HYDROXYNITRILE LYASE ACTIVITY IN NON-COMMERCIALISED PLANTS

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A dissertation submitted to the School of Molecular and Cell Biology, Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 2015

DECLARATION

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ABSTRACT

Cyanogenesis is defined as the hydroxynitrile lyase catalysed release of a cyanide group in the form of hydrogen cyanide and the corresponding aldehyde or ketone. When a plant is attacked, the hydrogen cyanide released is a self-defence mechanism. A special characteristic of enzymatic reactions is that all enzymatic reactions are reversible - hydroxynitrile lyases can also be used for the synthesis of enantiomerically pure cyanohydrins which are of great importance in industry. In this study, the hydroxynitrile lyase activity of 102 plants from 41 families was investigated, first by screening for cyanogenic activity, followed by a hydroxynitrile lyase activity assay. Six plants were found to be cyanogenic and exhibited specific hydroxynitrile lyase activity: *Adenia* sp. (0.44 U/mg), *Adenia firingalavensis* (2.88 U/mg), *Adenia fruticosa* (1.99 U/mg), *Adenia pechuelii* (2.35 U/mg), *Heywoodia lucens* (1.76 U/mg) and *Ximenia caffra* (1.84 U/mg). This is the first report of hydroxynitrile lyase activity in these plants.

RESEARCH OUTPUT

Kassim, M.A., Rumbold, K. (2014) HCN production and hydroxynitrile lyase: a natural activity in plants and a renewed biotechnological interest, *Biotechnology Letters*, vol. 36, pp. 223-228.

Kassim, M.A., Sooklal, S.A., Archer, R., Rumbold, K. (2014) Screening for hydroxynitrile lyase activity in non-commercialised plants, *South African Journal of Botany*, vol. 93, pp. 9-13.

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks and gratitude to:

My Supervisor, Dr. Karl Rumbold for all his expertise and constructive criticism throughout the course of this project,

Dr. Robert Archer of the South African National Biodiversity Institute for providing the plant material used during the course of this project,

Prof. Andreas Stolz of the Institute for Microbiology at the University of Stuttgart (Stuttgart, Germany) for his warm hospitality and teaching me the essential skills needed for enzyme study,

Prof. Rob Veale for granting me a staff bursary and Prof. Heini Dirr for his much appreciated assistance and guidance,

Staff and students - especially Mr. Ntutu Letseka at the School of Molecular and Cell Biology, University of the Witwatersrand for their assistance,

My partner Kevin Slaney, my family and my dear friend Andrew Mahas for their support, advice and encouragement throughout this project.

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LIST OF ABBREVIATIONS

BSA-Bovine Serum Albumin
cDNA-complementary Deoxyribonucleic Acid
mRNA-messenger Ribonucleic Acid
SANBI-South African National Biodiversity Institute

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Chapter One:

Introduction

1. INTRODUCTION

1.1 Cyanogenesis

Cyanogenesis is defined as the hydroxynitrile lyase mediated release of hydrogen cyanide and an aldehyde or ketone from a cyanohydrin (Sharma *et al.*, 2005). This two step process essentially involves two enzymes. In the first step, cyanogenic glycosides in the plant are broken down by β-glycosidase, to form glucose and a cyanohydrin. In the second step, this cyanohydrin is broken down by a hydroxynitrile lyase into hydrogen cyanide and an aldehyde or ketone (Zagrobelny *et al.*, 2008). A notorious and deadly poison, hydrogen cyanide exerts its toxic effects by binding to the ferric ion of cytochrome oxidase, an enzyme responsible for 90 % of the total oxygen uptake in most cells. Inactivation of this enzyme completely inhibits cellular oxygen utilisation and results in cytotoxic hypoxia and death (Erdman, 2003).

This ingenious self-defence mechanism protects the plant from microbial, fungal and animal attack and exists in over 3,000 plant species (Conn, 1981; Peterson *et al.*, 2000). Of these 3,000 species, cyanogenic plants are more commonly found in the following families: Linaceae, Gramineae, Euphorbiaceae, Clusiaceae, Olacaceae, Rosaceae, Passifloraceae and Filitaceae (Sharma *et al.*, 2005; Ueatrongchit *et al.*, 2010). Several important crop plants such as sorghum, maize, wheat, sugarcane, and, cassava, which is a staple food crop for most of sub-Saharan Africa are also known to be cyanogenic (Conn, 1981; Jones, 1998; Fokunang *et al.*, 2001). Perhaps our ancestors chose to grow those plants on a large scale as a staple food source because of their built in pest control mechanism which naturally protects them from voracious insect feeders and bacterial or fungal infection.

Apart from plants, a wide range of organisms such as fungi, bacteria, lichen, millipedes, arthropods and insects are also cyanogenic (Zagrobelny *et al.*, 2008).

In an interesting article by Kremer and Souissi (2001), an extraordinary method of making use of cyanogenic bacteria is revealed: hydrogen cyanide can also be used as an effective herbicide to inhibit the growth of weed seedlings. In their article, the hydrogen cyanide produced naturally by certain cyanogenic rhizobacteria strains occurring naturally on plant roots effectively inhibited the plants growth. These cyanogenic rhizobacteria, which are found naturally in the rhizosphere, show promise as an environmentally friendly, biological control method for weeds, a welcome alternative to the toxic herbicides in current use. Further research in this area is therefore welcome.

It is interesting though to note that at lower concentrations, cyanide is not toxic to a plant and does not inhibit respiration but it does still inhibit root hair development and germination of seeds. However, at higher concentrations, cyanide can inhibit respiration, production of adenosine triphosphate and other cellular processes such as ion uptake or phloem translocation which are dependent on adenosine triphosphate (Eisler, 2007; Garcia *et al.*, 2010).

Blom and co-workers (2011) found that volatile-mediated phytotoxicity involved oxidative stress and concluded that high concentrations of hydrogen cyanide produced by bacteria can kill plants.

1.2 Hydroxynitrile lyase

Since the late 90's, hydroxynitrile lyases have been receiving increasing attention from the fine chemical and pharmaceutical industries (Wajant and Mundry, 1993; Effenberger *et al.*, 1995; Effenberger and Jager, 1997). In nature, a cyanohydrin is broken down by a hydroxynitrile lyase into hydrogen cyanide and an aldehyde or ketone. However, in principle, each enzymatic reaction is completely reversible, and it is this reversible reaction (Fig 1.1) which is so desired by industry.

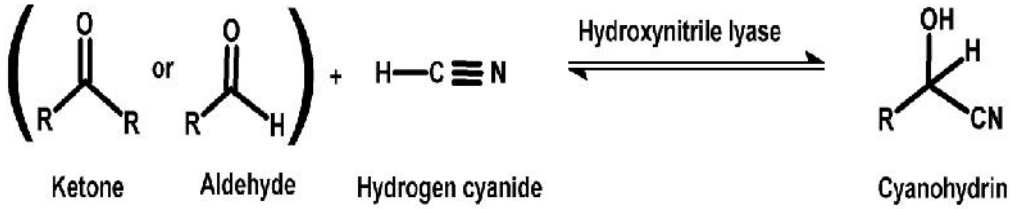

Figure 1.1 General reaction catalysed by hydroxynitrile lyase

The remarkable ability of these enzymes to produce a chiral cyanohydrin by the condensation of hydrogen cyanide with an aldehyde or ketone is quite advantageous, especially to the pharmaceutical and fine chemical industries. These chiral cyanohydrins are used as building blocks/intermediates for the production of pesticides, medicines, agrochemicals, etc (Dadashipour *et al.,* 2011). Hydroxynitrile lyase can also be used to detoxify cyanogenic food plants, especially cassava, which forms the staple diet in sub-Saharan Africa (Hasslacher *et al.,* 1996; Fokunang *et al.,* 2001).

1.3 Sources of hydroxynitrile lyase

The first discovery of hydroxynitrile lyase occurred in 1837, by two German chemists- Justus von Liebig and Friedrich Wohler. This was found in a bitter almond extract (Hosel, 1981).

