown nodulating bacteria species. Generally, the two commonly cited nitrogen-fixing bacteria include *Rhizobium* and *Bradyrhizobium* species, each with a wide range of associations from the wild (Gerding *et al.,* 2012). Several studies have shown that *Rhizobium*, a gram-negative N-fixing soil bacterium has a positive impact on most legume species (Laguerre *et al.,* 2007). *Bradyrhizobium*, a gram-negative bacterium, is one of the most cosmopolitan and diverse bacterial group nodulating a variety of legume species in Africa (Jaiswal and Dakora, 2019). The efficacy of the two Rhizobial strains is host- and location-specific (Hossain *et al*., 2019; Jaiswal and Dakora, 2019).

The first step in assessing the suitability of indigenous nodulation bacteria is comparing their efficacy with the existing commercial strains on plant growth variables and biosynthesis of nitrogen and proteins. Koskey *et al*. (2017) carried out greenhouse and field experiments to evaluate symbiotic efficiency, compare the effect of native rhizobia and commercial inoculant on nodulation, growth and yield parameters of mid-altitude climbing bean (MAC 13 and MAC 64) varieties. Results demonstrated a significant improvement in nodule dry weight and seed yields of MAC 13 and MAC 64 climbing bean

varieties upon rhizobia inoculation when compared to the non-inoculated controls (Koskey *et al.,* 2017). The symbiotic efficacy comparisons between native rhizobia and commercial inoculant on nodulation, growth and yield variables is a silver bullet in development of cost effective and environment-friendly inoculants. However, further characterisation and mapping of the native isolates would be imperative in development of effective and affordable commercial inoculants for the test plant species (Koskey *et al.*, 2017). Similar comparative studies would be important in *S. frutescens* since there is interest in the cultivation of this plant in smallholder farming systems (Makgato *et al.*, 2020). The development of an effective nodulation bacteria would provide a cheap inoculant and reduce the use of inorganic nitrogen fertilisers, which are costly and environment un-friendly (Zuluaga *et al.*, 2020). The objective of the study was to investigate whether the efficacies of natural *S. frutescens* nodulation bacteria would be similar to those of commercial nodulation bacteria on growth and chemical composition of *S. frutescens.* The null hypothesis stated that the efficacies of natural *S. frutescens* nodulation bacteria would not be similar to those of commercial nodulation bacteria on growth and chemical composition of *S. frutescens.*

4.2 Materials and methods

4.2.1 Description of the study site

The study was conducted at the University of Limpopo, Green Biotechnologies Research Centre (GBRCE), South Africa (23°53'10"S, 29°44'15"E) under microplot conditions. The location has mean annual rainfall below 500 mm, with the distribution being skewed towards summer months. The annual minimum/maximum temperatures averaged

10/38°C. Nodules from roots of *S. frutescens* plants were collected from Tubatse (Tubatse strain) and Sebayeng (Sebayeng strain) (Chapter 3) and prepared to generate the Tubatse and Sebayeng inoculation strains as described below.

4.2.2 Treatments and research design

The five treatments, namely, *Bradyrhizobium* spp. (Arachis) strain, *Rhizobium leguminosarum* strain, Tubatse strain, Sebayeng strain and untreated control, were laid-out in a randomised complete block design, with seven replications during the first season (Experiment 1) and with eight replications during the second season (Experiment 2). Blocking was done to minimise experimental error emanating from shading by windbreak trees in the morning and afternoon. Three days after transplanting, seedlings were fertilised with 2.5 g of NPK 2:3:2 (22) fertiliser mixture per plant to provide 186 N, 126 K and 156 P mg/ml water and 2 g NPK 2:1:2 (43) Multifeed (Nulandies, Johannesburg) fertiliser to provide a total of 0.35 N, 0.32 K and 0.32 P, 0.9 Mg, 0.75 Fe, 0.075 Cu, 0.35 Zn, 1.0 B, 3.0 Mn and 0.07 Mo mg/ml water (Tseke and Mashela, 2018). Control plants were irrigated weekly with 0.05% KNO3 solution to supply nitrogen (Koskey *et al.*, 2017). Plants were irrigated every other day with 250 ml chlorine-free tap-water to avoid leaching. Scouting and monitoring for insect pests were performed daily and none were observed.

Figure 4.1 Microplot layout in symbiotic efficacy trial of cancer bush at the Green Biotechnologies Research Centre of Excellence.

4.2.3 Procedures

The aseptic nodules from the two locations were shade-dried to allow for gradual water loss and for the bacteria to enter the dormant survival phase. Dried materials were ground separately. The two native strains (Tubatse and Sebayeng isolates) and the two commercial strains obtained from the Soygro Company (Potchefstroom, South Africa) were separately streaked on Yeast Extract Mannitol broth (YMB) and incubated at 25°C for the preparation of inoculants (Ndusha *et al*., 2017). The final *Rhizobium* cell density was adjusted to OD595 = 1.0 and then uniformly mixed with pre-autoclaved low peat soil at 12.5 ml/100 g peat soil and incubated for three days before treatment of *S. frutescens* seeds (Thilakarathna *et al*., 2019). The inoculum for each strain was prepared by mixing

bacterial suspension with low grade peat as the *Rhizobium* carrier material using the two-step method which involved adding a sticker to the seeds before the inoculant to support optimum growth and establishment of the rhizobia on the carrier (Burgos *et al.*, 1999; Chao and Alexander, 1984). The sticker was applied at the rate of 10 g per kg seed with 20% sugar-water (w/v) following the two-step inoculation method (Woomer *et al.*, 2011). Seeds were inoculated with the rhizobia at the rate of 10 g inoculant per 100 g seeds (Thilakarathna *et al.*, 2019).

Prior to inoculation, seeds were surface sterilised by immersing in 70% ethanol for 10 seconds to remove trapped air (Koskey *et al.*, 2017) and then immersed in 3% sodium hypochlorite solution for 3 minutes in an Erlenmeyer flask and rinsed six times in distilled pasteurised water (Ndusha, 2011). Seeds were left in the final rinsing water for 24 h to enhance imbibition and then air-dried for 30 min prior to manually sowing in 200-cone seedling trays, containing Hygromix-T (Hygrotech, Pretoria, South Africa) growing mixture. The 4-week-old seedlings, at four-leaf stage, were hardened-off outside the greenhouse using intermittent withdrawal of irrigation water and when 50% seedlings had wilted, they were taken to shade and irrigated to full capacity, with the ritual performed for two weeks. Thereafter, uniform seedlings were transplanted into 20-cm-diameter plastic pots, filled with approximately 2 700 ml steam-pasteurised loam soil and Hygromix-T at 3:1 (v/v) ratio. The soil comprised Hutton form (65% sand, 30% clay and 5% silt), containing 1.6% organic C, with EC at 0.148 DS/m and pH (H_2O) at 6.5. Microplots were established by inserting 20-cm diameter pots in 15-cm deep holes at 0.6 m × 0.6 m spacing.

4.2.4 Data collection

At 110 days after transplanting, plant height was measured from the crown to the tip of the flag leaf, were recorded. Branch numbers were counted, and shoots severed from roots, with stem diameter measured using a digital Vernier caliper at 5-cm above the severed end. Shoots were oven-dried at 52°C for 72 h and weighed. Roots were removed from pots, placed in polythene bags in cooler boxes and taken to the processing station where they were placed on a 75 mm opening sieve and rinsed with running tap-water to remove soil particles. Root nodule position on the root system was recorded, with healthy nodules removed and placed in sterile vials to record nodule number and nodule colour using the nodule colouration scale (Yates *et al*., 2016). Nodules and healthy mature leaves were oven-dried at 60ºC for 24-h for the determination of dry nodule mass (Ouma *et al*., 2016; Ulzen *et al*., 2016).

Approximately 0.40 g dried leaf material was digested in 40 ml 5% nitric acid (HNO_3) solution, followed by placing the container on a vortex to allow for complete wetting of the mixture (Thilakarathna *et al*., 2019). The material was magnetically stirred, thereafter incubated in a 95°C water-bath for 60 minutes. The samples were allowed to cool down at room temperature, filtered and then decanted into 50 ml tubes covered with a foil. Inductively Coupled Plasma Emission (ICPE-9000) was used to measure K and P in leaf tissue. Leaf tissues were analysed for N and protein (%) using the DUMAS Protein Content Analyser/LECO Nitrogen Analyser (Müller, 2014). Percent symbiotic efficiency SEF (%) was determined as outlined by Koskey *et al*. (2017). The symbiotic efficiency SEF (%) values were rated as > 80% = highly effective, 51-80% = effective, 35-50% =

lowly effective and < 35% = ineffective (Lalande *et al*., 1990).

4.2.5 Data analysis

The seasonal interactions (Experiment 1 × Experiment 2) on plant and nutrient elements were not significant ($P < 0.05$) and data for the two seasons were pooled ($n = 75$). Data for branch number and nodule number were transformed using $\log_{10}(x + 1)$ and subjected to Shapiro-Wilk test was performed on each dataset to determine the normality of distribution of the data (Ghasemi and Zahediasl 2012; Shapiro and Wilk, 1965), with the data depicting normal distribution. Data were then subjected to analysis of variance using Statistix 10.0 software. Mean sum of squares (MSS) were partitioned to establish the total treatment variation (TTV) on each variable (Little, 1981). Mean separation was accomplished using Fisher`s Least Significant Difference (LSD) test at the probability level of 5%. Unless otherwise stated, only means which were significant at the probability level of 5% were discussed.

4.3 Results

4.3.1 Plant growth variables

Treatments had highly significant effects on plant variables ($P \leq 0.01$, Appendix 4.1-4.2), contributing 87, 58, 66, 71 and 91% in (TTV) of plant height, root length, dry shoot mass, dry nodule mass and nodule number, respectively (Table 4.1). However, the treatments did not have significant effects on stem diameter, branch number and nodule position (Appendix 4.3-4.4). Relative to untreated control, *Bradyrhizobium* strain, *Rhizobium* strain, Tubatse strain and Sebayeng strain increased plant height, root length and dry

shoot mass by 31, 33, 44 and 40%, 30, 41, 40 and 42% and 48, 195 and 17%, whereas dry nodule mass and nodule number were reduced by 97, 98, 98 and 98% and 88, 89, 91 and 89%, respectively (Table 4.2). In some instances, the effects of native strains on plant growth variables were significantly better than those of commercial strains. Although not statistically different from Sebayeng strain, Tubatse strain recorded the highest plant height and higher dry shoot mass relative to the control and commercial strains (Table 4.2). Relative to the untreated control, *Rhizobia* increased root length, but the strains did not have significant differences (Table 4.2).

Table 4.1 Source of variation affecting plant height, root length and dry shoot mass of *Sutherlandia frutescens* at 110 days after transplanting under microplot conditions (n = 75).

		Plant height		Root length		Dry shoot mass		Dry nodule mass		Nodule number	
Source	Df	MS	%	MS	%	MS	%	MS	%	MS	%
Replication	14	150.1	9	59.582	27	267.22	24	1.090	15	0.032	5
Treatments	4	1431.9	87***	127.24	58***	733.85	66***	5.8088	77***	0.566	91***
Error	56	65.03	4	33.36	15	108.34	10	0.6237	8	0.026	4
Total	74	1647.03	100	220.18	100	1109.41	100	7.523	100	0.624	100

*** Highly significant at $P \leq 0.01$. TTV (%) = Total Treatment Variation.

Table 4.2 Effect of rhizobia inoculation on plant variables of *Sutherlandia frutescens* at 110 days after transplanting under microplot conditions (n = 75).

Strains	Plant height (mm)	RI (%)	Root length (cm)	RI (%)	Dry shoot mass (g)	RI (%)	Dry nodule mass (g)	RI (%)	Nodule number	RI (%)
Control	$56.22^{c} \pm 1.63$	–	$16.42^{b} \pm 1.23$	–	$8.38^{b} \pm 1.89$	–	$0.03^{b} \pm 0.02$	–	$0.05^{c} \pm 0.02$	–
Bradyrhizobium	$73.64^{b} \pm 2.55$	31	$21.39^{a} \pm 1.15$	30	$7.76^{a} \pm 1.40$	–99	$1.06^{a} \pm 0.29$	–97	$0.41^{b} \pm 0.05$	–88
Rhizobium strain	$74.68^{b} \pm 2.44$	33	$23.16^{a} \pm 2.16$	41	$12.37^{b} \pm 4.14$	48	$1.75^{a} \pm 0.20$	–98	$0.47^{ab} \pm 0.05$	–89
Tubatse strain	$81.05^{a} \pm 2.0$	44	$23.01^{a} \pm 1.59$	40	$24.70^{a} \pm 5.78$	195	$1.37^{a} \pm 0.26$	–98	$0.54^{a} \pm 0.14$	–91
Sebayeng strain	$78.68^{ab} \pm 2.81$	40	$23.27^{a} \pm 1.64$	42	$9.77^{b} \pm 1.84$	17	$1.42^{a} \pm 0.23$	–98	$0.46^{ab} \pm 0.05$	–89

[y]Column means ± standard error followed by the same letter were not different (P ≤ 0. 05) according to Fisher's Least Significant Different test. [z]Relative impact (RI %) = [(treatment/control) – 1] × 100.

4.3.2 Nutrient elements variables in *Sutherlandia frutescens* leaf tissues

Treatments had highly significant effects on N, protein and symbiotic efficancy (SEF), contributing 84, 74 and 31% in TTV of the respective variables (Table 4.3, Appendix 4.6-4.8). However, the treatments did not have significant effects on K and P in leaf tissues. Relative to untreated control, Tubatse, Sebayeng, *Rhizobium* and *Bradyrhizobium* strains increased N, protein and SEF (%) by 7, 25, 80 and 13%, 10, 24, 69 and 13% and 31, 133, 292 and 82%, respectively (Table 4.4). Relative to the untreated control and other rhizobia inoculates, Tubatse strain had the highest N in leaf tissues, while the lowest N was in leaf tissues of non-inoculated plants (Table 4.4).

Relative to commercial strains and the control, plants inoculated with Tubatse strain had the highest SEF (%) values with the relative mean value of 391%, but which was not significantly different from *Rhizobium* strain, whereas SEF (%) of Sebayeng strain was also not significantly different to that of *Bradyrhizobium* strain (Table 4). According to the accepted rating scale (Lalande *et al*., 1990), rhizobia inoculates used in the current study relative to the control were highly effective (SEF > 80%) in symbiotic nitrogen fixing efficiencies. Tubatse strain had the highest SEF (%), relative to the control and other rhizobia inoculates with percentage of 391% (Table 4.4). In the current study SEF (%) observed in native strains, commercial strains and the control ranged from 100 to 391%.

Table 4.3 Source of variation affecting shoot nitrogen, shoot protein and symbiotic efficiency of *Sutherlandia frutescens* at 110 days after transplanting under microplot conditions (n = 75).

		Shoot Nitrogen		Shoot Protein		Symbiotic efficiency	
Source	Df	MS	%	MS	%	MS	%
Replication	14	1.408	9	85.15	17	10.152	16
Treatments	4	13.35	84***	370.04	74***	19.695	31***
Error	56	1.134	7	42.98	9	33.645	53
Total	74	15.89	100	498.17	100	63.492	100

*** Highly significant at $P \leq 0.01$.

Table 4.4 Effect of rhizobia inoculation on nitrogen (N), protein (P) and symbiotic efficiency (SEF) (%) of *Sutherlandia frutescens* at 110 days after transplanting under microplot conditions (n = 75).

Strains	Shoot nitrogen (%)	RI (%)	Shoot protein (%)	RI (%)	Symbiotic efficiency (SEF) (%)	RI (%)
Control	2.94^{b} ± 0.26	–	18.40^{b} ± 1.65	–	100.00^{b} ± 0.00	–
Bradyrhizobium strain	3.14^{b} ± 0.20	7	20.15^{b} ± 1.17	10	131.38^{b} ± 23.96	31
Rhizobium strain	3.67^{b} ± 0.38	25	22.75^{a} ± 2.43	24	233.06^{a} ± 70.96	133
Tubatse strain	5.29^{a} ± 0.28	80	31.07^{a} ± 2.18	69	391.65^{a} ± 85.64	292
Sebayeng strain	3.32^{b} ± 0.25	13	20.77^{b} ± 1.54	13	182.86^{b} ± 37.86	82

[y]Column means followed by same letter were not different (P ≤ 0.05) according to Fisher`s Least Significance difference test (LSD). (SEF) (%) = Shoot dry weight (SDW) of inoculated plants/SDW of non-inoculated control plants supplemented with nitrogen × 100%. [z]Relative impact (RI %) = [(treatment/control) – 1] × 100.

