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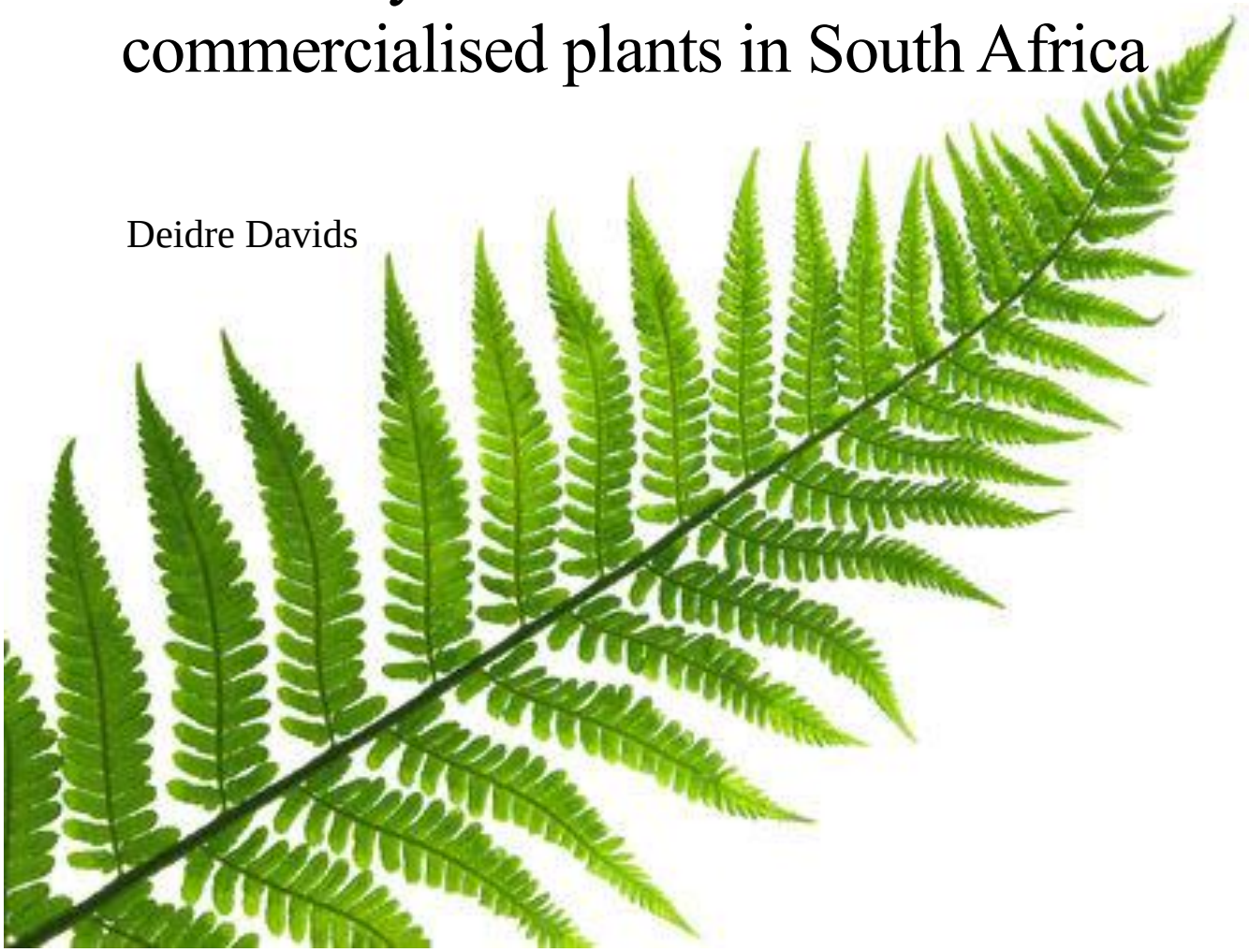
Bioprospection of hydroxynitrile lyase activity in ferns and other non-commercialised plants in South Africa

Deidre Davids



A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

27 May 2019, Johannesburg

Declaration

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Abstract

Hydroxynitrile lyases catalyse the decomposition of chiral cyanohydrins into a carbonyl compound and toxic hydrogen cyanide *in vivo*. The reverse reaction for the synthesis of chiral cyanohydrins, which are important precursors in the pharmaceutical, agrochemical and cosmetic industry, is desirable. This study aimed to discover novel hydroxynitrile lyase enzymes with superior biocatalytic properties to those currently used in industry by bioprospection using Feigl-Anger detection paper and novel hydrogen cyanide detection tubes. Forty-eight out of over 540 plant species (8.89%) were found to be cyanogenic. This is the first report of a hydroxynitrile lyase in the leaves of the African mulberry tree, *Morus mesozygia,* and in the fern family Thelypteridaceae. Hydroxynitrile lyases from three fern species namely, *Thelypteris confluens*, *Davallia trichomanoides* and *Phlebodium aureum mandaianum* were isolated and partially purified by anion exchange chromatography. Native polyacrylamide electrophoresis and an in-gel activity assay allowed for isolation of specific bands which were subjected to liquid chromatography tandem mass spectrometry. The hydroxynitrile lyase from *T. confluens* has good stability at pH 2.5 making it a promising candidate for industrial use, therefore, the transcriptome of *Tc*HNL was generated. The integration of the peptide data and the transcriptome is currently underway and will lead to the elucidation of the amino acid sequence of the enzyme. This will allow for heterologous expression and further enzymatic characterisation studies on the enzyme to determine the feasibility of industrial application.

Keywords: Chiral cyanohydrins, blue native PAGE, clear native PAGE, hydrogen cyanide detection tubes, bioprospection, transcriptome

Dedication

For Skyler

When I needed a hand, I found your paw

Acknowledgements

The National Research Foundation (NRF) and the Imperial Next Generation Bursary Programme are acknowledged and sincerely thanked for providing funding for the duration of this degree. Professor Karl Rumbold is acknowledged for providing supervision, advice and encouragement. The Austrian Centre of Industrial Biotechnology is acknowledged for their assistance with LC/MS-MS of my samples and with the integration of the transcriptome and proteome data obtained in this study. My colleague, Matthew du Plessis is acknowledged for his support provided in the laboratory. Finally, Mr Paulus Sebothoma from the South African National Biodiversity Institute (SANBI) is acknowledged for going above and beyond to assist me with the identification of my plant samples.

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List of abbreviations

ACE	Angiotension converting enzyme
ACIB GmbH	Austrian Centre of Industrial Biotechnology
APES	Animal Plant and Environmental Science
ARC-BTP	Agricultural Research Council Biotechnology platform
*At*HNL	HNL from *Arabidopsis thaliana*
*Bm*HNL	HNL from *Baliospermum montanum*
BN-PAGE	Blue native polyacrylamide gel electrophoresis
CSIR	Council for Scientific and Industrial Research
*Dt*HNL	HNL from *Davallia tyermannii*
*Dtm*HNL	HNL from *Davallia Trichomanoides*
*Ej*HNL	HNL from *Eriobotrya japonica*
FAD	Flavin adenine dinucleotide
FAO	The Food and Agricultural Organisation of the United Nations
GMC	Glucose-methanol-choline
*Hb*HNL	HNL from *Hevea brasiliensis*
HCN	Hydrogen cyanide
HNL	Hydroxynitrile lyase
IMBL	Industrial Microbiology and Biotechnology Laboratory
kDa	Kilo Dalton
LC-MS/MS	Liquid chromatography tandem mass spectrometry
*Lu*HNL	HNL from *Linum usitatissimum*
*Me*HNL	HNL from *Manihot esculenta*
NISE	Non-homologous isofunctional enzymes
*Pa*HNL	HNL from *Prunus amygdalus*
*Pat*HNL	HNL from *Prunus amygdalus turcomanica*
PCR	Polymerase chain reaction
*Pham*HNL	HNL from *Phlebodium aureum mandaianum*
Redox	Oxidation-reduction
*Sb*HNL	HNL from *Sorghum bicolor*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
*Tc*HNL	HNL from *Thelypteris confluens*

1 Literature review

1.1 Biocatalysis

In 2001 the Department of Science and Technology developed the National Biotechnology Strategy in response to the growing need to find greener alternatives for processes in industries such as pharmaceuticals, energy, agriculture and industrial sectors (Parker *et al*., 2001). The majority of South Africa's enzyme requirements are imported and the Bio-Economy Strategy (DST, 2013) strives to reduce the reliance on imports by increasing research and development efforts in biotechnology and implement its application in industry. Biocatalysis is defined as the use of biological enzymes, whole cells or cell extracts to produce value-added products (Bornscheuer *et al*., 2012). Biocatalysts are non-toxic, renewable and most importantly, biodegradable. Replacing current chemical synthesis methods with biocatalysis is a cleaner alternative which will decrease the burden of toxic waste products and the cost of their safe disposal. Enzymes are substrate specific and their high efficacy (in terms of reaction rates, enantioselectivity and product yield) combined with the mild conditions required to run biocatalytic reactions are an important advantage in the biocatalysis industry (Dadashipour *et al.*, 2015). Biotechnological approaches are becoming more attractive and therefore bioprospection is becoming more important for the advancement of these technologies and for the discovery of novel products that can be used in industry.

Plants represent a wealth of proteins, enzymes and metabolites which have potential for industrial application. Only a fraction of enzymes with biocatalysis potential in plants have been mined to date. One class of enzymes of industrial importance are the hydroxynitrile lyases (HNLs) which, *in vivo*, are involved the production of the toxic compound hydrogen cyanide. *In vitro*, the reverse reaction is valuable in industry.

1.2 Cyanogenesis

Cyanogenesis is the biological phenomenon observed when compounds called cyanohydrins are decomposed into hydrogen cyanide (HCN) and the corresponding carbonyl compound (aldehyde or ketone). This process has been observed in microbes, arthropods, and over 3000 plant species, including species from angiosperms (monocotyledons and dicotyledons), gymnosperms and pteridophytes (ferns) (Poulton, 1990).

Cyanogenesis occurs in a two-step process. Compounds called cyanogenic glycosides (CGs) are the glycosides of α-hydroxynitriles. The cyanogenic glycosides are a diverse group of secondary metabolites and to date about 65 types have been found in plants (Asano *et al.*, 2005). Most often the α-hydroxynitrile is β-linked to the glycoside moiety (Vetter, 1999). This sugar moiety can be a monosaccharide, a disaccharide (e.g. amygdalin, vicianin and linustatin) or a trisaccharide (xeranthin) (Poulton, 1990). Hydrolysis of cyanogenic disaccharides can occur via two pathways; sequentially or simultaneously. Sequential hydrolyses requires two different β-glucosidase enzymes as the two sugar moieties are removed stepwise. Examples of CGs that undergo sequential hydrolysis of their glycoside residues are amygdalin in *Prunus serotina* and linustatin and neolinustatin in *Linum usitatissimum.* In simultaneous hydrolysis the disaccharide is released as one unit. Simultaneous hydrolysis is observed in *Davallia trichomanoides* and *Vicia angustifolia* with the CG vicianin, and in *Hevea brasiliensis* with the CG linustatin (Poulton, 1990).

1.2.1 Biosynthesis of cyanogenic glycosides

Cyanogenic glycosides are derived from six amino acids; L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tyrosine and the non-proteinaceous amino acid cyclopentenyl-glycine (Zagrobelny *et al.*, 2004). These compounds can therefore be aromatic, aliphatic or cyclopentenoid, depending on the precursor (Poulton, 1990). Cyanogenic glycoside biosynthesis occurs in three broad steps (Figure 1).

In the first step, the precursor amino acid is converted to aldoxime through two successive N-hydroxylations of the amino group of the parent amino acid by an enzyme belonging to the

cytochrome-P450 family. The aldoxime is then converted into a nitrile by a dehydration reaction followed by the hydroxylation of the alpha carbon to form a cyanohydrin. This reaction is catalysed by another cytochrome-P450 enzyme. The enzyme UDP-glucosyltransferase then catalyses the glycosylation of the cyanohydrin to generate the cyanogenic glycoside (Zagrobelny *et al*., 2004).

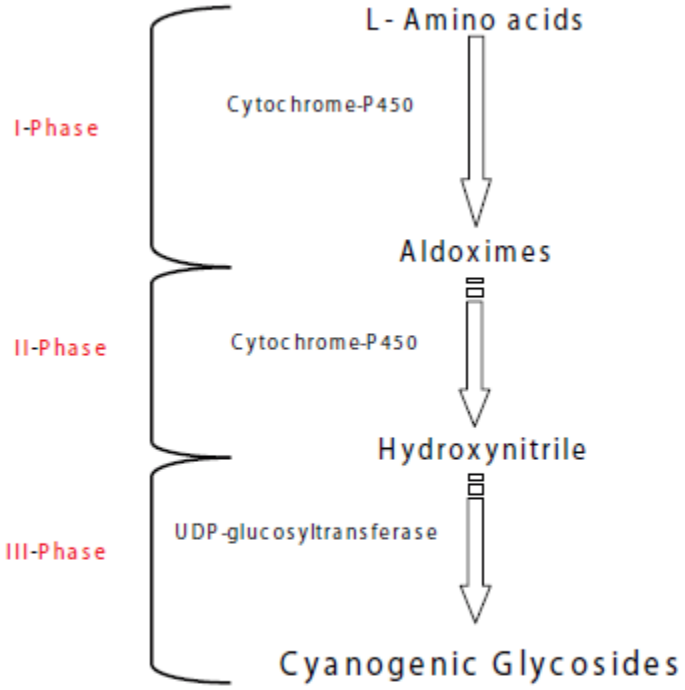


Figure 1. Biosynthesis of cyanogenic glycosides. Schematic of the pathway of cyanogenic glycoside biosynthesis from precursor amino acids (Ganjewala *et al*., 2010)

Cyanogenic glycosides and their subsequent degradation into the toxic HCN have long been considered to play a role in plant defence against herbivory and pathogenic attack by fungi or bacteria (Vetter, 1999). However, research has found that depending on the insect species, CGs can be a feeding deterrent (owing to its bitter taste) or a phagostimulant. For example, larvae of the economically important pest of stone fruit *Capnodis tenebrionis* (flatheaded woodborers) are deterred from feeding on bitter almond due to the high concentration of CGs (Malagon and Garrido, 1990). On the other hand, larvae of the pestilential moth *Spodoptera eridania* (southern army worm) thrive on feeding on CG-containing plants and have been shown to grow better when cyanide is present in their diet (Brattsen *et al*., 1983).

In addition to playing a role in plant defence, CGs are also a storage form of reduced nitrogen and can be broken down into amino acids (Wajant *et al*., 1995; Gebrehiwot and Beuselinck, 2001; Sharma *et al*., 2005). This process occurs following the hydrolysis of the cyanogenic glycoside into a sugar moiety and a cyanohydrin. This cyanohydrin is then cleaved into a carbonyl compound and HCN by the enzyme hydroxynitrile lyase. The HCN released can be fixed by the enzyme β-cyanoalanine synthetase to form β-cyanoalanine with L-cysteine. The enzyme β-cyanoalanine hydrolase acts to convert β-cyanoalanine into L-asparagine (Figure 2; Sharma *et al*., 2005). The refixation of one molecule of HCN, therefore, results in an amino acid which can be further utilized in protein synthesis within the plant.

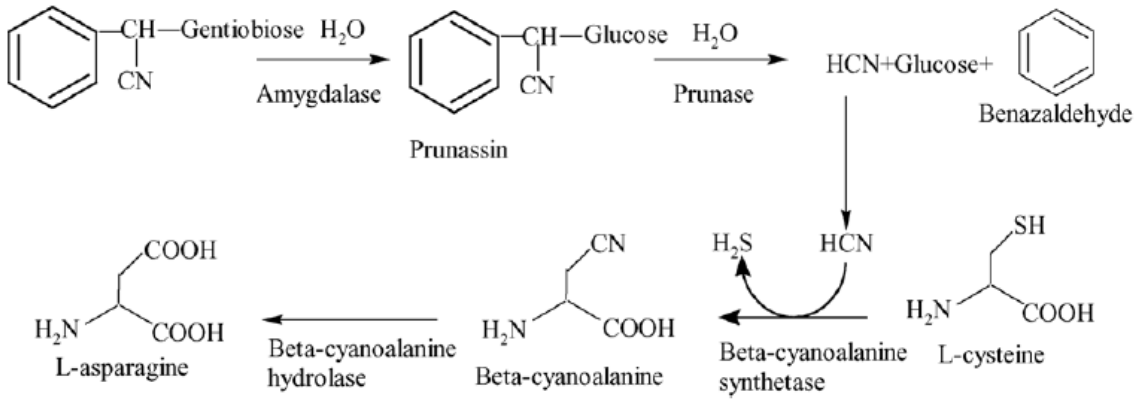


Figure 2. Hydrogen cyanide refixation into amino acids. The action of the enzymes β-cyanoalanine synthetase and β-cyanoalanine hydrolase lead to the production of the amino acid L-asparagine from the hydrogen cyanide produced in the cyanogenesis reaction (Sharma *et al*., 2005).

1.2.2 Effects of cyanogenesis on humans and animals

Several economically important plants such as almond (*Prunus amygdalus*), lima beans (*Phaseolus lunatus*), sorghum (*Sorghum bicolor*) and cassava (*Manihot esculenta*) are highly cyanogenic. Cassava is an important food crop in many developing countries and especially in sub-Saharan Africa, even though it contains very high levels of the cyanogenic glycoside linamarin (Siritunga and Sayre, 2004). Tissue disruption will result in deglycosylation of linamarin by linamarase to give acetone cyanohydrin which is further cleaved to produce HCN (Siritunga and Sayre, 2004). The Food and Agricultural Organisation of the United Nations (FAO) recommend a maximum level of 10 mg CN equivalents/ kg dry weight for CG containing foods. Cassava leaves contain between 200 -1300 mg CN equivalents/ kg dry weight and the root contains between 50 – 500 mg CN equivalents/ kg dry weight (Siritunga

and Sayre, 2004). These values are exceptionally high and proper processing of the leaves and roots of this crop is of utmost importance before consumption. Many cases of cyanide poisoning due to residual CGs in cassava have been reported over the years (Siritunga and Sayre, 2004). Risks of consuming cassava with high levels of CGs are neurological disorders and paralysis (Nicolson, 2007). Chronic low-level cyanide exposure can cause goiter and tropical ataxic neuropathy, whereas acute cyanide poisoning can cause the paralytic disorder Konzo and, in some cases, even be fatal (Howlett *et al*., 1990; Siritunga and Sayre, 2004; Nicholson, 2007). The lethal dosage of HCN for vertebrates is reported to be in the range of 35-150 µmol/ kg in a single dose (Zagrobelny *et al*., 2004). The toxic effects of cyanide are a result of the cyanide molecule having a high affinity for the ferric ion in cytochrome oxidase. This blocks the transfer of oxygen in the respiratory pathway leading to tissue anoxia (Zagrobelny, 2004; Nicholson, 2007). The ingestion of CG-containing cassava can interfere with biological functions such as gluconeogenesis, enzyme function, thyroid hormone activity and can also slow down the usual pattern of human growth and development (Vetter, 1999). People who are nutrient deficient, which unfortunately is common in developing countries, have a higher susceptibility to the negative effects of CGs (Delange *et al*., 1994).

Processing of cassava leaves and roots to remove CGs involve the maceration of plant tissue (Nweke *et al*., 2002). This allows linamarin to be converted into acetone cyanohydrin and subsequently HCN. The release of HCN can occur spontaneously at pH levels above 5 or at temperatures above 35 °C. Once the cyanide is free, it can be extracted with water or volatized into the atmosphere, completing the detoxification process (White *et al*., 1998; Siritunga and Sayre, 2004).

Siritunga and Sayre (2004) discovered another way in which the cyanogenic effects of high CG in cassava can be decreased through genetic engineering. It was found the leaves of cassava had high levels of both cyanogenic glycosides and hydroxynitrile lyases, whereas the root of cassava has no detectable hydroxynitrile activity. Researchers therefore genetically engineered the cassava root to express the leaf-specific HNL. This caused an increase in the rate of cleavage and elimination of cyanide.

Different animals have different responses to a significant amount of HCN. The response depends on factors such as age, fitness and nutritional status (Vetter, 1999). Ruminants are

more vulnerable to the toxic effects of CGs as the rumen microbial flora are able to rapidly hydrolyse cyanogenic glycosides into HCN. An example of this was seen in three geriatric cows which consumed the highly cyanogenic bird cherry (*Prunus padus*). Within hours the cows experienced signs of anorexia, weakness and depression, among others, and within 10 hours of consumption, the cows died. A more devastating report was that of 22 swamp buffalos dying within 18-36 hours after consuming the leguminous woody shrub *Mimosa invisa* var. *inermis* (Vetter, 1999). Fortunately, monogastric animals (including humans) have an acidic environment in the stomach which significantly slows down the breakdown of CGs, limiting our exposure to HCN (Nicholson, 2007).

1.2.3 Regulation of cyanogenesis in plants

Cyanogenic plants show high variation in the concentration of HCN. This variation may be due to differences in the concentration of cyanogenic glycosides and/or hydroxynitrile lyases (Vetter, 1999). Cyanogenesis is a genetically controlled trait. Two independent genes are involved in HCN production namely, Ac/ac for cyanogenic glycosides and Li/li for β-glucosidase production. Cyanogenic plants such as the *Lotus* spp. have a phenotype reflective of Ac/Li (Gebrehiwot and Beuselinck, 2001). Although the synthesis of CGs and the enzymes needed to decompose them are genetically controlled, environmental and abiotic stresses have a significant impact on the rate of expression (Harper *et al*., 1976; Vetter, 1999; Gebrehiwot and Beuselinck, 2001). The nutrient content of the plant, especially the nitrogen content, also affects CG concentration (Nicholson, 2007). El-Essawi and colleagues (1995) showed a positive increase in HCN correlating to the increase of nitrogen-phosphoric (NP) fertiliser in Sorghum.

Another important variable in cyanogenesis is temperature or seasonal variations. Three *Lotus* spp. were observed to have the highest concentration of HCN in spring and summer and the lowest concentration of HCN in winter (Gebrehiwot and Beuselinck, 2001). The high level of cyanogenic glycosides in the warmer seasons could be because these compounds are acting as storage methods of nitrogen which will be needed for survival in winter and growth in early spring.

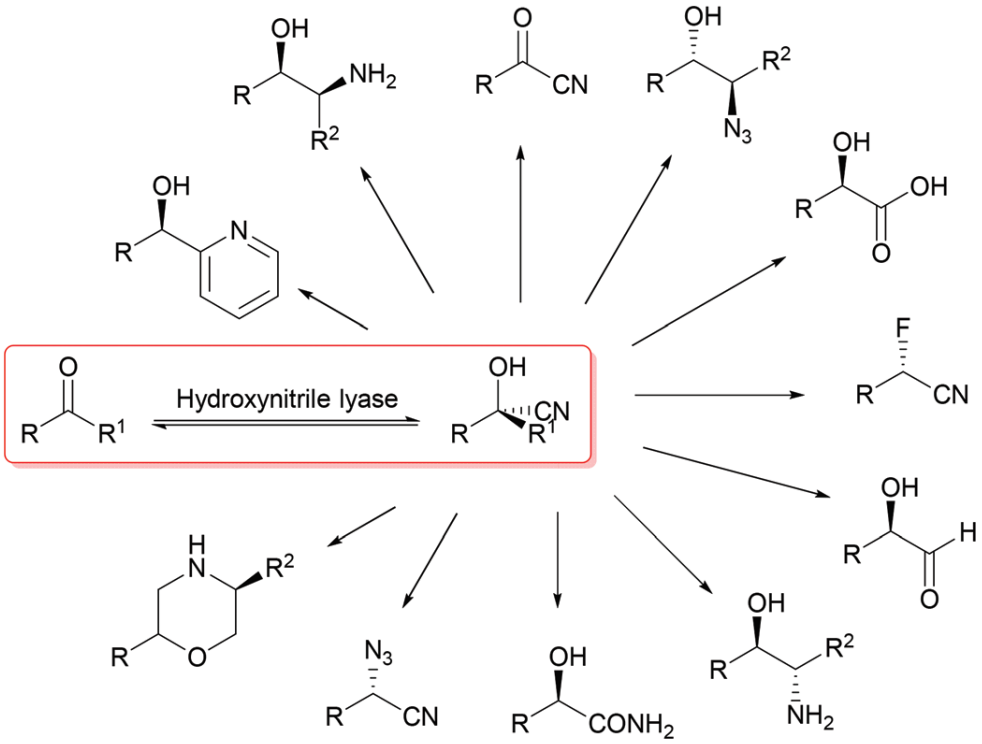
Cyanogenic glycoside content also varies with the age of the plant and the stage of growth. A higher concentration of CGs has been observed in seedlings of plants. During the first few days when plants undergo a phase of intensive growth, a higher rate of CG biosynthesis occurs (Vetter, 1999; Nicholson, 2007). This may occur as young seedlings are more vulnerable to insect attack and the high CG content deters herbivory. This was seen in seedlings of white clover in which the seedlings of cyanogenic variants had a higher survival rate compared to the acyanogenic seedlings (Pederson and Brink, 1998). As the plant grows and starts to produce fruit the CG levels decrease due to the change in the priorities of the plant (Briggs and Schultz, 1990). Most resources are utilised for fruit production and therefore a trade-off has to be made in order for the plant to successfully reproduce (Gebrehiwot and Beuselinck, 2001; Nicholson, 2007).

1.3 Cyanohydrins and their application in industry

All naturally occurring compounds are present in the form of one enantiomer only, that is, they are chiral. Synthetic chemicals, however, can exist in both enantiomeric forms. In pharmaceuticals, over half of the drugs on the market are chiral, however, the remaining percentage are sold as a racemic mixture (Nguyen *et al*., 2006). Although the enantiomers of a compound have the same chemical structure, most often the different stereoisomers have different biological activity, pharmacology, toxicology, pharmacokinetics and metabolism (Nguyen *et al*., 2006). In pharmaceuticals, most often only one of the stereoisomers have the desired pharmacological action and the use of a racemic drug in such cases can cause undesired and even toxic side effects. For example, only the *R*-selective enantiomer of adrenaline and the 1*R*, 2*S* configuration in ephedrine compounds are of pharmaceutical use (Effenberger and Jäger,1997). The chiral separation of racemic drugs, also known as resolution, is therefore necessary in the pharmaceutical industry for racemic mixtures which may cause undesirable side effects (Effenberger and Jäger,1997; Nguyen *et al*., 2006). An alternative to resolution techniques is biosynthetic methods which produce enantiomerically pure starting materials and precursors for pharmaceuticals.

Chiral cyanohydrins are precursors to compounds including but not limited to; 2-hydroxy carboxylic acids, 2-amino acids, α-hydroxy aldehydes, α-fluorocyanides, 2-amino alcohols,

α- hydroxy acids, α-hydroxy ketones and β-ethanolamines (Wajant *et al*., 1995; Sharma *et al*., 2005; Effenberger *et al*., 2007; Bracco *et al*., 2016). Figure 3 illustrates the various stereoselective follow-up reactions of chiral cyanohydrins. A brief overview of some of the pharmaceutical compounds derived from chiral cyanohydrins follows.



R = H R^1 = aliphatic (un)saturated aldehydes, (un)substituted benzaldehydes, heteroaromatic aldehydes
R ≠ H R^1 = methyl-, ethyl-, phenyl- and heterocyclic ketones, *cyclo*-hexanones

Figure 3. Stereoselective follow-up reactions of chiral cyanohydrins. Chiral cyanohydrins are important precursors to a variety of chiral compounds which find application in pharmaceutical, agrochemical and cosmetic industries. (Bracco *et al.*, 2016).

1.3.1 The 2-amino alcohols

Two-amino alcohols are an important building block in drug design. Synthesising enantiomerically pure 2-amino acids can be derived from using chiral cyanohydrins. Effenberger and Jäger (1997) showed that the bronchodilators (*R*)-terbutaline and (*R*)-salbutamol were able to be synthesized from optically active cyanohydrins. These chiral cyanohydrins were synthesized by the HNL-catalysed addition of HCN to the respective

benzaldehyde. This was an important discovery as it is now known that only the *R*-enantiomers of terbutaline and salbutamol are effective and the *S*- isomers may cause unpleasant side-effects (Nguyen *et al*., 2006).

1.3.2 (*R*)-pantolactone synthesis

(*R*)-pantolactone is a crucial starting material to the production of (*R*)-pantothenic acid (vitamin B5) which is a component of coenzyme A, the bactericide (*R*)-panthenol and the growth factor (*R*)-pantothein. Before the use of (*R*)-cyanohydrins, racemic products of (*R*)-pantolactone had to undergo tedious and time-consuming racemic resolution procedures to obtain enantiomerically pure (*R*)-pantolactone (Effenberger *et al.*,1995). The (*R*)-HNL catalysed synthesis of hydroxypivalaldehyde cyanohydrin, followed by acid-catalysed hydrolysis is a more attractive alternative (Pscheidt *et al.*, 2008). In 1995, Effenberger and colleagues used the *Pa*HNL from bitter almond (*Prunus amygdalus*), immobilsed on cellulose, to catalyse the addition of HCN to hydroxypivaldehyde. Following cyclisation by hydrolyses, and one recrystallisation an enantiomeric excess of 98% of (*R*)-pantolactone was observed (Effenberger *et al*., 1995). In a more recent study, the acid-stable *Pa*HNL isoenzyme 5 mutant (V317A) (*R*)-HNL was used in a pure aqueous reaction solution to catalyse the same reaction as above. The result was an enantiomeric excess exceeding 97% for (*R*)-pantolactone (Figure 4). This *Pa*HNL5 variant (V317A) has since become the most efficient biocatalyst in the synthesis pathway to vitamin B5 (Pscheidt *et al*., 2008) as the *Pa*HNL V317A mutant retains high enzyme activity at pH 2.5 and the non-enzymatic reaction is completely suppressed at this pH (Bracco *et al*., 2016).