Since then, more recent discoveries have been made, for example, in 1988, Xu and co-workers purified and characterised a hydroxynitrile lyase from *Linum usitatissimum* (commonly known as Flax).

Hughes and co-workers (1994) purified and characterised a hydroxynitrile lyase from cassava (*Manihot esculenta* Crantz). This enzyme was not glycosylated and did not contain a flavin group, and displayed activity against two natural substrates - acetone cyanohydrin and 2-butanone cyanohydrin.

Wajant and Forster (1996) purified and characterised a hydroxynitrile lyase from the rubber tree *Hevea brasiliensis*. This enzyme also showed activity toward acetone cyanohydrin. In the interim, several other hydroxynitrile lyases were discovered from *Prunus serotina, Prunus lyonii, Prunus capuli, Prunus amygdalus, Mammea americana, Malus communis, Phlebodium aureum, Sorghum bicolour, Sorghum vulgare, Ximenia americana* and *Sambucus nigra* (Sharma *et al.*, 2005). Most of the enzymes from these plants showed activity toward mandelonitrile and acetone cyanohydrin.

In 2010, Ueatrongchit and co-workers discovered, characterised, and purified a hydroxynitrile lyase from *Passiflora edulis* (passion fruit), a member of the Passifloraceae family, for the first time. The optimum pH for this enzyme was pH 4, and the optimum temperature was 10 ºC. They also found that the spontaneous non-enzymatic reaction which yields a racemic product was almost completely suppressed at the optimum pH and temperature.

In 2011, Dadashipour and co-workers discovered a novel hydroxynitrile lyase from *Baliospermum montanum*. This enzyme exhibited excellent pH stability in a wide pH range, showed activity in a wide temperature range (perfect for industrial applications) and showed activity toward aromatic aldehydes.

Also in 2011, Fukuta and co-workers discovered a new hydroxynitrile lyase from the Japanese apricot *Prunus mume*. It was found to be active over a broad pH and temperature range, and its amino acid sequence was found to be identical to the hydroxynitrile lyase from *Prunus dulcis*.

Despite the numerous discoveries made, the application of these naturally occurring enzymes in enantioselective biocatalytic synthesis was sadly limited due to the difficulty of obtaining a sufficient amount of enzyme. The presence and concentrations of various active secondary metabolites in a plant are also known to vary - their presence or concentration depending on season and age (Pichersky and Lewinsohn, 2011). A plant extract was therefore not a suitable enzyme source for the development of an industrial biocatalytic process of cyanohydrins.

1.4 Recombinant hydroxynitrile lyase

Many industrial and pharmaceutical production processes, which in the past were considered not feasible due to high production costs and limited amounts of starting material such as enzymes and other valuable compounds, are now feasible - thanks to biotechnology. Biotechnology/recombinant DNA technology has equipped us with the tools necessary to mass produce, in a heterologous host, these much needed enzymes which were previously only found in plants or animals.

Using recombinant DNA technology: cyanogenic plant samples are collected, their messenger ribonucleic acid (mRNA) is extracted, the gene coding for the hydroxynitrile lyase is located, cloned and over-expressed in a heterologous host/microbial expression system.

There are several advantages to using heterologous expression, the main being cost - enzymes can now be mass produced rapidly and sold at lower prices than those previously sourced from nature. Another important advantage is sustainability - 21st century industry has become more environmentally conscious - always researching cleaner, greener technologies and constantly seeking renewable and sustainable sources of enzymes and other valuable compounds (Terpe, 2006; Yesilirmak and Sayers, 2009; Chen, 2012).

Fukuta *et al.* (2011); Dadashipour *et al.* (2011); Semba *et al.* (2008a and 2008b); Breithaupt *et al.* (1999) and Hughes *et al.* (1994 and 1997) are several examples of researchers who were successful in the cloning of the hydroxynitrile lyase gene in either a bacteria or yeast species. Some found yeasts to be better hosts, producing the enzyme in an active, easily accessible form.

In 1996, Forster and co-workers reported the first recombinant hydroxynitrile lyase. The activity of this purified recombinant *Manihot esculenta* hydroxynitrile lyase (expressed in *Escherichia coli*) was reported to be 900 U/ml. Their 80 litre culture, comprising of just 150-200 g wet weight, gave a yield of 4,000 U of recombinant enzyme. They claimed that in order to obtain the same amount of enzyme from plant material, one would have required 100-200 kg of dried *Manihot esculenta* leaves. This study proved clearly the wonders of recombinant DNA technology.

Hasslacher *et al.* (1997) reported high level intracellular expression of a recombinant *Hevea brasiliensis* hydroxynitrile lyase in a microbial host. It was found that a yeast host was most suitable for protein expression, and that *Pichia pastoris* was the most efficient system. An exceptional yield of 22 g of pure enzyme per litre of culture broth was reported.

Semba *et al.* (2008a) expressed a *Manihot esculenta* hydroxynitrile lyase in a yeast and tested the application of this recombinant hydroxynitrile lyase in the production of (*S*)-mandelonitrile (a useful cyanohydrin) using an immobilised enzyme reactor. They also found that the *Manihot esculenta* hydroxynitrile lyase accepts a wider substrate range when compared with the *Sorghum bicolor* hydroxynitrile lyase, which is exactly what industry is currently searching for. Semba and co-workers (2008a) reported that productivity in *E. coli* was quite low, so they then decided to use a yeast, *Saccharomyces cerevisiae*. The yeast host expressed a recombinant hydroxynitrile lyase with a specific activity of 3.47 U/mg of soluble protein.

They also tested (*S*)-mandelonitrile production using the immobilised recombinant *Manihot esculenta* hydroxynitrile lyase. After testing a variety of supports, they found that inorganic supports showed better adsorption ability than organic supports. Silica gel was found to be the best - the enzyme immobilised on silica gel was successfully used for more than 20 batches and did not show any loss in conversion rate or enantioselectivity. Overall, after 22 batches, it was reported that 23.3 kg of benzaldehyde was converted to 28.6 kg of (*S*)-mandelonitrile, a useful cyanohydrin. This proved that production can be successful on a large scale for industrial purposes.

Semba *et al*. (2008b) then also experimented with the production of recombinant *Manihot esculenta* hydroxynitrile lyase using what they termed as low temperature cultivation. They found that when the temperature was reduced, the cell yield and ratio of soluble enzyme increased tremendously. The enzyme activity and yield at 17 ºC was found to be 850 times higher than those obtained at 37 ºC. Their rate of hydroxynitrile lyase production was 3,000 U/hour.

Perhaps in the future, researchers will find improvements in their yields if they test the effect of a lower temperatures in their fermentations.

Dadashipour *et al.* (2011) expressed a *Baliospermum montanum* hydroxynitrile lyase in *E. coli.* At first, the researchers experienced problems with expression at optimum temperature - having encountered a high level of inactive protein production as inclusion bodies.

They therefore decided to try a lower temperature for protein expression, as described by Semba *et al*. (2008b) and Qing *et al*. (2004), which allowed them to obtain at least a small amount of the soluble or native form of the enzyme. Aromatic aldehydes and ketones were found to be the preferred substrates for this enzyme (when compared with aliphatic substrates). The highest specific activity for this enzyme was 178 U/mg.

Fukuta *et al*. (2011) were able to express the gene coding for the hydroxynitrile lyase in *P. pastoris* as an active, highly glycosylated protein. An enzyme activity of 209 U/l of culture supernatant was measured in a cyanohydrin synthesis reaction, which corresponded to the amount of enzyme that can be isolated from 800 g of plant material. Their new enzyme was found to accept a wide range of substrates including aromatic and aliphatic carbonyl compounds.

1.5 Is the recombinant hydroxynitrile lyase as good as the native one?

A legitimate question, and rather well answered in an informative article by Hughes *et al*. (1997). The authors compared the secondary structures, specific enzyme activities, optimum temperatures, optimum pH, and isoelectric focusing of the recombinant *Manihot esculenta* enzyme with the native one. The recombinant and native enzymes were found to be similar in every way except one: isoelectric focusing. The recombinant enzyme was found to have a more basic mean isoelectric point (5.1), whilst the native enzyme was 4.5.