4.4 Discussion

4.4.1 Plant growth variables

The rhizobia inoculants varied in impact on growth of *S. frutescens* (Table 4.2). The differences among the treatments on different plant variables had also been encountered in other studies where indigenous nodulation bacteria were compared with the commercial strains (Gicharu *et al*., 2013; Ouma *et al*., 2016). The increased plant height in plants inoculated with Tubatse strain relative to commercial strains were consistent with observations elsewhere (Table 2), where native strains outperformed commercial strains on increasing plant height in three climbing bean cultivars (Gicharu *et al*., 2013). Kawaka *et al*. (2014) also observed similar results when comparing four native strains with two commercial strains (CIAT 899 and Strain 446) on plant growth of beans. Apparently, in the current study and others (Karaca and Uyanöz, 2012; Ouma *et al*., 2016), the differences in plant height and root length (Karaca and Uyanöz, 2012) were due to disproportionate accumulation of plant growth regulators which were induced by the native strains.

Inoculation increased nodule numbers and dry nodule mass, which resulted in increased dry shoot mass, although the observed performance in the native strains was not as high as reported elsewhere (Gyogluu *et al*., 2018; Irisarri *et al*., 2019; Ulzen *et al*., 2016). Due to lack of sufficient rhizobia in control plants, limited nodulation could have led to significant decrease in dry nodule mass when compared with observations on plants inoculated with nodule mass Tubatse strain as observed in other native strains (Karaca and Uyanöz, 2012). The presence of a higher nodule number and mass on plants

inoculated with native strains suggested that there was better combining and symbiotic relationship between native strains and *S. frutescens*, as described in another related study (Ndusha, 2011). Nodulation is an important symbiotic trait for effective symbiosis between *Rhizobium* species and legume host plants. Observations in the current study agreed with those of others Karaca and Uyanöz, 2012; Ouma *et al*. (2016), where rhizobia inoculate had similar effects on growth of soybean. In the current study, dry shoot mass was significantly increased in plants inoculated with Tubatse strain than other rhizobia strains and the untreated control. Koskey *et al*. (2017) observed similar results, were climbing beans inoculated with native isolate ELM3 had the highest dry shoot mass when compared with commercial strains and control treatments. Generally, dry matter can be used as an indicator for symbiotic effectiveness of nodulation isolates since the variable is significantly correlated with nitrogen fixation (Arafa *et al*., 2018).

4.4.2 Nutrient elements variables in *Sutherlandia frutescens* leaf tissues

The influence of rhizobia inoculants on chemical composition of *S. frutescens* relative to the control exhibited increased N and protein content in leaf tissues of plants inoculated with Tubatse strain (Table 4). In other studies (Koskey *et al*., 2017; Ouma *et al*., 2016), native strains also outperformed commercial strains on assimilation of certain nutrient elements in leaf tissues of test plants.

According to the rating scale developed by Lalande *et al*. (1990), relative to untreated control, rhizobia strains used in our study increased symbiotic nitrogen fixing efficiencies (SEF > 80%). The observed high symbiotic efficiency in plants treated with Tubatse strain,

concurred with observations where native strains were used in climbing beans, whereas Koskey *et al*. (2017) observed SEF (%) values that ranged from 86.17 to 123.72% when compared to those of commercial strains. Generally, the SEF (%) values were rated as being highly effective when SEF > 80%, effective when SEF was < 80%, but > 50%, lowly effective when SEF was 35%, but < 50% and ineffective when SEF < 35% (Lalande *et al*., 1990). Similarly, Kawaka *et al*. (2014) noted SEF (%) values due to native strains in Western Kenya that ranged from 67 to 170% when compared with values from commercial strains and control plants. Unfortunately, native strains are not as resilient as commercial strains under different conditions and could fail to compete successfully with commercial Rhizobia species in certain plant species (Kebede *et al*., 2020).

4.5 Synthesis and conclusion

Native rhizobia were potentially superior or had similar effects to those induced by commercial inoculants in relation to plant growth performance, symbiotic efficiency and assimilation of nutrient elements in leaf tissues of cancer bush. Consequently, the native strains in the current study have great potential of being further developed to provide cheap and efficient inoculum in the development of best agricultural practices for *S. frutescens*. The native rhizobia, as identified previously (Chapter 3), have an added advantage of being more adapted to the soils than the introduced commercial inoculant strains. The efficiency of utilisation of this strain as an inoculant needs further confirmation under field conditions. Response to inoculation remains highly site specific and depends on factors beyond the effectiveness and competitiveness of the strains used. Further adaptability studies of the strains would enhance chances of developing a cheap

inoculant that could be of great use in the introduction of *S. frutescens* on commercial scale of cultivation. In the current study, the null hypothesis, which stated that the efficacies of natural *S. frutescens* nodulation bacteria would not be similar to those of commercial nodulation bacteria on growth and chemical composition of *S. frutescens* was rejected. In the subsequent research chapter, the research focused on the development of the dosage model for cucurbitacin containing phytonematicides on *S. frutescens* under microplot conditions in order to manage nematode population densities without inducing phytotoxicity on the crop being protected against nematode damage.

CHAPTER 5

DOSAGE MODEL FOR CUCURBITACIN PHYTONEMATICIDES ON *SUTHERLANDIA FRUTESCENS*

5.1 Introduction

Cancer bush (*Sutherlandia frutescens* (L.R.) Br.) is highly susceptible to the root-knot (*Meloidogyne* species) nematodes, with most infected plants entering a permanent wilting phase (Raselabe, 2007). Traditionally, nematode population densities were managed using synthetic chemical nematicides, which were withdrawn from the agrochemical markets due to their environment-unfriendliness (Mashela *et al.*, 2017a). Fumigant nematicides contained halogens, which are elements in Group VII of the Periodic Table. Halogens are highly reactive with ozone (O_3) layer, with the interaction resulting in depletion of the layer, resulting in large quantities of ultraviolet rays arriving on earth. The other human-induced layer, the greenhouse layer, forming a sheath around the earth, allows the ultraviolet rays with the short wavelength to pass through to the earth. However, the earth-reflected rays, with long wavelength, cannot pass through the greenhouse layer, and become reflected to earth, resulting in global warming (MacCracken, 2001). The effects of global warming have been felt in various parts of the world in the form of flood, droughts, and excessive high temperatures (Trenberth, 2005).

Additionally, pests which were previously viewed as minor, are increasingly becoming major aggressive economic pests. Globally, *M. incognita* and *M. javanica* had been viewed as being the major thermophilic *Meloidogyne* species, with wide distribution,

where *M. incognita* was viewed as being more aggressive than *M. javanica* (Taylor and Sasser, 1978). In contrast, in South Africa, *M. javanica* was viewed as being more aggressive than *M. incognita* (Kleynhans *et al*., 1996). Another thermophilic *Meloidogyne* species, the guava root-knot nematode, *M. enterolobii*, is currently viewed as the most aggressive among the thermophilic *Meloidogyne* species, with the lifespan of 15 days (Collett, 2020). The nematode is not affected by the Mi resistance genes (the first isolate-specific insect resistance gene to be cloned and belongs to the nucleotide-binding, leucine-rich repeat family of resistance genes) and is gaining wide distribution in South Africa (Collett, 2020; Maleka, 2021).

Currently, in other crops the South African population densities of *Meloidogyne* species are consistently managed using the cucurbitacin phytonematicides, namely, Nemarioc-AL and Nemafric-BL phytonematicides, derived from fruit of two plants indigenous to Limpopo Province. However, the products have challenges of phytotoxicity, which was resolved through the empirically-based Mean Concentration Stimulation Point (MCSP), developed through a computer algorithm model, the Curve-fitting Alleochemical Response Dose (CARD), along with the related application interval and the application frequency, technically referred to as the dosage model, which is highly crop and nematode specific (Chapter 2; Mashela *et al*., 2017a). The dosage model had not been developed for *S. frutescens*. The objective of this study was to investigate whether the dosage models of Nemarioc-AL and Nemafric-BL phytonematicides could be established for *S. frutescens* under microplot conditions. The null hypothesis was that the dosage

models of Nemarioc-AL and Nemafric-BL phytonematicides could not be established for *S. frutescens* under microplot conditions.

5.2 Materials and methods

5.2.1 Description of the study site

The study for the establishment of the dosage model for Nemarioc-AL and Nemafric-BL phytonematicide was initiated under microplot conditions at the Green Biotechnologies Research Centre of Excellence (GBRCE), University of Limpopo, South Africa (23°53'10"S, 29°44'15"E) during autumn (February-April) 2017 and validated in 2018. The location has mean annual rainfall below 500 mm, with the distribution being skewed towards summer (October-January). The annual minimum/maximum ambient temperatures averaged 10/38°C. The determination of the MCSP, the application interval for each product was investigated using lifespan of *M. javanica* species under microplot conditions at the GBRCE during summer (November-January) 2018 and validated in 2019.

5.2.2 Research design

In MCSP experiments, seven treatments, namely, 0, 2, 4, 8, 16, 32 and 64% for each phytonematicide, were arranged in a randomised complete block design (RCBD), with 8 replicates. Blocking was done for afternoon shading from nearby windbreak trees (Gomez and Gomez, 1984). In application interval experiments, treatments, based on "weeks-per-month-of-30 days" for *M. javanica*, which translated to 1, 2, 3 and 4 weeks (Mashela *et al*., 2017b), were arranged in a RCBD, with 10 replicates.

Figure 5.1 Microplot layout experiement for mean concentration stimulation point of cancer bush at the Green Biotechnologies Research Centre of Excellence.

Figure 5.2 Microplot layout experiment for application interval experiment of cancer bush at the Green Biotechnologies Research Centre of Excellence.

5.2.3 Procedures

Purchased seeds of cancer bush (Mountain Herb Estate, Tzaneen, South Africa) were sown in 200-cone polystyrene seedling trays, containing Hygromix-T (Hygrotech, Pretoria, South Africa) growing mixture and placed on the greenhouse benches. At five leaf-stage, seedlings were hardened-off outside the greenhouse using intermittent withdrawal of irrigation water for two weeks under direct sunlight on a hardening-off stand (Tseke and Mashela, 2018). Briefly, when more than 50% seedlings had partially wilted, trays were removed to the shade and irrigated to full capacity. After recovering, the seedling trays were returned to the hardening-off site. Hardened-off seedlings were transplanted into 20-cm-diameter plastic pots, filled with 2 700 ml steam-pasteurised river

sand (300°C for 1 h) and Hygromix at 3:1 (v/v) ratio. The microplots were established by inserting the 20-cm-diameter plastic pots in 15-cm-deep holes at 0.6 m × 0.6 m spacing. Seedlings were irrigated using 250 ml every other day.

In the preparation of Nemarioc-AL and Nemafric-BL phytonematicides approximately 80 and 40 g ground material of dried fruits *Cucumis myriocarpus* Naude and *C. africanus* L., respectively, were fermented in 20 L-hermetically sealed plastic containers with 16 L chlorine-free tapwater as previously described (Mashela *et al*., 2017a). Briefly, allowance for the released CO_2 to escape from the containers was provided through an airtight 5 mm diameter tube with one end glued to an airtight hole on the lid of the 20 L containers. The outlet end dangled into a litre bottle half-filled with tapwater. Approximately 300 ml molasses, 100 g brown sugar and 300 ml (ZZ2) effective microorganisms (EM) were added into each container. After a 14-day fermentation period, when pH was at least ±3.7, the products were ready for use (Kyan *et al*., 1999). In the MCSP trials, phytonematicides were applied once a week as a substitute for irrigation. Seedlings were irrigated every other day with 250 ml chlorine-free tapwater. Three days after transplanting, each plant was fertilised with 2.5 g NPK at 2:3:2 (22) + 0.5% Zn + 5% S + 5% Ca to provide 74.3 g N, 111.4 g P, 74.3 g K, 5 g Zn and 50 g Ca per plant. Also, 1 g NPK 2:1:2 (43) Multifeed (Nulandies, Johannesburg) provided 0.47 N, 0.43 K, 0.43 P, 121 Mg, 1 Fe, 0.10 Cu, 0.47 Zn, 1.34 B, 4.02 Mn and 0.09 mg Mo per ml tapwater, without Ca (Mashela *et al.,* 2015). Scouting and monitoring for insect pests were performed daily, but none was observed. Manuel weeding was done among pots using hand hoes when necessary.

Meloidogyne javanica eggs and second-stage juveniles (J2) were extracted from roots of the greenhouse-grown nematode-susceptible tomato (*Solanum lycopersicum* L.) cv. 'Rodade' in 1% NaOCl solution (Hussey and Barker, 1973). In each phytonematicide experiment, seedlings were inoculated with 5 000 *M. javanica* eggs + J2 using 20 ml plastic syringe by placing into approximately 3-cm-deep holes on the cardinal points of the stem, with holes covered by growing mixture.

In application interval experiments, all procedures were similar to those in MCSP experiments except that, pots were at 1 m × 1 m spacing. Each seedling was inoculated with 5 000 *M. javanica* eggs and J2 at 7 days after transplanting. Treatments, developed using the concept “weeks-per-month-of-30 days” (Chapter 2), comprising 7.5, 15, 21 and 30 days, were initiated at 7 days after inoculation using the empirically derived MCSP values of Nemarioc-AL (3.43%) and Nemafric-BL (4.03%) phytonematicides. Cultural practices were similar to those in the MCSP experiments.

5.2.4 Data collection

At 56 days after inoculation, for both MCSP and application interval trials, branch number per plant were recorded and plant height measured from the crown to the tip of the flag leaf using a measuring stick. Shoots were severed at the crown and stem diameter measured using a digital Vernier calliper at 5-cm above the severed end of the stem. Shoots were dried in air-forced ovens at 52°C for 72 h weighed and recorded. Root systems were removed from pots, immersed in water to remove soil particles, blotted dry using laboratory paper towel to remove excess water and then weighed. Root galls were

assessed using the North Carolina Differential Rating scale, where 0 = no gall, 1 = 1−2 galls, 2 = 3−10 galls, 3 = 11−30 galls, 4 = 31−100 galls and 5 ≥ 100 galls (Taylor and Sasser, 1978), with fresh roots weighed and recorded. Roots from untreated control were dried in air-forced oven at 52°C for 72 h and weighed. Nematodes were extracted from total root systems per plant using the maceration and blending method in 1% NaOCl solution (Hussey and Barker, 1973). The materials were passed through nested 75 µm and 25 µm opening sieves, with contents from 25 µm opening sieve collected for further processing using the sugar floatation and centrifugation method to separate debris from nematodes (Jenkins, 1964). The soil from each pot was mixed and a 250 ml soil subsample collected for J2 extraction using the sugar floatation and centrifugation method (Jenkins, 1964). Eggs + J2 from roots and J2 from 250 ml soil subsamples were each placed in 100 ml containers, with water added to the mark, stored in the cold room at 5°C and then counted from a 5-ml aliquot at 60 × magnification under a stereomicroscope (Zeiss Stemi 2000, Model number: Z4SV240Vac) within 4 weeks. Nematodes were expressed as final nematode population densities (Pf).

5.2.5 Data analysis

In MCSP experiments, prior to running the plant variable means in the CARD model, the concentration with the geometric series 0, 2, 4, 8, 16, 32 and 64% Nemarioc-AL or Nemafric-BL phytonematicide were first converted to exponents (2°, 2^1, 2^2, 2^3, 2^4, 2^5 and 2^6) and then log-transformed to promote normality of measurements using $\log_2 2^x = x (\log_2 2)$, where $\log_2 2 = 1$, to generate the x-axis values of 0, 1, 2, 3, 4, 5 and 6% phytonematicide (Causton, 1977). The geometric series ensured that all phases of the

density-dependent growth (DDG) patterns induced by allelochemicals were covered (Mashela *et al.*, 2017a). In contrast, the log-transformation was performed to ensure that the curve-fitting challenges where the curves were skewed to the right (Liu *et al.*, 2003), thereby ensuring the normality of the datasets. The MCSP values were calculated using the biological indices of plant variables as previously described (Chapter 2). The indices included the sensitivity values (k-values), along with the calculated overall sensitivity (Σk) values (Table 5.1). Discrete nematode data were log-transformed through $\log_{10}(x + 1)$ to homogenise the variances, and then subjected to lines of the best fit (Gomez and Gomez, 1984).