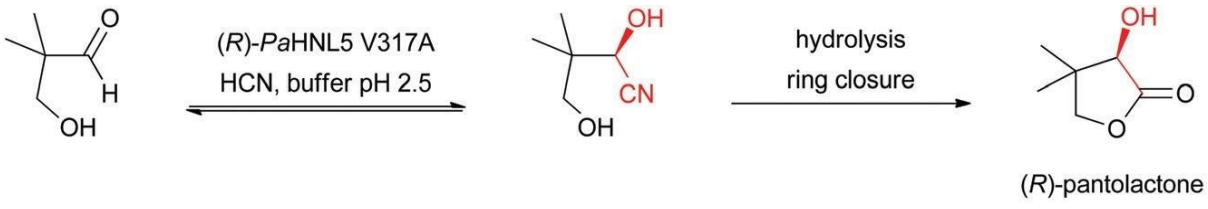


Figure 4. The synthesis of *R*- pantolactone. The *Pa*HNL isoenzyme 5 mutant (V317A) is the most efficient biocatalyst in the synthesis pathway to vitamin B5. The key chiral intermediate (*R*)-pantolactone is synthesized by the hydrocyanation of hydroxypivaldehyde at pH 2.5 with the complete suppression of the non-enzymatic reaction (Bracco *et al*., 2016).

1.3.3 The α-hydroxy-β amino acids

These amino acids are an important constituent of many available drugs and natural products. The most well-known products which are synthesized using these building blocks include the antimitotic Taxol, the immunostimulant Bestatin, angiotensin converting enzyme (ACE) inhibitors as well as the HIV-protease inhibitor named R-87366 (Tasić *et al*., 2004). Additionally, the chiral cyanohydrin (*R*, E)-2- hydroxypentenitrile is synthesised by the (*R*)-HNL-catalysed addition of HCN to 2-butenal. This optically active compound is a favourable precursor in amino acid synthesis (Warmerdam *et al*., 1996). These are just a few examples of the wide variety of compounds comprising or derived from α-hydroxy-β-amino acids. Using stereoselective HNL-catalysed synthesis of chiral cyanohydrins, the production of enantiomerically pure α-hydroxy-β amino acids is possible.

1.4 Hydroxynitrile lyases

The enzymes which catalyse the process of cyanogenesis are the hydroxynitrile lyases (HNLs). Hydroxynitrile lyases are enzymes and can therefore function in both directions (Figure 5). This means that chiral cyanohydrins which were discussed in section 1.2 are formed in an enantioselective manner given the right conditions. The first record of HNL activity was in 1837, when Freidrich Wöhler observed the synthesis of HCN and a carbonyl compound from a cyanohydrin using a protein extract from bitter almond, *P. amygdalus,* (Sharma *et al*., 2005). In 1908, Leopold Rosenthaler conducted the reverse reaction using a protein extract from *P. amygdalus* to produce enantiomerically pure mandelonitrile from benzaldehyde and HCN (Rosenthaler, 1908). This was, in fact, the first enantioselective synthesis reaction conducted (Bracco *et al*., 2016). This discovery had a major impact on an industrial level as HNLs are one of the few enzymes which can catalyse the formation of stereoselective carbon-carbon bonds and *Pa*HNL (HNL from *P. amygdalus*) has been used for this function for many decades (Zhao *et al*., 2011).

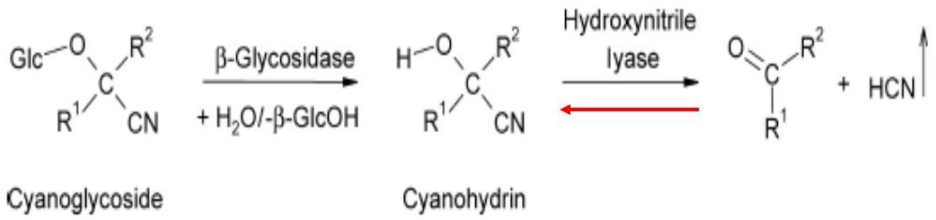


Figure 5. The process of cyanogenesis catalysed by hydroxynitrile lyases. Cyanogenic glycosides are hydrolysed by β- glucosidase enzymes to yield a chiral cyanohydrin. The cyanohydrin is cleaved by hydroxynitrile lyases into the corresponding carbonyl compound and gaseous hydrogen cyanide. The reverse reaction is desirable in industry (Effenberger *et al*., 2007).

Hydroxynitrile lyases fall into four categories based on the reactions they catalyse namely; mandelonitrile lyase [EC.4.1.2.10], P-hydroxymandelonitrile lyase [EC.4.1.2.11], acetone cyanohydrin lyase [EC 4.1.2.37] and hydroxynitrilase [EC 4.1.2.39] (Asano *et al*., 2005). Furthermore, these enzymes can be broadly grouped as flavin adenine dinucleotide (FAD)-dependent and as FAD-independent HNLs, hereafter referred to as HNL I and HNL II, respectively.

1.4.1 Hydroxynitrile lyases I

These HNLs have been isolated mainly from plants in the Rosaceae family specifically, from the subfamilies Prunoideae and Maloideae (Sharma *et al*., 2005). The co-factor FAD is not involved in the net oxidation-reduction (redox) reaction, but rather stabilizes the enzyme structure by binding to a hydrophobic patch near the active site (Sharma *et al*., 2005). Studies have proven that the removal of FAD inactivates the enzyme (Jorns, 1979). FAD-dependent HNLs are a homologous group of enzymes believed to have evolved convergently from an ancestral flavoprotein which lost the ability to catalyse redox reactions (Wajant *et al*., 1995). HNLs characterized in this group so far have been shown to be *R*-selective glycoproteins similar in size and serologically related (Wajant *et al*., 1995).

The HNLs from the Rosaceae family are related to the glucose-methanol-choline (GMC) oxidoreductase family of enzymes (Andexer *et al.*, 2012). The FAD-dependent *R*-(HNLs) show about 30% similarity to GMC oxidoreductases and within the HNL I group, highly conserved regions (89%) are shared between the HNLs (Sharma *et al.*, 2005).

The proposed reaction mechanism for *Pa*HNL is a general acid-base catalysis by imidazole of a conserved catalytic histidine. His496 acts as a base and deprotonates the hydroxyl group of the cyanohydrin. The negatively charged cyanide ion is neutralized by positive electrostatic potential at the active site due to two positively charged amino acids namely, arginine 300 and lysine 361 (Sharma *et al*., 2005).

Other HNLs of the same type as *Pa*HNL have been found in several plants from the Rosaceae family including, *Eriobotrya japonica, Prunus persica, P. avium, P. domestica, P. laurocerasus, P. mume, P. lyonia, P. serotina.* In addition, *Passiflora edulis, Chaenomles sinesis, Pouteria sapota, Cucumis melo* and *Cydonia oblonga* have been reported to have HNLs of this type (Ueatrongchit *et al*., 2008).

1.4.2 Hydroxynitrile lyases II

In contrast to HNL I, the FAD-independent HNLs are a heterologous group of enzymes which differ in size, extent of glycosylation, subunit composition and substrate specificity (Wajant *et al*., 1995). HNLs discovered in this group can be separated into five classes of enzymes. Two HNLs discovered from endophytic bacteria belong to the cupin family and the *R*-selective HNL from *Linum usitatissimum* (*Lu*HNL) shows homology to zinc-dependent alcohol dehydrogenases. In contrast, the *S*-selective HNL from *Sorghum bicolor* (*Sb*HNL) shares homology with the serine carboxypeptidases. The *S*-selective HNLs from *Hevea brasiliensis* (*Hb*HNL) *Manihot esculenta* (*Me*HNL), *Baliospermum montanum (Bm*HNL) and the *R*-selective HNL from *Arabidopsis thaliana* (*At*HNL) exhibit the α/β hydrolase fold (Andexer *et al*., 2007). Recently, a *R*-selective HNL from the fern *Davallia tyermannii* (*Dt*HNL) was reported to exhibit the Bet v1-like protein fold (Lanfranchi *et al*., 2017).

In general, the HNL catalytic mechanism has three main requirements; (1) deprotonation of the cyanohydrin's hydroxyl group by a strong base, (2) electrostatic stabilization of the negative charge at the cyano group resulting from C-C bond cleavage and (3) protonation of the cyanide ion to give hydrogen cyanide (Andexer *et al*., 2012).

Two of the most well-studied HNLs *Me*HNL and *Hb*HNL are highly homologous and the plants which they come from belong to the same family, Euphorbiaceae (Effenberger *et al*., 2007). Both *Me*HNL and *Hb*HNL have an α/β-hydrolase fold and have 77% sequence identity and the same molecular mechanism (Gruber *et al*., 2004; Andexer *et al*., 2012). *S*-selectivity was thought to be a characteristic of the α/β-hydrolase fold HNLs. However, *At*HNL was observed to have inversed enantioselectivity. The structures and reaction mechanism of *At*HNL and *Hb*HNL were compared to elucidate the reason for this enantio-complementarity. *At*HNL and *Hb*HNL were found to have high sequence similarity, with 45% identity and 67% similarity. HNLs in this group have a characteristic catalytic triad comprising of Ser-His-Asp (Andexer *et al*., 2012). The reactive complex structures between *At*HNL and *Hb*HNL show that the substrate's cyano- groups point in opposite directions. As a result, the substrate's hydroxyl groups interact differently in the two enzymes. In *Hb*HNL, the hydroxyl group bonds to the serine (Ser80) residue of the catalytic triad and threonine (Thr11). However, in *At*HNL the hydroxyl group bonds to the histidine (His236) residue of the triad and the amide group of asparagine (Asn12). These differences appear to be the result of two amino acid exchanges in the two enzymes: Thr11 in *Hb*HNL is replaced by Asn12 in *At*HNL and Lys236 in *Hb*HNL is replaced by Met237 in *At*HNL (Andexer *et al*., 2012). These small changes nearby the active site results in the inversion of enantioselectivity in the identically folded proteins (Andexer *et al*., 2012).

Differences in the electrostatic stabilization mechanisms of the two enzymes were also noted. In *Hb*HNL the Lys236 residue stabilizes the negative charge (Gruber *et al*., 2004). Because the positively charged Lys236 residue is replaced by the uncharged Met237 in *At*HNL, the electrostatic stabilization is due to hydrogen bonding with two main-chain amide groups which forms an acyl-enzyme intermediate. The dipole of the α-helix in *At*HNL (from Phe82 to Ile93) contributes to the stabilization by a cumulative effect (Andexer *et al*., 2012). The final step in the reaction mechanism is the protonation of the cyanide ion. In *Hb*HNL this is

achieved by His235 via direct protonation whereas, in *At*HNL, His236 indirectly protonates the cyanide ion with Ser81 acting as a mediator (Andexer *et al*., 2012).

Of the five HNLs currently used in industry, four belong to the HNL II group, namely *Me*HNL, *Hb*HNL, *Sb*HNL and *Lu*HNL illustrating the diversity of this group (Effenberger *et al*., 2007). It is interesting to note that over 3000 plants species have been reported to be cyanogenic but HNLs in only 20 species have been studied (Asano *et al*., 2005). This reveals a large gap in the knowledge of the different types of HNLs with the potential to be useful in industry. The advantages of using chiral cyanohydrins synthesised by HNLs over chemical synthesis is more environmentally friendly and yields excellent enantioselectivities (Bracco *et al*., 2016).

1.5 Rationale

Current limitations of the HNLs used in industry include low activity, low stability and low recombinant expression levels (Lanfranchi *et al*., 2017). Therefore, the need to bioprospect different plant species for novel HNLs with potentially superior catalytic and biochemical properties is appealing. Because the non-FAD dependent HNLs are non-homologous isofunctional enzymes (NISE) - a group of unrelated proteins which catalyse the same chemical reaction- the use of conventional protein discovery approaches is unsuitable (Lanfranchi *et al*., 2017). Conventional sequence-similarity and homology-based approaches for NISE yield disappointing results as these approaches are only appropriate for new genes which have high similarities to already deposited sequences on public sequence and protein databases.

In the case of HNLs, only a few amino acid sequences for known HNLs are available and given the heterologous nature of this group of enzymes a different approach is needed. Over the past few decades, the conventional HNL identification workflow used requires highly purified enzyme obtained from large volumes of the starting material. This is then followed by Edman degradation or with tryptic digestion and subsequent mass spectrometry. The amino acid sequence is then elucidated by the use of degenerate primers in the polymerase

chain reaction (PCR) of genomic or complementary DNA (Sturmberger *et al.*, 2016). This method is laborious and time-consuming.

An alternative method to discovering the gene/amino acid sequence of an HNL from scratch was proposed by Sturmberger *et al*. (2016) and applied by Lanfranchi and colleagues in 2017. This method combines the use of enzymatic screening and the integration of transcriptomics and proteomics for the elucidation protein sequences.

In a paper entitled 'Synergism of proteomics and mRNA sequencing for enzyme discovery' (Sturmberger *et al*., 2016), the generation of mRNA sequencing to produce a translated protein database combined with proteomic experiments is highlighted. The enzymatic workflow proposed is summarized in Figure 6.

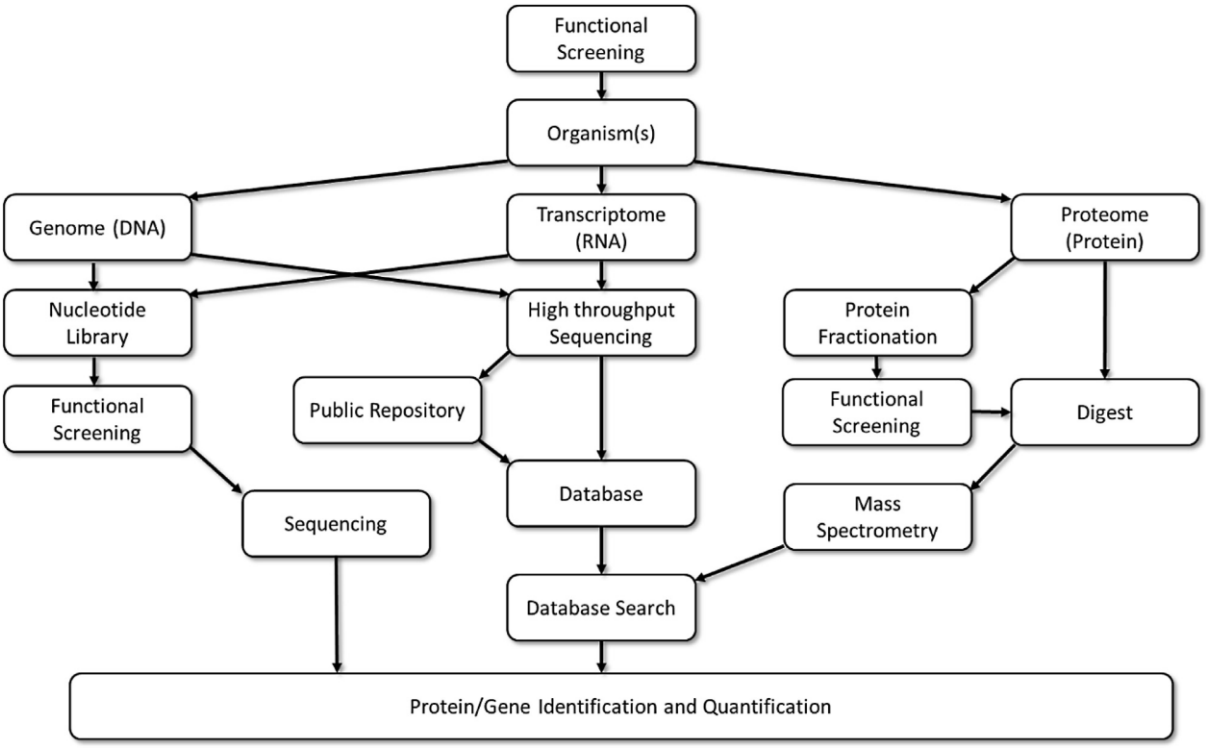


Figure 6. Novel enzyme sequence discovery approach. The combination of enzymatic screening, transcriptomics and proteomics can lead to the elucidation of an enzyme's amino acid or gene sequence (Sturmberger *et al*., 2016).

1.6 Aims and Objectives

The main aim of this study was to bioprospect fern genera and other non-commercialised plants from South Africa for hydroxynitrile lyase. The second aim was to determine the gene sequence of the HNLs using a combined omics and enzymatic screening approach if time and resources were available.

The specific objectives were to:

1. Screen plants for cyanogenic activity
2. Extract and partially purify HNL enzymes
3. Isolate plant HNL by BN-PAGE or CN-PAGE and in-gel activity assay
4. Obtain peptide data of the isolated protein by LC-MS/MS
5. Generate transcriptome of the plant(s) positive for HNL activity
6. Determine the gene sequence of the HNL(s) by transcriptome and proteome data integration

2 Methods and Materials

2.1 Plant collection

Samples of eight fern species were collected from the Animal Plant and Environmental Science (APES) garden on the East campus of the University of the Witwatersrand (Johannesburg, Gauteng) in March 2018. Leaves and croziers (if present) were collected in 50 ml falcon tubes and immediately flash frozen using liquid nitrogen.

More than 80 species of ferns were tested for cyanogenic activity at Fernhaven Nursery (Pretoria, Gauteng) using the in-field detection tubes described in section 2.2, in June 2018. Two fern species tested positive for cyanogenic activity and were purchased and transported back to the Industrial Microbiology and Biotechnology Laboratory (IMBL) at the University of the Witwatersrand.

Over 450 species of plants including angiosperms, gymnosperms and ferns were tested for cyanogenic activity at iSimangaliso Wetland Park (Kwa-Zulu Natal) in the first week of September 2018. Samples of the plants that tested positive for hydroxynitrile lyase activity were placed into 50 ml falcon tubes and immediately flash frozen and stored in liquid nitrogen. Upon arrival in the laboratory a week later, samples were transferred into a -80 °C freezer (Evosafe Series™ HF400-86, Snijders Labs). In addition, to assist with the identification of plant species, samples were collected and placed between two sheets of newspaper or a plastic bag in the field and pressed at a later stage. All specimens were labelled with the collector's name, sample number, date and GPS location. When possible, photographs were taken of the plant in its natural habitat to assist with identification.

In the region of Hluhluwe, Kwa-Zulu Natal, the highest rainfall occurs during the summer months (October to February). As such, at the time of collection in the first week of September most plants had not begun flowering or bearing fruit. This had an impact on the quality of the specimens collected as flowers and fruit are traditionally used in the characterisation and identification of herbarium specimens (Victor *et al.*, 2004).

2.2 Screening for cyanogenic activity in plants

Feigl and Anger (1966) developed a safe spot-test for the presence of HCN following the discovery of the carcinogenic nature of benzidine, which was previously used in several spot-tests. It was found that the non-toxic inner complex copper (II) ethylacetoacetate, dissolved in chloroform, delivered a suitable colourimetric reaction in the presence of gaseous HCN in the form of blue coloured spots. The blue salt is an oxidation product of the tetra base formed in the presence of copper (II) ethylacetoacetate and HCN (Krammer *et al.*, 2007). This method for the screening of HNL activity is versatile as it is applicable for both aliphatic and aromatic substrates. The Feigl-Anger detection paper was prepared by the addition of 1% (w/v) of the tetra base 4,4' -Methylenebis (*N, N* dimethyl-aniline) in chloroform to a 1% (w/v) solution of copper (II) ethylacetoacetate in chloroform. The two solutions were mixed together in a 1:1 ratio. This formed a dark green but clear solution in which Whatmann filter paper No1 (GE Healthcare) was soaked and dried under a fume hood.

A novel method for detecting cyanogenic plants in the field was developed by a member of the IMBL based on the Feigl-Anger HCN detection paper. The solution is prepared as above and approximately 30 µl of this mixture was added to the caps of microcentrifuge tubes and allowed to dry under a fume hood. Once dry, the tubes were closed and placed in a cool and dark area until use. The top of the tubes appeared slightly green similar to the detection paper.

In this study two levels of activity were tested. Level one tested for cyanogenic activity without the addition of substrate. In this case, the disruption of plant tissue would lead to the interaction of naturally occurring cyanogenic glycosides with β-glucosidase and HNL enzymes. If level one testing did not result in the observation of a blue spot after 5 minutes, 100 mM citrate buffer pH 4.00 with 13 mM racemic mandelonitrile was added to the disrupted plant tissue and the HCN detection paper or tube was observed for the development of blue spots. This is hereafter referred to as level two screening.

Level one activity assay

When using detection paper, a 96-well microtiter plate was routinely used to hold a small sample of the plant material (fresh plant material or from samples stored at -80 °C). Plant material was mashed with sterile toothpicks. The Feigl-anger detection paper was placed above, and the plate was closed with a transparent lid and a weight (e.g. glass bottle) was placed on it to ensure even distribution of HCN and allow for accurate determination of the source of HCN. The paper was observed for blue-spots which is indicative of cyanogenic activity. Using the detection tubes in the field, a plant of interest was mashed between fingers to ensure tissue disruption and placed at the bottom of the tube with forceps. The tube was kept in the dark and observed for any change in colour. A blue spot indicated possible cyanogenic activity

Level two activity assay

If no blue spots were detected after 5 minutes, approximately 100 µl of 100 mM citrate buffer with 13 mM racemic mandelonitrile (Technical grade, Sigma) was added to the mashed plants. A new detection paper was used as described above. Blue spots would indicate the presence of an HNL which catalyses the cleavage of mandelonitrile into a carbonyl compound and HCN. Cassava was used as a positive control and the buffer and substrate mixture with water was used as the negative control. When using the detection tubes, 100 µl of the buffer-substrate solution was added to the plant material in the tube and the cap was observed for a change in the colour. Samples of plants which tested positive on either level were collected in 50 ml falcon tubes and flash frozen in liquid nitrogen and stored at -80 °C for further testing in the laboratory.

2.3 Plant protein extraction

Plant protein was routinely extracted using the P-PER™ Plant Protein Extraction kit (Thermo Scientific) according to the manufacturer's instructions. This kit bypasses the need for mechanical lysis methods such as freeze-grinding in liquid nitrogen using a mortar and pestle (Thermo Scientific). Figure 7 summarises the protocol followed for total plant protein extraction.

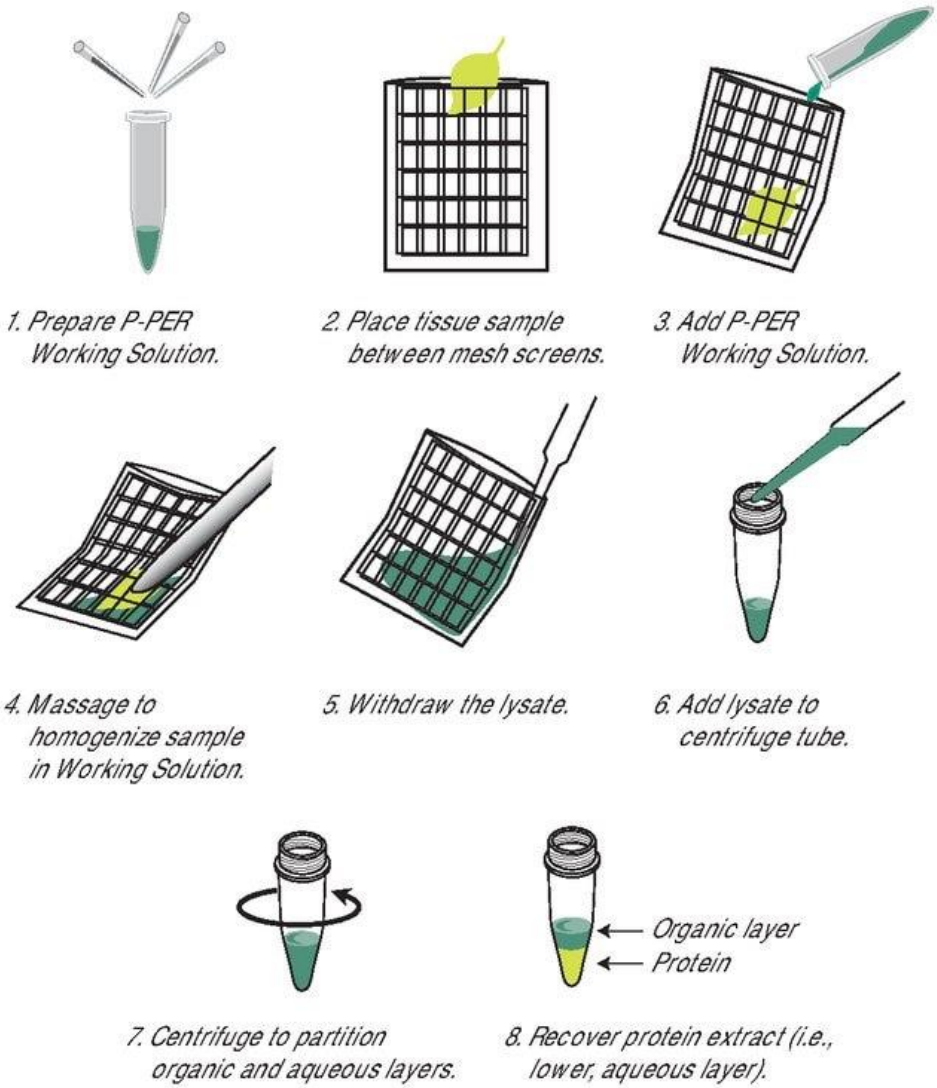


Figure 7. Plant protein extraction procedure. Schematic view of plant protein extraction using the P-PER™ Plant Protein Extraction kit (Thermo Scientific). This kit was routinely used for plant protein extraction.

After obtaining a crude plant protein extract, the samples were de-salted and buffer exchanged with 50 mM potassium phosphate pH 5.7 using 2 ml Zeba™ spin desalting columns, 7 K MWCO (Thermo Scientific). Protein concentration of the crude extract and the desalted-buffer exchanged sample was measured with a Qubit® 2.0 fluorometer (Invitrogen).

To test if the protein extracts were still active for HNL activity, an activity test was performed using 400 mM citrate buffer pH 4.0 and 13 mM mandelonitrile. One hundred microliters of the buffer containing substrate was added to 10 µl of the protein extracts. The plate was topped with a Feigl-Anger detection paper, closed and weighed down. The paper was observed for the presence of blue spots which would be indicative of the presence of a hydroxynitrile lyases which are able to use mandelonitrile as a substrate to form HCN and the corresponding carbonyl compound.

The positive control used was a crude protein extract obtained from the overexpression of the HNL from *D. tyermannii* (*Dt*HNL). The plasmid harbouring the gene encoding for recombinant *Dt*HNL was kindly donated by the Austrian team who elucidated the gene sequence for this HNL (Lanfranchi *et al*., 2017).

2.4 Protein isolation and partial protein purification

Previous HNLs have been found to have isoelectric points between 4 and 5 (Sharma *et al*., 2005). Therefore, anion exchange chromatography was chosen to partially purify HNLs. Protein was extracted, and buffer exchanged to 50 mM sodium phosphate pH 5.7 as described in section 2.3. Partial purification of the HNL proteins was achieved using the HiTrap DEAE Q- sepharose XL 1 ml column from the HiTrap IEX selection kit (GE Healthcare).

The HiTrap QXL column was regenerated with 20 mM sodium phosphate with 1 M NaCl (pH 5.7) in 10 column volumes and equilibrated with 20 mM sodium phosphate pH 5.7 in 10 column volumes. A linear gradient from 0 to 0.5 M NaCl in 12 column volumes was used to elute the protein. Elution fractions of approximately 1 ml were collected. All fractions were tested for HNL activity using the Feigl-Anger test paper and 100 mM citrate buffer pH 4.0 and 3 mM mandelonitrile in a 96-well plate. After approximately 20-30 minutes, paper was checked for blue spots.

Active fractions were pooled, concentrated, and buffer exchanged to 25 mM potassium phosphate pH 6.0, using Corning® spin X-UF concentrator 10 K MWCO (500 µl). After buffer exchange, concentrated fractions were tested again for HNL activity using the same buffer as above. The active, concentrated fractions were stored at -20 °C.