1.6 The search for new hydroxynitrile lyases

Although recombinant DNA technology has now provided a way to mass produce these desirable enzymes for industrial applications, the ever evolving world of pharmaceuticals and fine chemicals, the need for more environmentally friendly pesticides and herbicides, and greater environmental awareness with regard to industrial processes have now led researchers to search for hydroxynitrile lyases which have novel substrate specificity, which can lead to the manufacture of new types of medication, pesticides, etc. One of the main restrictions in the use of hydroxynitrile lyases today is their narrow substrate range and sometimes unstable behaviour under industrial conditions (Andexer *et al.,* 2009).

One solution to this is to identify multiple plants exhibiting hydroxynitrile lyase activity as there is the potential that each such plant may contain a hydroxynitrile lyase with novel substrate specificity, partially addressing the second issue. The aim of this study is to identify such new plants.

As can be seen in the previous sections, the majority of the hydroxynitrile lyases discovered to date show activity toward mandelonitrile and acetone cyanohydrin. The search for novel or improved hydroxynitrile lyases, which will hopefully have a novel or wider substrate specificity has therefore begun once again.

In 2004, Hernandez and co-workers decided to screen for hydroxynitrile lyase activity in the crude extracts of several edible plants. They reported that the seeds of *Cucumis melo, Cydonia oblonga* and *Annona cherimolia*, as well as the leaves of *Prunus persica, Prunus avium, Prunus domestica, Prunus serotina* var. *Capuli* and *Pouteria sapota* exhibited hydroxynitrile lyase activity. They were also the first to report hydroxynitrile lyase activity in the *Cucumis melo* seed. Until 2004, no hydroxynitrile lyase activity had been reported for any member of the Cucurbitaceae family.

Their crude extracts biocatalysed the transformation of benzaldehyde to mandelonitrile with varying degrees of enantioselectivity, some better than others. New alternative enzyme sources were found.

In 2005, Asano and co-workers screened for new hydroxynitrile lyases from plants using their own, in-house developed high performance liquid chromatography method, to screen for hydroxynitrile lyase activity. One hundred and sixty three plants, from 74 families, which included Rosaceae, Euphorbiaceae, Leguminosae, Passifloraceae, Araceae, and other families were tested. They discovered that partially purified (*R*)- hydroxynitrile lyases from *Passiflora edulis* and *Eriobotrya japonica* showed activity toward benzaldehyde and an aliphatic ketone. This proves that

hydroxynitrile lyases with varying substrate specificities are out there, and makes a great incentive to search for more.

Andexer *et al*. (2009) suggests the following two methods or strategies: the first being a functions based or activity based approach, and the second a sequence based approach. The first approach involves selecting a plant from a family which is known to contain cyanogenic species, and then screening their crude/tissue extracts for the desired activity. The second involves synthesising double stranded complimentary deoxyribonucleic acid (cDNA) from the plant's mRNA, sequencing the double stranded cDNA and then scanning this sequence (using various databases) for a sequence similar to available, known hydroxynitrile lyase sequences. The similar sequence can then be synthesised and cloned into a microbial host using recombinant DNA technology for over-expression.

However, a major limitation of the sequence based approach is that an undiscovered or novel hydroxynitrile lyase could be very easily missed, especially if one is only comparing a plant's cDNA sequence to those of already discovered enzymes.

Wajant and Forster, in 1996, found that there were no obvious sequence homologies among *Prunus serotina* hydroxynitrile lyase, *Manihot esculenta* hydroxynitrile lyase or the *Sorghum bicolor* hydroxynitrile lyase. Therefore, trying to look for a similar sequence will not be an ideal approach. As part of the renewed interest, two manuscripts offering alternative screening methods, one novel, and the other a modern take on a classical method are of particular interest and worth making note of:

Krammer *et al.* (2007) successfully developed a novel hydroxynitrile lyase screening assay, concentrating specifically on its application in high-throughput screening of *E. coli* mutant libraries. This method can also be used to discover improved hydroxynitrile lyase variants and makes use of a substrate.

Only colonies carrying the gene coding for the desired enzyme will be able to break down the substrate, resulting in a visible colorimetric reaction when a by-product is detected, for example, when the hydrogen cyanide produced is detected, the test paper will turn blue, as in the case of the hydroxynitrile lyase enzyme.

Takos *et al.* (2010) developed a cyanogenic screening test for plants. This method is based on the 1966 method by Feigl and Anger and makes use of a single freeze-thaw tissue cycle. A visible colorimetric reaction is produced on a specially prepared filter paper upon detection of hydrogen cyanide (a concise blue spot), as used by Krammer *et al.* (2007) as well. This method allows for the screening of plant samples for cyanogenic activity on a rapid and large scale (up to 96 samples or more at one time).

1.7 Plants used in this study

Non-commercialised plants were selected from the following families: Acanthaceae, Aizoaceae, Anacardiaceae, Apocynaceae, Asclepiadaceae, Asparagaceae, Asteraceae, Boraginaceae, Cactaceae, Cannabaceae, Celastraceae, Crassulaceae, Cucurbitaceae, Cycadaceae, Dichapetalaceae, Didiereaceae, Dioscoreaceae, Ebenaceae, Euphorbiaceae, Fabaceae, Geraniaceae, Gesneriaceae, Hernandiaceae, Icacinaceae, Juncaceae, Lamiaceae, Malvaceae, Meliaceae, Menispermaceae, Moringaceae, Olacaceae, Pandanaceae, Passifloraceae, Pedaliaceae, Phyllanthaceae, Polygalaceae, Pteridaceae, Rosaceae, Rubiaceae, Solanaceae and Zamiaceae.

The majority of the plants tested were from the Euphorbiaceae family as two of the most well known hydroxynitrile lyases are found in plants (*Hevea brasiliensis* and *Manihot esculenta*) from that family. Cyanogenesis is also prominent in the Olacaceae and Rosaceae families (Sharma *et al.*, 2005).

Chapter Two:

Materials and Methods

2. MATERIALS AND METHODS

This section describes the methods that were used to investigate plants that could contain a hydroxynitrile lyase. The experiments initially involved screening for cyanogenic activity using a specially prepared cyanide detection paper. Further analyses on those plants which exhibited cyanogenic activity involved extraction of the crude enzyme and finally measuring hydroxynitrile lyase activity spectrophotometrically.

The plants used in the study were selected based on the information gathered from reviewing literature. A number of the plants were selected from families which are known to contain cyanogenic plants and from which hydroxynitrile lyases have been extracted for commercial use.

All the reagents used were of analytical grade and were purchased from Sigma Aldrich (Germany) and Merck (Darmstadt, Germany).

2.1 Plant identification and collection

The 102 plant species, comprising 41 families used in this study were identified by a botanist, Dr. Robert Archer of the South African National Biodiversity Institute (SANBI) using taxonomic keys. Apical buds from each plant were collected in triplicate from the Pretoria National Botanical Gardens (National Herbarium, SANBI). Plants were selected according to the following criteria - plants which are non-commercialised but readily available, plants taxonomically related to other plants which contain hydroxynitrile lyase activity, and, plants from families in which the presence of cyanogenic activity has been well established (Hernandez *et al.,* 2004).

The plants were photographed at the time of collection and brought to the laboratory in microtitre plates. The microtitre plates containing the samples were kept on ice at all times during sample collection to prevent wilting and were stored at –80°C within 4 hours of collection. Pertinent details of each of the plants i.e., the scientific and family names, are listed in Table 2.1.