In application interval trials, the treatments 0. 7.5, 15, 22.5 and 30 days were expressed as 0, 1, 2, 3, and 4 weeks, with plant variables subjected to lines of the best fit (Gomez and Gomez, 1984). The relations were modelled by the regression curve estimates of the quadratic equation (Mashela *et al.,* 2015):

$$Y = b_2x^2 + b_1x + a$$

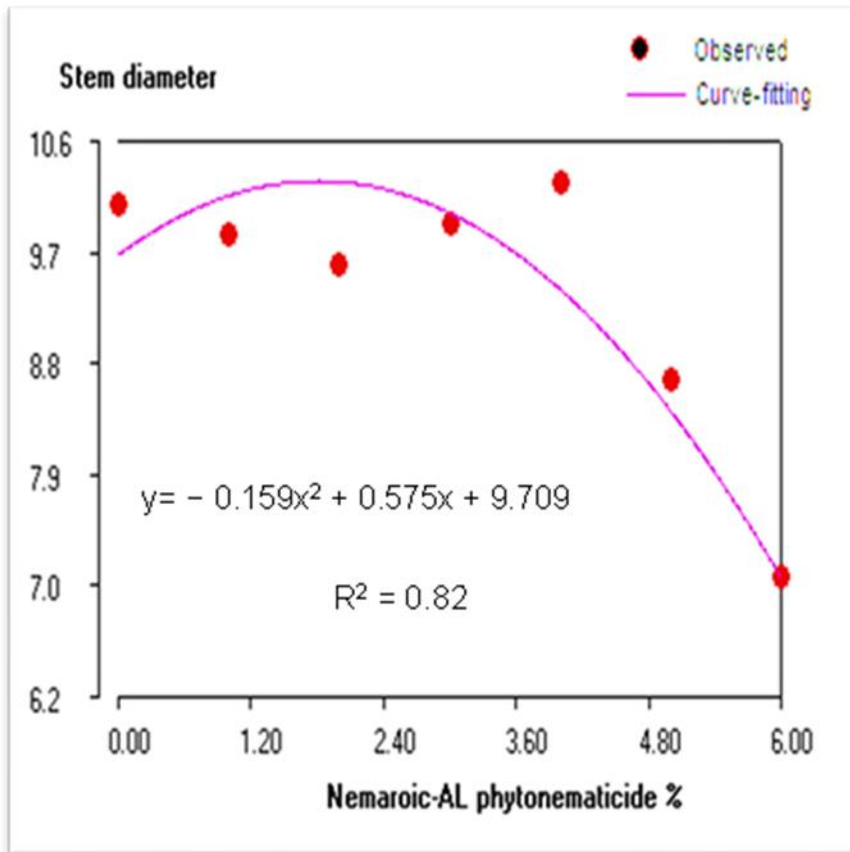
where Y = plant growth responses and x = application interval derived from $x = -b_1/2b_2$ relation for the optimum application time interval. Nematode data were analysed as in the MCSP trial.

5.3 Results

In both MCSP and the application interval trials, the seasonal interactions were not significant on all variables for both products. Hence, data for the two seasons were pooled for MCSP (n = 112) and the application interval (n = 100) (Gomez and Gomez, 1984).

5.3.1 Mean concentration stimulation point of phytonematicides on *Sutherlandia frutescens*

MCSP of Nemarioc-AL phytonematicide on *Sutherlandia frutescens*: The CARD-generated biological indices for the measured plant variables exhibited negative quadratic relations except for stem diameter and dry shoot mass. The negative quadratic variables were excluded in the calculation of MCSP since stimulation phases were not observed to allow for optimisation. The models for stem diameter and dry shoot mass versus increasing concentration of Nemarioc-AL phytonematicide were explained by 82 and 97% associations, respectively (Figure 5.3). The relation, MCSP = $[D_m + (R_h/2)]$ with D_m being the threshold stimulation point and R_h being the saturation point (Mashela *et al.*, 2017a), was used to compute the MCSP value. The MCSP of Nemarioc-AL phytonematicide on *S. frutescens* was established at 3.43% (Table 5.1). The sensitivity (k) values for stem diameter and dry shoot mass were each at 0 unit resulting in the overall sensitivity ($\sum k$) value of 0 unit. The measured stem diameter and dry shoot mass were stimulated at lower concentrations, with inhibition setting in at higher concentrations (Figure 5.3)



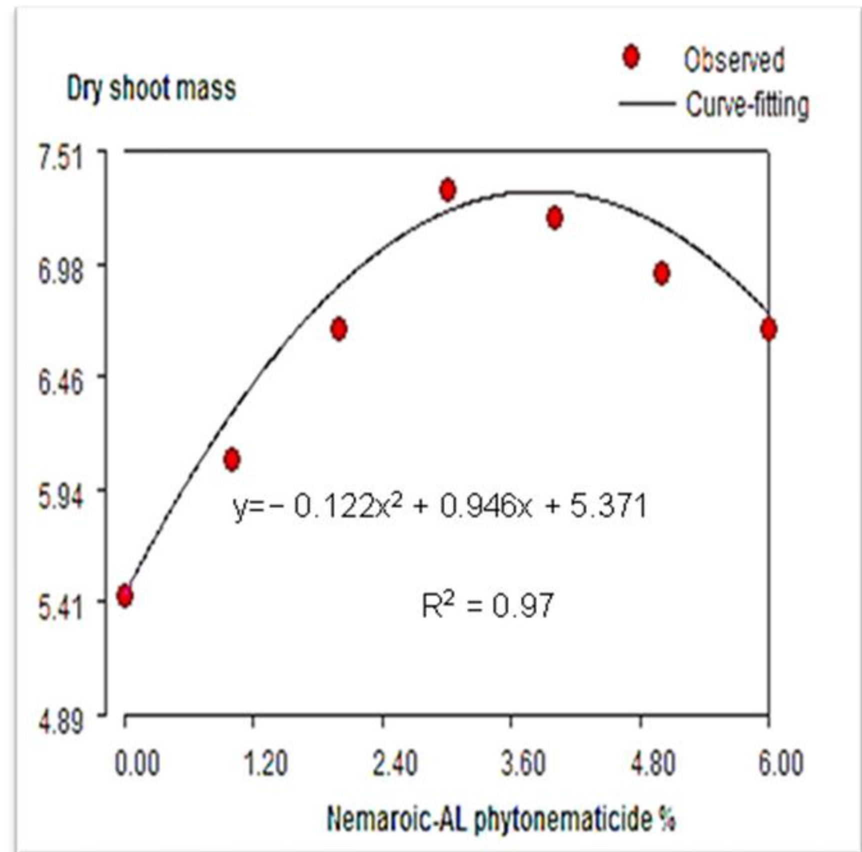


Figure 5.3 Responses of stem diameter and dry shoot mass of *Sutherlandia frutescens* to increasing concentration of Nemarioc-AL phytonematicide at 56 days after inoculation.

Table 5.1 Mean concentration stimulation point of Nemarioc-AL phytonematicide for *Sutherlandia frutescens* (n = 112).

Biological index[z]	Stem diameter	Dry shoot mass	Average
Threshold stimulation (D_m)	1.81	3.87	2.84
Saturation point (R_h)	0.52	1.83	1.175
0% inhibition (D_0)	3.62	7.74	5.68
50% inhibition (D_{50})	7.62	9.95	8.79
100% inhibition (D_{100})	9.80	11.5	10.65
k- value	0	0	
Overall sensitivity		$\Sigma k = 0$	

MCSP = D_m + (R_h/2) = 2.84 + (1.175/2) = 2.84+ 0.589 = 3.43 %.

Nematode response to MCSP of Nemarioc-AL phytonematicide on *Sutherlandia frutescens*: The J2 in root, eggs in root, J2 in soil and final population of nematodes versus Nemarioc-AL phytonematicide exhibited negative quadratic relationships. The respective models were explained by 55, 37, 44 and 74% associations (Figure 5.4), all being above the standard for significance, $R^2 = 0.25$ ($r = 0.5$).

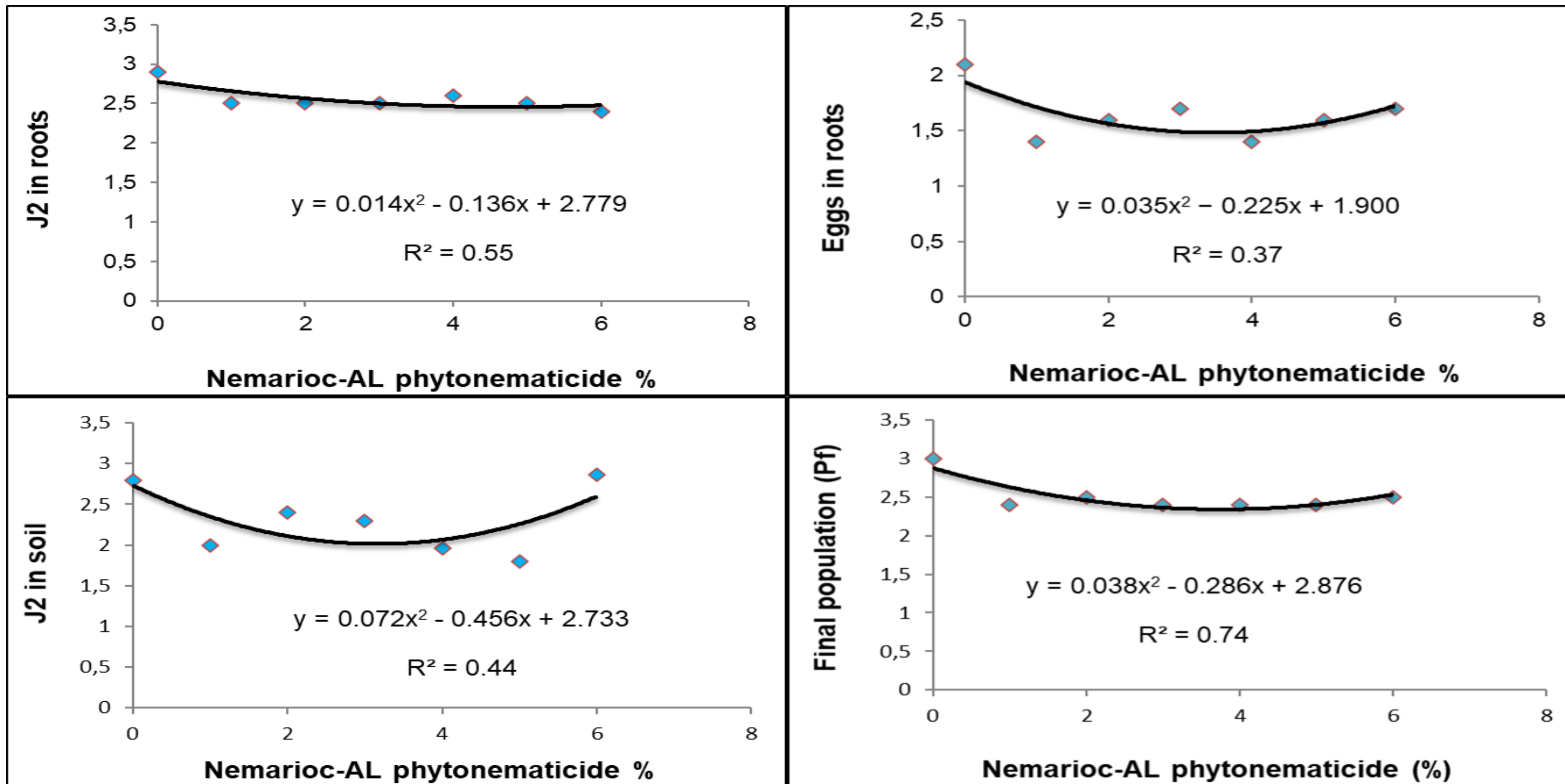


Figure 5.4 Responses of second stage juveniles in root, eggs in root, second stage juveniles in soil and final population of *Sutherlandia frutescens* to increasing concentration of Nemarioc-AL phytonematicide at 56 days after inoculation.

MCSP of Nemafric-BL phytonematicide on *Sutherlandia frutescens*: Plant height, branch number, dry root mass and dry shoot mass versus increasing Nemafric-BL phytonematicide exhibited positive quadratic relations, with the respective models explained by 89, 71, 82 and 90% associations (Figure 5.5). Stem diameter and root galls versus Nemafric-BL phytonematicide each exhibited negative quadratic relations and were therefore, excluded in the determination of the MCSP value, which was at 4.03% (Table 5.2). The k values of *S. frutescens* dry shoot mass, dry root mass, branch number and plant height were 1, 1, 20 and 0 units, respectively, for the ∑k value of 22 units (Table 5.2).

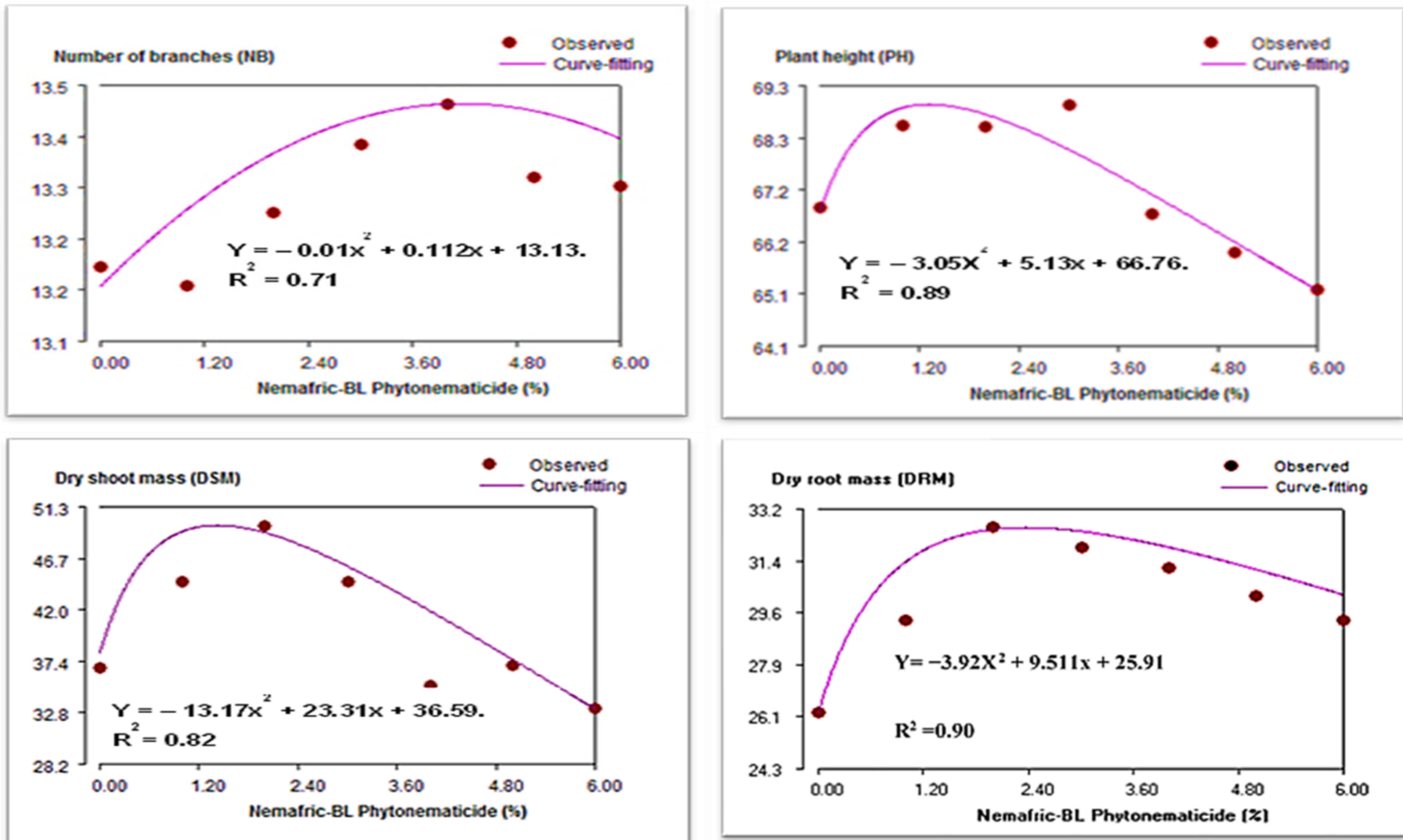


Figure 5.5 Responses of branch numbers, plant height, dry root mass and dry shoot mass of *Sutherlandia frutescens* to increasing concentration of Nemafric-BL phytonematicide at 56 days after inoculation

Table 5.2 Mean concentration stimulation point of Nemafric-BL phytonematicide for *Sutherlandia frutescens* (n = 112).