The purity and approximate molecular weight of the partially purified HNLs was analysed according to the method by Laemmli (1970) on 16% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Sodium dodecyl sulphate is a strongly denaturing anionic detergent which denatures proteins by forming an SDS-protein complex with an overall negative charge. The proteins are then subjected to an electrical charge and migrate according to their molecular weight only. PageRuler™ unstained protein ladder (Thermo Scientific) was used to determine approximate molecular weight. The gels were stained using the rapid Fairbanks method of Coomassie staining. This method uses three staining solutions, each containing 10% acetic acid and a steadily decreasing concentration of Coomassie R-250 and isopropanol. After the gel was placed in each stain namely, Fairbanks A (25% isopropanol and 0.05% Coomassie R-250), Fairbanks B (10% isopropanol and 0.005% Coomassie R-250) and Fairbanks C (0.002% Coomassie R-250), it was heated in a microwave until boiling point. The gel was then allowed to cool while shaking for 15 minutes. The final solution (Fairbanks D) comprised of only 10% acetic acid. After heating the solution to boiling point in the final solution and subsequent cooling for 30 minutes, bands were visible against a clear background (Fairbanks *et al*., 1970; Wong *et al*., 2000).

In some cases, Coomassie staining was followed by silver staining for better visualisation of the bands. Silver staining was done after the Coomassie dye was removed from the SDS-gel by de-staining overnight in 10% acetic acid. This double staining method increases the sensitivity up to 4 -fold compared to silver staining alone.

2.5 Blue native or clear native polyacrylamide gel electrophoresis

Retaining enzymatic activity after separation by electrophoresis is crucial to the success of detecting HNL activity. Blue native polyacrylamide gel electrophoresis (BN-PAGE) was developed by Schägger and von Jagow (1991) for the isolation of native membrane proteins

and protein complexes in the size range of 10 kDa to 10 MDa. The exclusion of detergents maintains the native form of the protein and therefore activity is retained. The principle of BN-PAGE is based on the charge shift caused by the Coomassie blue G-250 dye. This charge shift causes acidic and basic proteins alike to migrate towards the anode. Proteins are separated based on size only and retain their enzymatic activity. The native proteins appear as blue bands on the gel (Wittig *et al*., 2006).

For some of the enzymes in this study clear native PAGE provided a better result albeit at a lower resolution compared to BN-PAGE. CN-PAGE is a milder method than BN-PAGE and it includes the neutral detergent digitonin which does not compromise the native form of the proteins (Wittig, and Schägger, 2006). In contrast to BN-PAGE, proteins electrophoresed by CN-PAGE migrate according to their intrinsic charge and estimating molecular weight is unreliable, however, with CN-PAGE different oligomeric states can be detected which would not be visible on BN-PAGE. The running buffer (pH 7.5) used in CN and BN-PAGE were identical and the only difference was the composition of the cathode buffer, which in the case of BN-PAGE contained Coomassie G-250 dye. Hydroxynitrile lyase enzymes which have been well characterised have all been shown to have isoelectric points below 7, therefore in CN-PAGE these proteins will naturally migrate towards the anode (Wittig, and Schägger, 2006).

Pre-cast NativePAGE™ Novex® 4-16% gels (Life Technologies) were used with NativeMark™ unstained protein standard (Life Technologies) for molecular weight estimation. The buffers and samples were prepared as recommended by the manufacturer. Electrophoresis was done at 4°C and 150 V for 60 minutes (8- 15 mA) followed by 250 V for 90 minutes (2- 4 mA) using an Xcell SureLock® Mini cell connected to a Consort EV243 programmable power supply. After electrophoresis, the gel was carefully removed and washed twice with ultra-pure (Milli-Q) water in preparation for the in-gel activity assay.

2.6 In-gel hydroxynitrile lyase activity assay

The native gel was incubated in 100 mM citrate buffer at pH 4.5 for 30 minutes at 4 °C to allow for equilibration (Lanfranchi *et al*., 2015). The sandwich configuration for the in-gel HNL activity assay is shown in Figure 8. At the bottom lies Whatmann filter paper No1 (GE

Healthcare) dampened with 100 mM citrate buffer (pH 4.5). The buffer-soaked paper is then covered with the native gel. Whatmann filter paper No1 (GE Healthcare) soaked in a buffer solution (100 mM citrate buffer pH 4.5) containing 20 mM mandelonitrile was placed onto the native gel to ensure direct contact with the fractions (Krammer *et al*., 2007). A mosquito net or nylon tissue which is gas permeable was placed above the substrate-soaked paper to separate it from the Feigl-Anger detection paper placed above it. The "sandwich" was then topped with a transparent lid and a transparent bottle as a weight to provide uniform contact between all layers. The transparent equipment allows for the visual detection of blue spots without disruption of the sandwich configuration. The in-gel activity assay was performed at room temperature.

The development of blue spots was carefully monitored as it has been noted that upon prolonged exposure, the blue spots spread rapidly, making it difficult to determine the source and subsequently the corresponding band (Lanfranchi *et al*., 2015). Using a fresh HCN detection paper approximately every 10 minutes facilitates in isolating the source of the blue spot. This, however, will depend on the amount of enzyme present and the activity of the particular HNL. It is therefore crucial to monitor the development of spots carefully in the first few minutes to know when to change HCN papers.

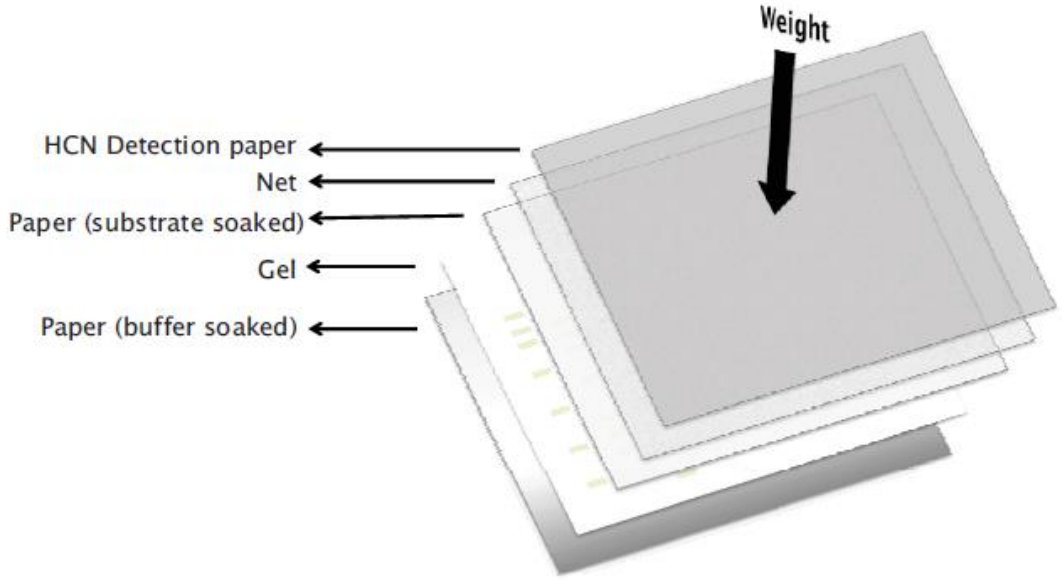


Figure 8. Sandwich assembly for in-gel hydroxynitrile lyase activity assay. The buffer and substrate soaked Whatmann filter paper No1 is in direct contact with the native gel while the net acts to separate the Feigl-Anger HCN detection paper from the damp paper. A weight is needed to ensure equal distribution and the accurate detection of the position of the blue spots (Lanfranchi *et al*., 2015).

2.7 Liquid chromatography tandem mass spectrometry

After the in-gel activity assay, the BN-PAGE or CN-PAGE was silver stained using ProteoSilver™ Silver Stain Kit (Sigma) according to the manufacturer's protocol. The silver stained gel was then examined alongside the HCN detection paper used in the in-gel activity assay to determine which band(s) were potentially HNL-containing.

The silver staining kit was chosen for staining of BN-PAGE and CN-PAGE gels due to its high sensitivity (detection limit of 0.1 ng of BSA/mm^2). It has also been found that silver staining produces a lower signal-to-noise ratio in the mass spectrum compared to the more commonly used Coomassie stained proteins. In addition, no chemical modifications such as oxidative damage occurs during silver staining of gels. (Shevchenko *et al*. 1996).

Protein bands exhibiting HNL activity were excised and stored at -20°C in 10% ethanol and sent to the Austrian Centre of Industrial Biotechnology (ACIB GmbH) for liquid chromatography tandem mass spectrometry (LC-MS/MS) and processed as follows: The protein bands were reduced, alkylated and digested with Promega modified trypsin according to the method of Shevchenko *et al.* (1996). Resulting peptides were acidified with formic acid (final concentration of 0.1 % (v/v)) and analysed by nano-HPLC (Dionex Ultimate 3000) equipped with an Aurora (Ionoptics) nanocolumn (C18, 1.6 µm, 250 x 0.075 mm). Separation was carried out on the nanocolumn at a flow rate of 150 nl/min at 35 °C using the following gradient, where solvent A is 0.1 % (v/v) formic acid in water and solvent B is acetonitrile containing 0.1 % formic acid: 0-0.1 min: 2 % B, 150 µL/min; 0.1-18 min: 2% 150 µL/min – 300 µL/min; 18-100 min 2%-25 % B; 100 -107 min: 25-35 % B, 108 – 118 min: 35 - 95 % B; 118-118.1 min: 95 % - 2% B; 118.1-133 min: 2% B. The maXis II ETD mass spectrometer (Bruker) was operated with the captive source in positive mode with following settings: mass range: 200 - 2000 m/z, 2 Hz, capillary 1600 V, dry gas flow 3 L/min with 150 °C, nanoBooster 0.2 bar, precursor acquisition control top20 (CID).

2.8 Transcriptome generation

Total RNA was extracted using the method described by Xu *et al.* (2010) in which a modified cetyltrimethylammonium bromide (CTAB) extraction method was developed to extract high quality total RNA. All lab equipment, plasticware, glassware and lab surfaces were decontaminated of RNase enzymes using RNase AWAY® (Sigma). Mortars and pestles were baked overnight at 200 °C to degrade any traces of RNases and other contaminants. All reagents were prepared with diethylpyrocarbonate (DEPC) treated water (0.01% v/v) which was autoclaved three times to remove any unreacted DEPC which may interfere with the RNA.

The CTAB extraction buffer comprised of 100 mM tris-HCL (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB (w/v), 2% polyvinylpyrrolidone (PVP) (w/v) and 2% β-mercaptoethanol, which was added just before use. Other reagents used in the protocol includes 5 M NaCl, chloroform, isopropanol, 70% ethanol and a high salt solution comprised of 0.8 M trisodium citrate dihydrate and 1.2 M NaCl.

The CTAB extraction buffer was prewarmed in a water bath set at 65 °C. Plant material weighing approximately 0.1 g was crushed in liquid nitrogen using a mortar and pestle on ice and immediately transferred into 0.6 ml of the warmed CTAB buffer. The solution was mixed by inverting the tube several times until a homogenate formed. The homogenate was incubated in the 65 °C water bath for 15 minutes and vortexed several times during the incubation period. The solution was removed from the water bath and 0.5 ml of chloroform was added, mixed well and centrifuged at 9600 x *g* for 10 minutes at 4 °C.

The supernatant was transferred into a new tube and 0.1 ml of 5 M NaCl and 0.3 ml of chloroform was added, mixed well and centrifuged as above. The unequal volumes of salt and chloroform promotes the separation of aqueous and organic material and the chloroform also removes residual protein (Xu *et al.*, 2010). The supernatant was transferred into a new tube and the step was repeated. After the supernatant was transferred into a new tube again, a half volume of isopropanol and a half volume of high salt solution was added and mixed gently and left at room temperature for 15 minutes to precipitate the RNA and remove polysaccharides. The RNA was recovered by centrifugation at 9600 x *g* for 10 minutes at

4 °C. The supernatant was discarded, and the RNA pellet washed twice with 70% ethanol by spinning at 8.5 x *g* for 5 minutes. Any residual ethanol was removed using gel loading tips. The RNA was dissolved in DEPC-treated water and 1 µl of Ribolock™ (Thermo Scientific). RNA concentration was measured with the Qubit™ RNA assay, and purity was measured with a Nanodrop® spectrophotometer (Thermo Scientific). The RNA sample was stored at -80°C.

Normalised transcriptome sequencing was obtained by the commercial service provided by the Agricultural Research Council Biotechnology Platform (ARC-BTP). The total RNA prepared above was transported in liquid nitrogen and immediately placed into a -80 °C freezer upon arrival at the ARC. The library preparation was achieved using the Illumina Truseq stranded mRNA protocol according to manufacturer's instructions. Total RNA of ± 1 µg starting material was used. A summary of the method follows: The total RNA integrity was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies). The poly-A containing mRNA in the sample was purified using poly-T oligo attached magnetic beads. The mRNA was then fragmented with divalent cations at high temperature. First strand cDNA was synthesised using Superscript II Reverse Transcriptase and random hexamer primers using the following parameters on the thermal cycler: 25 °C for 10 minutes, 42 °C for 15 minutes and 70 °C for 15 minutes.

Second strand synthesis was achieved using DNA polymerase I and RNase H. The RNase H serves to degrade the RNA strand in the RNA/DNA hybrid and allow for double strand DNA synthesis. The 3' end of the fragments were adenylated, and a corresponding 'T' nucleotide was added to the corresponding adapter which promotes the ligation of the adapter to the fragments. PCR was then performed to enrich the products resulting in the final cDNA library. Cluster generation and read sequencing followed. Sequencing was performed on the Illumina Hiseq 2500 instrument using the Illumina sequencing by synthesis (SBS) version 4 chemistry with paired end, 125 bp x 125 bp sequencing format.

Due to budget constraints, only one transcriptome was able to be generated. The three ferns *Davallia trichomanoides, Phlebodium aureum mandaianum* and *Thelypteris confluens* (Thunb.) Morton were found to have HNL activity. Previous research on HNLs in the genera *Davallia* and *Phlebodium* has been reported (Lanfranchi *et al*., 2017; Wajant *et al*. 1995) however, no reports on HNL activity in in the Thelypteridaceae family has been found in the

literature to date. For this reason, the transcriptome of *T. confluens* was generated to fill the gap in the knowledge about this plant.

2.9 Hydroxynitrile lyase activity assay

HNL activity was measured spectrophotometrically by monitoring the decomposition of racemic mandelonitrile into benzaldehyde at 280 nm. A control reaction resulting from the spontaneous decomposition of mandelonitrile was measured and the linear slope of absorbance for the spontaneous reaction was subtracted from the linear slope of absorbance of HNL- catalysed reaction. Racemic mandelonitrile at a concentration of 2 mM was used as the substrate (previously dissolved in 3 mM sodium citrate phosphate buffer pH 3.5).

A total reaction volume of 1 ml was used in a quartz cell (10 mm, Agilent Technologies). The partially purified and concentrated enzyme solution was added to 50 mM citrate phosphate buffer pH 5.5. For *Tc*HNL and *Pham*HNL 5 µl of the enzyme solution was added, however, for *Dtm*HNL 10 µl of the enzyme was added to account for the reduced protein concentration of the final extract which was almost half that of *Tc*HNL and *Pham*HNL. The substrate was always added last and the solution was mixed gently by pipetting up and down three times. The spectrophotometer (S-22 UV/Vis. Boeco, Germany) was blanked 10 seconds after addition of the enzyme and the increase in absorbance was monitored for 10 minutes. All assays were done in triplicate. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 µmol/min of benzaldehyde from mandelonitrile under the assay conditions

Activity was calculated as follows:

$$Activity\left(\frac{U}{ml}\right) = \frac{\Delta OD}{\varepsilon} \times \frac{V_t}{V_e}$$

Where $\Delta OD = A_{280\,nm}/min_{Partially\ purified\ enzyme} - A_{280\,nm}/min_{Spontaneous\ reaction}$

Equation 1. Calculation of enzyme activity

The ΔOD represents the difference in the maximum linear rate between the partially purified enzyme and the spontaneous decomposition reaction in the first minute, Ɛ (1. 3761 ml/

µmol/cm) is the absorption coefficient of benzaldehyde at 280 nm. V_t and V_e are the total volume of the reaction and enzyme volume used respectively.

2.10 Enzyme stability at acidic pH

The effect of pH on the activity of the three enzymes were assayed by using HCN detection paper. The cyanogenesis reaction was run in a 96-well plate with a total reaction volume of 100 µl at 25 °C. The reaction buffer comprised of 50 mM sodium citrate phosphate buffer, pH 2.5 - 6.5 (in 1 pH unit increments). The cyanohydrin substrate stock solution comprised of 20 mM racemic mandelonitrile dissolved in 3 mM sodium citrate phosphate pH 3.5.

The reaction mixture comprised of 85 µl of the sodium citrate phosphate buffer at the respective pH with 5 µl of the partially purified enzyme added (in the case of *Dtm*HNL, 80 µl buffer and 10 µl of enzyme). After an incubation period of five minutes, the cyanohydrin solution was added at a volume of 10 µl to give a final concentration of 2 mM. The reaction solution was gently mixed by pipetting up and down three times. The 96-well plate was topped with Feigl-Anger detection paper. A transparent lid was placed on the plate and a weight was added. The detection paper was observed for 15 minutes for the development of blue spots. Colour intensity and time of blue spot appearance was used as a qualitative and semi-quantitative measurement of enzyme activity. The spontaneous decomposition of racemic mandelonitrile (2 mM) was observed at the different pH values and compared to the results obtained.

2.11 Herbarium specimen collection

For identification purposes, most of the plants used in this study were collected and pressed according to the instructions in the Southern African Herbarium User Manual from the National Botanical Institute (Victor *et al.*, 2004). In addition to identifying the plant species, having an herbarium specimen collection would be helpful in potential future studies on cyanogenic plants. As each plant has a GPS location attached to it, revisiting the place where

the plant was originally collected is possible and can be compared to the herbarium specimen for confirmation of identity.

Ideally plants should be pressed immediately after collection, whilst in the field, to decrease the chance of damage to the plant and mixing of different plants species, however, due to the large volume of plant material collected at iSimangaliso Wetland Park, pressing was done once back in the laboratory.

Figure 9 shows the construction of a plant press as set up in this study. Newspaper was used as a drying sheet as well as a flimsy. Drying sheets were changed daily and the straps on the plant press were tightened regularly to keep plants flattened. Corrugated cardboard sheets were used as ventilators, positioned every five to eight specimens allowing air to flow between the flimsies and the drying paper to accelerate the drying process (Victor *et al.*, 2004).

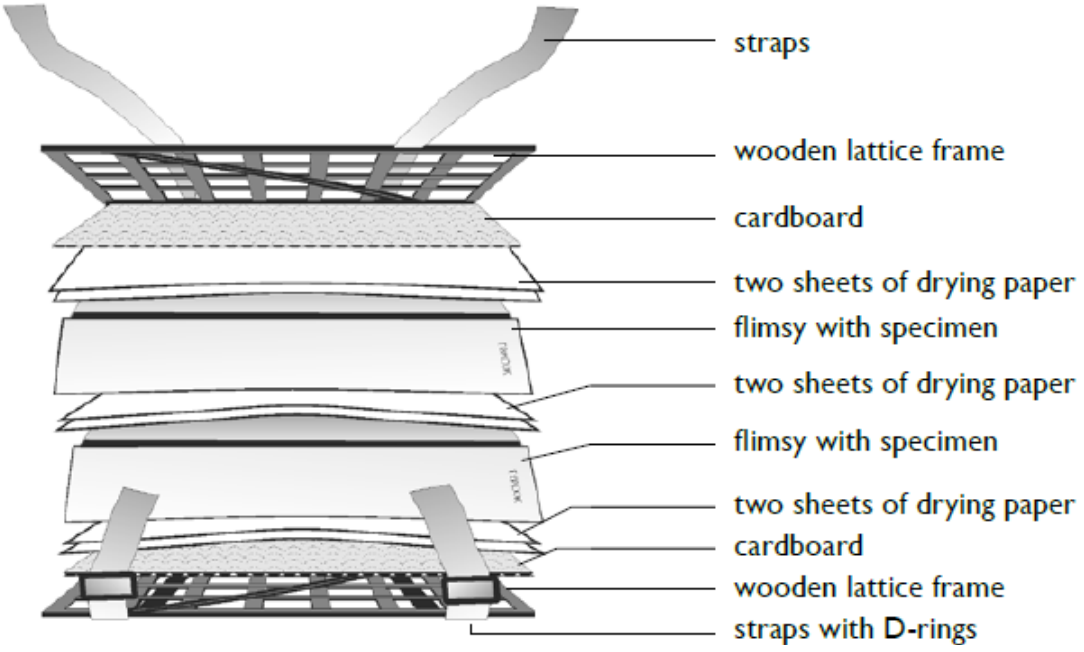


Figure 9. Arrangement sequence of a plant press. This arrangement allows for efficient drying and flattening of plant material for subsequent identification (Victor *et al.*, 2004).

The plant press was kept in a warm, dry and well-ventilated area. Once dry, plant specimens were mounted onto plain-white paper and secured with wood glue. Identification was done at the South African National Biodiversity Institute (SANBI) in the national herbarium at the Pretoria National Botanical Gardens, Gauteng. Before the specimens were identified they were microwaved for up to 75 seconds to prevent insect damage and contamination of other herbarium specimens. An electronic copy of the herbarium specimen collection can be viewed in appendix A.

3 Results

3.1 Screening for cyanogenic plants

In the context of this study, screening can be divided into two different levels. The first level is screening for cyanogenesis by crushing plant material and covering it with Feigl-Anger HCN detection paper, alternatively, crushed plant material can be added to HCN detection tubes. A blue spot is indicative of cyanogenesis occurring in the plant.

The second level of screening occurs when no blue spot is detected in the first level. Racemic mandelonitrile in buffer (13 mM mandelonitrile in 100 mM citrate buffer pH 4.0) is added to the crushed plants and topped with a new detection paper. In the case of the detection tubes, the substrate solution is added to the same tube as used in level 1. The appearance of a blue spot will indicate mandelonitrile lyase activity. It is important to note that a plant may not be cyanogenic at a given time but may still possess hydroxynitrile lyase enzymes and the addition of a cyanohydrin substrate activates the enzyme.

Fern specific screening

In the APES garden, eight species of fern were tested by addition of racemic mandelonitrile to crushed plants in a 96-well plate (Figure 10). In addition, fern A1, identified as *Pteridium aquilinum* along with three plants which was shown to possess cyanogenic activity in a prior field trip to iSimangaliso Wetland Park (in December 2017) was included in the assay. Row A and B contain species collected from the APES garden, row D contains the positive control, *Manihot esculenta* (cassava), row F contains samples from iSimangaliso Wetland

Park (*P. aquilinum*, *Vigna sp.*, unknown and *Gomphocarpus sp*.). Row H contains the negative control which comprises of racemic mandelonitrile in citrate buffer pH 4.0 and 20 µl of distilled water. Three intense blue spots were observed for three of the ferns tested, namely *Pteris sp.*, *T. confluens*, and *P. aquilinum*. This is indicative of the presence of hydroxynitrile lyase enzymes and more specifically mandelonitrile lyase [EC.4.1.2.10] in these ferns.

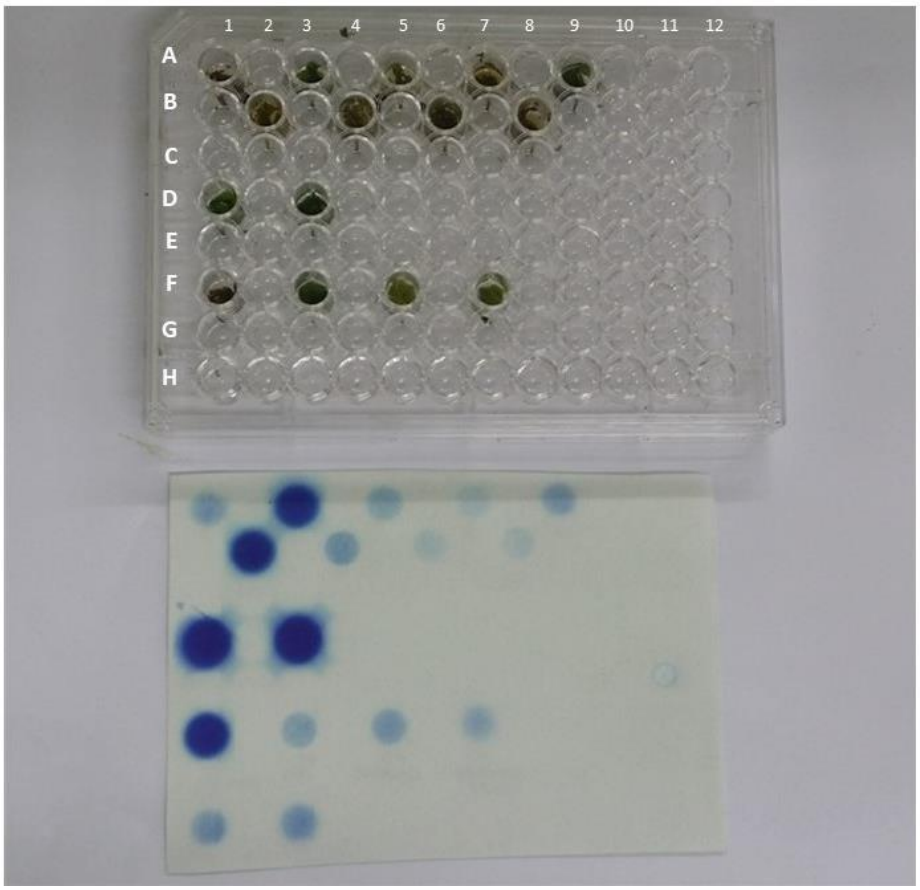


Figure 10. Screening for cyanogenic activity in ferns using Feigl- Anger HCN detection paper. Ferns were collected from the School of APES garden (Johannesburg) and iSimangaliso Wetland Park (KZN). Citrate buffer (100 mM) pH 4.0 with 13 mM racemic mandelonitrile was added to the mashed plants. Intense blue spots indicate the presence of gaseous hydrocyanic acid and putatively suggests hydroxynitrile lyase activity. Wells B2 (*Pteris sp*.) and A3 (*Thelypteris confluens*) and F1 (*Pteridium aquilinum*) show intense blue spots. *Manihot esculenta* (cassava) was used as a positive control in D1 and D3. Negative control included buffer with substrate and water in H1 and H3.