Table 2.1 Plants used in this study

Family	Species
Acanthaceae	*Barleria obtusisepala* C.B. Clarke
Aizoaceae	*Ruschia* sp. C.f. *indurata,* L.c. *Trichodiadema* sp.
Anacardiaceae	*Searsia lancea* (L.F) F.A. Barkley
Apocynaceae	*Orbea melanantha* (Schltr.) Bruyns, *Pachypodium namaquensis* (Wyley ex Harv.) Welw., *Pachypodium lamerei* Drake, *Strophanthus amboensis* (Schinz) Engl. & Pax, *Acokanthera oblongifolia* (Hochst.) Benth. & Hook.f. ex B.D. Jacks., *Catharanthus roseus* (L.) G. Don, *Rauvolfia caffra* Sond.
Asclepiadaceae	*Huernia zebrina* (Phillips) L.C. Leach
Asparagaceae	*Asparagus densiflorus* (Kunth) Jessop
Asteraceae	*Kleinia stapeliiformis* (E. Phillips) Stapf , *Senecio barbertonicus* Klatt
Boraginaceae	*Ehretia rigida* (Thunb.) Druce
Cactaceae	*Rhipsalis baccifera* (J.S. Muell.) Stearn

Cannabaceae	*Celtis africana* Burm. f.
Celastraceae	*Elaeodendron croceum* (Thunb.) DC., *Gymnosporia tenuispina* (Sond.) Szyszyl., *Putterlickia verrucosa* (E. Mey. Ex Sond.) Szyszyl.
Crassulaceae	*Kalanchoe beharensis* Drake , *Adromischus* sp., *Adromischus filicaulis* (Eckl. & Zeyh.) C.A. Sm. *subsp. filicaulis*, L.c *Adromischus diabolicus* Toelken
Cucurbitaceae	*Xerosicyos danguyi* Humbert
Cycadaceae	*Cycas thouarsii* R. Br.
Dichapetalaceae	*Dichapetalum cymosum* (Hook.) Engl.
Didiereaceae	*Alluaudiopsis fiherenensis* Humbert & Choux, *Alluaudia procera* (Drake) Drake, *Alluaudia dumosa* (Drake) Drake, *Alluaudia humbertii* Choux, *Decarya madagascariensis* Choux
Dioscoreaceae	*Dioscorea dregeana* (Kunth) T. Durand & Schinz
Ebenaceae	*Euclea* sp. (Pretoria National Botanical Gardens 18236/73)
Euphorbiaceae	*Euphorbia tortirama* R.A. Dyer, *Euphorbia fruticosa* Forssk, *Euphorbia platyclada* Rauh, *Euphorbia stellispina* Haw., *Euphorbia leistneri* R.H. Archer, *Euphorbia pseudocactus* A. Berger, *Euphorbia cylindrica* Marloth ex A.C. White, R.A. Dyer & B. Sloane, *Euphorbia clivicola*

	R.A. Dyer, *Euphorbia buruana* Pax, *Euphorbia jansenvillensis* Nel, *Euphorbia fusca* Marloth, *Euphorbia gummifera* Boiss., *Euphorbia* C.f. *aeruginosa*, *Euphorbia bupleurifolia* Jacq., *Euphorbia lignosa* Marloth, *Euphorbia pulvinata* Marloth, *Euphorbia aeruginosa* Schweick, *Euphorbia monteiroi* Hook., *Euphorbia antso* Denis., *Euphorbia invenusta* (N.E.Br.) Bruyns, *Euphorbia schubei* Pax, *Euphorbia lugardae* (N.E.Br.) Bruyns, *Euphorbia ritchiei* (P.R.O Bally) Bruyns, *Euphorbia guentheri* (Pax) Bruyns, *Euphorbia virosa* Willd., *Euphorbia dregeana* E. Mey. Ex Boiss., *Euphorbia cupularis* Boiss., *Spirostachys africana* Sond., *Sclerocroton ellipticus* Hochst., *Croton sylvaticus* Hochst., *Croton gratissimus* subsp. *Gratissimus*, L.c. *Jatropha curcus*
Fabaceae	*Burkea africana* Hook., *Philenoptera violaceae* (Klotzch) Schrire, *Peltophorum africanum* Sond., *Bauhinia galpinii* N.E.Br., *Colophospermum mopane* (Benth.) Leonard,
Geraniaceae	*Pelargonium ceratophyllum* L'Her., *Pelargonium klinghardtense* R. Knuth, *Pelargonium crassicaule* L'Her.
Gesneriaceae	*Streptocarpus* sp. Pink cultivar
Hernandiaceae	*Gyrocarpus americanus* Jacq.
Icacinaceae	*Pyrenacantha cordata* Villiers.

Juncaceae	*Juncus effusa* L.
Lamiaceae	*Tetradenia fruticosa* Benth.
Malvaceae	*Grewia flavescens* Juss.
Meliaceae	*Turraea obtusifolia* Hochst.
Menispermaceae	*Tinospora fragosa* Verdoorn & Troupin
Moringaceae	*Moringa drouhardii* Jum.
Olacaceae	*Ximenia caffra var. Caffra* Sond.
Pandanaceae	*Pandanus epiphyticus* Martelli
Passifloraceae	*Adenia* sp. (Pretoria National Botanical Gardens 14638/69), *Adenia* sp., *Adenia firingalavensis* (Drake ex Jum.) Harms, *Adenia fruticosa* Burtt Davy, *Adenia pechuelii* (Engl.) Harms, *Adenia gummifera* (Harv.) Harms
Pedaliaceae	*Ceratotheca triloba* (Bernh.) Hook.f.
Phyllanthaceae	*Bridelia catharthica* subsp. *Carthartica, Heywoodia lucens* Sim
Polygalaceae	*Polygala myrtifolia* L.
Pteridaceae	*Adianthum* sp.
Rosaceae	*Leucosidea sericea* Eckl. & Zeyh.
Rubiaceae	*Vangueria infausta* Burch., *Xeromphis obovata* (Hochst.) Keay
Solanaceae	*Solanum tomentosum* L., *Solanum seaforthianum* Andrews
Zamiaceae	*Encephalartos friderici-guilielmi* Lehm.

2.2 Cyanogenic activity

Several methods exist in order to determine cyanide and cyanogenic compounds in biological systems. These include titrimetric, chromatographic and colorimetric assays. When looking for cyanogenic compounds in plants however, two qualitative and semi-qualitative methods are used - Sodium Picrate paper and Feigl-Anger paper (Curtis *et al.,* 2002; Harborne, 1972). Sodium Picrate paper makes use of sodium bicarbonate and picric acid and relies on the endogenous enzyme linamarase which is liberated when cells are ruptured to release cyanide - the colour of the paper changes from yellow-brown to red-brown in the presence of hydrogen cyanide. Although the Picrate paper test is easy to use, the long sample incubation time is inconvenient (Alonso-Amelot and Oliveros, 2000).

It is also prone to interferences such as the breakdown of glucosinates which result in false positive results (Brinker *et al.,* 1992). Carbonyls are also known to influence the reaction and this alters the colour of the Picrate paper (Alonso-Amelot and Oliveros, 2000; Curtis *et al.,* 2002). It is also important to note that picric acid is a health/safety hazard - many picrates are explosive.

In this study, cyanogenic activity was tested using the Feigl-Anger test (Feigl and Anger, 1966; Takos *et al.,* 2010) which relies on the oxidation of a tetrabase in the presence of hydrogen cyanide (a by-product of cyanogenesis) to create a distinct blue spot on a specially prepared detection paper after tissue disruption by a single freeze-thaw cycle. Feigl-Anger papers were found to be better than Picrate papers and are preferred as they are quick, both qualitative and semi-quantitative, useful in high-throughput screening and allow for on site testing. It is also more sensitive and less prone to giving false results (Ganjewala *et al.,* 2010; Haque and Bradbury, 2002). The Feigl-Anger method also does not need much equipment such as corks, vials, etc (as needed by the Picrate test).

Whatman 3MM filter paper, cut to the dimensions of 8 cm x 11 cm to fit the microtitre plate was used. The solution was then prepared by separately dissolving 75 mg of copper ethylacetoacetate (Sigma-Aldrich) and 75 mg of the tetrabase 4,4-methylenebis (N,N-dimethylalanine) (Sigma-Aldrich) in 7.5 ml of chloroform (Merck) each and then combining both solutions. The filter paper was then saturated with this combined solution and allowed to dry. After drying, the resultant pale green detection paper was stored in a dark, dry place at 4°C until required.