Biological index[z]	Dry shoot mass	Dry root mass	Branch number	Plant height	Average
Threshold stimulation (D_m)	1.42	2.34	0.12	2.72	1.65
Saturation point (R_h)	10.31	5.77	1.01	1.9	4.75
0% inhibition (D_0)	4.87	10.31	140.2	5.4	50.16
50% inhibition (D_{50})	9.58	29.00	-	14.6	12.09
100% inhibition (D_{100})	15	56.9	-	19.3	17.15
k- value	1	1	20	0	
Overall sensitivity: $\Sigma k = 22$					
MCSP = $D_m + (R_h/2) = 1.65 + (4.75/2) = 1.65 + 2.38 = 4.03$ %.					

Nematode response to Nemafric-BL phytonematicide on *Sutherlandia frutescens*: The J2 in root, eggs in root, J2 in soil and Pf versus increasing Nemafric-BL phytonematicide exhibited negative quadratic relations (Figure 5.6). The models were explained by 55, 91, 50 and 66% associations, respectively (Figure 5.6).

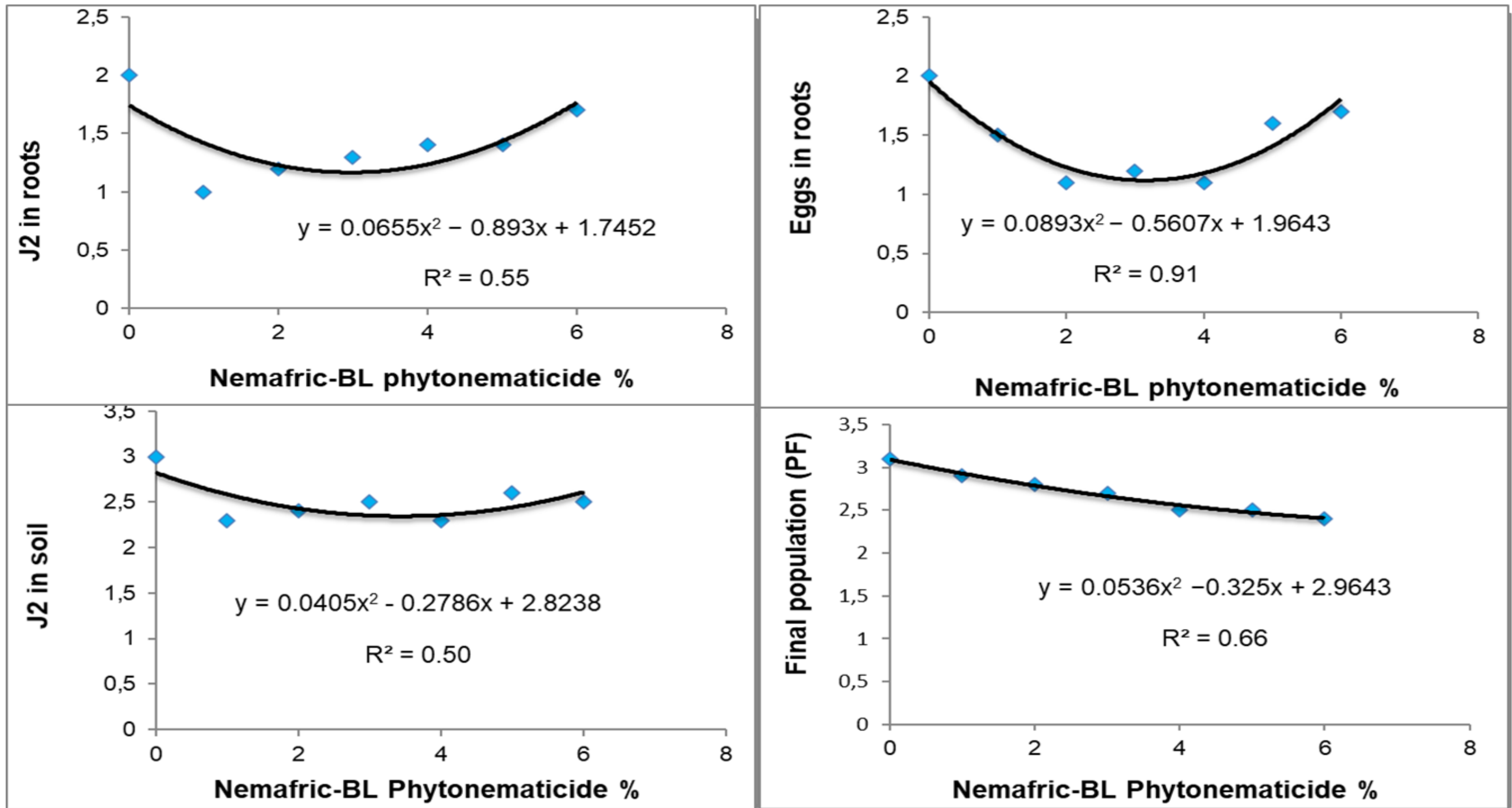


Figure 5.6 Responses of second stage juveniles in root, eggs in root, second stage juveniles in soil and final population of *Sutherlandia frutescens* to increasing concentration of Nemafric-BL phytonematicide at 56 days after inoculation

5.3.2 Application interval on *Sutherlandia frutescens*

Application interval of Nemarioc-AL phytonematicide on *Sutherlandia frutescens*: Dry shoot mass and gall rating versus application interval of 3.43% Nemarioc-AL phytonematicide, exhibited positive quadratic relations, with the models explained by 98 and 60% associations, respectively (Figure 5.7). Plant variables were optimised using $x = -b_1/2b_2$ relation at different application intervals in weeks, with the mean application interval of 3.86 weeks for Nemarioc-AL phytonematicide, which was converted to 29 days the relation MSCP/4 weeks of nematode life cycle (3.86 weeks/4 weeks × 30 days) (Table 5.3).

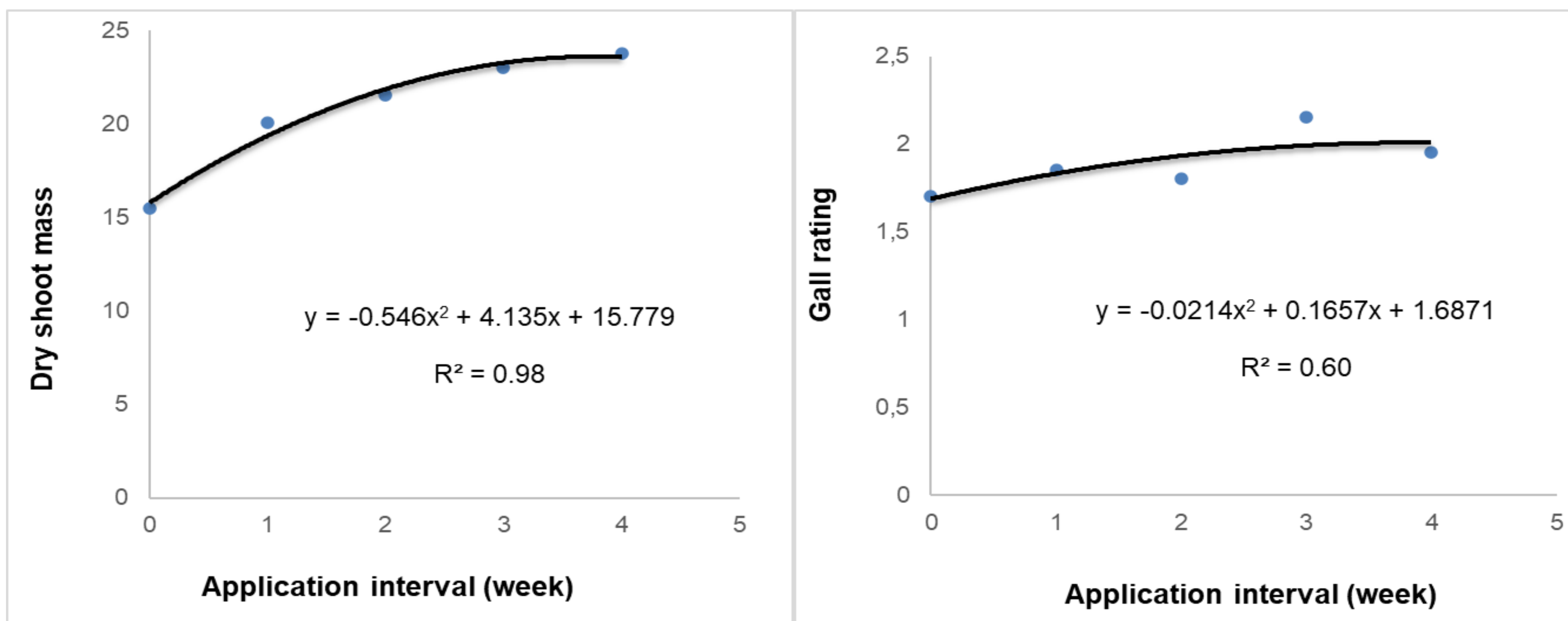


Figure 5.7 Influence of application interval of Nemarioc-AL phytonematicide on dry shoot mass and gall rating of *Sutherlandia frutescens*.

Table 5.3 Quadratic relationship, coefficient of determination and computed optimum Nemarioc-AL phytonematicide application interval for dry shoot mass and gall rating of *Sutherlandia frutescens.*

Variable	Quadratic relation	R^2	x^z
Dry shoot mass	$y = 0.546x^2 + 4.135x + 15.78$	0.98	3.79
Gall rating	$y = 0.021x^2 + 0.1657x + 1.69$	0.60	3.93
Mean			3.86

$^z x = -b_1/2b_2$, where x is optimum concentration, where b_1 = coefficient of x and b_2 = coefficient of x^2 on the quadratic equation, then x was the optimum planting densities.

Nematode response to application interval of Nemarioc-AL phytonematicide on *Sutherlandia frutescens*: The J2 in root, eggs in root, J2 in soil and final nematode population when plotted against application intervals of Nemarioc-AL phytonematicide, exhibited negative quadratic relations, with the model explained by 58, 78, 91 and 60% associations, respectively (Figure 5.8).

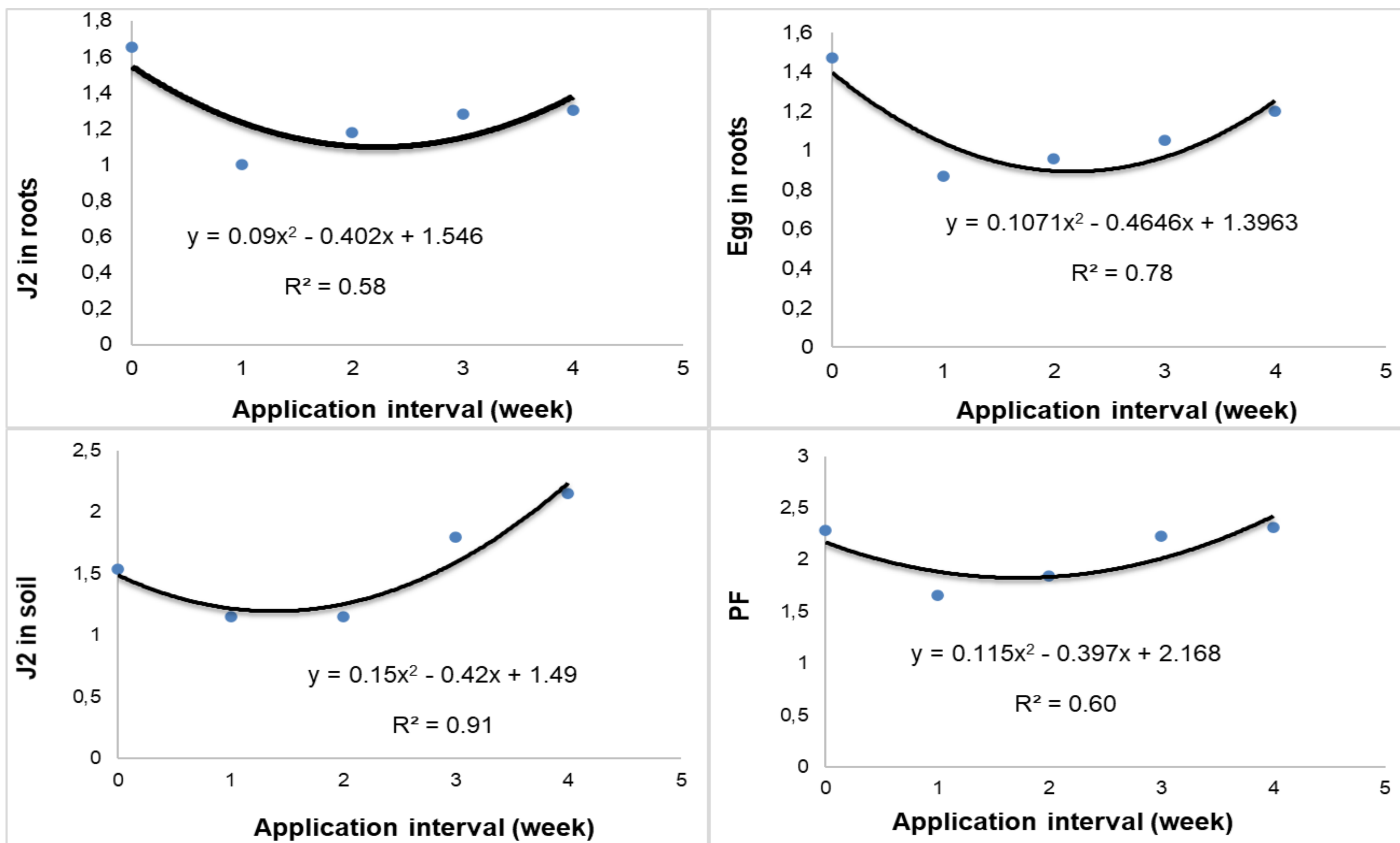


Figure 5.8 Influence of application interval of Nemarioc-AL phytonematicide on second stage juveniles in root, eggs in root, second stage juveniles in soil and final population of *Sutherlandia frutescens.*

Application interval of Nemafric-BL phytonematicide on *Sutherlandia frutescens*: Plant height, dry shoot mass and stem diameter versus increasing application interval of Nemafric-BL phytonematicide exhibited positive quadratic relations with the models explained by 66, 98 and 51% associations, respectively (Figure 5.9). The relation $x = -b_1/2b_2$ was used to optimise plant variables at different application intervals in weeks, with the mean application interval of 2.29 weeks for Nemafric-BL phytonematicide which was estimated to an application interval of 17 days (2.29 weeks /4 weeks × 30 days) (Table 5.4).