At Fernhaven Nursery (Pretoria) only level one screening was conducted in the field. Ferns showing strong level one positive were purchased and taken back to the laboratory for further testing. Two out of more than 80 fern species showed cyanogenic activity and when tested

with the substrate mandelonitrile, intense blue spots were observed (Figure 11). The two ferns were identified as *Davallia trichomanoides* and *Phlebodium aureum mandaianum.*

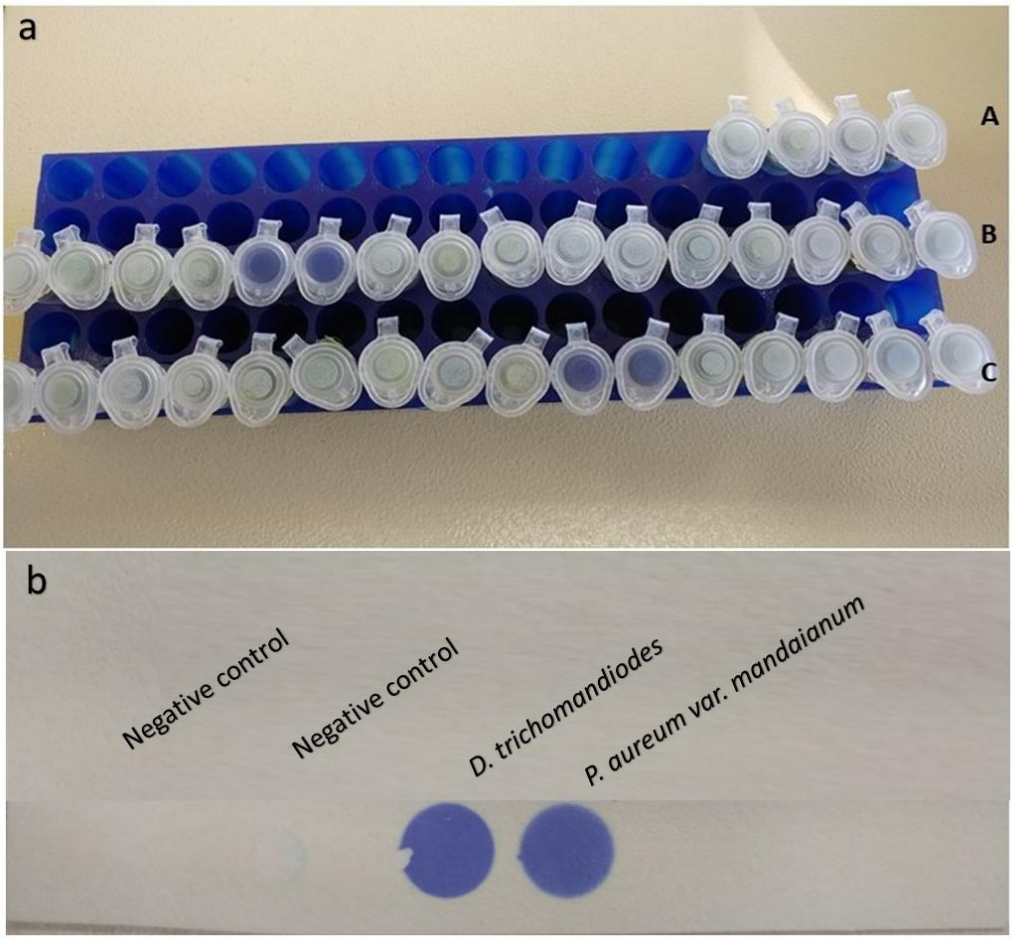
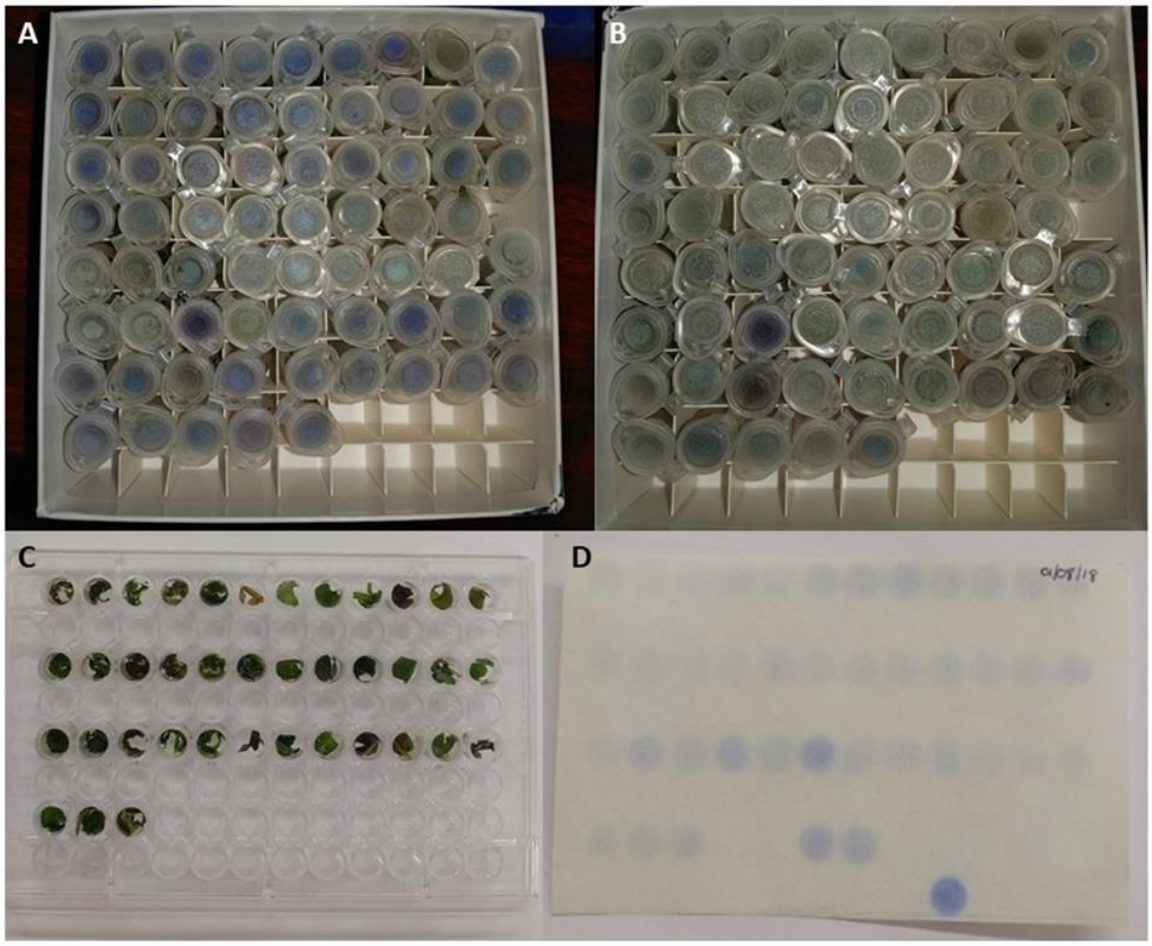


Figure 11. Screening for cyanogenic activity in ferns from Fernhaven Nursery, Pretoria. Only two out of more than 80 species of ferns exhibited cyanogenesis (a) Positive level one screening. In row B, from the left, tubes 5 and 6 are duplicates of the fern *Davallia trichomanoides.* Row C, tubes 10 and 11 are duplicates of the fern *Phlebodium aureum mandaianum*. (b) Level two screening of the ferns confirm HNL activity.

Non-commercialised plants

Non-fern specific screening was conducted at iSimangaliso Wetland Park in the first week of September 2018. To date, this is the biggest HNL bioprospection campaign ever conducted in South Africa. Over 450 species of plants were tested for hydroxynitrile lyase activity over a period of five days. Level one (cyanogenic activity) and level two (mandelonitrile lyase activity) were screened in the field using HCN detection tubes. Out of the 450 plants

screened, five showed level one positive results. A further 39 were observed to be level two positive and these species potentially express mandelonitrile lyase enzymes. The protein extracts from the 39 level two positive plants were re-tested for HNL activity upon return to the laboratory (1 week later) in a 96-well plate with buffer and substrate added (Figure 12). A significant decrease in HNL activity was observed in the crude protein extracts.



A	1	2	3	5	6	7	10	24	103	105	113	116
B												
C	142	164	171	185	188	207	214	216	219	241	263	267
D												
E	283	284	286	290	295	302	323	324	325	336	337	338
F												
G	343	349	350			- ve	- ve					
H									+ ve			

Figure 12. Screening for cyanogenic plants in iSimangaliso Wetland Park. A and B show the results obtained in the field using hydrogen cyanide detection tubes. C and D show the results obtained upon return to the laboratory a week later. The schematic shows the position of the different plant species in the 96- well plate. Water was used as the negative control whereas *Dt*HNL crude protein extract was used as the positive control.

3.2 Protein extraction

Routine protein isolation was done with the P-PER™ plant protein extraction kit (Thermo Scientific). After testing for activity in the P-PER™ protein extract, the crude enzyme was de-salted and buffer exchanged to 50 mM potassium phosphate pH 5.7 with 2 ml Zeba™ spin desalting columns, 7 K MWCO (Thermo Scientific).

Ferns

The protein concentrations for the three ferns collected from the APES garden and Fernhaven Nursery namely *T. confluens, D. trichomanoides* and *P. aureum mandaianum*, are shown in Table 1. In all cases, after desalting and buffer exchange protein concentrations decreased. HNL activity was observed in both the P-PER™ protein extract and the desalted protein extract for the three ferns.

Table 1. Protein concentration and activity status for three ferns exhibiting HNL activity

Fern species	P-PER™ extract protein concentration (mg/ml)	HNL activity	Desalted protein extract concentration (mg/ml)	HNL activity
Thelypteris confluens	7. 95	+	5.61	+
Davallia trichomanoides	10.4	+	8.68	+
Phlebodium aureum mandaianum	22.4	+	17.5	+

Non-commercialised plants

The five plants from iSimangaliso Wetland Park, which showed cyanogenic activity without the addition of mandelonitrile were labelled as 163, 308, 318, 327 and 342. All five plants had no HNL activity in the P-PER™ extraction solution, however, after desalting and buffer exchange, HNL activity was noted (Table 2). Plant 308 has been identified as *Morus mesozygia* (Family Moraceae) and the identification of the remaining four species is still in progress.

Table 2. Protein concentration and HNL activity status for the five cyanogenic plants from iSimangaliso Wetland Park, KZN (September 2018)

Plant ID	P-PER™ extract protein concentration (mg/ml)	HNL activity	Desalted extract protein concentration (mg/ml)	HNL activity
163	16.1	–	14.3	+
308- *Morus mesozygia*	9.46	–	4.06	+
318	18.5	–	17.7	+
327	10.8	–	6.76	+
342	12.4	–	9.38	+

Total plant protein was extracted from the 39 plants which exhibited HNL activity after the addition of racemic mandelonitrile (level-two positive). Due to limited resources, these protein extracts were not de-salted or buffer exchanged. An HNL activity assay was done on the 39 P-PER™ protein extracts one week after the extraction procedure, and no significant HNL activity was observed for any of the 39 species. This disappointing result led to the progression of further studies on the three ferns from Gauteng, namely *T. confluens*, *D. trichomanoides* and *P. aureum mandaianum* only. Table 3 shows the total protein concentration measured and the activity status for the 39 plants. Only 19 of the plants have been identified to date, two of which are ferns, namely, 336 – *Microsorum scolopendria* from the family Polypodiaceae (commonly known as Monarch fern) and 350- *Cheilanthes viridis* belonging to the Pteridaceae family (also known as the common lip fern).

Table 3. Concentration of P-PER™ plant protein extracts and hydroxynitrile lyase activity status of 39 plants exhibiting mandelonitrile lyase (MDL) activity from iSimangaliso Wetland Park, KZN.

Plant ID	Family	Active in-field (MDL)	Total protein concentration (mg/ml)	Active in-lab (MDL)
1		+	13.3	–
2		+	15.9	–
3- *Tephrosia polystachya*	Fabaceae	+	17	–
5- *Sida cordifolia*	Malvaceae	+	5.86	–
6- *Searsia natalensis*	Anacardiaceae	+	8.18	–
7- *Garcinia livingstonei*	Clusiaceae	+	3.82	–
10		+	21.6	–
24- *Acalypha sp.*	Euphorbiaceae	+	3.92	–
103		+	15.9	–
105- *Ochna natalitia*	Ochnaceae	+	4.44	–
113		+	18.6	–
116		+	17.4	–
142- *Fabaceae sp.*	Fabaceae	+	15.8	–
164		+	18.9	–
171		+	15.7	–
185		+	18.1	–
188		+	16.96	–
207- *Searsia sp.*	Anacardiaceae	+	4.16	–
214		+	14.6	
216		+	8.38	–
219- *Cola sp.*	Malvaceae	+	8.12	–
241		+	18.2	–
263- *Fabaceae sp.*	Fabaceae	+	19.0	–
267- *Fabaceae sp.*	Fabaceae	+	16.6	–
283		+	12.5	–

Table 3. Continued				
Plant ID	Family	Active in-field (MDL)	Total protein concentration (mg/ml)	Active in-lab (MDL)
284		+	12.0	–
286		+	13.3	–
290- *Indigofera oxalidea*	Leguminosae	+	11.8	–
295		+	14.4	–
302		+	10.2	–
323		+	14.2	–
324		+	12.9	–
325- *Brachylaena discolor*	Asteraceae	+	14.2	–
336- *Microsorum scolopendria*	Polypodiaceae	+	11.4	–
337- *Ranunculus Dregei*	Ranunculaceae	+	9.08	–
338- *Solanum sp.*	Solanaceae	+	15.5	–
343- *Clausena anisate*	Rutaceae	+	14.3	–
349- *Tragia sp.*	Euphorbiaceae	+	14.8	–
350- *Cheilanthes viridis*	Pteridaceae	+	9.78	–

3.3 Protein isolation and partial protein purification

Based on the low isoelectric points observed in known HNLs from different enzyme families, anion exchange chromatography was chosen to partially purify the HNL enzymes using a 1 ml HiTrap QXL column (GE healthcare). A linear gradient from 0 to 0.5 M sodium chloride was used to elute the proteins. All fractions, including the flow-through were collected and tested for HNL activity in 100 mM citrate buffer, pH 4.0 and 13 mM racemic mandelonitrile as the substrate. SDS-PAGE was used to produce a purification profile and analyse the purity and molecular weight of the partially purified HNLs from the three ferns.

3.3.1 *Thelypteris confluens*

The hydroxynitrile lyase from *T. confluens* (*Tc*HNL) eluted between 0.2 and 0.3 M sodium chloride. The SDS-PAGE gel was stained with Coomassie brilliant blue using the Fairbanks staining method. *Tc*HNL is estimated to have a size of 20 kDa (Figure 13).

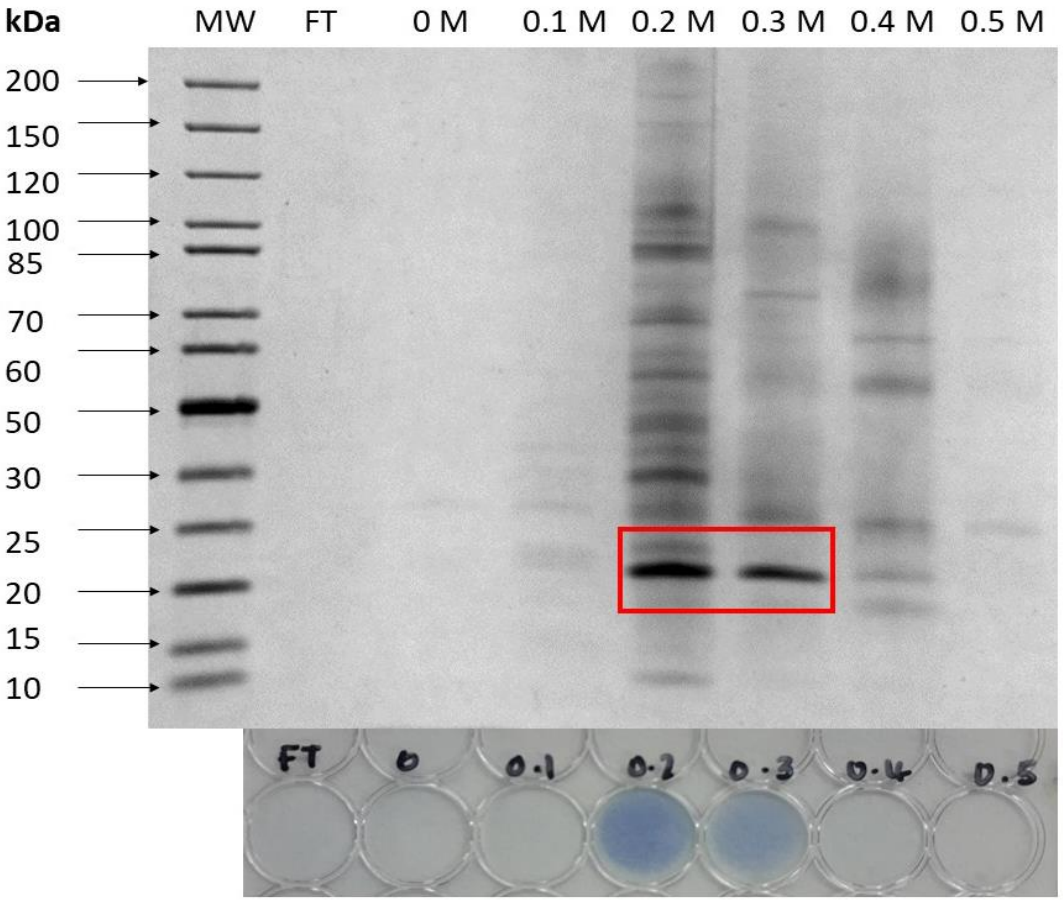


Figure 13. SDS-PAGE purification profile and fraction activity assay for the hydroxynitrile lyase from *Thelypteris confluens*. Anion exchange chromatography of the desalted protein extract was used to partially purify *Tc*HNL. Protein was eluted by a linear gradient from 0 to 0.5 M NaCl. *Tc*HNL eluted between 0.2 and 0.3 M with an approximate molecular weight of 20 kDa. A 16% polyacrylamide gel was used to analyse the protein fractions.

3.3.2 *Davallia trichomanoides*

The hydroxynitrile lyase from *D. trichomanoides* (*Dtm*HNL) eluted mostly at 0.2 M sodium chloride, however, some activity was also observed in the fraction eluted at 0.1 M sodium chloride and to a lesser extent the 0.3 M fraction. An SDS-PAGE gel which was double-stained (Coomassie and silver stained) shows a band between 20 to 25 kDa which is suspected to contain *Dtm*HNL (Figure 14).

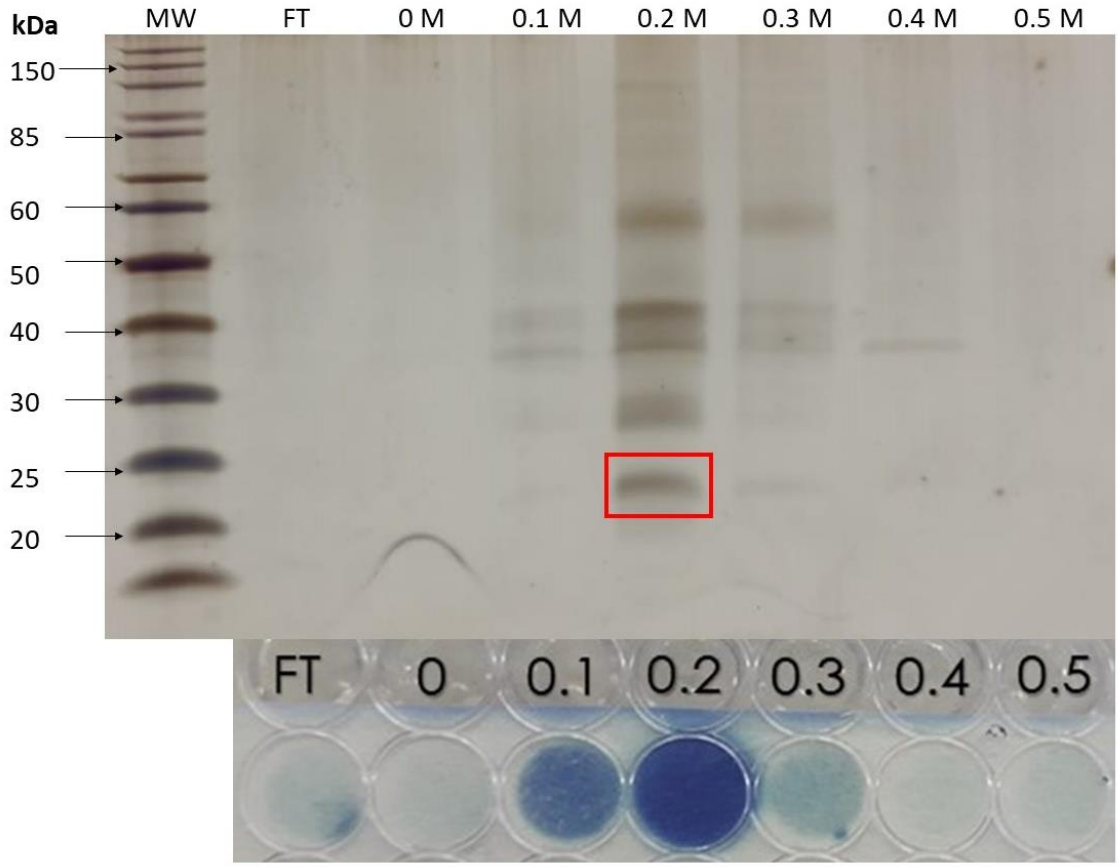


Figure 14. Purification profile and fraction activity assay of the hydroxynitrile lyase from *Davallia trichomanoides*. A linear gradient from 0 to 0.5 M NaCl was used to elute the HNL from a DEAE QXL column. The HNL eluted at 0.2 M NaCl with some residual activity in the 0.1 M and 0.3 M fractions. The double-stained 16% polyacrylamide gel reveals a band between 20 – 25 kDa containing *Dtm*HNL.

3.3.3 *Phlebodium aureum mandaianum*

The hydroxynitrile lyase from *P. aureum mandaianum* (*Pham*HNL) eluted at 0.2 M sodium chloride. A clear, broad band can be seen in the silver-stained SDS-PAGE gel at approximately 40 kDa (Figure 15). The broad band may be indicative of glycosylation of the enzyme as reported by Ueatrongchit *et al*. (2008) and Alagöz *et al.* (2014).

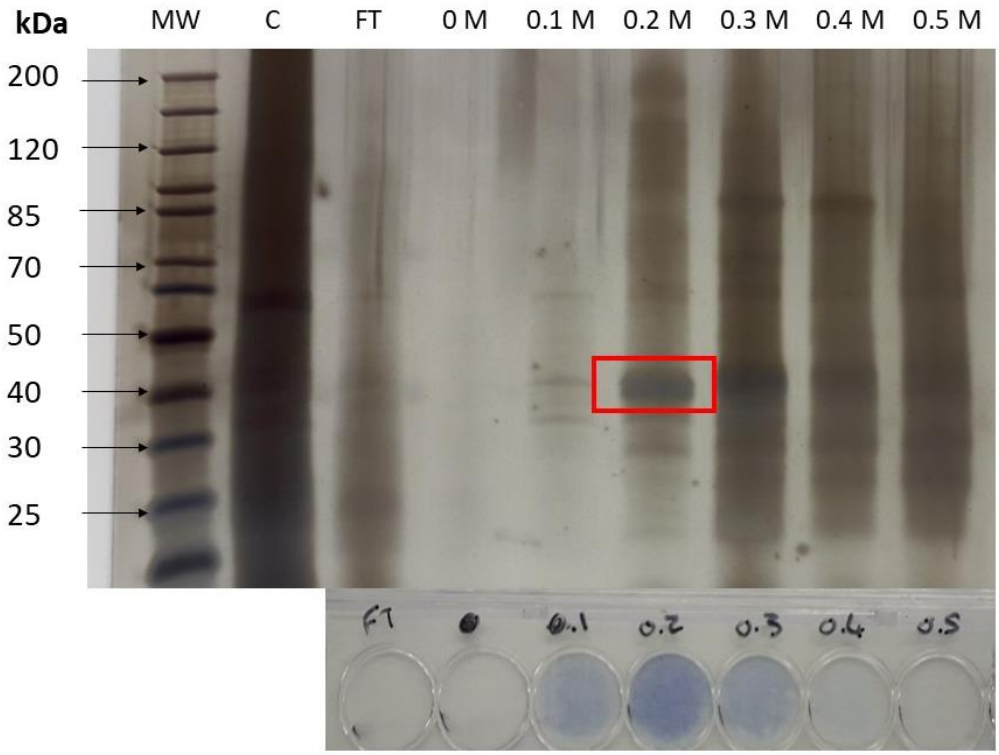


Figure 15. Purification profile and fraction activity assay of the hydroxynitrile lyase from *Phlebodium aureum mandaianum*. Partial purification of *Pham*HNL was achieved using anion exchange chromatography of the desalted protein extract and a linear gradient from 0 to 0.5 M NaCl. Silver-stained 16% SDS-PAGE gel and HNL activity assay of all fractions show *Pham*HNL eluted at 0.2 M NaCl. The estimated size of *Pham*HNL is 40 kDa.

Fractions which showed HNL activity were pooled and concentrated with Corning® Spin X-UF concentrator (10 K MWCO) spin columns. The concentrations of the combined HNL-active fractions are shown in Table 4.

Table 4. Summary data of partially purified and concentrated HNL enzymes after anion exchange chromatography

Plant species	Active fraction (s) (NaCl concentration)	Pooled fraction concentration (mg/ml)
Thelypteris confluens	0.2 M and 0.3 M	0.708
Davallia trichomanoides	0.1 M and 0.2 M	0.399
Phlebodium aureum mandaianum	0.2 M and 0.3 M	0.736

3.4 Native polyacrylamide gel electrophoresis and in-gel activity assay

Blue native PAGE or clear native PAGE was followed by in-gel activity assays. Racemic mandelonitrile at a concentration of 20 mM was used as the substrate for the activity assay. Bands corresponding to blue spots on the detection paper were excised and stored in 10% (v/v) ethanol at -20 ℃.

The partially purified protein extract from *T. confluens* was run in parallel with *Adenia* species previously identified as cyanogenic and three other ferns which indicated low mandelonitrile lyase activity. The positive control was a crude protein extract of *Dt*HNL. As shown in Figure 16, only the positive control (*Dt*HNL) and *T. confluens* gave a positive result for hydroxynitrile lyase activity. The blue spot corresponding to *T. confluens* was a result of two bands located very close together. These bands were excised separately and sent for liquid chromatography tandem mass spectrometry analysis.

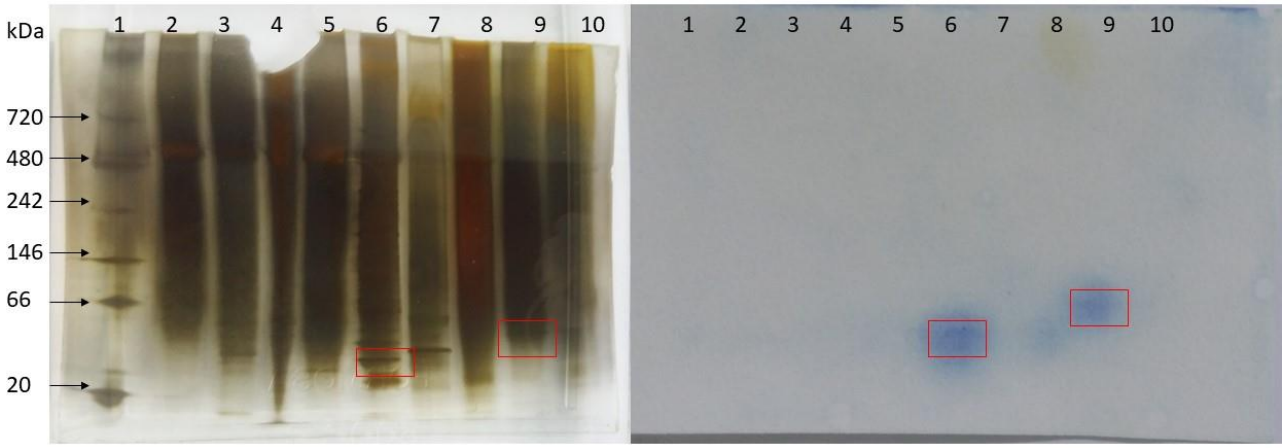


Figure 16. Blue native PAGE and corresponding in-gel activity assay for plant protein isolated from *Thelypteris confluens*. Pre-cast NativePAGE™ Novex® 4-16% gels were used to separate proteins in their native state. Lane 1, NativeMark™ unstained protein standard; lane 2, *Adenia fruticosa* leaves; lane 3, *Adenia fruticosa* stem; lane 4, *Kiggelaria Africana*; lane 5, *Adenia firingalavensis*; lane 6, positive control *Dt*HNL (*Davallia tyermannii*); lane 7, *Pteridium aquilinum*; lane 8, *Pteris sp*.; lane 9, *Thelypteris confluens* and lane 10, *Davallia solida.*

D. trichomanoides and *P. aureum mandaianum* did not show any activity after BN-PAGE. However, CN-PAGE and subsequent in-gel activity assay for all three ferns yielded positive results. The milder conditions of CN-PAGE allowed for the HNLs from all three ferns to remain active. Figure 17 shows the clear native gel after silver staining, and the corresponding detection paper from the in-gel activity assay. The two consistent blue spots observed for *P. aureum mandaianum* may be indicative of different isoforms of the HNL as CN-PAGE can detect different oligomeric states of proteins which may not be visible on BN-PAGE. The bands corresponding to a blue spot were excised and sent for liquid chromatography tandem mass spectrometry analysis.

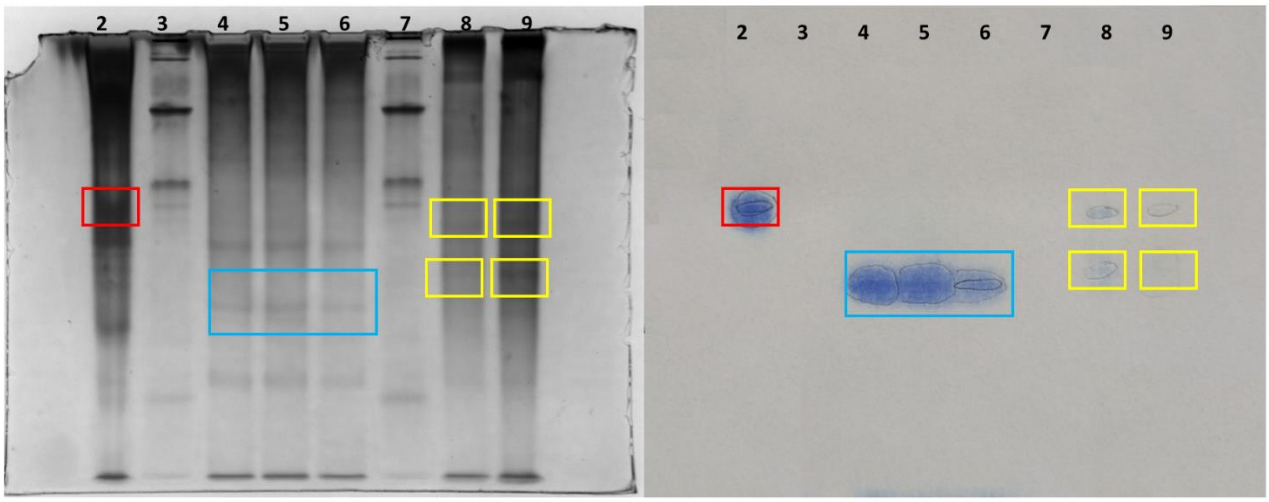


Figure 17. Clear native PAGE and corresponding in-gel activity assay for plant protein isolated from three cyanogenic ferns. Pre-cast NativePAGE™ Novex® 4-16% gels were used to electrophorese proteins in their native state. Lane 2, *Thelypteris confluens*; Lane 3, NativeMark™ unstained protein standard; lane 4-6 *Davallia trichomanoides*; lane 7, NativeMark™ unstained protein standard; lane 8 and 9, *Phlebodium aureum mandaianum*.