The trays containing the samples were removed from the -80ºC freezer and the detection paper was immediately overlaid on the plate. The plate was then covered, and the lid weighed down to create a tight fit between plate, detection paper and lid to prevent diffusion of hydrogen cyanide from individual wells. The tissue was then allowed to thaw and disrupt on the laboratory bench at room temperature. Results were recorded within three hours (assessed after one, two and three hours) in order to detect any hydrogen cyanide released.

Apical buds from *Manihot esculenta* Crantz (cassava), were used as a positive control whilst distilled water was used as a negative control. A positive result was indicated by a change in colour of the detection paper from white to blue. No change in colour indicated a negative result. An image of each result was captured immediately as the colour is known to fade with time.

2.3 Hydroxynitrile lyase activity

2.3.1 Crude enzyme extraction from cyanogenic plants

In order to study specific activity, substrate specificity or conformation of proteins, the protein of interest will have to be isolated and purified.

The purity of the isolated protein will depend on its intended use - for certain applications, a crude extract is sufficient, but for use in the food or pharmaceutical industries, a higher level of purity is essential. In order to obtain a pure product, several protein purification methods are used (Johnson and Hecht, 1994; Zubay, 1988).

The preparation of a crude extract is considered as the first step when purifying intracellular or extracellular proteins. This extract will contain a mixture of all the proteins, macromolecules and co-factors present in the cell. In general, crude extracts are prepared firstly by cell lysis - lysis is either achieved by chemical or mechanical methods. The resulting cellular debris is then removed by centrifugation and the supernatant containing the protein of interest is recovered. In the case of extracellular proteins, these proteins may be obtained simply by centrifugation and recovering the supernatant (Zubay, 1988; Amersham Pharmacia Biotech, 1999).

Extraction of the crude enzyme was performed according to Ueatrongchit et al (2010) with slight modifications. Young leaves (1 g) were frozen in liquid nitrogen and homogenized by mortar and pestle to form a fine powder. The powder was re-suspended in 1 ml of 50 mM sodium citrate buffer (pH 5.0) and vortexed vigorously for 5 minutes. The resultant slurry was centrifuged at 20 000 x *g* for 10 minutes. The supernatant was used as the crude enzyme extract.

2.3.2 Protein assay

Several methods for measuring proteins exist and are based on either spectrophotometric or chemical methods - comparing the colour produced in the reaction with that of a known protein standard. The commonly used standard is Bovine Serum Albumin (BSA).

The three most widely used chemical methods are: the Biuret method - this method produces a purple colour with alkaline copper, the Lowry method - this method also makes use of alkaline copper but produces a stronger colour by oxidation of residues in the protein with a phosphotungstomolybdate reagent, and, the Bradford method. The Bradford method relies on the interaction between the protein and a dye at a very low pH - the dye changes spectral properties when it binds to the protein (Bradford, 1976; Lowry *et al.*, 1951).

There are both advantages and disadvantages to the above methods and potential issues regarding sensitivity, simplicity, accuracy and interference must be taken into account. Fluorometry is more sensitive, highly specific for a single analyte, simple to use and cheaper than the above mentioned methods.

Total protein was quantified in a Qubit Fluorometer (Life Technologies) using a Qubit protein assay kit (Life Technologies) according to manufacturer's instructions - the reagent was diluted using the buffer provided, a 2 µl sample was added and the concentration was read in a fluorometer. The Qubit protein assay kit (Life Technologies) can be used with any fluorometer. Based on the BSA method, this method is highly selective for proteins and is designed to be accurate in the presence of reducing agents or common contaminants.

2.3.3 Hydroxynitrile lyase activity assay

Enzyme assays are used to study the rate of an enzyme catalysed reaction. This assay can serve two purposes - to identify an enzyme or prove its presence or absence in a particular organism, or, to determine the amount of enzyme present in a sample (Biswanger, 2014; Conn and Stumpf, 2009).

Enzyme activity however is a measure of the quantity of the enzyme present in a reaction. Measurement of enzyme activity can be achieved either by monitoring the appearance of a product over time or the disappearance of a substrate over time. Monitoring the appearance of a product over time is however more accurate.

Two types of assays exist - fixed-time assays and continuous assays. A fixed-time assay measures the enzyme concentration in a fixed period of time. The advantage of conducting a fixed-time assay is that many assays can be run concurrently (Biswanger, 2014; Conn and Stumpf, 2009).

The continuous assay on the other hand uses a spectrophotometer to measure, in real time either the appearance of a product or the disappearance of a substrate. Continuous assays are more advantageous as they are convenient - reaction rates can be easily measured and the linearity of the assay can also be measured. One disadvantage of this method is that only one reaction can be run at any one time. (Biswanger, 2014). This however will depend on the number of spectrophotometers available.

When enzyme activity is measured, the parameters selected are almost always at optimum pH, saturating substrate concentrations and a controllable temperature. Several factors are known to effect enzyme activity and they include temperature, pH, substrate concentration, nature of salts present and ionic strength (Biswanger, 2014, Conn and Stumpf, 2009).

Hydroxynitrile lyase specific activity was measured spectrophotometrically as described by Krammer *et al*. (2007) and Zhao *et al.* (2011) by following the formation of benzaldehyde from a racemic mixture of mandelonitrile (Fig 2.1).

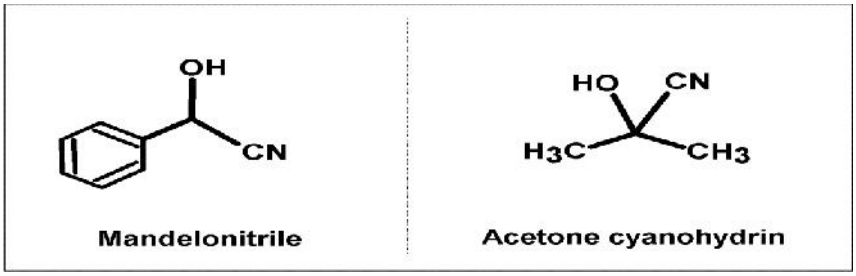

Figure 2.1 Two products of hydroxynitrile lyase mediated catalysis

The reaction mixture comprised 50 mM sodium citrate buffer (pH 5), 150 µl of the crude enzyme extract and 10 mM mandelonitrile (Sigma-Aldrich) to a final volume of 3 ml in a Quartz cell. The substrate was always added last.

Twenty seconds after the substrate was added, the spectrophotometer (Jenway 6305 UV/Vis, Staffordshire, United Kingdom) was blanked using the reaction mixture and the formation of benzaldehyde was tracked by monitoring the increase in absorbance at 280 nm for 10 minutes at room temperature.

Due to the fact that mandelonitrile breaks down spontaneously at a pH >5, a control reaction was run in parallel with the samples and comprised 50 mM sodium citrate buffer (pH 5) and 10 mM mandelonitrile in a final volume of 3 ml.

The linear slope of absorbance resulting from the spontaneous decomposition of mandelonitrile (control) was subsequently subtracted from the slope of absorbance obtained for the crude enzyme assays in order to avoid false indications of hydroxynitrile lyase activity in the plant macerates. All assays were carried out in triplicate.

Hydroxynitrile lyase activity was then calculated using the equation:

$$\text{Activity (U/ml)} = \frac{\Delta OD}{\varepsilon} \times \frac{V_t}{V_s}$$

$$\Delta OD = \Delta A_{280}/\min_{crude\ enzyme} - \Delta A_{280}/\min_{spontaneous}$$

where ΔOD represents the difference in the maximum linear rate between the crude enzyme extract and the spontaneous decomposition reaction, ε is the absorption coefficiency (1.3761 ml/μmol/cm) at 280 nm, V_t and V_e are the total volume of the reaction and enzyme volume used respectively. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol/min of benzaldehyde from mandelonitrile under the assay conditions.