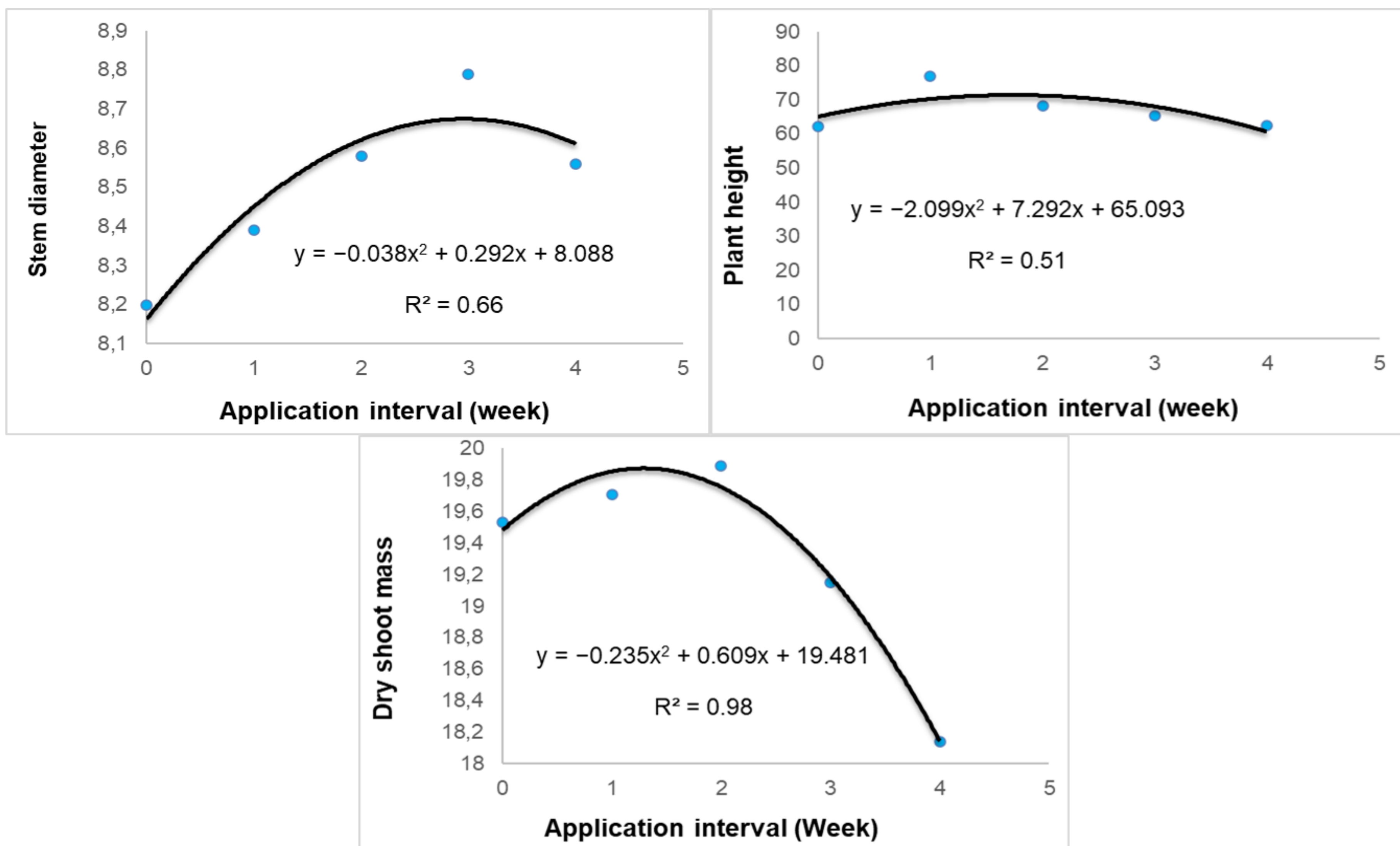


Figure 5.9 Influence of application interval of Nemafric-BL phytonematicide on stem diameter, dry shoot mass and plant height of *Sutherlandia frutescens*.

Table 5.4 Quadratic relationship, coefficient of determination and computed optimum Nemafric-BL phytonematicide application interval for plant height, dry shoot mass and stem diameter of *Sutherlandia frutescens.*

Variable	Quadratic relation	R^2	x^z
Plant height	$y = -2.099x^2 + 7.292x + 65.09$	0.51	1.74
Dry shoot mass	$y = -0.236x^2 + 0.609x + 19.48$	0.98	1.29
Stem diameter	$y = -0.038x^2 +0.292x + 8.09$	0.66	3.84
Mean			2.29

$^z x = -b_1/2b_2$, where x is optimum concentration, where b_1 = coefficient of x and b_2 = coefficient of x^2 on the quadratic equation, then x was the optimum planting densities.

Nematode response to application interval of Nemafric-BL phytonematicide on *Sutherlandia frutescens*: The J2 and eggs in roots, J2 in soil and final nematode population density of *M. javanica* over increasing application interval of Nemafric-BL phytonematicide exhibited negative quadratic relationships, with the models explained by 84, 86, 82 and 90% associations, respectively (Figure 5.10).

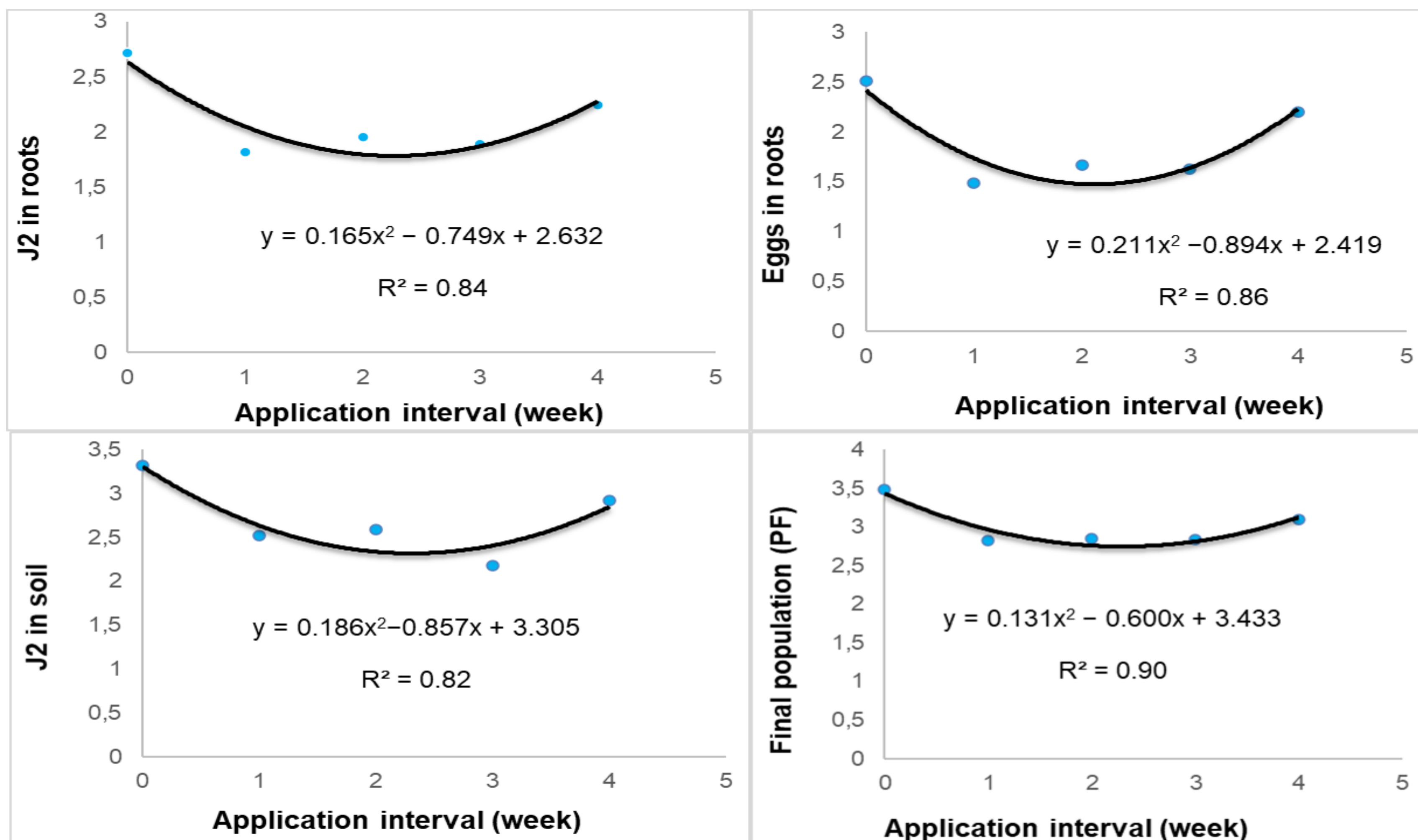


Figure 5.10 Influence of application interval of Nemafric-BL phytonematicide on second stage juveniles in root, eggs in root, second stage juveniles in soil and final population of *Sutherlandia frutescens.*

5.3.3 Application frequency of phytonematicides on *Sutherlandia frutescens*

Application frequency for Nemarioc-AL phytonematicides on *Sutherlandia frutescens*: After deriving the application interval, the application frequency (T_f) defined as the proportion of the crop cycle to the application interval was computed. The application frequency, which is a unit-less factor of Nemarioc-AL phytonematicide on *S. frutescens* would be:

$$T_f = \text{crop cycle (days)/application interval (days)}$$

$$T_f = 56 \text{ days }/29 \text{ days} = 1.93$$

Application frequency for Nemafric-BL phytonematicides on *Sutherlandia frutescens*

The application frequency, which is a unit-less factor of Nemafric-BL phytonematicide on *S. frutescens* would be:

$$T_f = \text{crop cycle (days)/application interval (days)}$$

$$T_f = 56 \text{ days}/17 \text{ days} = 3.29$$

5.3.4 Dosage model of phytonematicides on cancer bush

Dosage model for Nemarioc-AL phytonematicides on *Sutherlandia frutescens*: The dosage model for Nemarioc-AL phytonematicide for the managing *M. javanica* population densities in *S. frutescens* would be:

$$D\ (\%) = MCSP\ (\%) \times T_f$$

$$D (\%) = (3.43\% \times 1.93) = 6.62\%$$

Dosage model for Nemafric-BL phytonematicides on *Sutherlandia frutescens*: The dosage for Nemafric-BL phytonematicide for managing *M. javanica* population density in *S. frutescens* would be:

$$D (\%) = MCSP (\%) \times T_f$$

$$D (\%) = (4.03\% \times 3.29) = 13.26\%$$

5.4 Discussion

5.4.1 The dosage model of phytonematicides on *Sutherlandia frutescens*

The dosage model for Nemarioc-AL and Nemafric-BL phytonematicides, as derived in the current study was a function of the MCSP (i.e. concentration), application interval (time) and the application frequency. In order to put the discussion of the dosage model in context of what was studied in the current research chapter, one needs to distinguish the differences among concentration, dosage and dose in context of the CARD model (Figure 5.11). Concentration (x-axis) is an input to the CARD model, applied to generate the relevant doses. In order to subscribe to normal distribution and therefore, inference, prior to the CARD, the concentration level should straddle the three dose-response phases, namely, the stimulation phase, the neutral phase and the inhibition phase (Mashela *et al.*, 2017a). In the outputs of the CARD-generated curves, the y-axis depicts five doses, from D_m, R_h, D_0 D_{50}, to D_{100}, whereas the x-axis designates the dosage – all as the model output information. By definition, a dose (y-axis) is the amount of an active ingredient that

induces a specific response on the variable, but in addition to the active ingredient that was already inside the test organ. For instance, the CARD model value for D_{100} by definition is the dose that would result in 100% inhibition of the test variable. Presumably, administration of D_{100} would customarily not achieve 100% inhibition since this is just the dose that confers that response, which then takes the discussion to the important concept of dosage as depicted in the CARD model.

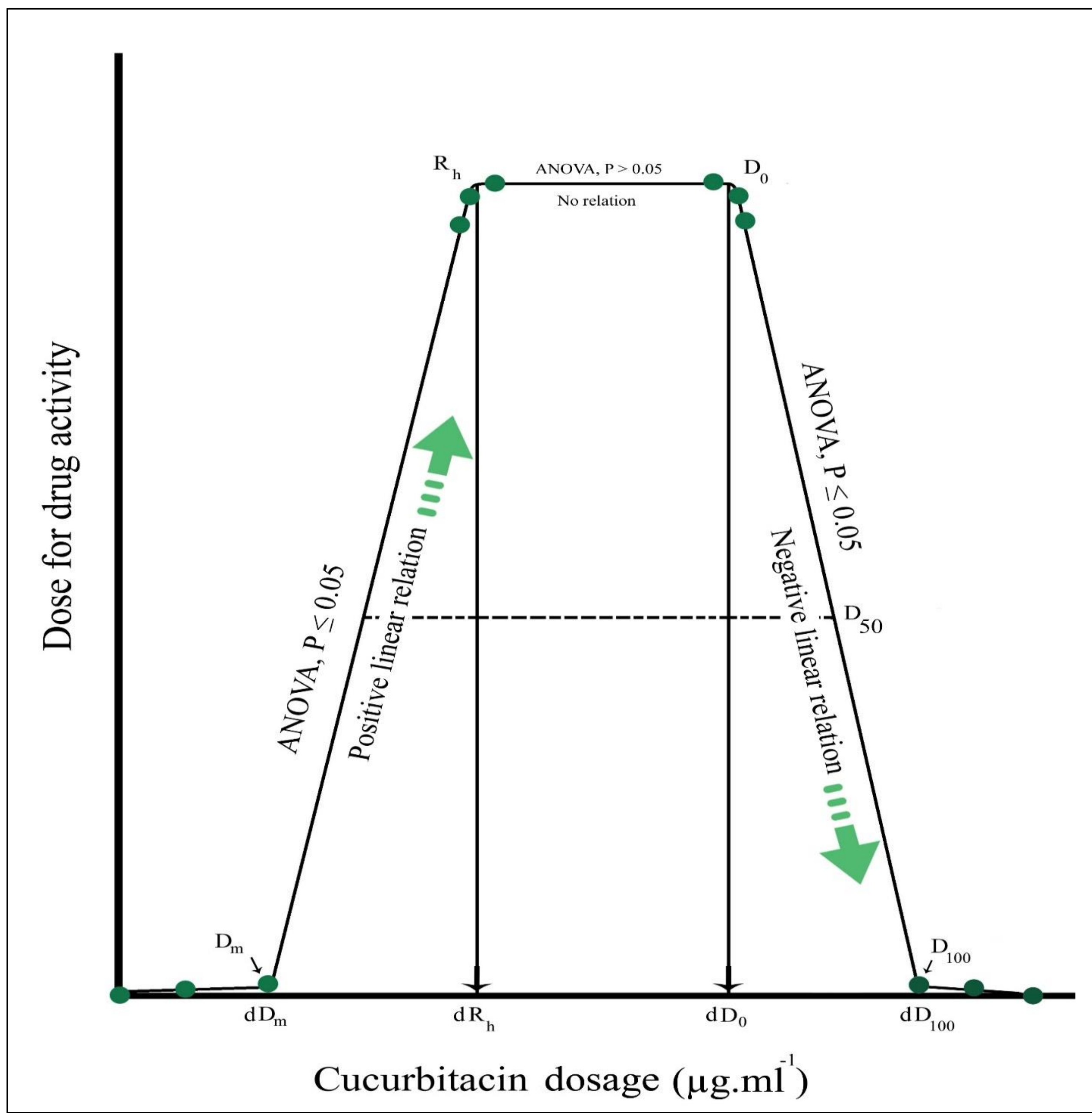


Figure 5.11 Conceptualised outputs of CARD model: Dosage and doses (Mashela *et al.*, 2017a).

Noling (2019) described dosage as the product of concentration and time (D = % × time), which implied that the unit of the product would be percent days, a unit that is totally

different to that of the dosage model, namely, percent (Mashela *et al.*, 2017a). Obviously, in the CARD-generated dosage, the dosage is completely different to the generally accepted dosage in nematology (Noling, 2019). Arguably, the dosage model as derived in the current study is exactly similar to Noling's definition. However, in the study the dosage unit was reduced to percent, instead of percent day. The latter was achieved by using algorithm modelling, the nematode ontogeny in application interval, with the elimination of time derived from developing the application frequency as a proportion of the life cycle of the test crop (days) to the application interval (days). Let us briefly look at the dosage concept as generated by the CARD algorithm computer-based model in order to distinguish this from the dosage model as described by Liu *et al.* (2003). Dosage is that amount of the active ingredient that should be administered to provide a specific dose-response and is, therefore, it is an important constituent of the phytonematicide (Figure 5.11). The doses D_m, R_h, D_0, D_{50} and D_{100} are each induced by a specific dosage (d), arbitrarily abbreviated as dD_m, dR_h, dD_0, dD_{50} and dD_{100}, respectively. Generally, for any specific response, the dosage must always be greater than the dose. In the analysis of primary and secondary datasets (Mafeo *et al.*, 2011; Liu *et al.*, 2003), it was noticed that the addition of previous doses to the dose of interest provided the required dosage (Mashela *et al.,* 2017a). For instance, if the dose of interest was D_{100}, its dD_{100} would be (Figure 5.11):

$$dD_{100} = D_m + R_h + D_0 + D_{50} + D_{100}$$

In short, this dD_{100}, is the dosage that would completely inhibit growth of the plant being protected against nematode damage. In contrast, the dosage model as used in the current study provided the information on the amount of the active ingredients of the phytonematicide that would be placed in a given environment for a given crop in relation to the derived MCSP, application interval days) and application frequency. The latter, just to emphasise, is a function of the proportion of the life cycle of the crop (days) to the application interval (days), which is a unit-less proportion. In short, the dosage models with the percent unit, provides information on the quantity of the active ingredient which would be placed into a particular environment by the time the crop is harvested, but without inducing phytotoxicity to the test plant, while intentionally breaking the life cycle of the test nematode during the production cycle of the test crop.