3.5 RNA extraction and transcriptome generation

Limited resources only allowed for the generation of one transcriptome. As no reports of HNLs have been published for the fern family Thelypteridaceae the transcriptome of *T. confluens* was generated. High quality total RNA was extracted from young leaves of *T. confluens* which exhibited HNL activity (Table 5). Transcriptome sequencing was done on the Illumina® Hiseq 2500 using the Sequencing by Synthesis (SBS) version 4 chemistry.

Table 5. Total RNA extraction from hydroxynitrile lyase active *Thelypteris confluens* leaves

Variable	Value
RNA concentration (ng/µl)	227.10
260/ 280 ratio	2.15
260/230 ratio	2.54

3.6 Hydroxynitrile lyase activity assay

HNL activity was measured spectrophotometrically by observing the increase in absorbance at 280 nm, as mandelonitrile is cleaved into benzaldehyde at pH 5.5. All assays were done in triplicate and the absorbance resulting from the spontaneous degradation of mandelonitrile at pH 5.5 was subtracted. The partially purified HNL from *D. trichomanoides* showed the highest specific activity, followed by the partially purified *Tc*HNL. The HNL from *P. aureum mandaianum* had the lowest specific activity indicating a relatively low degree of enzyme purity (Table 6).

Table 6. Hydroxynitrile lyase activity assay from three cyanogenic fern species

Plant species	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
Thelypteris confluens	7.71 ± 0.22	0.708	10.88 ± 0.31
Davallia trichomanoides	6.86 ± 0.28	0.399	17.18 ± 0.69
Phlebodium aureum mandaianum	6.15 ± 0.44	0.736	8.36 ± 0.60

3.7 Enzyme stability at acidic pH

The effect of pH on the three enzymes, *Tc*HNL, *Dtm*HNL and *Pham*HNL were qualitatively and semi-quantitatively measured using a colourimetric HCN detection assay. Colour intensity and time of blue spot appearance was used as a measurement of enzyme activity.

A pH range from 2.5 to 6.5 was tested in 1 pH unit increments and a pH profile for each enzyme was developed including a control reaction showing the spontaneous decomposition of 2 mM mandelonitrile at the different pH values (Figure 18). At pH 5.5 and 6.5 spontaneous decomposition can be observed after 5 minutes. After 15 minutes, no HCN was detected for pH 4.5 and lower, indicating the inhibition of the non-enzymatic reaction at the more acidic pH values. The higher intensity of colour at pH 6.5 compared to pH 5.5 indicates a higher rate of spontaneous decomposition.

*Tc*HNL was the only enzyme which exhibited HNL activity at pH 2.5. The colour intensity at pH 2.5 compared to the higher pH values indicates the lowest rate of HNL activity. In addition, activity at pH 2.5 was observed after 10 minutes whereas for pH 3.5 and above, activity was noted after 5 minutes or less (Table 7).

After 10 minutes of reaction time *Dtm*HNL showed activity at pH 3.5 and *Pham*HNL showed activity pH 4.5. This indicates that *Pham*HNL has remarkably slower reaction rates at pH 4.5 and no detectable HNL activity below this pH.

At pH 6.5 all assays showed an intense blue spot which started to form approximately 3 minutes into the reaction. This can mainly be attributed to spontaneous decomposition of mandelonitrile and is not a reliable indicator of HNL activity at this pH. Mandelonitrile degrades at pH values above 5.5 which is visible from the control reaction.

Based on the intensity of the blue spots, the pH optimum for *Dtm*HNL and *Pham*HNL appear to be between pH 4.5 and 5.5. It is possible that for *Tc*HNL the pH optimum lies between pH 3.5 and 5.5 because the blue colour at pH 3.5 and 4.5 are of similar intensity. Upon comparison of the intensity of the blue spot at pH 4.5 for the three enzymes it is clear that *Tc*HNL has the highest activity at this pH.

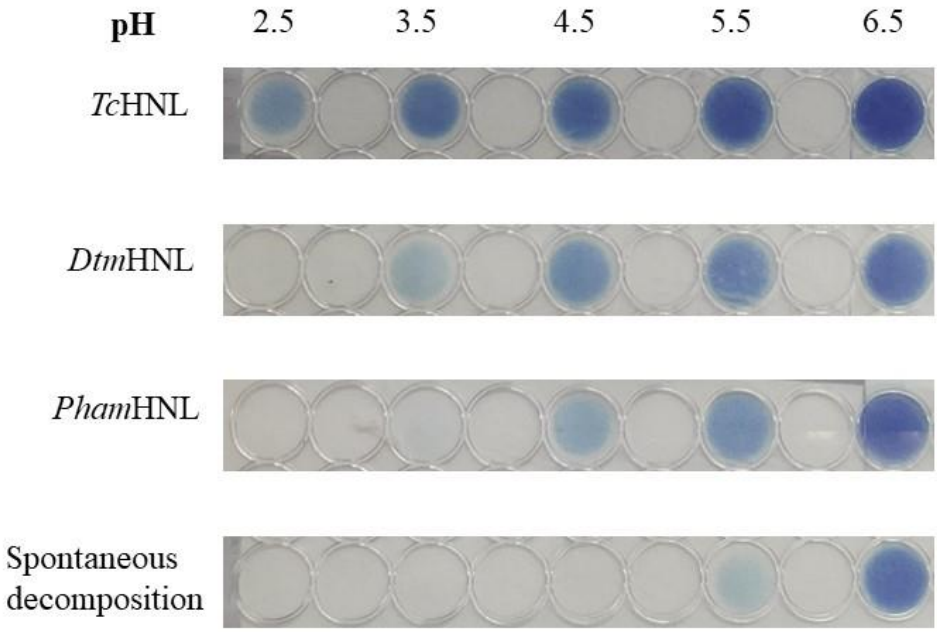


Figure 18. pH stability of *Tc*HNL, *Dtm*HNL and *Pham*HNL. HNL activity was tested at five acidic pH values. The intensity of the blue colour was used as an indication of the amount of hydrogen cyanide produced and inferably a semi-qualitative measure of HNL activity.

Table 7. Rate of hydrogen cyanide production at various pH values for *Tc*HNL, *Dtm*HNL and *Pham*HNL

Plant species	*Thelypteris confluens*			*Davallia trichomanoides*			*Phlebodium aureum mandaianum*		
pH	**Time (min)**								
	5	**10**	**15**	**5**	**10**	**15**	**5**	**10**	**15**
2.5	-	**+**	+	-	-	-	-	-	-
3.5	**+**	+	+	-	**+**	+	-	-	-
4.5	**+**	+	+	**+**	+	+	-	**+**	+
5.5	**+**	+	+	**+**	+	+	**+**	+	+
6.5	**+**	+	+	**+**	+	+	**+**	+	+

+ indicates HCN detection

− indicates no HCN detected

Grey blocks highlight the first observation of activity at each pH

3.8 Liquid chromatography tandem mass spectrometry

The LC-MS/MS data were analysed by searching against the transcriptome database of *P. aquilinum* (L.) Kuhn (PRJEB10897 on the EMBL-EBI European Nucleotide Archive) and *D. tyermannii* (T. Moore) Backer (PRJEB10896). In addition, the data was analysed by searching against the transcriptome database generated in section 2.8 for *T. confluens* (Thunb) Morton and the public databases SwissProt (all species) and Trembl plants with Proteome Discoverer 1.3 (Thermo Electron) and Mascot 2.3 (Matrix Science).

Detailed search criteria were used as follows: trypsin, max. missed cleavage sites: 2; search mode: MS/MS ion search with decoy database search included; precursor mass tolerance +/- 10 ppm; product mass tolerance +/- 0.7 Da; Carbamidomethylation on Cys was entered as fixed modification, and oxidation on methionine was entered as variable modification. Acceptance parameters for identification: 1% protein FDR and minimum 2 peptides per protein.

4 Discussion

4.1 Screening for cyanogenic plants

Ferns

This study embarked on the largest bioprospection campaign for hydroxynitrile lyase activity in plants of South Africa. The initial aim of the study was to bioprospect fern species in South Africa, however, the search was later expanded to include any plant species. Over 90 fern species were screened for HNL activity. Seven fern species (12% of the total) tested positive for HNL activity, from the families Thelypteridaceae (*T. confluens*), Pteridaceae (*Pteris sp.* and *Cheilanthes viridis*), Davalliaceae (*D. trichomanoides*), Polypodiaceae (*P. aureum mandaianum* and *Microsorum scolopendri*a) and Dennstaedtiaceae (*Pteridium aquilinum*).

This is the first report of a hydroxynitrile lyase in the family of Thelypteridaceae and more specifically in the genus *Thelypteris*. This finding contributes to the knowledge base of HNLs in ferns which is known to be an uncommon occurrence (Harper *et al*., 1976). *T. confluens* is considered to be a rare fern species in many countries including New Zealand (Brownsey and Perrie, 2016), South India (Sara and Manickam, 2007) and Australia (Fensham, 1998). In South Africa it is an indicator species for escarpment peat wetlands and is mainly found in the Mpumalanga escarpments as it requires permanently wet conditions on peat (Sieben *et al*., 2016). The requirement of permanently wet conditions by *T. confluens* was observed in this study. In the winter months (dry season in Johannesburg), significant wilting and browning of the fern fronds were noted and no new growth was observed. In addition, no HNL activity was observed in the winter months, although, as soon as the first rains occurred in September, HNL activity returned (data not shown). This is a clear example of trade-offs made in *T. confluens* to survive the lack of water in the winter months by decreasing or halting the biosynthesis of cyanogenic glycosides and/or their degradative enzymes to reserve resources (Vetter, 2000; Gebrehiwot and Beuselinck, 2001).

In 1976, Harper and colleagues embarked on a study to determine the occurrence of cyanogenesis in fern species. The group screened 298 fern species for cyanogenic activity collected from the Royal botanical gardens in Kew (United Kingdom). Only 15 out of the 298

species screened (5%) exhibited HNL activity, including species belonging to the families; Davalliaceae (*D. trichomanoides*, *D. epiphylla*, *D. solida*, *Humata griffithlana* and *H. pusilla*), Polypodiaceae (*Campyloneurum angustifolia* and *Phlebodium decumanum*), Pteridaceae (*Actiniopteris semiflabelata*), Sinopteridaceae (*Cheilanthes notholaenoides* and *C. pulchella*), Dennstaedtiaceae (*Microlepia setosa*, *M. strigosa* and *Pteridium aquilinum*), Aspidiaceae (*Pteridrys olivacea*) and Blechnaceae (*Stenochlaena tenuifolia*).

Interestingly, some of the plants screened by Harper *et al*. (1976) which tested negative for cyanogenesis were found to exhibit HNL activity in this and other studies. Most notably, *Phlebodium aureum* did not exhibit HNL activity when tested by Harper *et al.* (1976) however, it is now one of the most well-known cyanogenic ferns and has been extensively studied by Wajant and colleagues (1995). In addition, a cultivar of this fern, *P. aureum mandaianum* was used in this study and it also exhibited excellent HNL activity. Two of the ferns found to have HNL activity in this study namely, *M. scolopendria* (#336) and *C. viridis* (#350) from iSimangaliso Wetland Park also tested negative for cyanogenesis in the study by Harper *et al*. (1976). On the other hand, the cyanogenic ability of *D. trichomanoides* and *Pteridium aquilinum* correlates with the results in this study. Harper *et al*. (1976) also observed cyanogenesis in *Davallia solida* but in this study no HNL activity was detected.

An explanation for the different results achieved in the two studies is the fact that plants are polymorphic for cyanogenesis (Gebrehiwot and Beuselinck, 2001). It may be that Harper *et al*. (1976) tested an acyanogenic form of *P. aureum*, *M. scolopendria* and *C. viridis*, among others and that the *D. solida* plant in the APES garden expresses an acyanogenic phenotype. The alternative phenotypes displayed by plants for cyanogenesis depends on a number of factors such as the age of the plant, the part of the plant that was tested, the season of sampling (which is unknown for the study by Harper *et al.*), environmental factors, gene variation and stress conditions (Vetter, 1999; Gebrehiwot and Beuselinck, 2001; Hernández *et al*., 2004; Nicholson, 2007; Kassim *et al*., 2014). In addition, the plants collected by Harper *et al*. were grown in a botanical garden and this may not be a true reflection of the natural habitat of these plants. In this study, plants were randomly sampled from their natural habitats and this may be the reason that we observed cyanogenic activity for some species whereas Harper and colleagues did not.

Thirty-one species belonging to the Thelypteridaceae family was screened by Harper *et al.*, (1976), including six ferns in the genus *Thelypteris* (*T. asplenioides*, *T. asterothrix*, *T. dentata*, *T. erbescens*, *T. uliginosa* and *T. viridifrons*). Until now, no report of cyanogenesis has been found in the family Thelypteridaceae and therefore the discovery of HNL activity in *T. confluens* is an exciting find.

Non-commercialised plants

Over 450 randomly selected non-commercialised plants were screened for HNL activity. Five species were observed to be cyanogenic without the addition of a cyanohydrin substrate. The identity of only one of these species is currently known, that is, *Morus mesozygia* commonly known as the African Mulberry belonging to the Moraceae family. *M. mesozygia* has been used for many centuries in traditional medicine to treat ailments such as stomach disorders, inflammation, ulcers, depression and rheumatoid arthritis (Adediwura and Bola, 2013; Oshiomah *et al.*, 2016). No reports on cyanogenesis in the leaves of *M. mesozygia* have been found in the literature to date. In addition, studies on the secondary metabolites present in the stem bark of *M. mesozygia* have detected compounds such as anthraquinone, tannins, flavonoids, saponins, alkaloids and cardiac glycosides but have not detected the presence of cyanogenic glucosides (Adediwura and Bola, 2013; Oshiomah *et al.*, 2016). Previously, ten species in the Moraceae family were screened for cyanogenic activity and all results were negative (Miller *et al.*, 2006; Kadiri and Ajayi, 2009). A suitable method of protein extraction should be developed for *M. mesozygia* leaves as it has been shown to be incompatible with the P-PER™ plant protein extraction kit. Following successful protein extraction and purification, the HNL from *M. mesozygia* should be further investigated for its potential in industrial use.

After the addition of racemic mandelonitrile, 39 additional plants showed HNL activity. Of the plants identified to date, 13 families were found to have species which were cyanogenic in the field. Cyanogenesis has been previously reported in most of these families, namely; Solanaceae (Adsersen *et al.*, 1988), Rutaceae (Miller *et al.*, 2006; Nahrstedt and Schwind, 1991), Ranunculaceae (Siegler, 1975; Siegler *et al.*, 2005; Da-Cheng *et al.*, 2015), Clusiaceae (Lanfranchi *et al.*, 2013), Euphorbiaceae (Wajant *et al.*, 1995; Wajant *et al.*, 1996), Leguminosae (Siegler, 1975), Malvaceae (Siegler *et al.*, 2005), Fabaceae (Vetter, 2000),

Asteraceae (Nahrstedt and Schwind, 1991), Polypodiaceae (Harper *et al*., 1976; Vetter, 2000) and Pteridaceae (Harper *et al*., 1976). However, no reports of cyanogenesis was found for the remaining two families, Anacardiaceae and Ochnaceae.

4.2 Comparing methods of screening for hydroxynitrile lyase activity

Most studies over the past few decades relied on detecting HNL activity by spectrophotometry. The cleavage reaction of cyanohydrins is conducted and the increase in the production of benzaldehyde is monitored by measuring the increase in absorbance at 280 nm, alternatively, the synthesis reaction is observed spectrophotometrically (Hickel *et al*., 1997; Asano *et al*., 2005, Hernández *et al*., 2004). Although the spectrophotometric method is an accurate way of detecting HNL activity, it is not suitable for high throughput screening and cannot be conducted in the field.

Harper *et al*. (1976) screened for cyanogenesis by adding 2-3 drops of toluene to plant material in a sealed tube. Filter paper pre-treated with sodium picrate or copper (II) ethylacetoacetate in chloroform was suspended from the stopper. The tubes were incubated at 35 °C for 3 hours and observed for a change in colour. This method may be accurate and possible to conduct in the field; however, it is time consuming and makes use of the toxic compound toluene.

In 2006, Andexer and colleagues developed a high throughput colourimetric method for detecting HNL activity. This assay requires the use of microtiter plates which are transparent in the UV range. The cyanohydrin cleavage reaction is run, and the reaction is stopped after 5 minutes by the addition of 100 mM *N*-chlorosuccinimide with 10-fold excess of succinimide for stabilisation. This results in the oxidation of the CN^- to CN^+. After 2 minutes the colourimetric reaction is initiated by the addition of 30 µl of 65 mM isonicotinic acid, 125 mM barbituric acid in 0.2 M NaOH. The cyanide cation reacts with the isonicotinic acid to form a dialdehyde which is further coupled to two molecules of barbituric acid to form the dye. The rate of colour formation is observed at 600 nm for 20 minutes. This method is suitable for testing many samples simultaneously and is advantageous in that the colour

development (faint blue to purple) is proportional to the HCN concentration and as a result specific activity of the enzyme can be calculated (Andexer *et al*., 2006). The disadvantage of this method is the cost involved and its inapplicability for field testing. When screening a large amount of different species in the field, a reliable and fast method for cyanogenesis detection is needed to eliminate negative samples early in the bioprospection process and allow researchers to proceed only with samples which show positive results.

Krammer *et al.* (2007) developed a high throughput method for detecting HNL activity in hundreds of mutant colonies by using Feigl-Anger detection paper which detects gaseous HCN released during the cleavage reaction of cyanogenesis. This method was then routinely used by Lanfranchi and colleagues (2015 and 2017) and Kassim *et al*. (2014) for the screening of plants for HNL activity. This technique is suitable for field screening and only requires a 96-well plate and the detection paper. HCN release can be detected within minutes (Lanfranchi *et al*., 2015). This principle was further developed during this study by the development of HCN detection tubes using the same solution used to prepare the Feigl-Anger detection paper. These detection tubes work reliably in the field and allow for mass screening of plants. As with the detection paper a blue spot indicates HCN release and is indicative of cyanogenesis. This is the first time that detection tubes have been used and it has proved to be a fast and reliable high-throughput screening method which allows for the mass bioprospection screening in the field and requires only a small amount of plant material.

4.3 Plant protein extraction and purification

The P-PER™ plant protein extraction kit (Thermo Scientific) was routinely used for plant protein extraction in this study because it requires as little as 80 mg of plant material and in many cases not a lot of plant material was available, especially for plants collected at iSimangaliso Wetland Park. All 39 plants which tested level-two positive for HNL activity had no HNL activity a few hours after treatment with P-PER™. However, the five plants from iSimangaliso Wetland Park which showed level-one cyanogenic activity (activity without the addition of mandelonitrile) were also inactive in the P-PER™ extraction buffer and after desalting and buffer exchange, activity returned. The 39 potentially cyanogenic P-

PER™ protein extracts could not be desalted due to limited resources and future studies could focus on new protein extraction methods for these plants.

It is herein postulated that some component of P-PER™ may inactivate HNLs if stored for more than a few hours in the P-PER™ buffer. To test this hypothesis, four *Adenia* species (family Passifloraceae) which were previously proven to be cyanogenic by a member of our lab (Kassim *et al*., 2014) were treated with P-PER™ and tested for HNL activity after 24 hours. All four *Adenia* species exhibited no HNL activity in the P-PER™ extract (data not shown).

No studies on the inhibitory effects of P-PER™ on enzymes have been reported to date, but upon research into the components of the P-PER™ kit it was found that reagent B contains between 10-20% of aliphatic carboxylic acid. The decomposition products of this compound include carbon dioxide, carbon monoxide, phosphorous oxides and halogenated compounds. The mechanism of action for HNL requires the deprotonation of the cyanohydrin's hydroxyl group, resulting in a negative charge on the cyanide ion (Andexer *et al*., 2012). It is possible that the halogenated compounds produced by the decomposition of reagent B are electrostatically attracted to the nucleophilic CN^- as the C-X bond is electrophilic where the halogen atom (X) acts as the electron acceptor. The nucleophilic substitution reaction results in a halogen-substituted ligand which may act as an inhibitor of the HNL enzyme because halogen-substituted ligands have a high affinity for the carbonyl oxygen of some protein residues and may also alter the conformation of the enzyme, thereby affecting the HNL function (Lu *et al*., 2012).

An alternative method of protein extraction is therefore required in such cases. For example, Kassim *et al*. (2014) extracted plant protein by grinding young leaves into a fine powder in liquid nitrogen using a mortar and pestle. The plant powder was added to 50 mM sodium citrate buffer pH 5.0 and vortexed vigorously before centrifugation at 20 000 ×g for 10 minutes. The supernatant was used as the crude protein extract. Consequently, different protein extraction methods should be attempted for plants which show HNL inactivation after treatment with P-PER™. Most often these methods will require more plant material than

needed for P-PER™ and as a result when in the field, researchers need to collect a substantial amount of plant material and store these samples appropriately to ensure no protein degradation occurs because of decreased plant quality.

Not all 39 plants may have been affected by the components of the P-PER™ buffer. As these protein extracts stored in P-PER™ buffer were not kept in a cool place during transportation back to the laboratory, this may have caused inactivation of some HNLs. Another factor may be the result of false positives using the HCN detection tubes. It is recommended that these tubes be kept in a dark and cool place to avoid false positives due to the reaction of the components in the Feigl-Anger solution in response to heat or light stimulation (Kassim *et al*., 2014; Lanfranchi *et al*., 2015). However, in the field, unavoidable high temperatures were experienced which may have contributed to the high number of positives observed due to both the HCN tubes and spontaneous decomposition of mandelonitrile, which occurs at temperatures above 35 °C. Hence, it is essential for future field trips using HCN detection tubes and mandelonitrile that these tubes be stored in a portable cooler/ fridge to avoid any erroneous HNL activity detection.

4.4 Native and sodium dodecyl sulfate PAGE and in-gel activity assays

To maintain enzyme functionality native polyacrylamide gel electrophoresis was conducted to separate the protein fractions and obtain individual bands corresponding to HNLs. The BN-PAGE coupled to an in-gel activity assay was successful in isolating functional HNL enzymes from *Prunus domestica* kernels by Lanfranchi *et al*. (2015). This method is advantageous in that the native enzyme size and substrate specificity may be determined. With the addition of mass spectrometry, a partial amino acid sequence for the enzyme can also be elucidated.

The five level-one-positive plants, and the 39 level-two-positive plants collected from iSimangaliso Wetland Park were subjected to BN-PAGE (data not shown). The plant proteins in the P-PER™ extraction buffer were loaded onto the native gel and electrophoresed. No individual blue spots were detected, and these plants were not proceeded with in further

studies. However, these plants do represent a wealth of potential novel HNL enzymes and are currently stored at -80 °C for use in future studies.

Because of the lack of resources available to repeat protein extraction and purification procedures on the 44 plants from isiMangaliso, it was decided that the three most promising fern species would be further investigated namely, *T. confluens*, *D. trichomandiodes* and *P. aureum mandaianum*.

Of the three HNLs from ferns investigated, only *Tc*HNL exhibited HNL activity in the in-gel activity assay after BN-PAGE. A similar result was reported by Lanfranchi *et al*. (2015) for the cyanogenic plant *Passiflora capparidifolia.* Plant protein was extracted using the P-PER™ kit and HNL activity was observed after extraction. However, after electrophoresis on BN-PAGE, no activity was observed in the in-gel activity assay hence no individual band could be attributed to the HNL activity observed in the P-PER™ protein extract (Lanfranchi *et al*., 2015). No explanation for this observation was postulated, however, it is possible that the protein concentration was not sufficient for HNL activity detection or the HNL from *P. capparidifolia* does not accept mandelonitrile or acetone cyanohydrin as substrates. This may also be the case for some of the plants collected from iSimangaliso Wetland Park.

Blue native PAGE of the desalted protein extract of *T. confluens* revealed the size of the native *Tc*HNL enzyme to be between 20 and 66 kDa. The exact size was not determined, however on SDS-PAGE the enzyme appeared at around 20 kDa and it is therefore possible that *Tc*HNL exists as a homodimer in its native form which would give it a native molecular weight of 40 kDa which is within the range observed on the BN-PAGE.

The unsuccessful result for *Dtm*HNL and *Pham*HNL on BN-PAGE led to separation on CN-PAGE which operates under milder conditions. In the case of CN-PAGE, the molecular weight of the native enzyme cannot be determined. However, *Dtm*HNL appeared as one band after comparison with the in-gel activity assay detection paper. The SDS-PAGE purification profile for *Dtm*HNL shows a polypeptide band at around 25 to 30 kDa.

On the other hand, *Pham*HNL appeared as two bands, indicating the possibility of two isoforms for this HNL. This seems like a likely conclusion given that *Pha*HNL (HNL from *Phlebodium aureum*) occurs as at least three isoforms and given that *Phlebodium aureum mandaianum* is a cultivar of this species, it is a reasonable inference that *Pham*HNL may have different isoforms.

*Pha*HNL is a homodimer comprised of subunits of 20 kDa. From the SDS-PAGE purification profile of *Pham*HNL a broad band at 40 kDa is observed. This significant difference in the size of the single polypeptides of these two HNLs from the same genus and species but different cultivars has been observed previously. For example, the HNL from *Prunus amygdalus* (*Pa*HNL) has a molecular mass of 72 kDa and exists as a monomer. Whereas, the cultivar *Prunus amygdalus turcomanica* has an HNL which is a homotetramer with a subunit molecular mass of 25 kDa and is possibly glycosylated (Alagöz *et al*., 2014). It is therefore plausible to hypothesise that the cultivar *P. aureum mandaianum* contains a novel HNL.

4.5 Activity assays and pH stability

Specific activity is a measure of the purity of an enzyme (Foustoukos, 2014). In this study low specific activity values were observed for the three fern species when compared with the specific activity of highly purified *Me*HNL which has been reported as 91.6 U/mg (Wajant *et al*., 1995). This high specific activity was recorded after a 241-fold purification of the initial crude protein extract which had a specific activity of only 0.38 U/mg (Wajant *et al*., 1995). The low specific activity values observed for *Tc*HNL (10.88 ± 0.31 U/mg), *Dtm*HNL (17.18 ± 0.69 U/mg) and *Pham*HNL (8.36 ± 0.60 U/mg) is not unexpected given that the protein extract used in the activity assay was only partially purified. A more intensive purification procedure would significantly increase the specific activity of the respective HNLs.

For application in industry, HNL enzymes need to be able to function at pH values below 4 and low temperatures (0 to 5 °C) to suppress the chemical reaction which decreases the

enantiomeric excess of the cyanohydrins (Guterl *et al*., 2009). The ability of *Tc*HNL to catalyse the cleavage reaction at pH 2.5 is remarkable. An illustration of just how exciting this is can be found in the production of (*R*)- pantolactone. Even at pH 4.0 the synthesis of chiral hydroxypivaldehyde cyanohydrin was hindered by the background reaction which produced a racemic product. After site directed mutagenesis on *Pa*HNL5, the reaction could take place at pH 2.5 and the background reaction was completely suppressed (Bracco *et al*., 2016). The natural stability of *Tc*HNL at pH 2.5 makes this enzyme industrially relevant as it is possible that extensive enzyme engineering techniques to increase stability for industrial working conditions is not necessary.

The pH optima for the most well-studied HNLs currently used in industry are *Me*HNL (3.5 to 5.4), *Hb*HNL (5.5 to 6.0) *Pha*HNL (6.2), *Sb*HNL (5.5), *Pa*HNL (5.5 to 6.0), *Lu*HNL (5.5) and 5.5 for *Ej*HNL (Wajant *et al*., 1995; Effenberger *et al*., 2000; Sharma *et al*., 2005; Ueatrongchit *et al*., 2008). The optimum pH for *Tc*HNL was estimated to be around 3.5 to 5.5 comparable to the industrially important *Me*HNL. The HNLs from the ferns *D. trichomanoides* and *P. aureum mandaianum* have pH optima between 4.5 and 5.5. Furthermore, the stability of *Dtm*HNL at pH 3.5 also makes this a promising candidate for industrial applications. HNL activity assays in which the synthesis reaction is performed at different pH values will be more informative to the applicability of these enzymes in industry (Wiedner *et al*., 2015). The HNL stability at acidic pH for the synthesis of chiral cyanohydrins will be focused on in future studies.

The use of enzymes as biocatalysts in industry has three main challenges, that is (1) enzyme stability and activity under certain conditions, (2) narrow substrate range, and (3) the non-enzymatic reaction which negatively affects the enantio-purity of the final product. The implementation of enzyme engineering combined with reaction engineering can improve the properties of the enzyme and allow it to function better than the wild type (Andexer *et al*., 2009). Although these methods for improving enzyme capabilities exist and are becoming less expensive, it is still desirable to find novel enzymes which have superior biochemical properties in the wild type form. As such the process of bioprospection for novel proteins with superior capabilities is an important research area.

4.6 Bioprospection and the search for novel HNLs

Bioprospection is the science of discovering new biological compounds or proteins which can be exploited to produce value added products (Sampath, 2005). A lot of controversy has surrounded the field of bioprospection mainly concerning benefit sharing with indigenous communities in exchange for access to biodiversity (Artuso, 2002). In South Africa, a bioprospecting collaboration with traditional healers and the Council for Scientific and Industrial Research (CSIR) has been recognised and admired worldwide. This collaboration includes a database created using information from traditional healers which serves to prioritise certain plants for the bioprospection of potentially useful compounds (Artuso, 2002). The inside information from traditional healers provides an advantage by focusing efforts and resources on plants used in traditional medicine.