Chapter Three:

Results

3. RESULTS

3.1 Cyanogenic activity

Of the 102 plants tested, six were found to be cyanogenic: *Adenia* sp., *Adenia firingalavensis*, *Adenia fruticosa*, and *Adenia pechuelii*, all from the family Passifloraceae (Fig 3.1). *Heywoodia lucens* and *Ximenia caffra* were also found to be cyanogenic (Fig 3.2).

(a) (b)

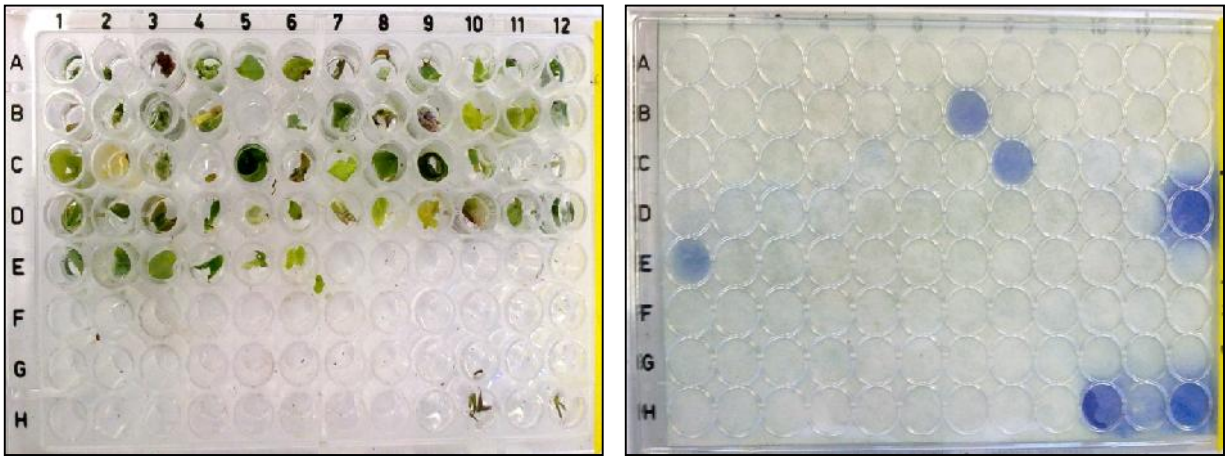

Figure 3.1 Screening for cyanogenic activity using Feigl-Anger paper (a) A representative microtitre plate containing samples in wells A1 to E6, *Manihot esculenta* (positive control) in wells H10 and H12 and distilled water (negative control) in wells F10 and F12. (b) A blue spot indicates cyanogenic activity due to hydrogen cyanide detection, whilst the absence of a blue spot indicates a negative test for cyanogenesis. *Adenia* sp. was in well B7, *Adenia firingalavensis* in C8, *Adenia fruticosa* in D12, and *Adenia pechuelii* in E1

(a) (b)

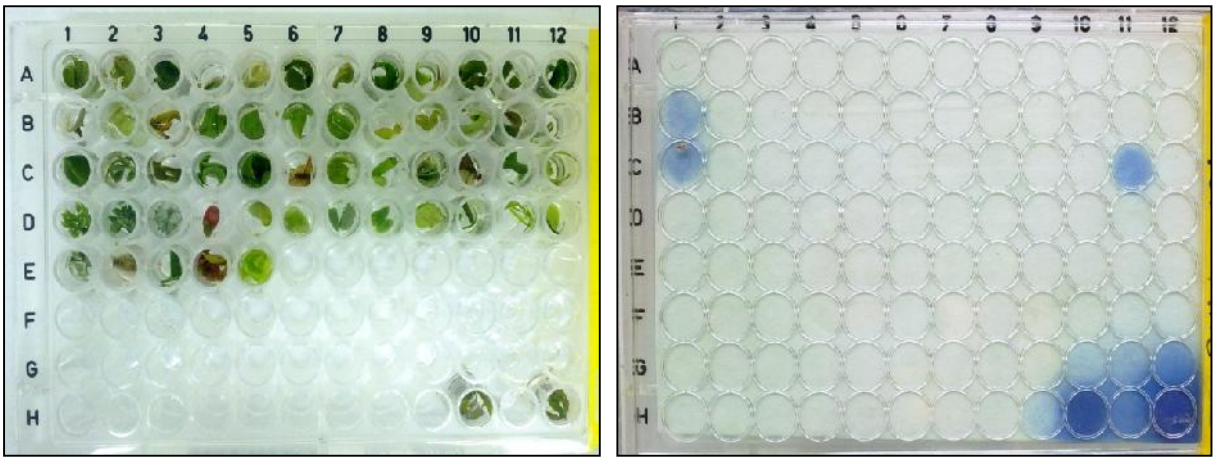

Figure 3.2 Screening for cyanogenic activity using Feigl-Anger paper (a) A representative microtitre plate containing samples in wells A1 to E5, *Manihot esculenta* (positive control) in wells H10 and H12 and distilled water (negative control) in wells F10 and F12. (b) A blue spot indicates cyanogenic activity due to hydrogen cyanide detection, whilst the absence of a blue spot indicates a negative test for cyanogenesis. *Ximenia caffra* was in well B1, *Heywoodia lucens* in C1, and *Adenia fruticosa* again in C11

3.2 Rate of cyanogenic activity

All six plants exhibited cyanogenic activity within two hours. Four of the six plants exhibited cyanogenic activity after just one hour (Table 3.1). These plants can therefore be classified as cyanogenic in the field.

Table 3.1 Rate of cyanogenesis

Family	Species	Cyanogenic activity		
		After 1 hour	After 2 hours	After 3 hours
Passifloraceae	*Adenia* sp.	-[a]	+[b]	+
	Adenia firingalavensis	+	+	+
	Adenia fruticosa	+	+	+
	Adenia pechuelii	+	+	+
Olacaceae	*Ximenia caffra var. Caffra*	+	+	+
Phyllanthaceae	*Heywoodia lucens*	-	+	+
Euphorbiaceae	*Manihot esculenta* (positive control)	+	+	+

[a] (-) indicates hydrogen cyanide was not detected after elapsed time

[b] (+) indicates hydrogen cyanide was detected after elapsed time

3.3 Hydroxynitrile lyase activity

Hydroxynitrile lyase specific activity (Table 3.2) was calculated for the crude enzyme extracts of *Adenia* sp., *Adenia firingalavensis*, *Adenia fruticosa*, *Adenia pechuelii*, *Ximenia caffra* and *Heywoodia lucens* which were identified as cyanogenic. The total activity represents the yield of enzyme at each step, whereas the specific activity is a measure of the purity of the enzyme. *Adenia firingalavensis*, *Adenia fruticosa* and *Adenia pechuelii* exhibited the highest hydroxynitrile lyase specific activities.

Table 3.2 Hydroxynitrile lyase activity in the crude extracts of cyanogenic plants

Plant species	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
Adenia sp.	0.304 ± 0.169	0.692	0.440 ± 0.190
Adenia firingalavensis	0.559 ± 0.265	0.194	2.884 ± 0.240
Adenia fruticosa	0.878 ± 0.094	0.440	1.998 ± 0.214
Adenia pechuelii	1.917 ± 0.630	0.815	2.353 ± 0.200
Ximenia caffra	0.763 ± 0.346	0.657	1.843 ± 0.231
Heywoodia lucens	0.661 ± 0.074	0.510	1.761 ± 0.216

Chapter Four:

Discussion

4. DISCUSSION

To our knowledge, cyanogenic properties and hydroxynitrile lyase activity have not been reported for any of the selected plants, until now, and no study of this nature has been performed previously in South Africa.