In the current study, results suggested that the dosage models for Nemarioc-AL and Nemafric-BL phytonematicides were 6.62% and 13.26%, respectively. In other words, at 56 days after applying each of the test phytonematicides, Nemarioc-AL phytonematicide left almost half of the active ingredients of Nemafric-BL phytonematicide in the treated environment. The observation agreed with the known chemistry of cucurbitacin A ($C_{32}H_{46}O_9$), which is slightly polar, but with limited solubility in water (Jeffrey, 1978). The molecule rapidly breaks down into cucumin ($C_{36}H_{46}O_9$) and leptodermin ($C_{36}H_{46}O_8$) chemical molecules (Chen *et al.*, 2005). In contrast, cucurbitacin B ($C_{32}H_{46}O_8$) is non-polar and therefore insoluble in water, with the molecule being highly stable (Jeffrey, 1978). All the listed attributes could, to the certain degree, provide an explanation to some of the differences observed in the dosage models of the two products.

During shelf-life studies of cucurbitacin phytonematicides, Mashela *et al*. (2020b) demonstrated that under chilled (5ºC at 95-98% RH) conditions neither the Arrhenius nor the newly derived quadratic model could predict the shelf-life of Nemarioc-AL phytonematicide. In contrast, under fixed tropical (38ºC at 90% RH) conditions, Nemarioc-AL and Nemafric-BL phytonematicides had, from the quadratic model the shelf lives of 35 and 825 weeks, respectively (Mashela *et al*., 2020b). Apparently, with the temperature playing a role in explaining the differences between the observed differences in shelf lives. Cucurbitacins have extremely high boiling points, with cucurbitacin A boiling at 731°C at 760 mmHg (Krieger, 2001), where 760 mmHg implies at 1 atmosphere or at sea level. Similarly, cucurbitacin B boils at 699°C at 760 mmHg (Krieger, 2001). Shadung (2016) was the first to demonstrate that temperature played a drastic role in explaining the differences in concentration of Nemarioc-AL and Nemafric-BL phytonematicides, with refrigeration not being recommended for storage of the products.

In various crops, observations suggested that the two phytonematicides had almost the same application interval, on average being approximately 14 days when the test nematode was a *Meloidogyne* species, which results in breaking the life cycle several times depending on the crop cycle of the test crop (Mashela *et al*., 2017a). The relatively shorter application intervals, which were empirically-derived, which could imply that in addition to inherent chemical breakdown processes as outlined in cucurbitacin A (Jeffrey, 1978), and temperature related effects (Shadung, 2016), there could be other factors, which play some roles in the dosage model of the two phytonematicides as observed in the current study. In most other pesticides such as carbamates and organophosphates

with short application intervals (Noling, 2019; Van Gundy and McKenry, 1975), most active ingredients are removed from the environment through living entities, with the process referred to as bioremediation (Mashela *et al.*, 2021). Traditionally, bioremediation factors that have been widely reported include bacteria, fungi and plants (Mashela *et al.*, 2021). In instances where plants were involved in bioremediation of the pesticides, the active ingredients were detected as residual chemicals in plant produce. A good example was the carbamate, aldicarb, which had acropetal movements in plants, and accumulated in large quantities in aboveground organs such as fruit of watermelon (*Citrulus lanatus* L.), where the residues were far above the permissible level and actually killing more than a thousand consumers within three years in the USA (Essumang *et al.*, 2017). The latter could be viewed as bioremediation of pesticides by plants (Mashela *et al.,* 2021). In contrast, concerted efforts in assessing cucurbitacin residues from cucurbitacin phytonematicides in various crops demonstrated that there was hardly any bioremediation of cucurbitacins by plants since cucurbitacin residues were hardly detected in plant produce (Dube, 2016, Mashela *et al.*, 2017a; Shadung *et al.*, 2016). Generally, non-polar chemicals like cucurbitacins from phytonematicides cannot move through the symplastic pathway that is conferred by the pericycle and the endodermis in the root systems (Campbell, 1990). In addition to plants, bacteria and fungi release enzymes, which include reductases that are responsible for bioremediation of most soil-drenched pesticides (Mashela *et al.*, 2021). However, to date reductases that have the capability of breaking down the cucurbitacin chemical compounds have not been detected in microorganisms, but have been detected in the biosynthesis pathway of cucurbitacins (Chen *et al.*, 2005).

Cuticle moulting animals in the Ecdozoan, which is the largest superphylum in the Animalia Kingdom, comprise billions of billion animals, including nematodes, in the soil (Halanych, 2004). Mashela *et al*. (2021) in their attempt to investigate the role of cuticle moulting animals in bioremediation of cucurbitacin phytonematicides, subjected second-stage juveniles (J2) of *M. incognita* to a geometric series of Nemafric-BL phytonematicide. In the study, protein content of J2 versus phytonematicide exhibited negative quadratic relations; but with protein content increasing after the minimum point was reached. The decrease and increase in protein content were explained using the chemical properties of cucurbitacins and the nematode cuticles through two distinct processes. The decrease and increase depicted isoprenylation (protein breakdown) and farneslation (protein synthesis), respectively (Mashela *et al.,* 2021). Due to the size of the Ecdozoan superphylum in the soil, bioremediation of cucurbitacins could play an indispensable role in explaining the observed reduced dosage of cucurbitacin phytonematicides as observed in the current research chapter.

5.5 Synthesis and conclusion

The dosage model for Nemarioc-AL and Nemafric-BL phytonematicides as described in the study, provided an integrated approach that could be useful in determining the amount of cucurbitacins that could be placed in specific environments during protecting crops against nematode damage. In the derivation of the model, the CARD-generated dose responses of various plant organs are used to derive the MSCP, which is the concentration that would stimulate plant growth, while suppressing nematode population density. The MCSP, along with the ontogeny of the test nematode, are then used to

establish the application interval, which brought in the second biological entity in the model. Thirdly, the application frequency brought the life cycle of the crop and the application time, resulting in a unit less proportion, which enables the generation of the dosage model with the unit of percent (concentration) as opposed to % days.

The primary focus in the discussion of the dosage model of Nemarioc-AL and Nemafric-BL phytonematicides centred on the vast differences between the values of the two products on *S. frutescens*. Clearly, there were inherent chemical differences, which were affected by both abiotic and biotic factors. Primarily, the major abiotic factor was temperature, whereas biotic factors with the ability to induce bioremediation were identified as the cuticle moulting animals, through isoprenylation (protein breakdown) and farneslation of cucurbitacins. Since the dosage of Nemarioc-AL and Nemafric-BL phytonematicides were different, the null hypothesis which stated that the dosage model of the two products on *S. frutescens* would not be similar under microplot conditions was therefore accepted, which supported the view that operationally, the two phytonematicides should be viewed as different entities, with Nemarioc-AL phytonematicide having limited concern regarding environmental contamination. The latter view could certainly not be valid, since the breakdown constituents of cucurbitacin A, cucumin and leptodermin could be having bioactivities on nematodes and other animals within the Ecdozoan superphylum. In the next research chapter, the researcher investigated the nutritional water productivity of secondary metabolites in *S. frutescens*.

CHAPTER 6

NUTRITIONAL WATER PRODUCTIVITY OF PHYTOCHEMICALS IN *SUTHERLANDIA FRUTESCENS*

6.1 Introduction

Cancer bush (*Sutherlandia frutescens)* is a drought-tolerant indigenous crop with the potential to serve as a future crop under context of climate-smart agriculture inland South Africa, where predictions suggested extremes climatic conditions for most exotic crops (Carr *et al.*, 2016). Climate-smart agriculture entails the adoption of good management practices, which comprise the adoption of empirical-developed best strategies and innovations that would enhance matching the selected crops to extremes such as water deficiency as per the envisaged predictions, where water-scarcity would be among the main limiting factors in crop husbandry. Nutritional water productivity (NWP) had been established as one of the descriptors that can be used to identify the suitability of a crop for a given area in relation to the amount of water used for specific physiological activity, which are collectively responsible for enhancing the nutritional value of plants (Nyathi *et al.*, 2016; Renault and Wallender, 2000). The nutritional value of the crop is dependent upon certain macronutrient elements, micronutrient elements, vitamins or secondary metabolites that the crop can provide in large quantities, which had been determined using the concept of NWP of a given crop (Li *et al.*, 2020; Mabhaudhi *et al.*, 2017). In the current study, since *S. frutescens* is viewed as being a medicinal plant of repute, it would be reasonable to compute its NWP on the basis of phytochemicals by manipulating cultural practices that can result in high production of the target descriptor of NWP. For example, in medicinal plants, where the descriptors are secondary metabolites, planting

density can be manipulated to affect the accumulation of secondary metabolites through plant competition for resources. The objective of the study, therefore, was to determine whether planting density of *S. frutescens* would affect NWP of phytochemicals to enhance the cultivation of this medicinal plant under field conditions in semi-arid regions. The null hypothesis was that planting density of *S. frutescens* would not affect NWP of phytochemicals to enhance the cultivation of this medicinal plant under field conditions in semi-arid regions.

6.2 Material and Methods

6.2.1 Description of the study site

Two separates experiments to determine NWP of *S. frutescens* were conducted at the Green Biotechnologies Research Centre (GBRCE), University of Limpopo, South Africa (23°53'10"S, 29°44'15"E). The experiments were conducted during early to mid-autumn (March-April) in 2018 and validated (March-April) in 2019. The environmental conditions are similar to those reported in Chapter 4 and 5 at the GBRCE.

6.2.2 Treatments and research design

The study used nine treatments designated as T1, T2, T3, T4, T5, T6, T7, T8 and T9, respectively, consisting of 1, 2, 3, 4, 5, 6, 7, 8 and 9 seedlings/hole of drip irrigation transplanted using a 3S planter under field conditions. The plots were irrigated using drip irrigation, where transplanted seedling were used. A 3S planter was used under field conditions, arranged in a randomised complete block design (RCBD) with nine replications (n = 81) in both seasons. An Integrated Drip Irrigation System (IDIS) and a

3S planter were developed by the GBRCE founder to save water and increase planting densities/drip hole, respectively, in the semi-arid regions of Limpopo Province. A 3S planter has capabilities of planting one to nine plants/hole of drip irrigation system with the spacing between the seedlings being 5 cm (Figure 6.2). Each drip hole can cover 50 cm around the drip hole.

Figure 6.1 Layout of nutritional water productivity of *Sutherlandia frutescens* under field conditions at the Green Biotechnologies Research Centre of Excellence.

Figure 6.2 3S planting tool used for transplanting *Sutherlandia frutescens* seedlings at the Green Biotechnologies Research Centre of Excellence.

6.2.3 Procedures

Purchased seeds (Mountain Herb Estate, Pretoria, South Africa) were sown in 200-cone polystyrene seedling trays, containing Hygromix-T growing mixture (Hygrotech, Pretoria, South Africa) and placed on the greenhouse benches. At five leaf-stage, seedlings were hardened-off outside the greenhouse using intermittent withdrawal of irrigation water for two weeks under direct sunlight (Tseke and Mashela, 2018). Seedlings were transplanted under field conditions at 0.5 m × 1.0 m spacing, with nine plant densities (T1 = 1, T2 = 2, T3= 3, T4 = 4, T5 = 5, T6 = 6, T7 = 7, T8 = 8 and T9 = 9) seedlings/hole of drip irrigation. Three days after transplanting, each plant was fertilised with 2.5 g NPK at 2:3:2 (22) + 0.5% Zn + 5% S + 5% Ca to provide 74.3 g N, 111.4 g P, 74.3 g K, 5 g Zn and 50 g Ca

per plant. Also, 1 g NPK 2:1:2 (43) Multifeed (Nulandies, Johannesburg) provided 0.47 N, 0.43 K, 0.43 P, 121 Mg, 1 Fe, 0.10 Cu, 0.47 Zn, 1.34 B, 4.02 Mn and 0.09 mg Mo per ml tapwater, without Ca (Mashela *et al.,* 2015). Scouting and monitoring for insect pests were performed daily, but none was observed. Manuel weeding was done when weeds when necessary. Plants were irrigated every other day using drip irrigation at 2 litre water/hole.

6.2.4 Data collection

At 60 days after transplanting, shoots were severed from roots, shoots were oven-dried at 52°C for 72 h and weighed. Branches per plant were recorded and plant height measured from the crown to the tip of the flag leaf using measuring tape. Stem diameter was measured using a digital Vernier caliper at 5-cm above the severed end of the stem. In the laboratory, the aboveground biomass was oven-dried at 52ºC for 72 h, and then weighed to determine dry biomass. The dry shoot mass was weighed and grounded to pass through 0.75 mm sieve for quantifying the secondary metabolites total phenol content, total tannin content and total flavonoid content, which were used in computation of NWP.

Total phenol content: The total phenol content (TPC) of the plant extracts was determined using the Folin-Ciocalteu assay. A volume of 1 ml of the plant extract was mixed with 9 ml of distilled water in a volumetric flask (25 ml). Approximately 1 mm Folin-Ciocalteu phenol reagent was added to the mixture in a 25 ml volumetric flask and then shaken. Approximately 10 ml 7% sodium carbonate (Na_2CO_3) solution added, with the

mixtures brought to 25 ml mark using distilled water. Gallic acid was used as a standard in concentration of 1, 0.5, 0.25, 0.125, and 0.0625 mg/ml prepared in the same manner as the test samples. The mixtures were then incubated for 90 minutes at room temperature in the dark room. The absorbance for the test and standard solutions was determined against the blank reagent using a UV visible spectrophotometer (Beckman Coulter-DU730) at 765 nm. The total phenol content was expressed as mg of gallic acid equivalents (GAE) per g of the extract (mg GAE /g). The total phenolic content was determined using the linear regression formula from a gallic acid calibration curve standard (Singleton *et al.*, 1999). All samples were analysed in three replicates.

Total tannin content: The Folin-Ciocalteu assay was used to determine the total tannin content (TTC) of the plant extracts. Approximately 0.1 ml plant extract was mixed with 7.5 ml distilled water in a 10 ml volumetric flask, into which 0.5 ml Folin-Ciocalteu phenol reagent was added. Approximately 1 ml 35% $Na_2CO_3$was added, with the mixture diluted with 10 ml distilled water. The mixture was shaken and also incubated in the dark at room temperature for 30 minutes. Gallic acid was used as reference standard in 1, 0.5, 0.25, 0.125, 0.0625 mg/ml prepared using the same procedure as the test samples. The absorbance for the standard and the test samples was determined against the blank reagent at 725 nm using UV/Visible spectrophotometer (Beckman Coulter-DU730). The tannin content was expressed as mg GAE/g extract (mg GAE /g) (Singleton *et al.*, 1999). The tannin content was determined by the linear regression formula from a gallic acid standard calibration curve. All samples were analysed in three replicates.

Total flavonoids content: The total flavonoid content (TFC) was determined using the aluminium chloride colorimetric assay. The plant extract (1 ml) and distilled water (4 ml) were added into a 10 ml volumetric flask, 0.30 ml 5% sodium nitrite ($NaNO_3$) added and after 5 minutes, 0.3 ml 10% aluminium chloride ($AlCl_3$) also added and mixed well. Approximately 2 ml 1 M sodium hydroxide (NaOH) was added after 5 minutes and then the mixture diluted to 10 ml using distilled water. Quercetin was used as reference standard in 1, 0.5, 0.25, 0.125, 0.0625 mg/ml prepared in the same manner as the test samples. The absorbance of the test and standard solutions were determined against the reagent blank at 506 nm using UV/Visible spectrophotometer (Beckman Coulter-DU730). The total flavonoid content was expressed as mg of quercetin equivalents (QE) per g extract (mg GAE /g), total flavonoid content determined by the linear regression formula from a quercetin calibration standard curve (Har and Intan, 2012). All samples were analysed in three replicates.