In the case of HNLs, no such prior knowledge exists, and it is up to the researcher to decide which plants to focus on. Previous studies have focused on plants families in which HNLs have been previously discovered (Hickel *et al*., 1997; Hernández *et al*., 2004; Kassim *et al*., 2014), but this limits the possibilities of finding truly novel HNL enzymes. In this study, no specific family was targeted, and all plants were treated as potential HNL sources. This allowed us to discover potentially cyanogenic species in families which have not been thoroughly investigated for HNL activity such as the Anacardiaceae and Ochnaceae plant families. This study has therefore increased the knowledge base of plant families with potential cyanogenic activity which can be focused on in future studies.

The process of bioprospection needs to be carefully planned prior to field testing. The climate and rainfall season are one of the most important factors that affect the success of HNL bioprospection. Many studies, including this one, have shown that a higher concentration of HNLs or cyanogenic glycosides are observed in warmer seasons (Gebrehiwot and Beuselinck, 2001), therefore, the timing of field bioprospection is crucial. Furthermore, testing younger plants will give a more accurate result on the cyanogenic status of the plant tested (Vetter, 2000). The high proportion of negative results obtained at iSimangaliso Wetland Park may be a result of this, as we did field testing in the first week of September,

before the first rains had occurred. Consequently, mostly older plant material which survived the dry and cold season were screened.

The method of screening used in this study is reliable, however, one major limitation in this study was the lack of plant material available to be transported back to the laboratory for further testing. As mentioned, all the plant extracts did not exhibit HNL activity upon return to the lab, and not enough plant material or resources was available to attempt different plant protein extraction methods. The collection of sufficient material and the appropriate storage conditions en route to the lab is crucial to maintaining enzymatic activity. The correct identification of plants is also important so that the plant of interest may be located elsewhere if more plant material is needed. Many of the plants collected from isiMangaliso have not yet been identified because of the poor quality of the specimens. Most of the plant material used in the herbarium collection was very dry and the reproductive organs were not present which are essential in obtaining correct identifications (Victor *et al*., 2004). In addition, the herbarium specimens were all subjected to microwaves for 75 seconds before identification by SANBI and this destroyed some of the plant material. During the bioprospection process, high quality images of plants in their natural habitat will contribute to successful identification and this should be a priority in the future.

5 Conclusion

In this study, only a fraction of the biodiverse flora in South Africa was bioprospected for novel HNL activity. Forty-eight out of over 540 plant species (8.89%) were found to be cyanogenic. Hydroxynitrile lyases from the three most promising species namely, *Thelypteris confluens*, *Davallia trichomanoides* and *Phlebodium aureum mandaianum* were partially purified by anion exchange chromatography. The results indicate that *Tc*HNL is a homodimer with a subunit molecular weight of 20 kDa, and it is the only HNL in this study which exhibits activity at pH 2.5, making it a good candidate for industrial applications. In addition, this is the first report of an HNL in the plant family Thelypteridaceae and in the leaves of the African mulberry tree, *Morus mesozygia*. The second aim of this study was to elucidate the gene sequence of the HNLs found. Mass spectrophotometry of the bands containing the HNLs were conducted and the transcriptome of *T. confluens* was generated. Data integration

for amino acid sequence determination is currently underway in collaboration with the Austrian Centre of Biotechnology. Future studies will involve HNL purification techniques to increase specific activity and alternative protein extraction methods for plants which are incompatible with the P-PER™ plant protein extraction kit. Synthesis of chiral cyanohydrins catalysed by *Tc*HNL, *Dtm*HNL and *Pham*HNL need to be assayed and the optimal temperature needs to be determined. Improvement of the field techniques as suggested above will be helpful in future bioprospection campaigns. Finally, after the elucidation of the amino acid sequence, heterologous expression of the HNLs will be done to examine the feasibility for industrial scale application.

6 Appendix

Herbarium specimens of plants bioprospected for hydroxynitrile lyase activity in South Africa

Herbarium specimens prepared by Deidre Davids

V001- *Thelypteris confluens*
Family: Thelypteridaceae



A1: *Pteridium aquilinum*

Family: Dennstaedtiaceae



Z001- Tree fern



BL001- *Pteris sp.*
Family: Pteridaceae

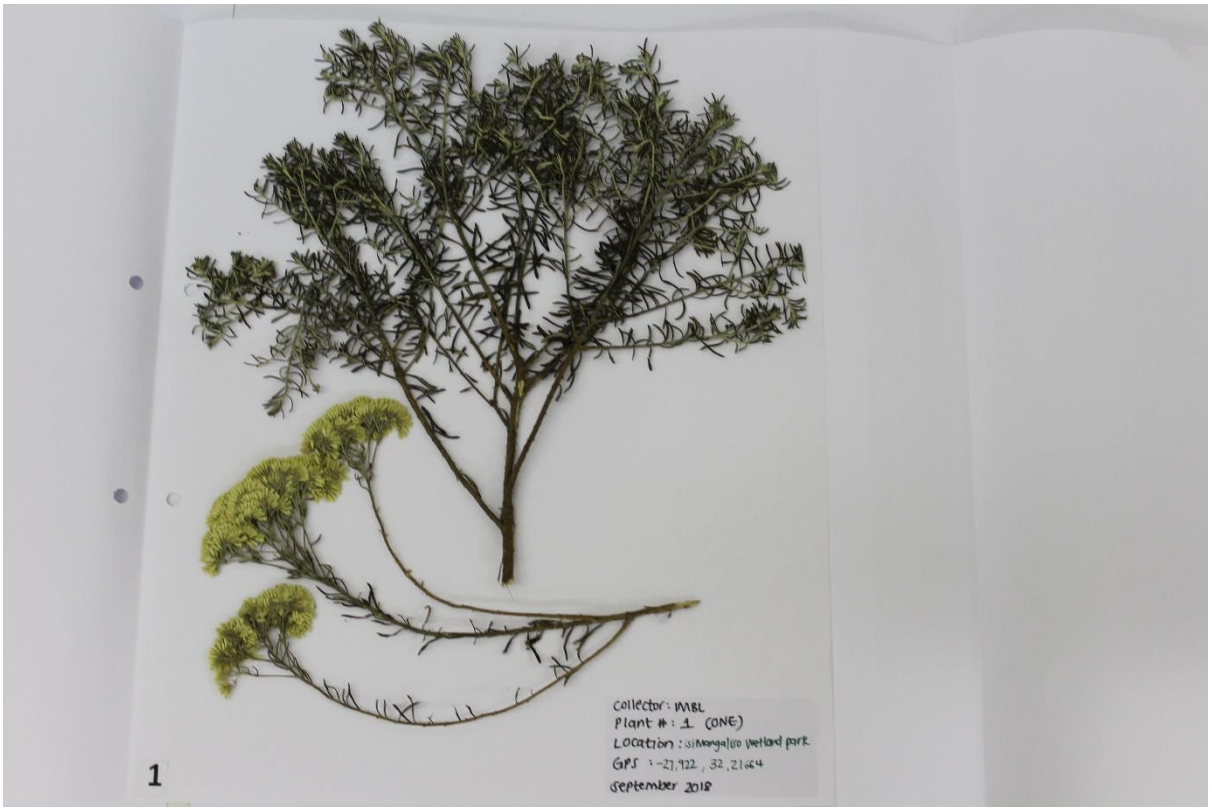


W001- *Davallia solida*

Family: Davalliaceae



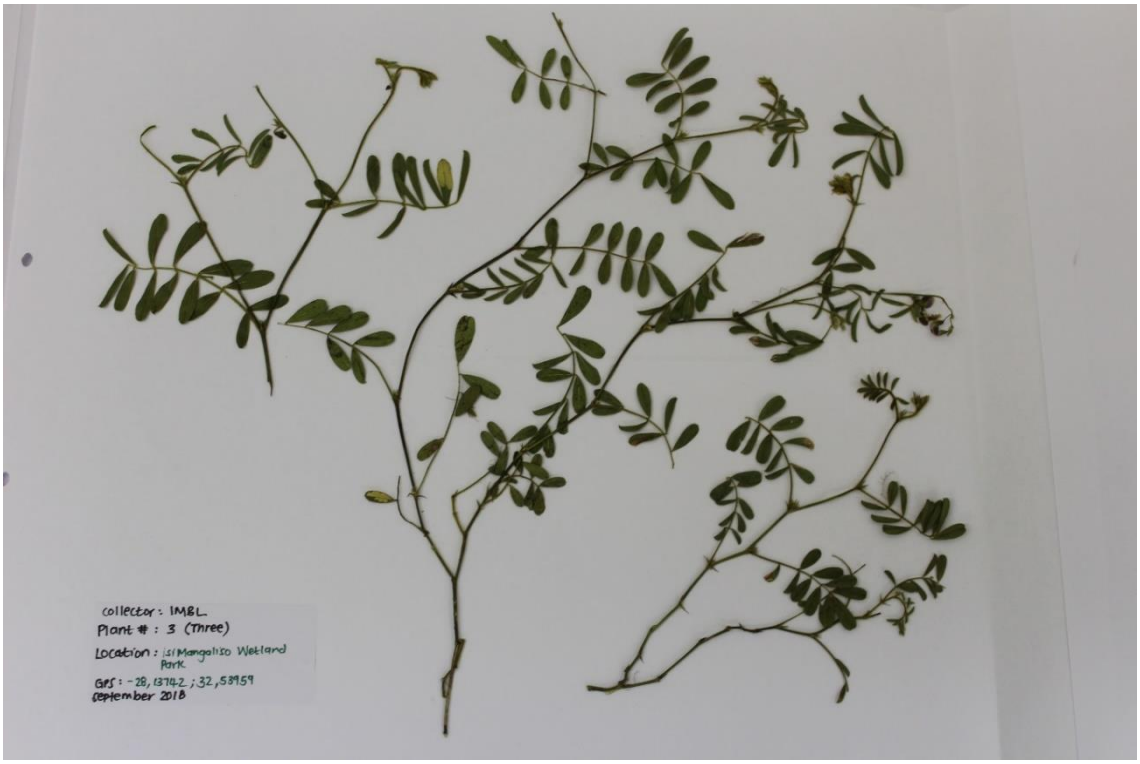
1-Identification pending



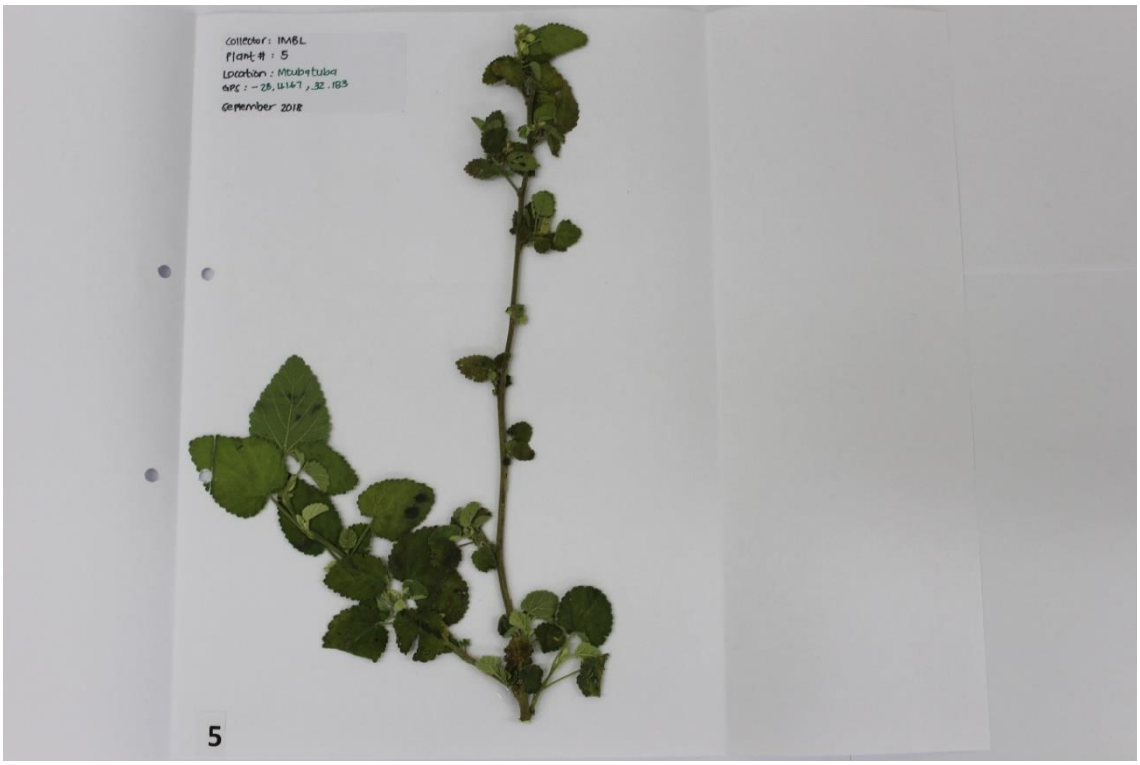
2- Identification pending



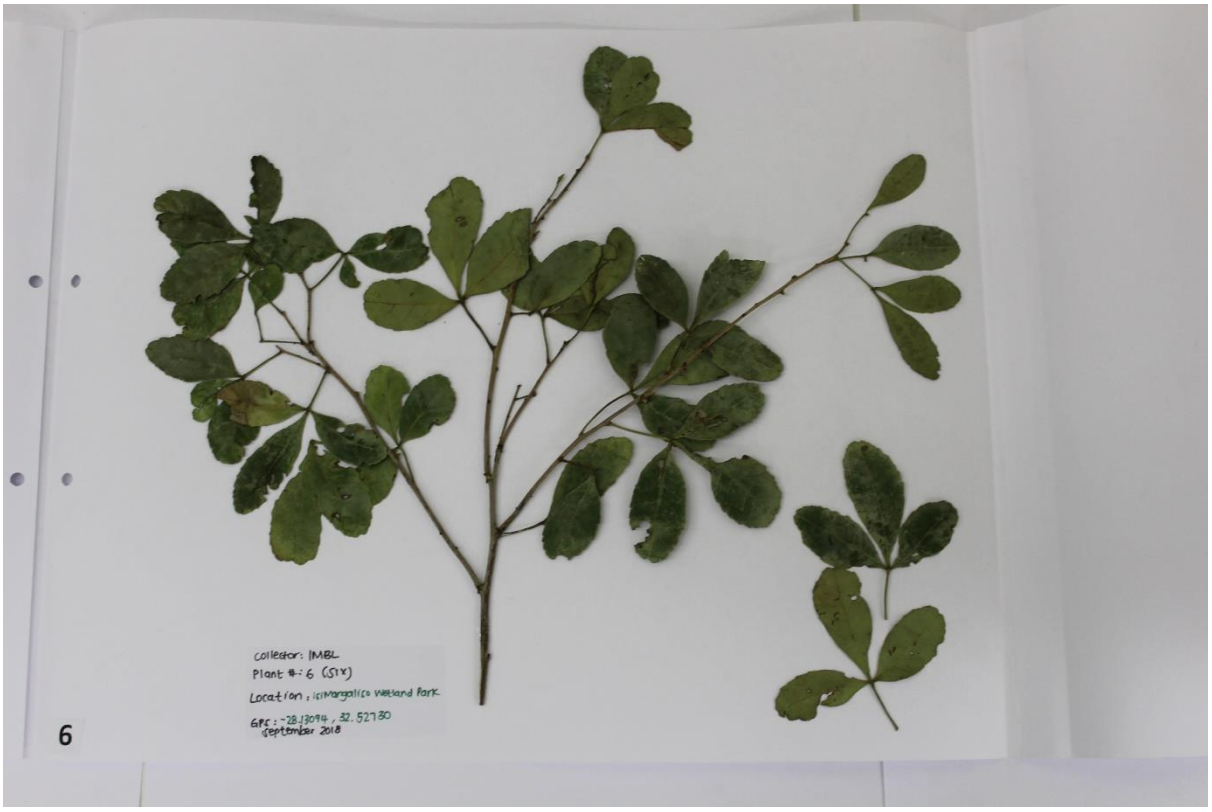
3- *Tephrosia polystachya*
Family: Fabaceae



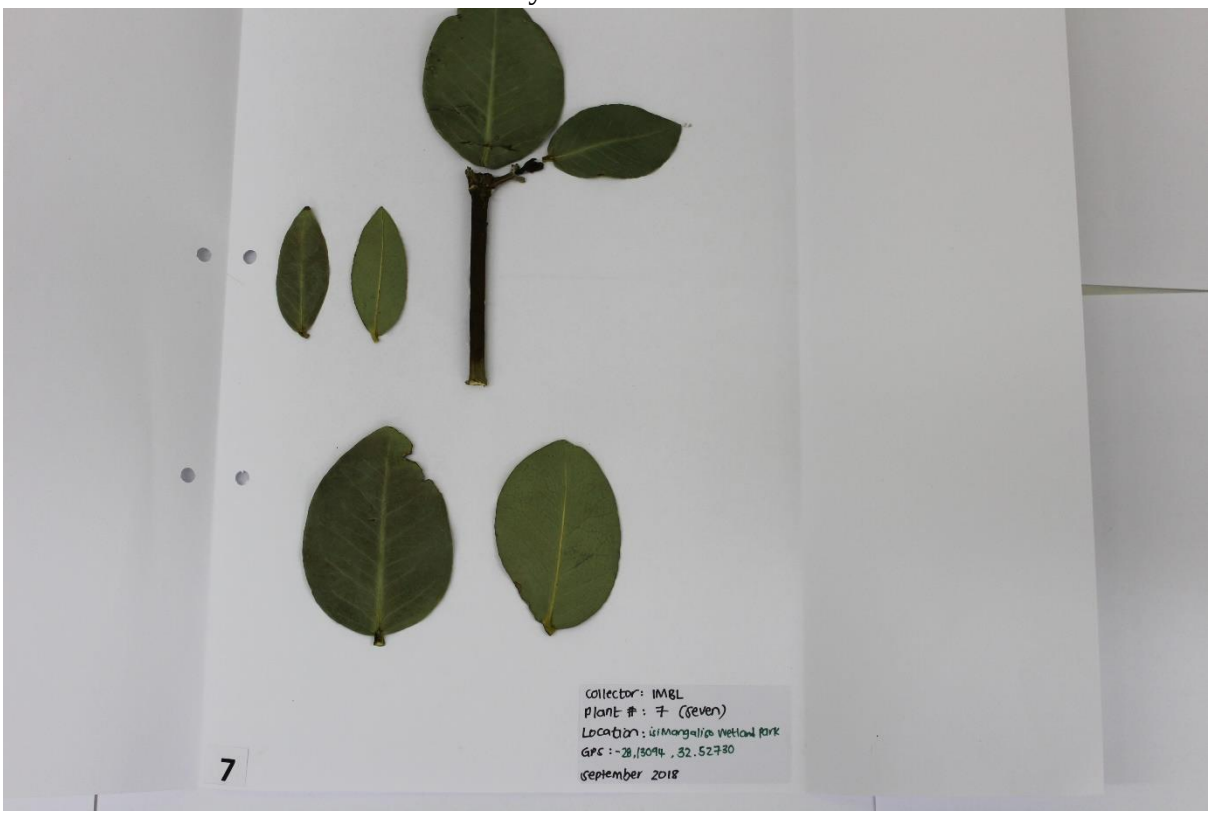
5- *Sida cordifolia*
Family: Malavaceae



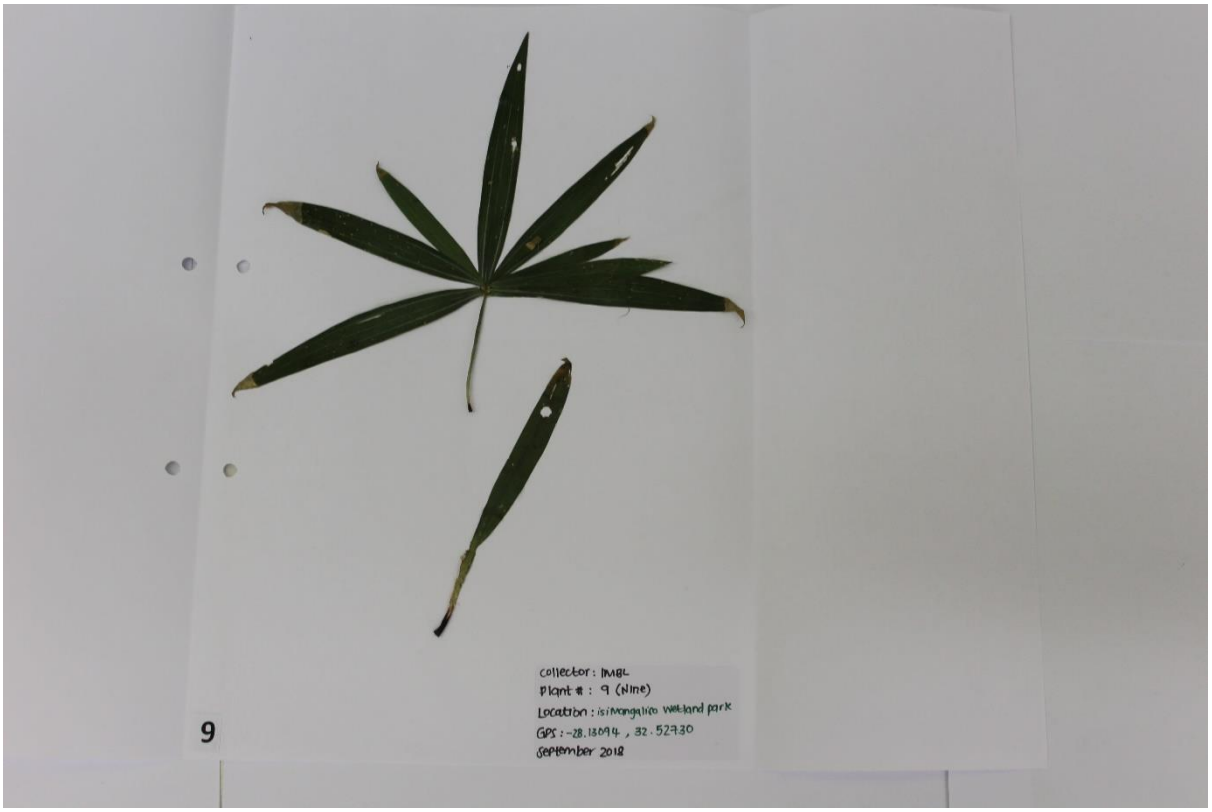
6- *Searsia natalensis*
Family: Anacardiaceae



7- *Garcinia livingstonei*
Family: Clusiaceae



9- *Cyperus sp.*
Family: Cyperaceae



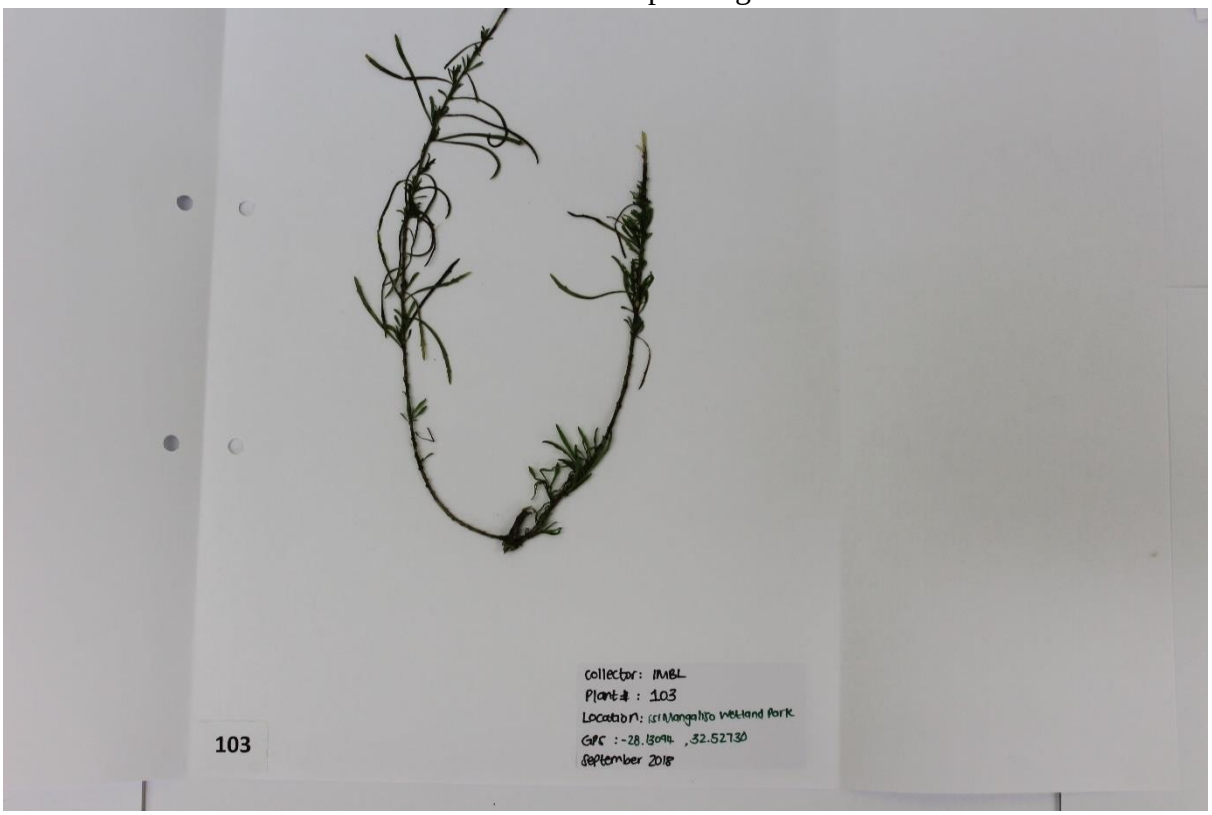
10- Identification pending



24- Acalypha sp.
Family: Euphorbiaceae



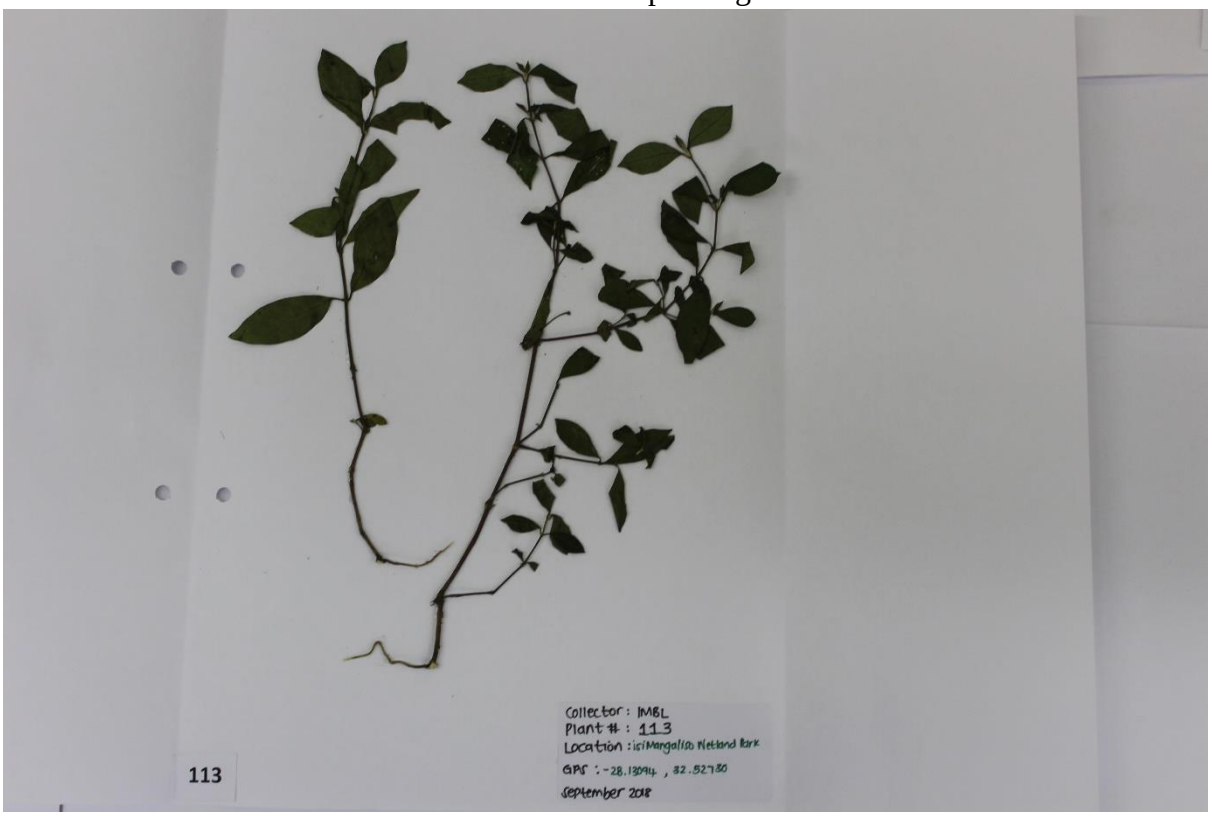
103- Identification pending



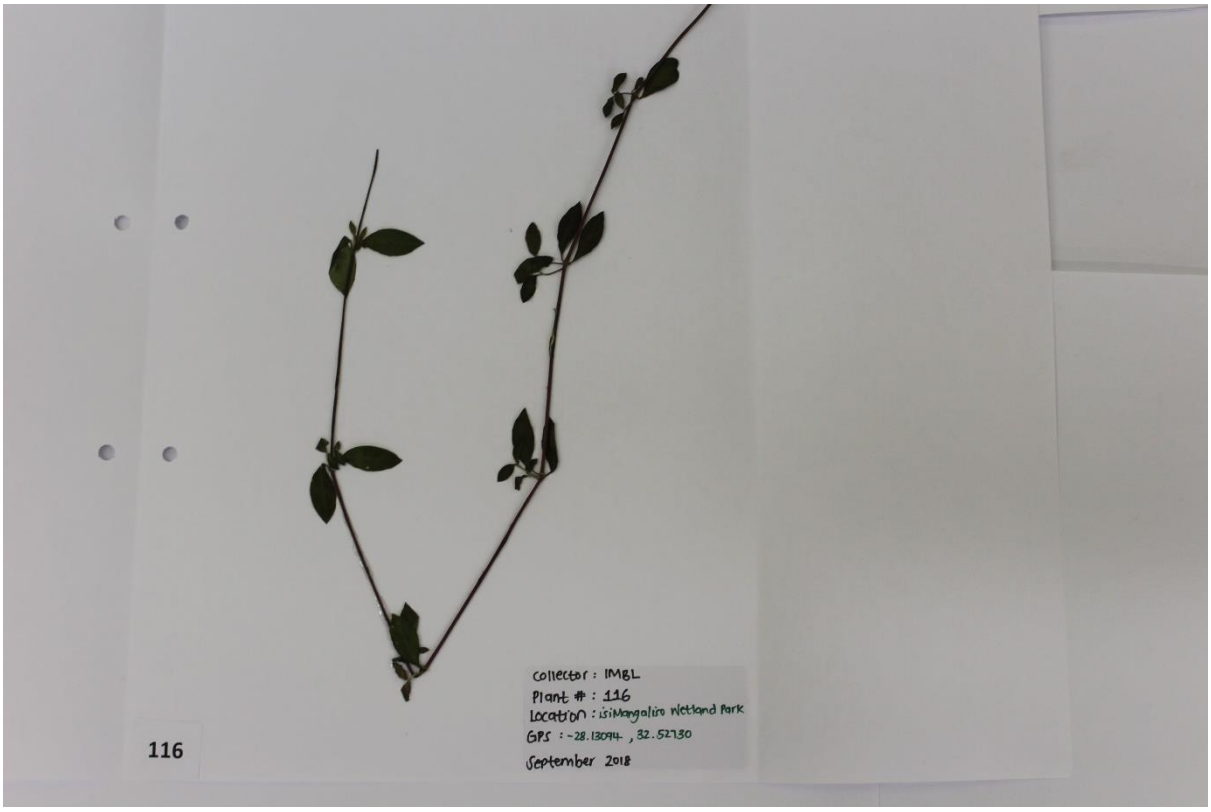
105- *Ochna natalitia*
Family: Ochnaceae