All six novel cyanogenic plants demonstrated a rapid rate of cyanogenesis. This is a clear indicator of the presence of both the cyanogenic glycoside and the hydroxynitrile lyase. Francisco and Pinotti (2000) concluded that if the colour change occurred within two hours, it was due to the presence of the cyanogenic glycoside and the respective hydrolytic enzyme/hydroxynitrile lyase. The plant could therefore be classified as cyanogenic in the field. If the colour change occurred slowly (after two hours), it indicated that the hydrogen cyanide detected was released slowly by the cyanogenic glycoside without the action of an enzyme. This plant could therefore not be classified as cyanogenic in the field.

Of the six new cyanogenic plants discovered in this study, four were of the genus *Adenia,* from the Passifloraceae family. Certain species within the Passifloraceae are known to be cyanogenic (Asano *et al.*, 2005, Hernandez *et al.*, 2004, Ueatrongchit *et al.*, 2010) however, no studies are known to the authors that report cyanogenic activity in *Adenia*.

In 2005, Asano and co-workers were the first to report (*R*)-hydroxynitrile lyase activity in *Passiflora edulis* Sims. Thereafter, in 2010, Ueatrongchit and co-workers characterised and purified a hydroxynitrile lyase from *Passiflora edulis*, a member of the Passifloraceae family, for the first time. They reported a specific activity of 2.47 U/mg in the crude extract from the leaves of *Passiflora edulis.* This specific activity is not dissimilar from three of the findings in the current study, indicating that the enzymes present in the currently studied plants have similar levels of activity to those found in other plants from the same family.

However, it may also indicate that these enzymes have the same characteristics as those found in *Passiflora edulis*. Further study is thus needed to exclude this possibility and confirm the presence of novel substrate specificity.

In 1989, Kuroki and Conn reported hydroxynitrile lyase activity in the leaves of *Ximenia americana* L. - a member of the Olacaceae family. This enzyme catalysed the dissociation of mandelonitrile to benzaldehyde and hydrogen cyanide. The enzyme from *Ximenia* was however only investigated once but the investigation focused only on structural organisation and substrate specificity. To our knowledge, there has been no previous report of hydroxynitrile lyase activity in any member of the Phyllanthaceae family.

The specific activities measured in this study were not unexpected as hydroxynitrile lyase activity is usually low in the crude homogenate of leaves. This can be attributed to the low concentration of hydroxynitrile lyase present relative to the higher concentrations of other contaminating proteins in the crude extract. Hydroxynitrile lyase activity also largely depends on the pH of the plant macerates requiring an acidic pH to be active (Dadashipour *et al.,* 2011). Ueatrongchit *et al.* (2008) isolated and purified to homogeneity a hydroxynitrile lyase from the seeds of *Eriobotrya japonica* (Thunb). They reported a specific activity of 0.8 U/mg in the crude extract. However, after affinity column Concanavalin A Sepharose 4B purification, the specific activity had increased to 40.9 U/mg.

Although the remaining 38 families studied are known to contain cyanogenic species, none of the selected plants from these families exhibited cyanogenic activity in this study. It is therefore important to note that because a plant family contains cyanogenic plant species, it cannot be presumed that every member of that family will be cyanogenic. This cyanogenic variation amongst species may be attributed to a cyanogenic polymorphism (Goodger and Woodrow, 2002).

However, it should be noted that a negative test for cyanogenic activity is not necessarily evidence that a plant is not cyanogenic. It is possible that the age or stage of growth of the plant, the part of the plant that was tested, environmental conditions, climate and seasonal variation could have very well contributed to the results of this study.

It is known that the proportion of cyanogenic plants declines in colder climates - most plants found at high elevations are acyanogenic whilst those in lower elevations and warmer climates are cyanogenic. The two suggested reasons for this are autotoxicity - the frost will result in cell rupture releasing cyanide within the tissue, and, the presence of fewer herbivores in cold climates or higher altitudes-the plant does not have to utilise energy to produce compounds for cyanogenesis and can focus instead on essential flowering and seed production (Olsen *et al.*, 2007 and 2008). Johannesburg has an approximate elevation of 1700 meters above sea level and during winters can experience frost. The samples in this study were however collected in autumn.

In this study, it was only the apical bud of each plant that was tested as the apical bud and younger leaf tissues are known to contain the highest concentration of cyanogenic glycosides. Gleadow and Woodrow (2000) found that the concentration of cyanogenic glycosides decreases as the leaves mature. They also found that the cyanogenic glycoside concentration in the young leaf tips varied seasonally, and propose that this seasonal variation in cyanogenic glycoside concentration may be due to the availability of nitrogen in the soil, climate, temperature, etc.

Using field and greenhouse studies in 2001, Gebrehiwot and Beuselinck confirmed seasonal variation in hydrogen cyanide concentrations. They found that plants in spring and summer had a 50% greater concentration of hydrogen cyanide than the same plants in autumn or winter, with the lowest concentrations observed in winter.

The presence and concentrations of various compounds in a plant are known to vary with regard to season and age (Pichersky and Lewinsohn, 2011).

Hernandez and co-workers (2004) found that the different parts (leaves, seeds, etc) of the same plant gave a different result. They found in some plants that the seeds were cyanogenic, but the leaves were not, and *vice-versa*.

Chapter Five:

Conclusion

5. CONCLUSION

Hydroxynitrile lyases, a concept or technology which was thought, by some, to be outdated, having had its 'hey-day' in the 1980s and 1990s, is slowly gaining a new interest in this new millennium. Recombinant DNA technology has solved the initial concerns regarding lack of availability and insufficient amounts of this enzyme for industrial application. Pharmaceutical giants have realised how essential this enzyme is, and we make use of numerous medicines and pesticides produced using this enzyme in our everyday lives.

But, the search is on again, this time not for a way to produce more hydroxynitrile lyase for industrial applications, but to find a previously undiscovered hydroxynitrile lyase, and more importantly, to find a hydroxynitrile lyase which has novel substrate specificity, to meet the demands of our ever changing needs.

This study expands the limited knowledge of the frequency of cyanogenesis in the natural plant community and includes novel reports of cyanogenesis - six new cyanogenic plants exhibiting hydroxynitrile lyase activity have been reported. The crude extracts of these plants exhibited specific activities similar to that found in the crude extract of *Passiflora edulis*. These findings also expand the number of plants known to exhibit hydroxynitrile lyase activity and there is the potential that these plants may contain an enzyme with novel substrate specificity. The enzyme activity found in these plants is important because they can provide a source of biocatalysts naturally. Several of the selected plants are also non-seasonal - meaning availability will not be an issue.

The remaining 96 plants which tested negative cannot however be confirmed as non cyanogenic due to the effects of seasonal variation.

The plants described in this study are expected to draw the interest of scientists and industrialists because of their cyanogenic properties. Indeed, a hydroxynitrile lyase from these plants could have a major share in the future pharmaceutical market.

Therefore the immediate future challenge lies on the growing of these plants in bulk quantities to extract their enzymes at low cost and to conduct research on their characteristics - industrial applications of the current study’s findings will then be better understood.

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APPENDIX

Appendix A — Kassim, M.A., Rumbold, K. (2014) HCN production and hydroxynitrile lyase: a natural activity in plants and a renewed biotechnological interest, *Biotechnology Letters*, vol. 36, pp. 223-228.

Appendix B — Kassim, M.A., Sooklal, S.A., Archer, R., Rumbold, K. (2014) Screening for hydroxynitrile lyase activity in non-commercialised plants, *South African Journal of Botany*, vol. 93, pp. 9-13.

Biotechnol Lett (2014) 36:223–228
DOI 10.1007/s10529-013-1353-9

REVIEW

HCN production and hydroxynitrile lyase: a natural activity in plants and a renewed biotechnological interest

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Received: 4 July 2013 / Accepted: 6 September 2013 / Published online: 24 September 2013

Abstract Over 3,000 plant species are cyanogenic. Cyanogenesis is defined as the hydroxynitrile lyase catalysed release of a cyanide group in the form of HCN and the corresponding aldehyde or ketone. When a plant is attacked, HCN released is a self defence mechanism. A special characteristic of enzymatic reactions is that all enzymatic reactions are reversible—hydroxynitrile lyases can also be used for the synthesis of enantiomerically pure cyanohydrins which are of great importance in industry. This article presents a comprehensive review of the role of hydroxynitrile lyases, both in nature and industry, and an insightful. Areas covered include: history, discovery and natural sources of the hydroxynitrile lyase. Molecular cloning for mass production of this enzyme, including detailed information about several successful recombinant hydroxynitrile lyases is also included.