Determination of evapotranspiration: Evapotranspiration for each treatment was calculated as the residual of a soil water balance (Nyathi *et al.*, 2016; Renault and Wallender, 2000);

$$ET = P + I - D - R - \Delta SWC,$$

where ET = evapotranspiration (mm), P = precipitation (mm), I = irrigation (mm), D = drainage (mm), R = runoff (mm), and ΔSWC = changes in soil water content (mm).

Rainfall (mm) data was obtained from weather station at the University of Limpopo, weather station less than 9 km radius from the sites. Changes in soil water content (SWC) were measured using a PR2/6 profile probe connected to an HH2 handheld moisture meter (Delta-T, UK). The sensors of the PR2/4 profile probe are positioned to measure volumetric water content at six depths (0.10, 0.20, 0.30, 0.40 m along the probe) (Chibarabada *et al*., 2017).

Drainage was considered as negligible. Runoff (R) was not quantified during the trials. Chibarabada *et al*. (2017) postulated that to account for its effect, the United States Department of Agriculture–Soil Conservation Service (USDA-SCS) procedure is adopted to estimate the monthly effective rainfall that is stored in the root zone after subtracting the amount of rainfall lost to runoff (USDA-SCS, 1967). The soil water balance was therefore simplified to

$$WU = ER + I - \Delta SWC$$

where WU = water use = evapotranspiration (mm), ER = effective rainfall (mm), I = irrigation (mm), and ΔSWC = changes in soil water content (mm). Values of ET in mm (depth) were then converted to m^3 (volume) using the formula;

Volume (m^3) = Area (m^2) × Depth (m)

Calculation of nutritional water productivity (NWP): The NWP was calculated based on the formula by Renault and Wallender (2000) using three secondary metabolites, namely, total phenol content, total tannin content and total flavonoids content:

$$NWP = (Y/ET) \times SMC$$

where NWP was expressed as secondary metabolite m^{-3} water evapotranspiration, Y is the harvested dry shoot mass ($kg \cdot ha^{-1}$), ET is the actual evapotranspiration (m^{-3} ha^{-1}), and SMC is the nutritional content per kg of product (nutrition unit$\cdot kg^{-1}$).

6.2.5 Data analysis

The seasonal interactions (Experiment 1 × Experiment 2) on NWP variables were significant ($P < 0.05$). The Shapiro - Wilk test was performed to determine the normality of distribution of the collected data (Ghasemi and Zahediasl, 2012; Shapiro and Wilk, 1965) with the data depicting normal distribution. Data were then subjected to analysis of variance using Statistix 10.0 software. The degree of freedom and their mean sum of squares (MSS) were partitioned to provide the total treatment variation (TTV), in the event when the treatment was significant ($P \leq 0.05$), mean comparisons were achieved using Waller-Duncan multiple range test at the probability level of 5%. Significant NWP variables were subjected to lines of the best fit, with the generated quadratic equations used to compute optimum planting density for NWP of selected secondary metabolites. Unless stated otherwise, treatment effects were discussed at the probability level of 5%.

6.3 Results

The treatment, planting densities, had significant effects ($P \leq 0.05$) on NWP of total phenol content (TPC), total tannin content (TTC) and total flavonoid content (TFC), during the 2018 and 2019 growing seasons (Appendix 6.1-6.5). Means of NWP of TFC, TTC and TPC under various planting densities were summarised (Table 6.1). Subjecting NWP variables to the lines of the best fit, positive and negative quadratic relations were

exhibited during the 2018 and 2019 season (Figure 6.3 and 6.4). The models for NWP of TFC, TTC and TPC were explained by 73, 87, and 29%, during the 2018 growing season (Figure 6.3), whereas during the 2019 growing season, the models explained by 90, 55, and 45% relations, respectively (Figure 6.4).

The optimum values for NWP of TFC, TTC and TPC during the 2018 planting season were attained at treatment 9 (9 planting densities), treatment 2 (2 planting densities) and treatment 7 (7 planting densities) respectively (Table 6.2), whereas, the optimum values during the 2019 planting season were attained at treatment 6 (6 planting densities), treatment 8 (8 planting densities) and treatment 4 (4 planting densities), respectively (Table 6.2).

Table 6.1 Mean nutritional water productivity of selected secondary metabolites in *Sutherlandia frutescens* over planting densities during the 2018/19 season.

	2018 season				2019 season			
Plant density	ET m^{-3}	NWP_{TFC} $Kg\text{-}m^{-3}$	NWP_{TTC} $Kg\text{-}m^{-3}$	NWP_{TPC} $Kg\text{-}m^{-3}$	ET m^{-3}	NWP_{TFC} $Kg\text{-}m^{-3}$	NWP_{TTC} $Kg\text{-}m^{-3}$	NWP_{TPC} $Kg\text{-}m^{-3}$
1	9.162	0.111^{e}	0.033^{c}	0.199^{c}	18.21	0.040^{d}	0.009^{e}	0.262^{b}
2	10.10	0.115^{e}	0.034^{c}	0.170^{c}	17,86	0.069^{c}	0.045^{bcd}	0.069^{c}
3	9.119	0.159^{d}	0.083^{c}	0.185^{c}	18.09	0.084^{bc}	0.031^{cde}	0.125^{bc}
4	8.719	0.186^{bc}	0.068^{c}	0.529^{ab}	20.67	0.087^{abc}	0.014^{de}	0.188^{bc}
5	10.11	0.167^{cd}	0.077^{c}	0.242^{bc}	18.10	0.098^{ab}	0.061^{abc}	0.175^{bc}
6	10.38	0.161^{d}	0.089^{bc}	0.638^{a}	18.04	0.106^{a}	0.071^{ab}	0.119^{bc}
7	9.943	0.165^{cd}	0.174^{a}	0.433^{abc}	18.25	0.093^{ab}	0.082^{a}	0.212^{bc}
8	8.839	0.189^{ab}	0.145^{ab}	0.203^{c}	18.39	0.083^{bc}	0.067^{abc}	0.460^{a}
9	8.952	0.208^{a}	0.150^{ab}	0.459^{abc}	18.38	0.091^{ab}	0.051^{abc}	0.270^{b}

*** Highly significant at $P \leq 0.05$. ET = evapotranspiration, Nutritional Water Productivity of total tannin content (TTC), total flavonoid content (TFC) and total phenolic content (TPC).

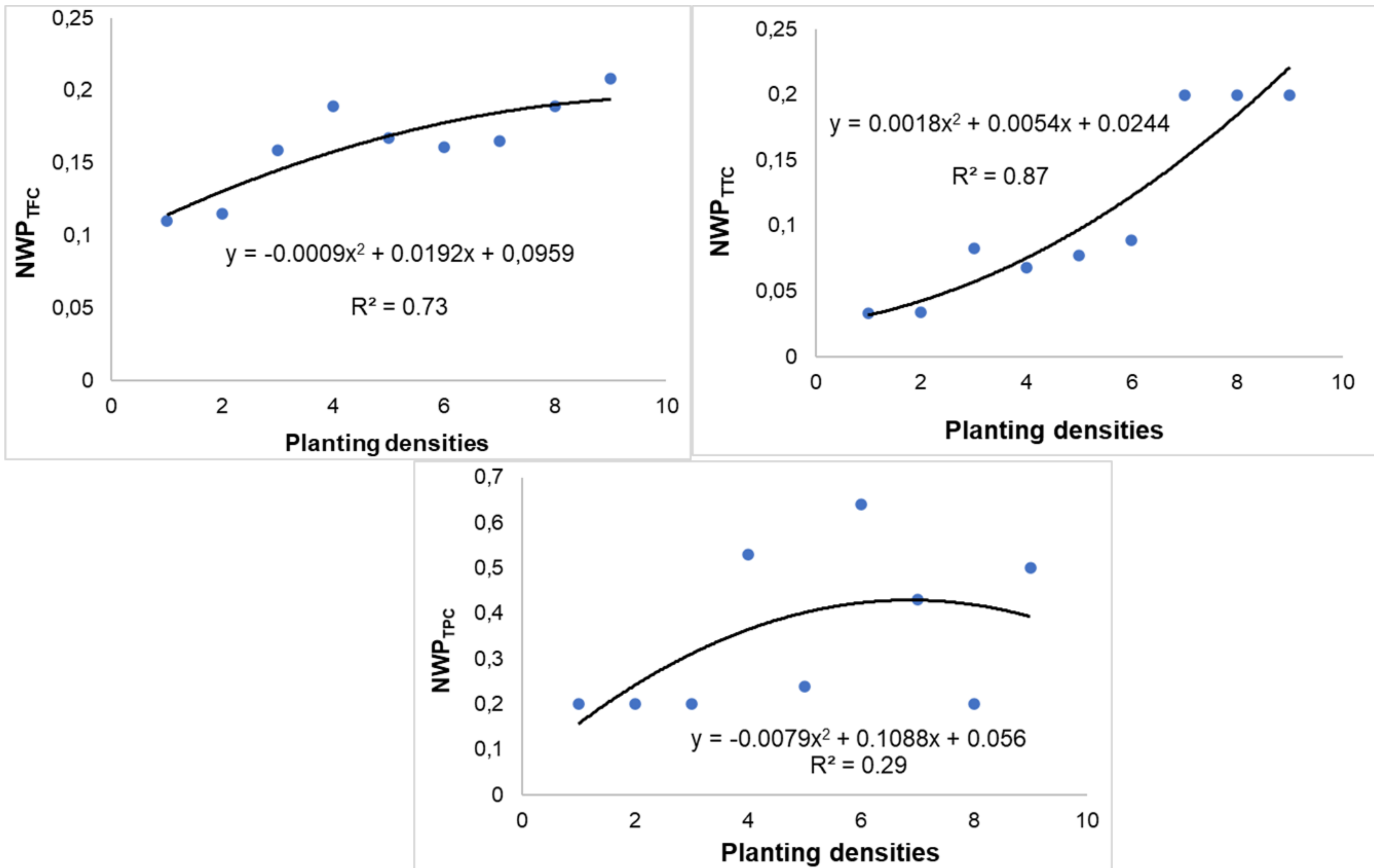


Figure 6.3 Effect of planting densities on nutritional water productivity of TFC, TTC and TPC during 2018 season.

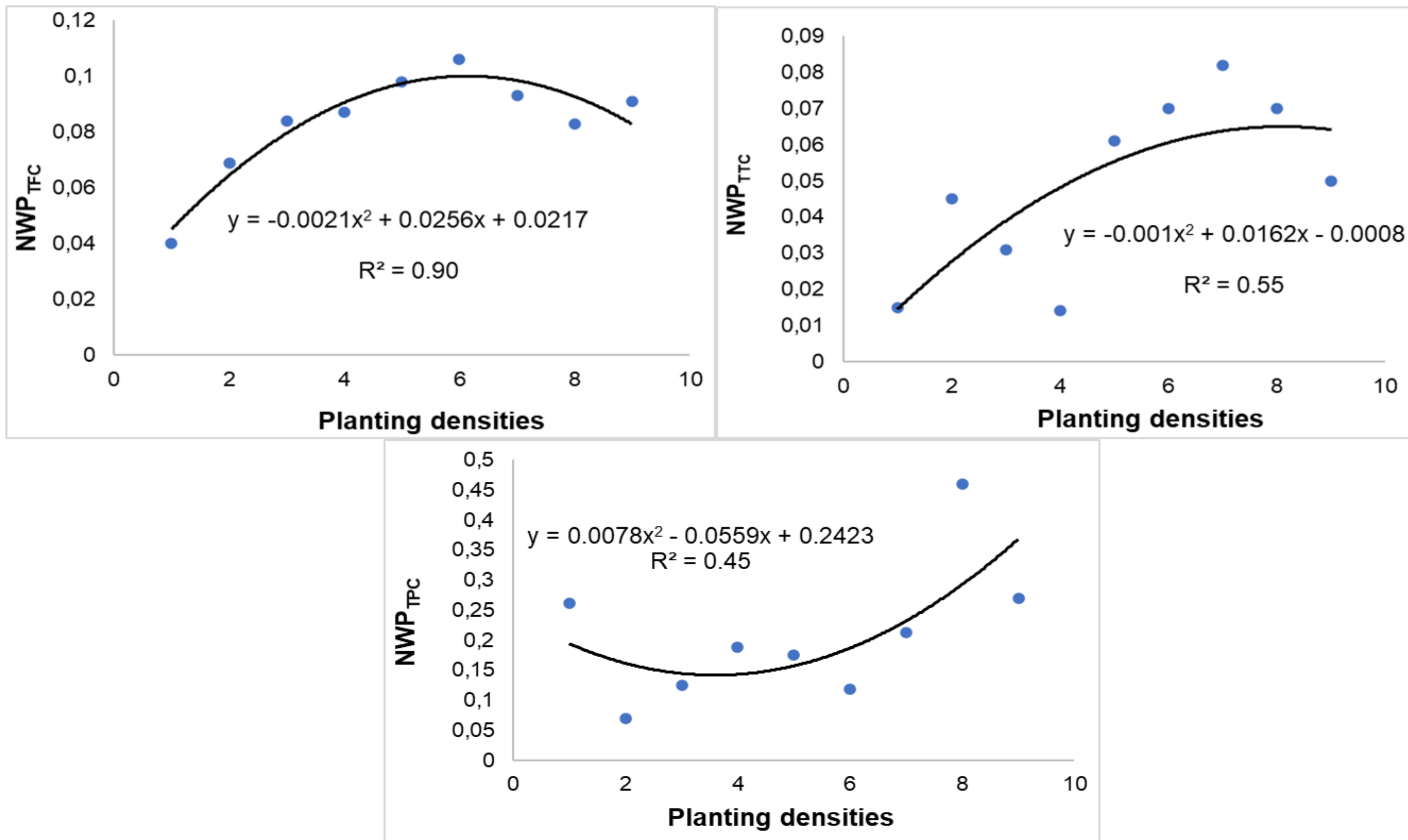


Figure 6.4 Effect of planting densities on nutritional water productivity on TFC, TTC and TPC during 2019 season.

Table 6.2 Quadratic relations for nutritional water productivity of secondary metabolites over planting densities during the 2018/19 season.

	Quadratic relations	R^2	x^X	Y^Z
2018 season				
NWP_{TFC}	$Y = -0.009x^2 + 0.192x + 0.0959$	0.73	11	1
NWP_{TTC}	$Y = 0.0018x^2 - 0.0054x + 0.0244$	0.87	2	0.02
NWP_{TPC}	$Y = -0.0079x^2 + 01088x + 0.056$	0.29	7	1
2019 season				
NWP_{TFC}	$Y = -0.0021x^2 + 0.0256x + 0.217$	0.92	6	0.3
NWP_{TTC}	$Y = -0.0002x^2 + 0,0122x + 0.0079$	0.52	8	0.1
NWP_{TPC}	$Y = 0.0078x^2 - 0.0559x + 0.2423$	0.45	4	0.4

^{x}Calculated optimum treatment level (planting densities, $x = -b_1/2b_2$, where b_1 = coefficient of x and b_2 = coefficient of x^2 on the quadratic equation, then x was the optimum planting densities.

^{z}Nutritional water productivity at optimum level.

6.4 Discussion

The concept of NWP implies that any factor influencing yield, nutrient content and water uptake would affect NWP directly or indirectly (Ramputla, 2019). In the current study, plant densities influenced NWP of phytochemicals in *S. frutescens*. The results of this study showed that as planting densities increased under drip irrigation there was stimulation of NWP for TFC, TTC and TPC during both growing seasons. Several studies have focused on NWP for grain legumes and vegetables such as sweet potato, nightshade and tomato (Chibarabada *et al*., 2017; Li *et al*., 2021; Nyathi *et al*., 2019a; Nyathi *et al*., 2016; Renault and Wallender, 2000). A study by Li *et al*. (2021) is among the few or the only one that explored NWP of phytochemicals. In that study, the team focused on the influence of irrigation methods on NWP of phytochemicals in tomato. The results suggested that under drip irrigation, the mass concentration of lycopene, β-carotene and total phenols increased by 10.8%, 14.5% and 21.4%, respectively, and the NWP of phytochemicals increased by 8.5%, 12.1% and 18.9%, respectively, which was higher than in furrow irrigation. The results of Li *et al*. (2021) showed that the selection of cultural practices can play an important role in determining the balance between yield and nutritional quality of crops. Hadebe *et al*. (2021) explored the suitability of sorghum genotypes to alleviate protein, Zn and Fe deficiency in SSA under water scarcity in dryland agriculture through evaluation of NWP. The concept was suitable to serve as a descriptor for suitability of sorghum in water-scarce regions. This also support the use of NWP principles concept to evaluate adaptability of cancer bush in water-scarce areas of Limpopo Province.