113- Identification pending



116- Identification pending

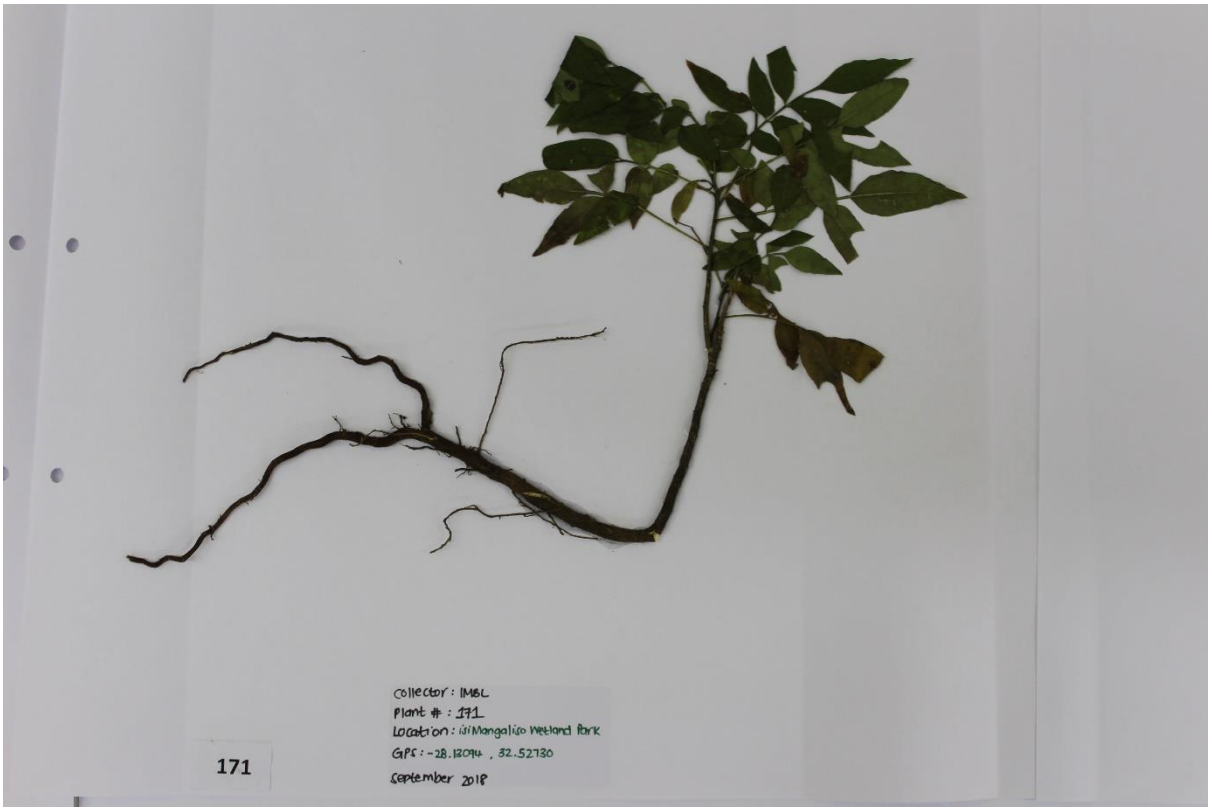


142- *Fabaceae sp*

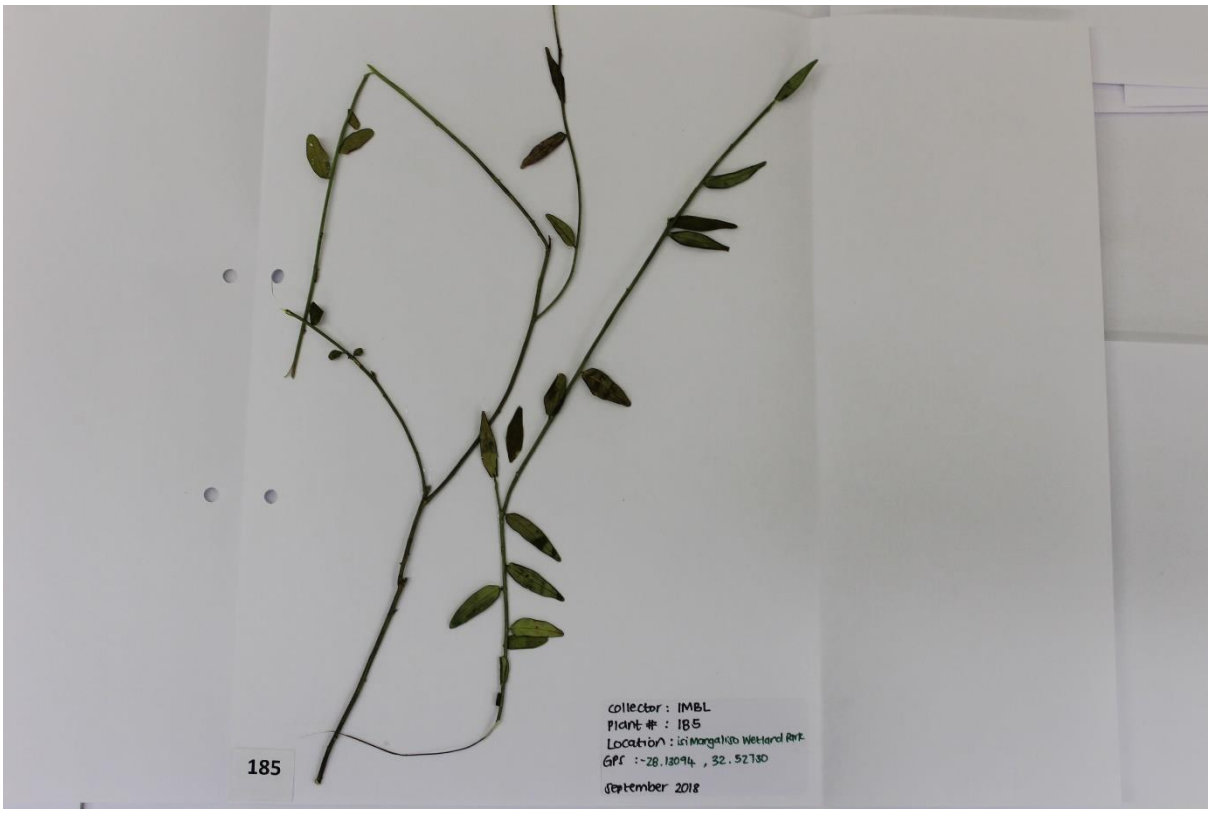
Family: Fabaceae



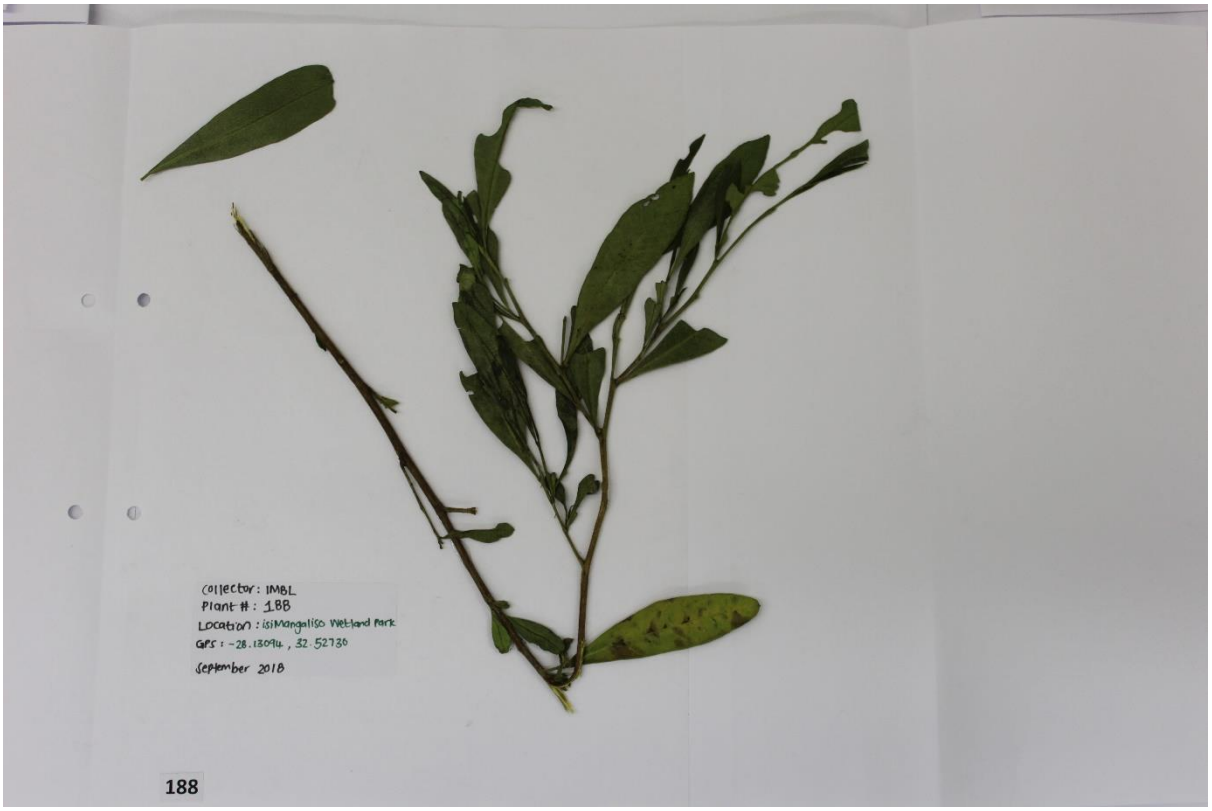
171- Identification pending



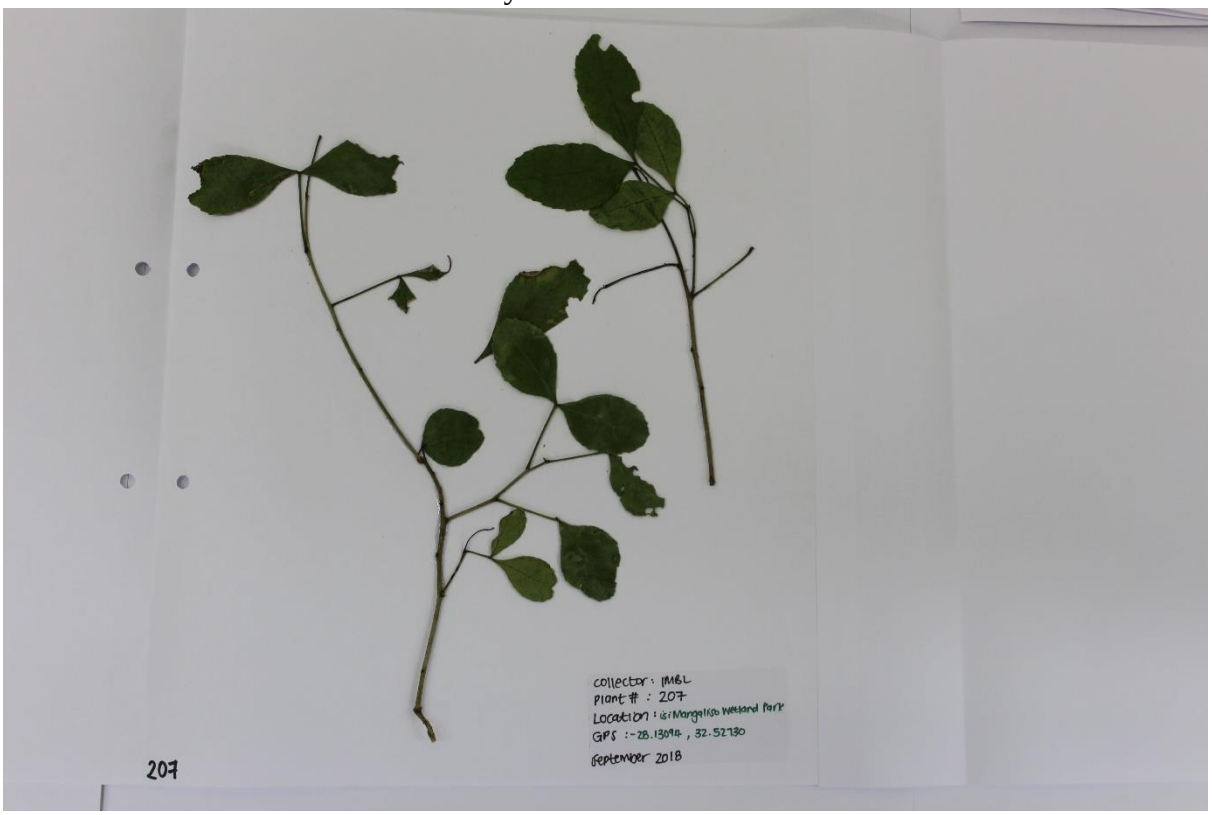
185- Identification pending



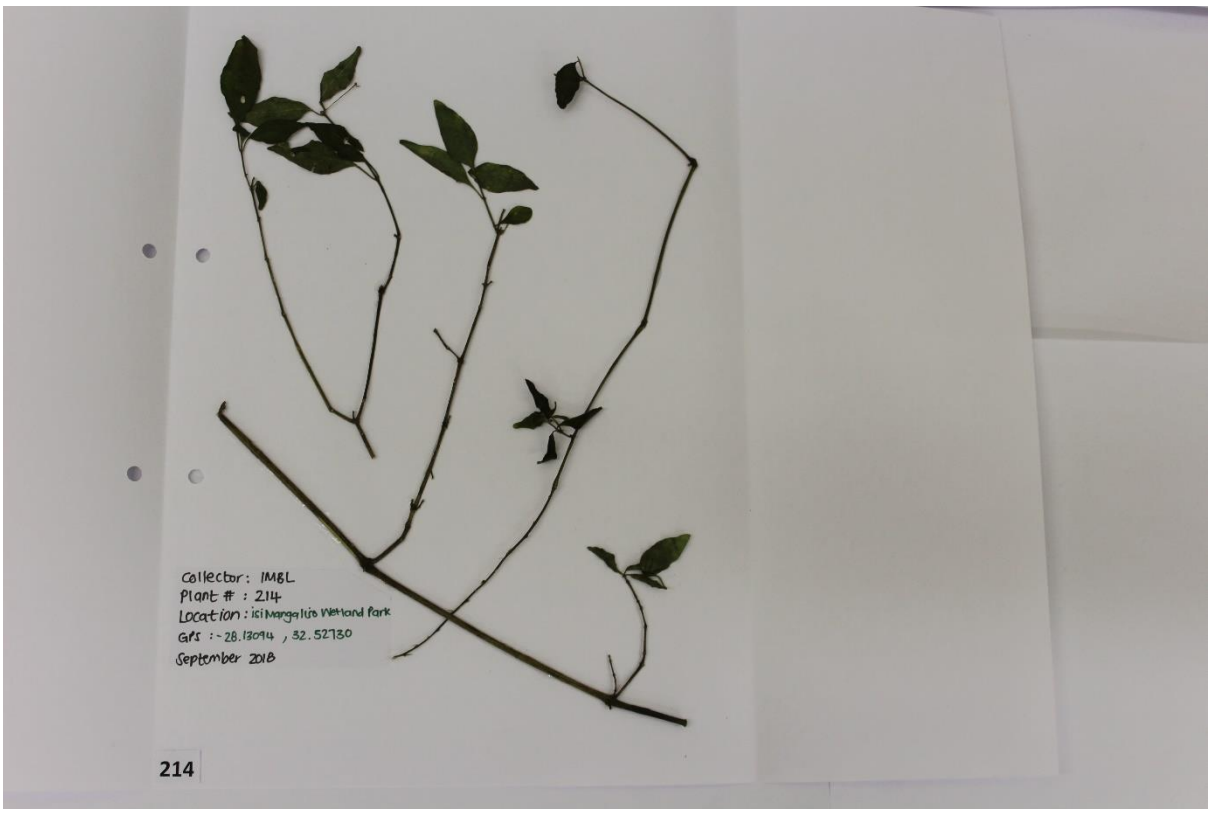
188- Identification pending



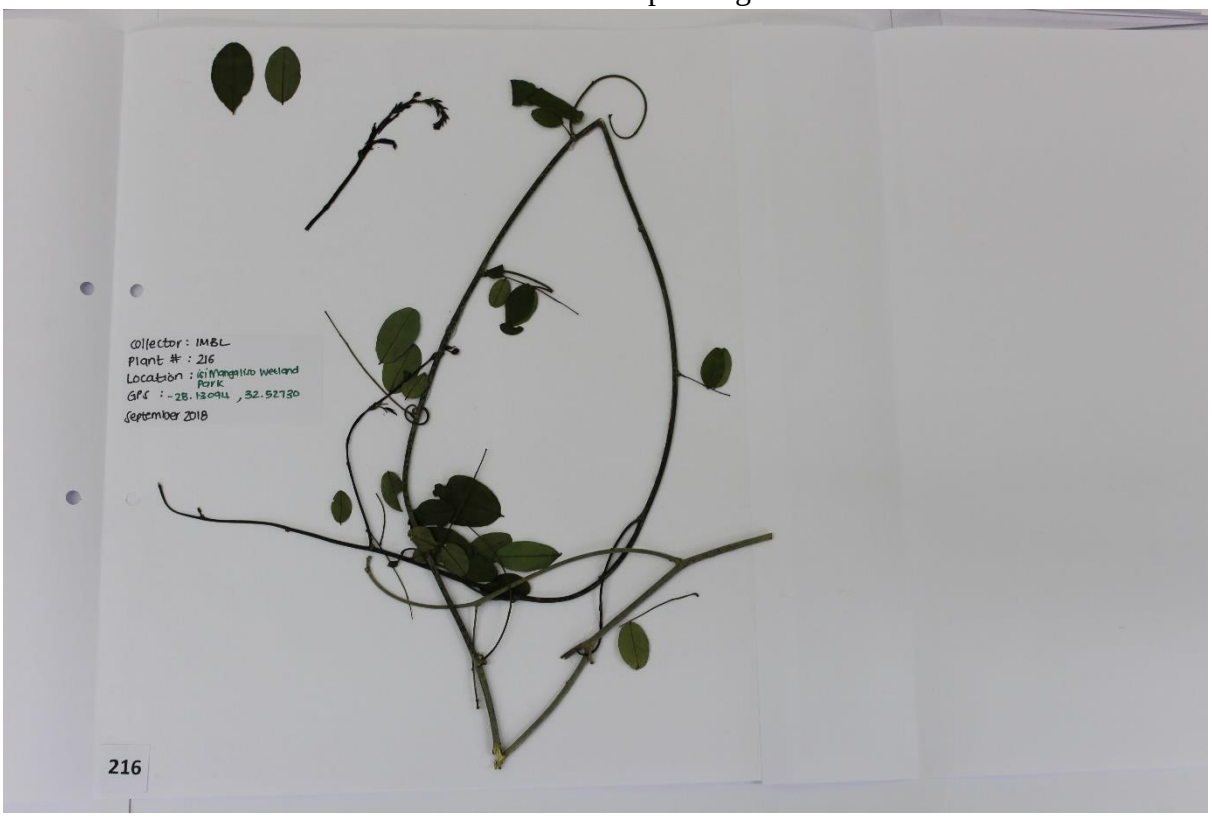
207- *Searsia sp.*
Family: Anacardiaceae



214- Identification pending

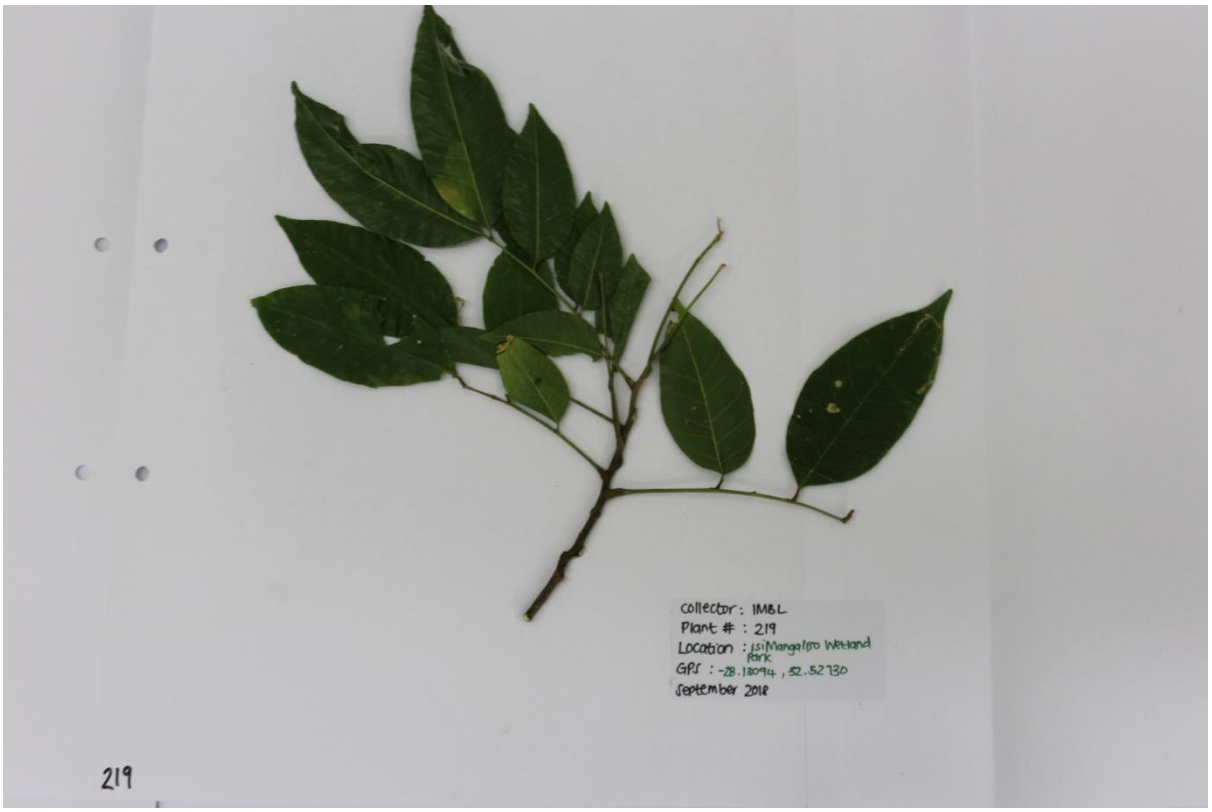


216- Identification pending



219- *Cola sp.*

Family: Malvaceae



263- *Fabaceae sp.*

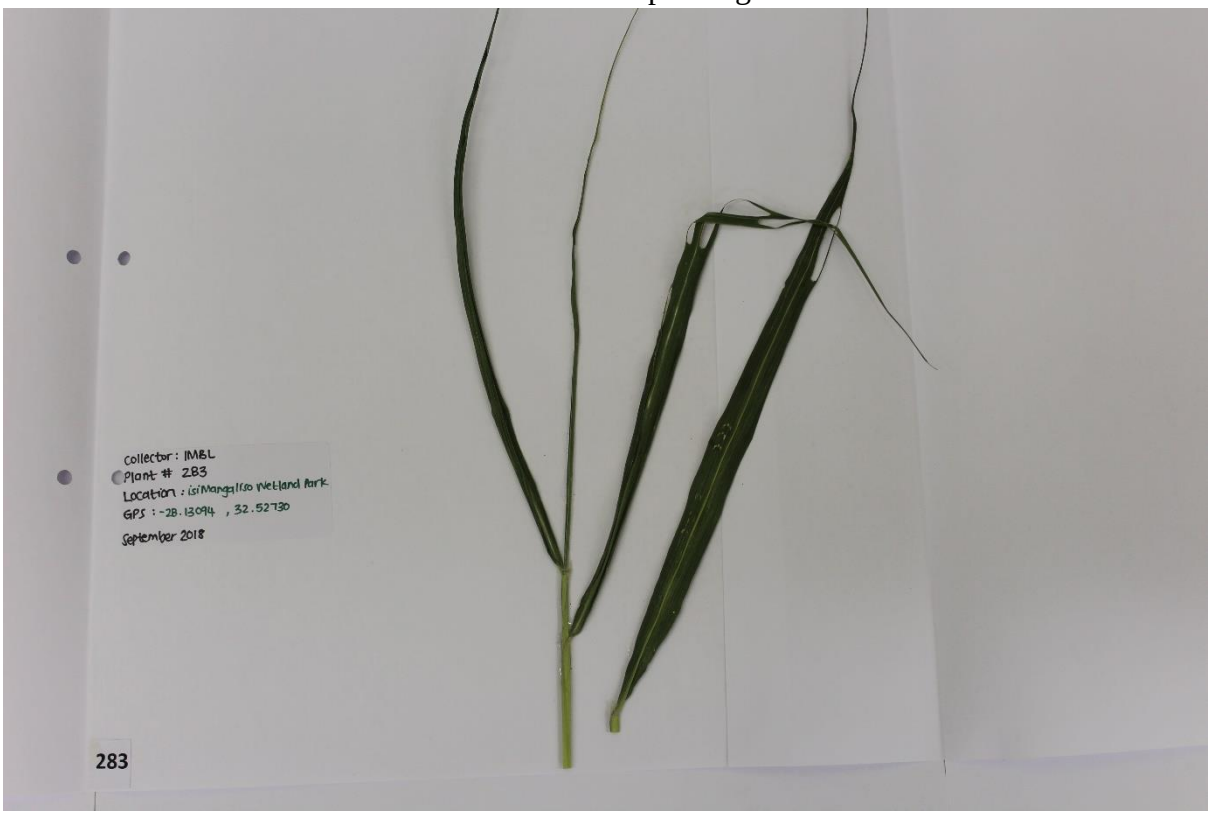
Family: Fabaceae



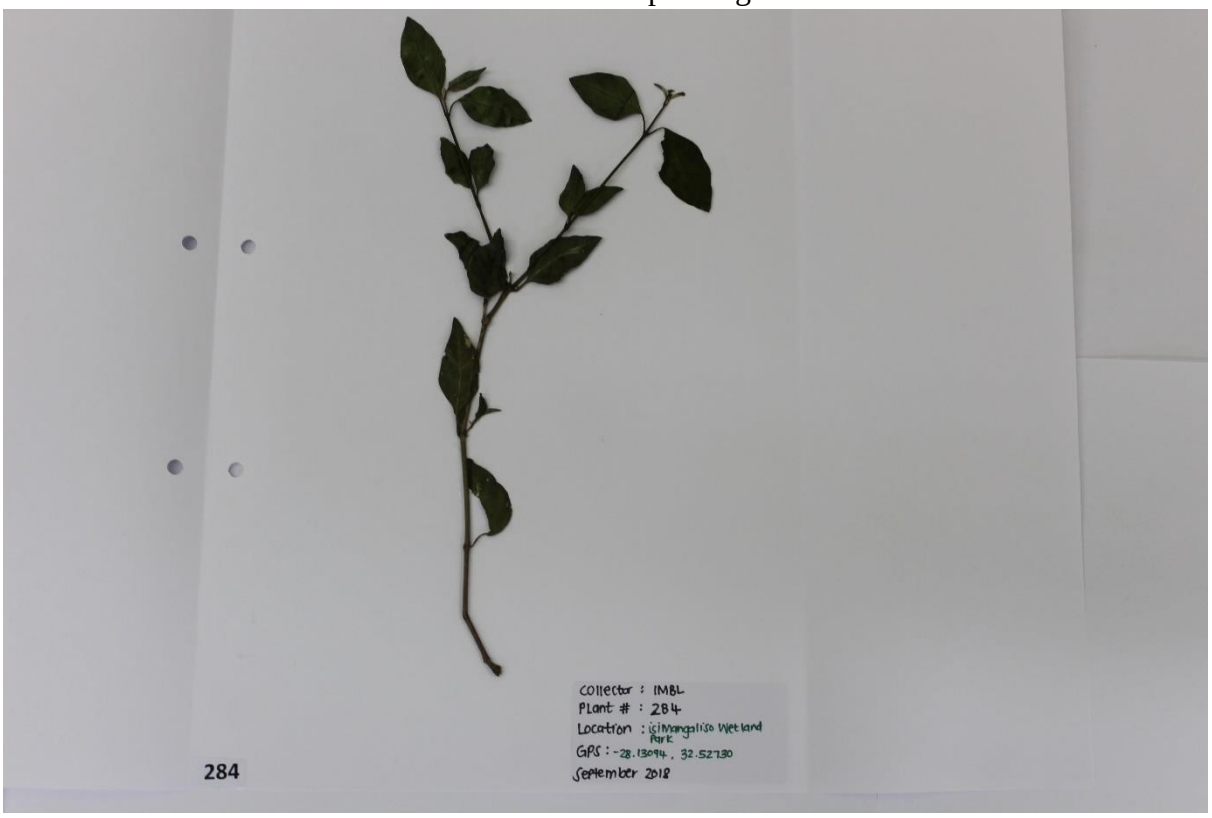
267- *Fabaceae sp*.
Family: Fabaceae



283- Identification pending



284- Identification pending



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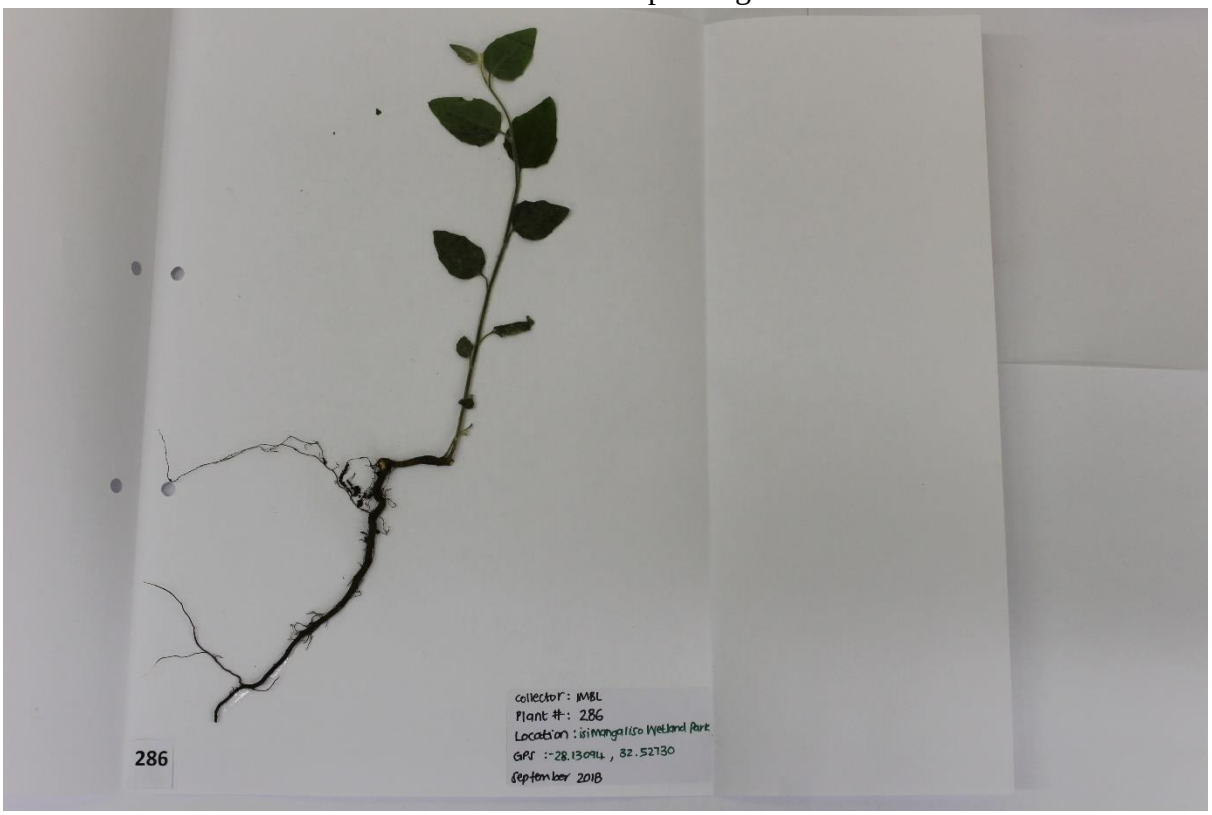
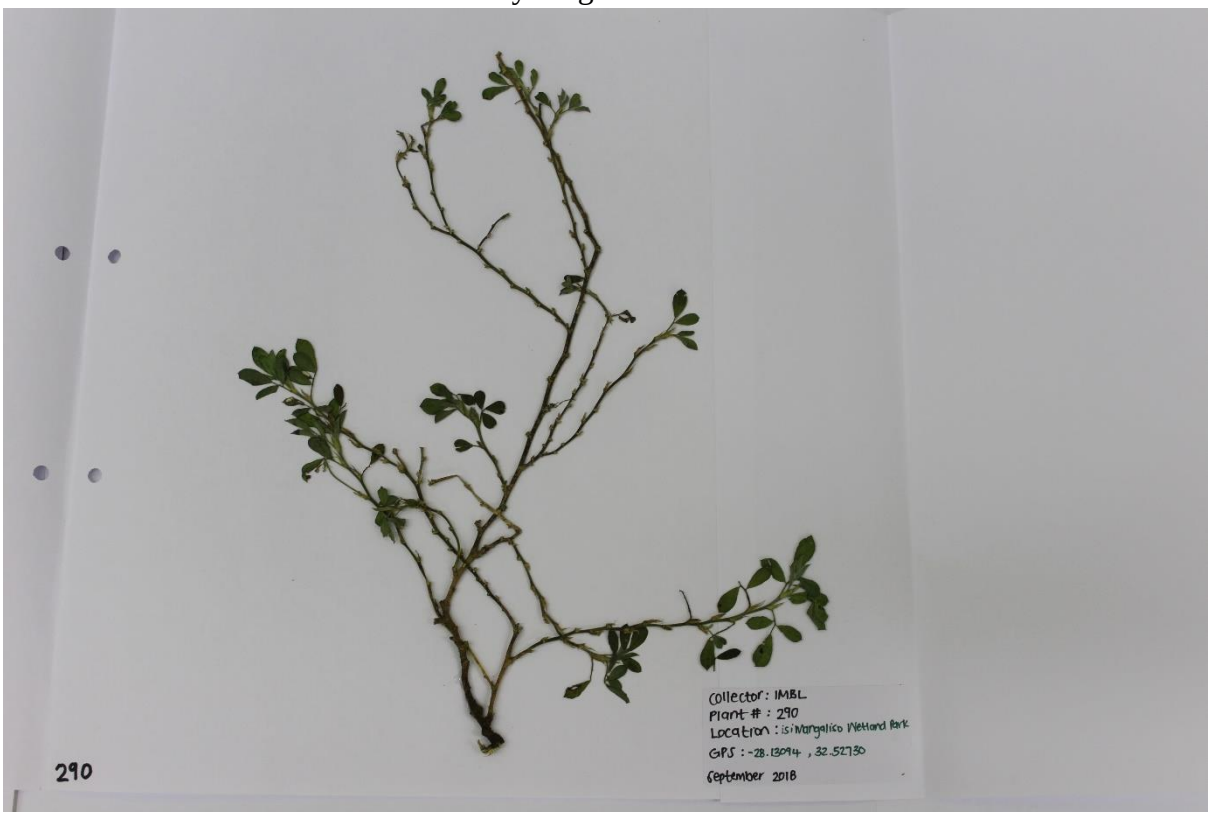
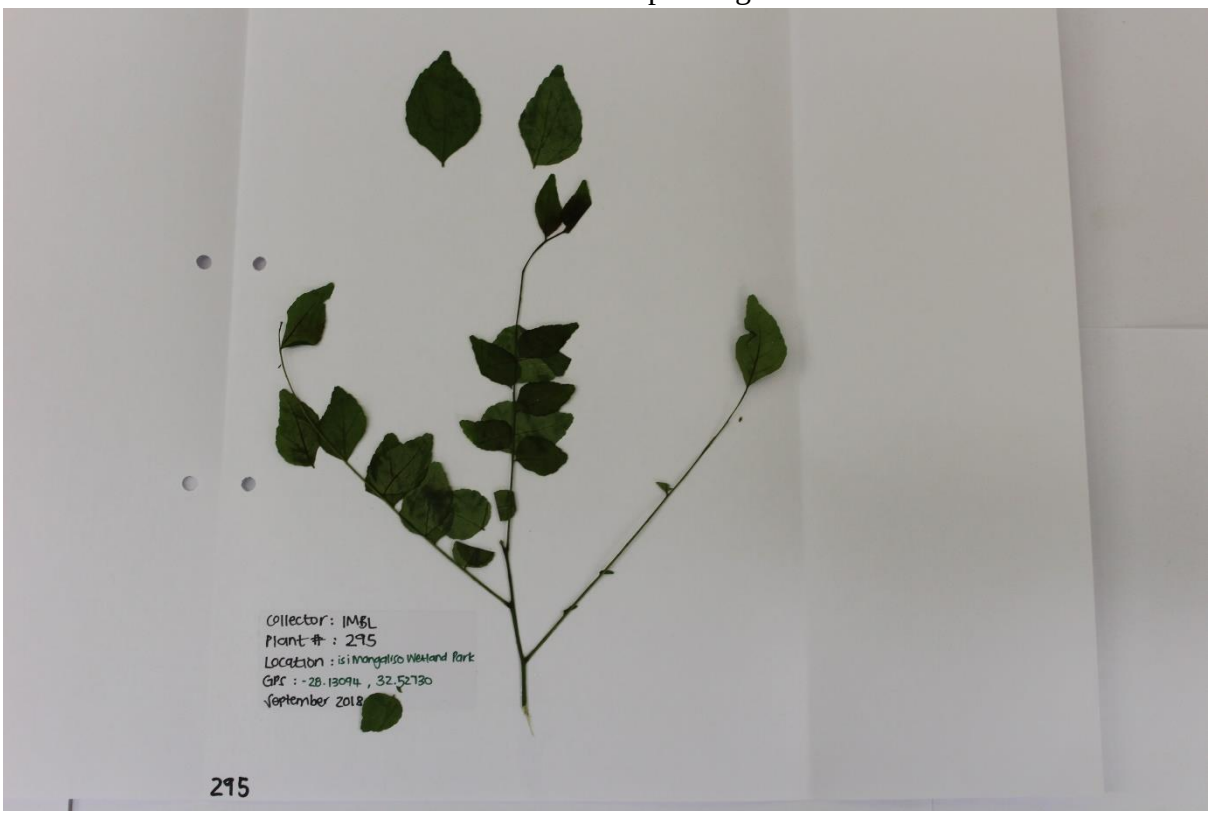


290- *Indigofera oxalidea*
Family: Leguminosae



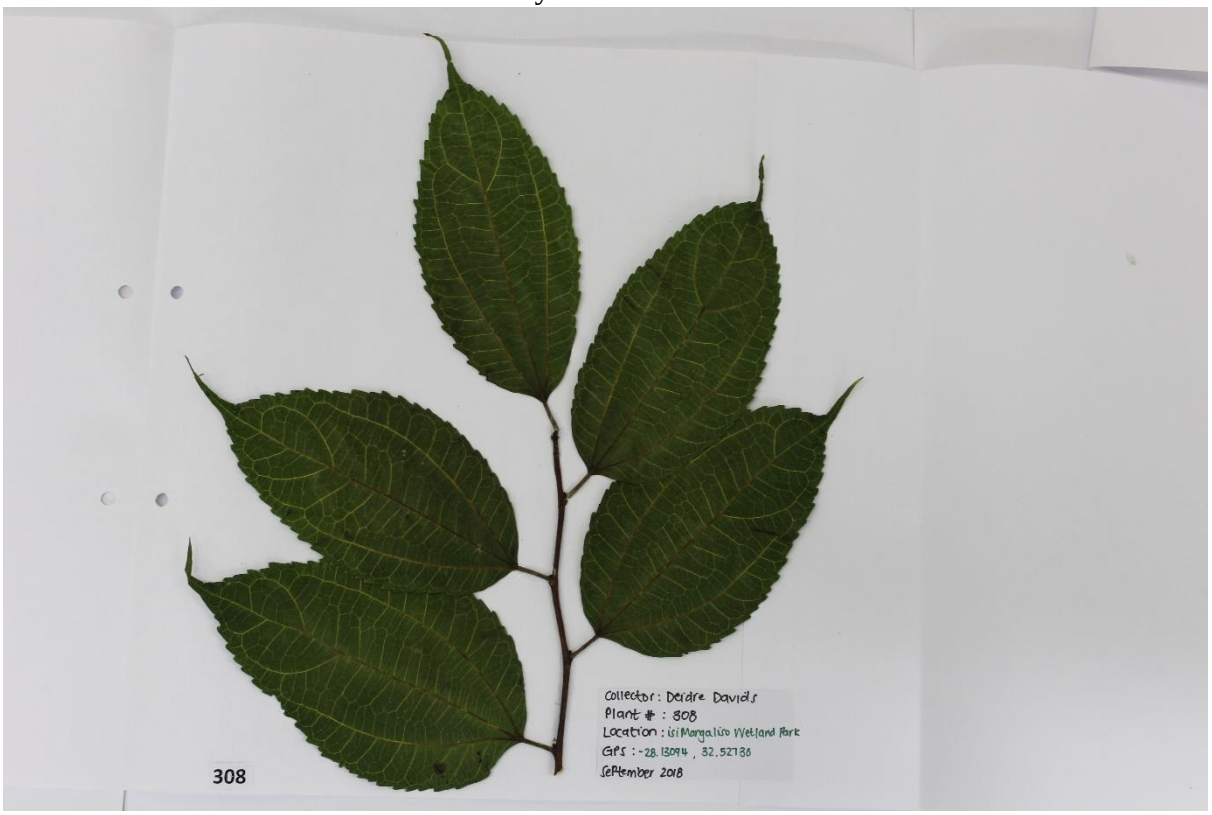
295- Identification pending



302- Identification pending



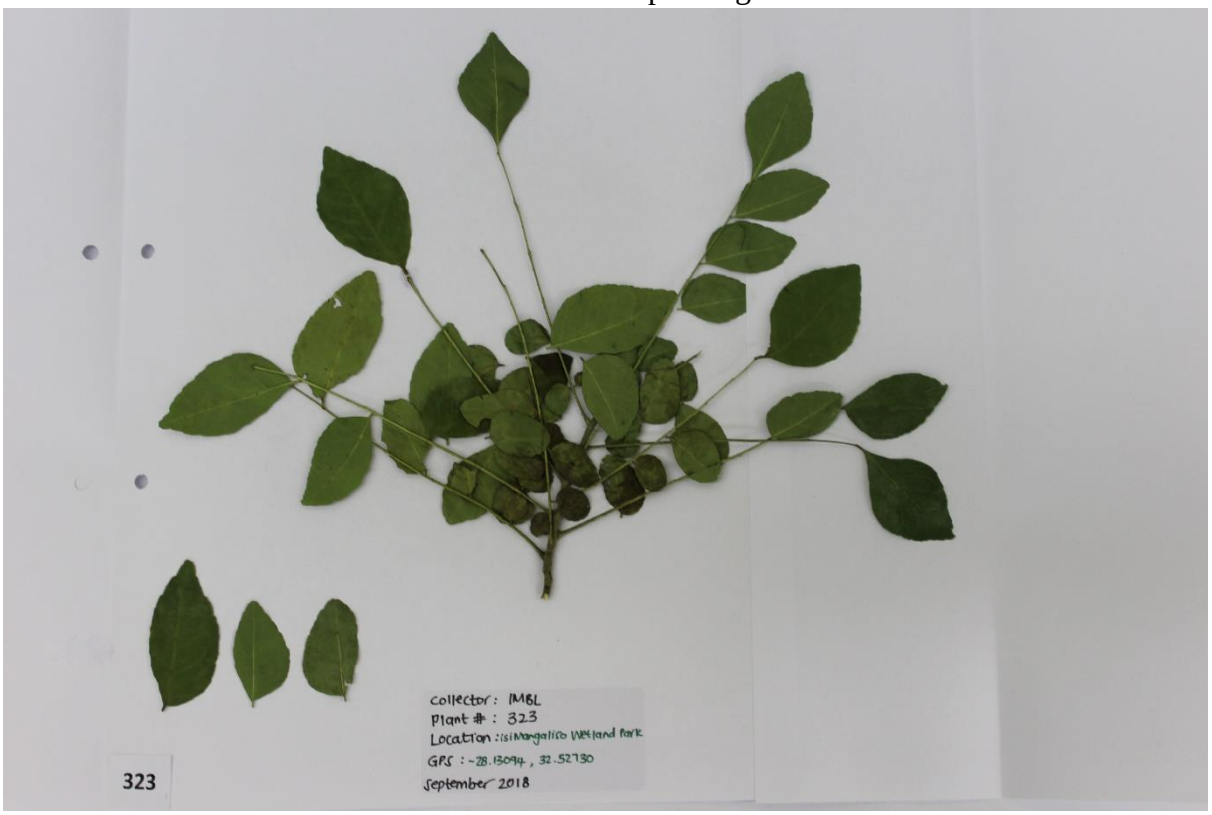
308- *Morus mesozygia*
Family: Moraceae



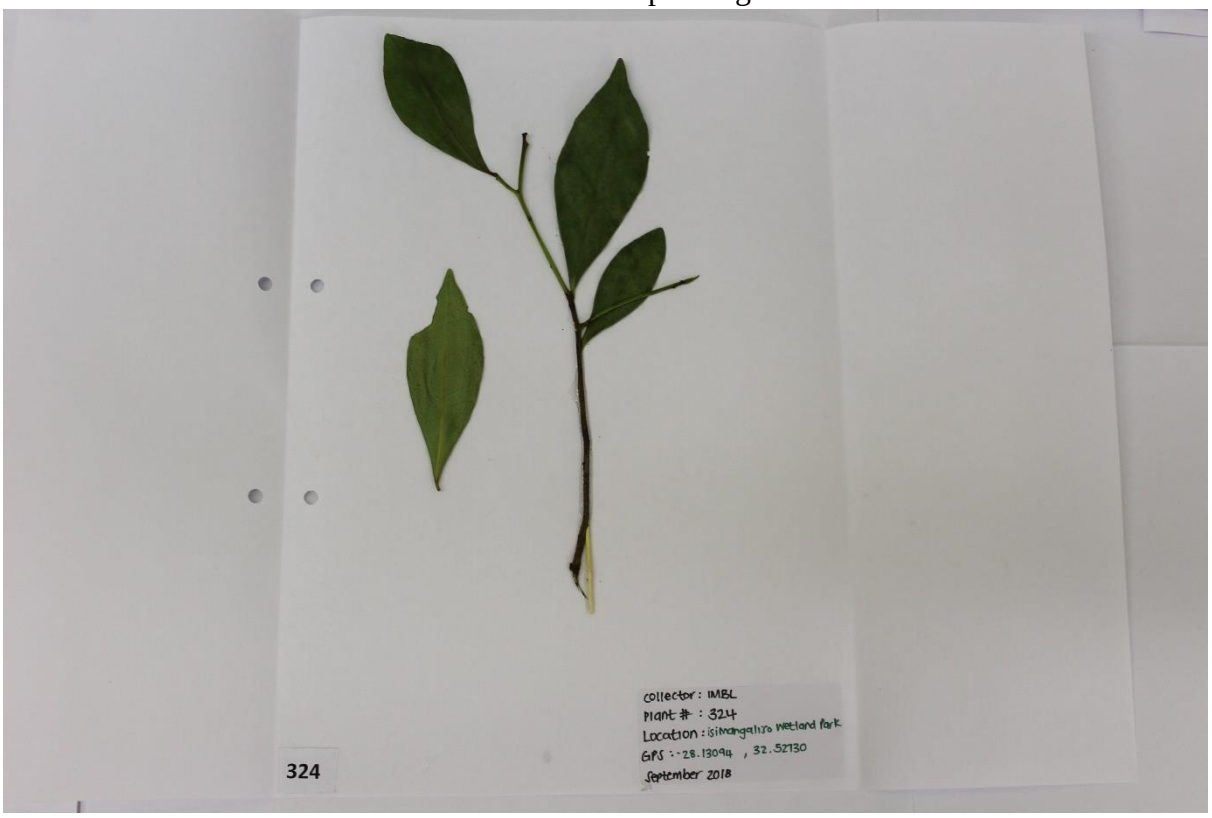
318- Identification pending



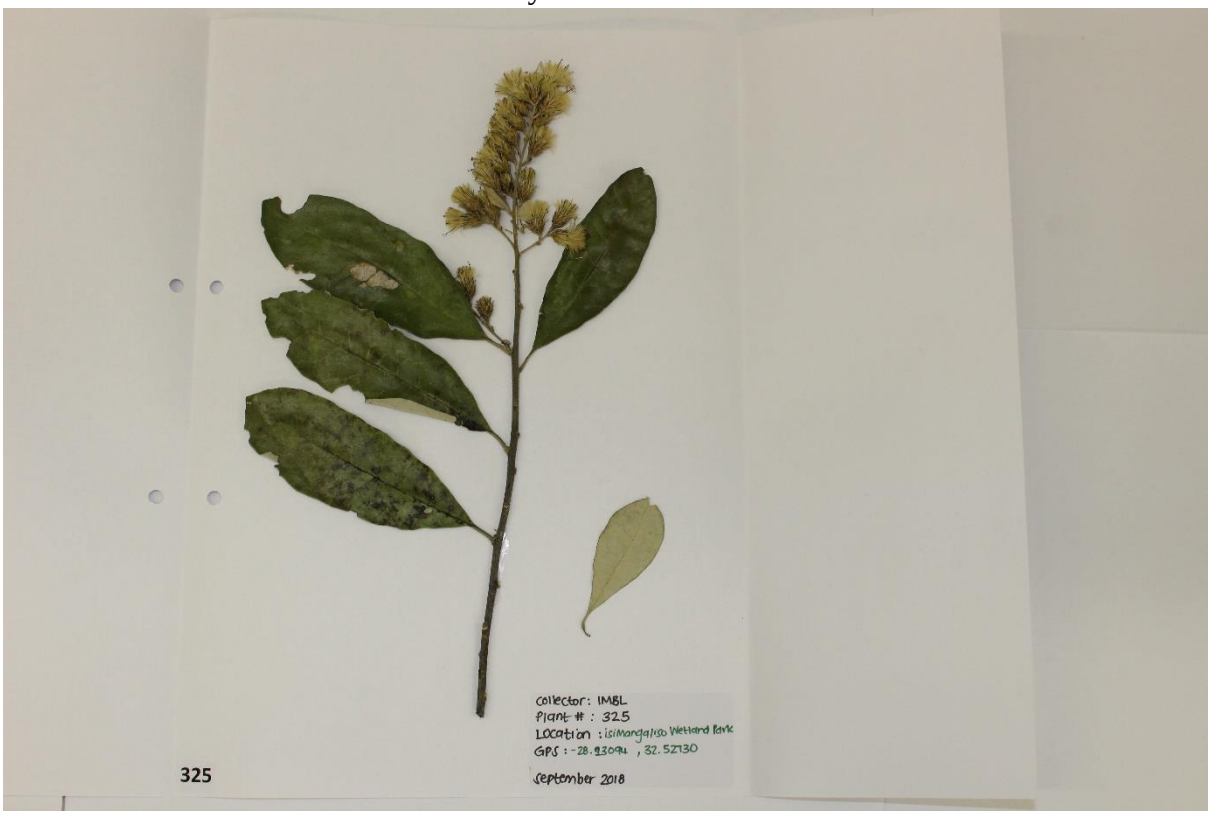
323- Identification pending



324- Identification pending



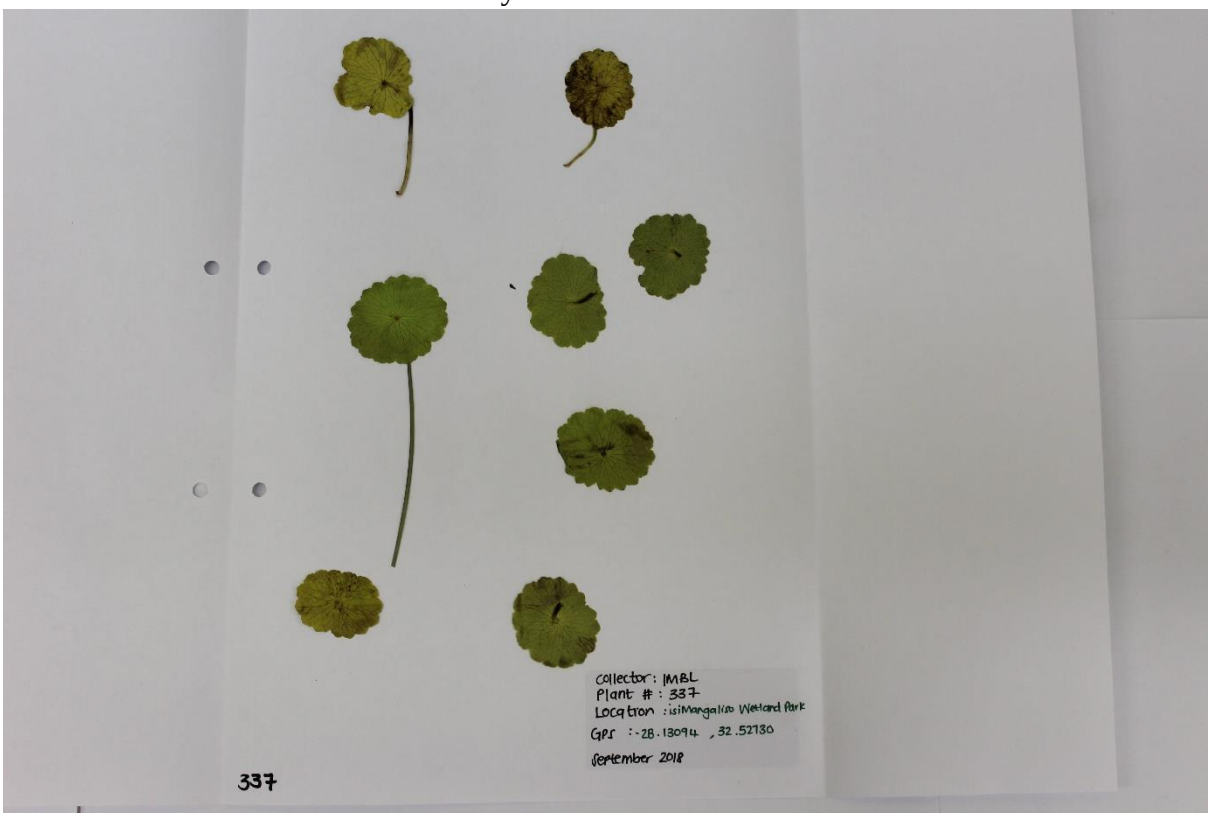
325- *Brachylaena discolor*
Family: Asteraceae



336- *Microsorum scolopendria*
Family: Polypodiaceae



337- *Ranunculus Dregei*
Family: Ranunculaceae



338- *Solanum sp.*
Family: Solanaceae



343- *Clausena anisate*
Family: Rutaceae



349- *Tragia sp.*
Family: Euphorbiaceae



350- *Cheilanthes viridis*
Family: Pteridaceae



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