Ramputla (2019) investigated the influence of two root-knot nematodes, *Meloidogyne incognita race 2* and *M. javanica* on NWP, in hot chilli and observed the nematodes interfered with NWP. In that study, it was shown that increasing nematode population densities increased NWP in chilli plants, which supported observations in the current study, were increasing planting density increased NWP of phytochemicals. In Ramputla (2019) and the current study, NWP of nutrient elements versus nematodes or NWP of phytochemicals versus planting density, exhibited density-dependent growth (DDG) patterns, where stimulation of NWP was observed as the independent variables increased, with the eventual levelling off, which was followed by the gradual inhibition phase as the variable increased. Such relationships are common in biological entities subjected to allelochemical-containing phytonematicides (Mashela *et al*., 2017a).

6.5 Synthesis and conclusion

The NWP of the three phytochemicals and planting density of *S. frutescens* exhibited positive quadratic relations, which was one among the recent records which are demonstrating the importance of empirically-establishing the best agricultural practices in context of climate-smart agriculture. The optimum NWP of phytochemicals occurred when planting density was at least five seedlings per drip hole of irrigation using the 3S planter, which was ascribed to competition for the resources. Other cultural practices that exacerbate competition for resources such as irrigation interval and the use of filamentous fungi could improve various aspects of NWP of phytochemicals. The null hypothesis, planting density of *S. frutescens* would not affect NWP of phytochemicals to enhance the cultivation of this medicinal plant under field conditions in semi-arid regions was therefore

rejected. In the next chapter, the researcher provided a summary of the findings, along with the significance of the findings, the existing gaps, future recommendations and the conclusion of the study.

CHAPTER 7

SUMMARY, SIGNIFICANCE OF FINDINGS, RECOMMENDATIONS AND CONCLUSIONS

7.1 Summary

The current study through isolation and characterisation of nodule bacteria identified the genera *Raoultella ornithinolytica* and *Enterobacter cloacae spp. Dissolvens* in Tubatse location and the genera *Sphingomonas paucimobills*, *R. ornithinolytica* and *E. cloacae* were found in the root nodules of *S. frutescens* in Sebayeng location. This is the first report of these effective microorganisms' existence in roots of *S. frutescens*. The identification of genera *R. ornithinolytica* and *E. cloacae* in both locations suggest that the species were common in roots of *S. frutescens.*

Relative to untreated control, Tubatse strain, Sebayeng strain, *Rhizobium* strain and *Bradyrhizobium* strain increased plant height, root length and dry shoot mass of *Sutherlandia frutescens* by from 31-44%, 30-42% to 48-195%, respectively, whereas nodule number and dry nodule mass were reduced from 88-91% to 97-98%, respectively. Treatments had highly significant effects on nitrogen, protein and symbiotic efficiency (SEF%), contributing 84, 74 and 31% in TTV of nitrogen, protein and SEF%, respectively, but without having significant effects on potassium and phosphorus in shoot tissues. Relative to untreated control, Tubatse, Sebayeng, *Rhizobium* and *Bradyrhizobium* strains increased nitrogen by 80, 13, 25 and 7%, proteins by 69, 13, 24 and 10%, whereas SEF (%) by 292, 82, 133 and 31%, respectively. Relative to the control and other rhizobia

inoculates, Tubatse strain had the highest nitrogen shoot, while the lowest nitrogen shoot was observed in non-inoculated plants which reflected low shoot.

Sutherlandia frutescens plants were highly sensitive to Nemarioc-AL phytonematicide, but were highly tolerant to Nemafric-BL phytonematicide. The Mean Concentration Point (MCSP) for the two products was 3.43% and 4.03%, respectively, with application intervals of 29 and 17 days, respectively. Also, the dosage values for the two products were 6.62 and 13.26%, respectively. The products reduced various stages of root-knot (*Meloidogyne Javanica)* nematodes and could therefore be used to manage population densities of this nematode.

Increasing planting density promoted the nutritional water productivity of three phytochemicals, total phenol content, total tannin content and total flavonoid content in *S. frutescens*. The ideal planting density per hole of drip irrigation were, was at least five planting densities of S. *frutescens* plants, which improved the nutritional productivity of secondary metabolites in the test plant.

7.2 Significance of findings

The isolated and identified nodulation bacteria had the economic potential in the successful cultivation of *S. frutescens* under field conditions. Also, since the test plant was highly susceptible to *Meloidogyne* species, while it is highly tolerant to cucurbitacin phytonematicides, the phytonematicides could be applied at the empirically-derived MCSP values and the application intervals. Findings in the study also showed that the

desired secondary metabolites could be improved by manipulating planting density, which suggested that other cultural practices which could increase intraspecific competition could achieve similar attributes.

7.3 Recommendations

The influence of soil type and soil pH should be tested on the identified native strains of *S. frutescens*, along with their potential mass culturing with the necessary shelf-life. Also, the potential cucurbitacin residues in leaf tissues of the test plant should be assessed prior to recommending the use of cucurbitacin phytonematicides on *S. frutescens.* Additionally, the impact of the two cucurbitacin phytonematicides on the efficacy of the three identified nodulation bacteria should be assessed.

7.4 Conclusion

Findings in the current study demonstrated that nodulation bacteria from the wild could have the potential for commercialisation for use in the production of *S. frutescens*. Additionally, empirically-based information suggested that cucurbitacin phytonematicides could be used successfully in the cultivation of *S. frutescens* in areas with high population densities of M*eloidogyne* species.

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APPENDICES

Appendix 4.1 Analysis of variance on the effect of rhizobia inoculation on plant height of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	2100.8	150.06		
Treatment	4	5727.7	1431.93	22.02	0.0000
Error	56	3641.7	65.03		
Total	74	11470.2	549.01		

Appendix 4.2 Analysis of variance on the effect of rhizobia inoculation on root length of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	834.15	59.582		
Treatment	4	508.95	127.236	3.81	0.0082
Error	56	1867.9	33.356		
Total	74	3211.03	73.39		

Appendix 4.3 Analysis of variance on the effect of rhizobia inoculation on nodule number of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	19.247	1.3748		
Treatment	4	57.013	14.253	10.97	0.0000
Error	56	72.787	1.2998		
Total	74	149.05	5.875		

Appendix 4.4 Analysis of variance on the effect of rhizobia inoculation on dry nodule mass of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	15.255	1.0896		
Treatment	4	23.235	5.8088	9.31	0.0000
Error	56	34.925	0.6237		
Total	74	73.415	13.941		

Appendix 4.5 Analysis of variance on the effect of rhizobia inoculation on dry shoot mass of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	3741.4	267.22		
Treatment	4	2935.4	733.85	6.77	0.0002
Error	56	6067.3	108.34		
Total	74	12743.7	369.80		

Appendix 4.6 Analysis of variance on the effect of rhizobia inoculation on shoot nitrogen (%) of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	19.709	1.4078		
Treatment	4	53.411	13.353	11.78	0.0000
Error	56	63.482	1.1336		
Total	74	136.60	5.2981		

Appendix 4.7 Analysis of variance on the effect of rhizobia inoculation on shoot protein of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	1192.06	85.147		
Treatment	4	1480.16	370.04	8.61	0.0000
Error	56	2407.08	42.984		
Total	74	5079.29	166.06		

Appendix 4.8 Analysis of variance on the effect of rhizobia inoculation on symbiotic efficacy of *Sutherlandia frutescens* 110 days after planting (n = 75).

Source	DF	SS	MS	F	P
Replication	14	14243	10.152		
Treatment	4	78781	19.695	5.85	0.0005
Error	56	18841	33.645		
Total	74	40932	63.492		

Appendix 5.1 Analysis of variance for second stage juveniles (J2) in roots inoculated with *Meloidogyne javanica* under increasing concentrations of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	2.479	0.1652		
Treatment	6	3.033	0.5054	3.75	0.0022
Error	90	12.12	0.1347		
Total	111	17.633	0.8053		

Appendix 5.2 Analysis of variance for second stage juveniles (J2) in soil inoculated with *Meloidogyne javanica* under increasing concentrations of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	19.606	1.307		
Treatment	6	16.261	2.710	1.86	0.0970
Error	90	131.37	1.459		
Total	111	167.241	5.476		

Appendix 5.3 Analysis of variance of eggs in roots inoculated with *Meloidogyne javanica* under increasing concentrations of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	13.794	0.9156		
Treatment	6	5.8788	0.9798	1.56	0.1679
Error	90	56.502	0.6278		
Total	111	76.116	2.5232		

Appendix 5.4 Analysis of variance for final population (PF) of *Meloidogyne javanica* under increasing concentrations of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	2.0186	0.1346		
Treatment	6	2.8310	0.4718	3.99	0.0014
Error	90	10.634	0.1182		
Total	111	15.484	0.7247		

Appendix 5.5 Analysis of variance for second stage juveniles (J2) in roots inoculated with *Meloidogyne javanica* under increasing concentrations of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	1192.06	0.3916		
Treatment	6	1480.16	1.6061	3.42	0.0044
Error	90	2407.08	0.4695		
Total	111	57.765	2.4672		

Appendix 5.6 Analysis of variance for second stage juveniles (J2) in soil inoculated with *Meloidogyne javanica* under increasing concentrations of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	10.559	0.7040		
Treatment	6	15.341	2.5568	3.17	0.0072
Error	90	72.498	0.8055		
Total	111	98.398	4.0663		

Appendix 5.7 Analysis of variance Analysis of variance of eggs in roots inoculated with *Meloidogyne javanica* under increasing concentrations of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	7.4570	0.4971		
Treatment	6	9.8518	1.6419	3.84	0.0019
Error	90	38.519	0.4279		
Total	111	55.828	2.5669		

Appendix 5.8 Analysis of variance for final population (PF) of *Meloidogyne javanica* under increasing concentrations of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 112).

Source	DF	SS	MS	F	P
Replication	15	2.6953	0.1797		
Treatment	6	6.0483	1.008	4.18	0.0009
Error	90	21.692	0.2410		
Total	111	30.436	1.4287		

Appendix 5.9 Analysis of variance for dry shoot mass of *Sutherlandia frutescens* to Nemarioc-AL phytonematicide at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	744.34	39.176		
Treatment	4	859.69	214.92	14.59	0.0000
Error	76	1119.42	14.729		
Total	99	2723.45	268.827		

Appendix 5.10 Analysis of variance for gall rating of *Sutherlandia frutescens* to Nemarioc-AL phytonematicide at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	19.910	0.9953		
Treatment	4	2.660	0.6650	0.44	0.7817
Error	76	115.74	1.5229		
Total	99	1.5229	3.1832		

Appendix 5.11 Analysis of variance for second stage juveniles (J2) in roots inoculated with *Meloidogyne javanica* at application interval of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	5.6347	0.2966		
Treatment	4	4.3453	1.0863	3.60	0.0096
Error	76	22.6035	0.3014		
Total	90	32.5835	1.6843		

Appendix 5.12 Analysis of variance for second stage juveniles (J2) in soil inoculated with *Meloidogyne javanica* at application interval of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	31.137	1.6388		
Treatment	4	14.858	3.7145	3.05	0.0218
Error	76	92.494	1.2170		
Total	99	138.49			

Appendix 5.13 Analysis of variance of eggs in roots inoculated with *Meloidogyne javanica* at application interval of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	7.3047	0.3845		
Treatment	4	4.3565	1.0891	3.47	0.0117
Error	76	23.8348	0.3136		
Total	99	35.4961			

Appendix 5.14 Analysis of variance for final population (PF) of *Meloidogyne javanica* at application interval of Nemarioc-AL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	11.017	0.5799		
Treatment	4	6.9556	1.7389	2.85	0.0296
Error	76	46.448	0.6112		
Total	99	64.421	2.930		

Appendix 5.15 Analysis of variance for plant height of *Sutherlandia frutescens* to Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	2924.2	153.90		
Treatment	4	6230.1	1557.53	8.83	0.0000
Error	76	13401.1	176.33		
Total	99	22555.4	1887.76		

Appendix 5.16 Analysis of variance for dry shoot mass of *Sutherlandia frutescens* to Nemafric-BL phytonematicide at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	3049.4	160.496		
Treatment	4	1728.6	432.152	5.10	0.0011
Error	76	6436.6	84.692		
Total	99	11214.6	677.34		

Appendix 5.17 Analysis of variance for stem diameter of *Sutherlandia frutescens* to Nemafric-BL phytonematicide at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	10	96.789	5.0941		
Treatment	4	6.738	1.6846	0.49	0.7453
Error	76	262.96	3.4599		
Total	99	366.49	10.239		

Appendix 5.18 Analysis of variance for second stage juveniles (J2) in roots inoculated with *Meloidogyne javanica* at application interval of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	14	4.5276	0.2383		
Treatment	4	10.923	2.7307	8.23	0.0005
Error	76	25.231	0.3319		
Total	99	40.682	3.3008		

Appendix 5.19 Analysis of variance for second stage juveniles (J2) in soil inoculated with *Meloidogyne javanica* at application interval of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	6604623	347612		
Treatment	4	3.8671	96678	20.44	0.0000
Error	76	3.5941	472908		
Total	99	8.1221	917198		

Appendix 5.20 Analysis of variance for eggs in roots inoculated with *Meloidogyne javanica* at application interval of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	6.1109	0.3216		
Treatment	4	15.151	3.7878	10.86	0.0000
Error	76	26.517	0.3489		
Total	99	47.779	4.4583		

Appendix 5.21 Analysis of variance PF of *Meloidogyne javanica* at application interval of Nemafric-BL phytonematicide on *Sutherlandia frutescens* at 56 days after initiation of treatments (n = 100).

Source	DF	SS	MS	F	P
Replication	19	2.3119	0.1217		
Treatment	4	6.6650	1.6663	19.52	0.0000
Error	76	6.4861	0.0853		
Total	99	15.463	1.8733		

Appendix 6.1 Analysis of variance of nutritional water productivity (total flavonoid content) of *Sutherlandia frutescens* under field conditions during the 2018 season (n = 81).

Source	DF	SS	MS	F	P
Replication	8	0.0144	1.8030		
Treatment	8	0.0748	9.3550	17.11	0.0000
Error	64	0.0350	5.4670		
Total	80	0.1243			

Appendix 6.2 Analysis of variance of nutritional water productivity (total tannin content) of *Sutherlandia frutescens* under field conditions during the 2018 season (n = 81).

Source	DF	SS	MS	F	P
Replication	8	0.0867	0.0108		
Treatment	8	0.155	0.0194	4.73	0.0001
Error	64	0.2621	0.0041		
Total	80	0.5039	0.0343		

Appendix 6.3 Analysis of variance of nutritional water productivity (total phenol content) of *Sutherlandia frutescens* under field conditions during the 2018 season (n = 81).

Source	DF	SS	MS	F	P
Replication	8	1.8642	0.2330		
Treatment	8	2.2395	0.02799	2.70	0.0127
Error	64	6.6409	0.1038		
Total	80	10.745	0.1216		

Appendix 6.4 Analysis of variance of nutritional water productivity (total flavonoid content) of *Sutherlandia frutescens* under field conditions during the 2019 season (n = 81).

Source	DF	SS	MS	F	P
Replication	8	0.0058	7.225		
Treatment	8	0.0266	3.327	7.22	0.0000
Error	64	0.0295	4.608		
Total	80	0.0619	5.053		

Appendix 6.5 Analysis of variance of nutritional water productivity (total tannin content) of *Sutherlandia frutescens* under field conditions during the 2019 season (n = 81).

Source	DF	SS	MS	F	P
Replication	8	0.0524	6.550		
Treatment	8	0.0454	5.801	4.08	0.0006
Error	64	0.0910	1.422		
Total	80	0.1898	4.591		

Appendix 6.6 Analysis of variance of nutritional water productivity (total phenol content) of *Sutherlandia frutescens* under field conditions during the 2019 season (n = 81).

Source	DF	SS	MS	F	P
Replication	8	0.1460	0.0183		
Treatment	8	0.9509	0.1189	4.52	0.0002
Error	64	1.6817	0.0263		
Total	80	2.7785	0.0545		