Keywords Biocatalysis · Cyanide · Cyanogenesis · Cyanogenic glycoside · HCN · Hydrogen cyanide · Hydroxynitrile lyase

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Cyanogenesis

Cyanogenesis is defined as the hydroxynitrile lyase mediated release of HCN and an aldehyde or ketone from a cyanohydrin (Sharma et al. 2005). This two-step process essentially involves two enzymes. In the first step, cyanogenic glycosides in the plant are broken down by β-glycosidases to form glucose and a cyanohydrin. In the second step, this cyanohydrin is broken down by a hydroxynitrile lyase into HCN and an aldehyde or ketone (Zagrobelny et al. 2008). A notorious and deadly poison, HCN exerts its toxic effects by binding to the ferric ion of cytochrome oxidase, an enzyme responsible for 90 % of the total O_2 uptake in most cells. Inactivation of this enzyme completely inhibits cellular oxygen utilisation and results in cytotoxic hypoxia and death (Erdman 2003).

This ingenious self-defence mechanism protects the plant from microbial, fungal and animal attack and exists in over 3,000 plant species (Conn 1981; Peterson et al. 2000). Of these 3,000 species, cyanogenic plants are more commonly found in the following families: *Linaceae, Gramineae, Euphorbiaceae, Clusiaceae, Olacaceae, Rosaceae, Passifloraceae* and *Filitaceae* (Sharma et al. 2005; Ueatrongchit et al. 2010). Several important crop plants such as sorghum, maize, wheat, sugarcane and cassava, which is a staple food crop for most of sub-Saharan Africa, are also known to be cyanogenic (Conn 1981; Jones 1998; Fokunang et al. 2001). Perhaps our ancestors chose to grow those plants on a large scale as a staple food

source because of their built-in pest control mechanism which naturally protects them from voracious insect feeders and bacterial or fungal infections.

Apart from plants, a wide range of organisms, such as fungi, bacteria, lichen, millipedes, arthropods and insects, are also cyanogenic (Zagrobelny et al. 2008). In an interesting article by Kremer and Souissi (2001), an extraordinary method of making use of cyanogenic bacteria is revealed: HCN can also be used as an effective herbicide to inhibit the growth of weed seedlings. In their article, HCN produced naturally by certain cyanogenic rhizobacteria strains occurring on plant roots effectively inhibited the growth of plants. These cyanogenic rhizobacteria, which are found naturally in the rhizosphere, show promise as an environmentally friendly, biological control method for weeds, a welcome alternative to the toxic herbicides in current use. Further research in this area is therefore welcome.

It is interesting, though, to note that at lower concentrations, cyanide is not toxic to a plant and does not inhibit respiration but it still inhibits root hair development and the germination of seeds. However, at higher concentrations, cyanide can inhibit respiration, production of ATP and other cellular processes such as ion uptake or phloem translocation which are dependent on adenosine triphosphate (Eisler 2007; Garcia et al. 2010). Blom et al. (2011) found that volatile-mediated phytotoxicity involved oxidative stress and concluded that high concentrations of HCN produced by bacteria can kill plants.

Hydroxynitrile lyase

Since the late 1990s, hydroxynitrile lyases have been receiving increasing attention from the fine chemical and pharmaceutical industries (Effenberger et al. 1995; Effenberger and Jager 1997). In nature, a cyanohydrin is broken down by a hydroxynitrile lyase into HCN and an aldehyde or ketone. However, in principle, each enzymatic reaction is completely reversible and it is this reversible reaction (Fig. 1) which is so desired by industry. The ability of these enzymes to produce a chiral cyanohydrin by the condensation of HCN with an aldehyde or ketone is advantageous, especially to the pharmaceutical and fine chemical industries. These chiral cyanohydrins are used as building blocks/intermediates for the

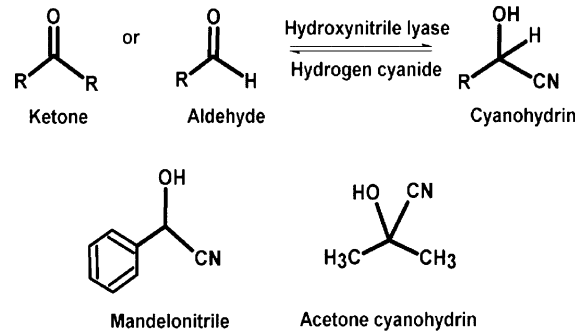

Fig. 1 General reaction catalysed by hydroxynitrile lyase and two products of hydroxynitrile lyase mediated catalysis

production of pesticides, medicines, agrochemicals, etc. (Dadashipour et al. 2011). Hydroxynitrile lyase can also be used to detoxify cyanogenic food plants, especially cassava, which forms the staple diet in sub-Saharan Africa (Hasslacher et al. 1996; Fokunang et al. 2001).

Sources of hydroxynitrile lyase

The first discovery of hydroxynitrile lyase occurred in 1837 by two German chemists Justus von Liebig and Friedrich Wohler. This was found in a bitter almond extract (Hosel 1981). Since then, discoveries have been made: for example, Xu et al. (1988) purified and characterised a hydroxynitrile lyase from *Linum usitatissimum* (commonly known as Flax). Hughes et al. (1994) purified and characterised a hydroxynitrile lyase from cassava (*Manihot esculenta* Crantz). Their enzyme was not glycosylated and did not contain a flavin group but did display activity against two natural substrates: acetone cyanohydrin and 2-butanone cyanohydrin. Wajant and Forster (1996) purified and characterised a hydroxynitrile lyase from the rubber tree *Hevea brasiliensis*. Their enzyme also showed activity toward acetone cyanohydrin. Several other hydroxynitrile lyases have been discovered in *Prunus serotina, Prunus lyonii, Prunus capuli, Prunus amygdalus, Mammea americana, Malus communis, Phlebodium aureum, Sorghum bicolour, Sorghum vulgare, Ximenia americana* and *Sambucus nigra* (Sharma et al. 2005). Most of these enzymes showed activity toward mandelonitrile and acetone cyanohydrin. Ueatrongchit et al. (2010) discovered, characterised, and purified a hydroxynitrile lyase from

Passiflora edulis (passion fruit), a member of the *Passifloraceae* family, for the first time. The optimum pH for their enzyme was pH 4, and the optimum temperature was 10 °C. They also found that the spontaneous non-enzymatic reaction which yields a racemic product was almost completely suppressed at the optimum pH and temperature. Dadashipour et al. (2011) discovered a novel hydroxynitrile lyase from *Baliospermum montanum*. Their enzyme exhibited excellent pH stability in a wide pH range, showed activity in a wide temperature range (perfect for industrial applications) and showed activity toward aromatic aldehydes. Fukuta et al. (2011) discovered a new hydroxynitrile lyase from the Japanese apricot *Prunus mume* that was active over a broad pH and temperature range. Its amino acid sequence was identical to the hydroxynitrile lyase from *Prunus dulcis*.

Despite the numerous discoveries, the application of these naturally-occurring enzymes in enantioselective biocatalytic synthesis was sadly limited due to the difficulty of obtaining them in sufficient amounts. The presence and concentrations of various active secondary metabolites in a plant are also known to vary—their presence or concentration depending on season and age (Pichersky and Lewinsohn 2011). A plant extract was therefore not a suitable enzyme source for the development of an industrial biocatalytic process of cyanohydrins.

Recombinant hydroxynitrile lyase

Many industrial and pharmaceutical production processes, which in the past were considered not feasible due to high production costs and limited amounts of starting material such as enzymes and other valuable compounds, are now feasible—thanks to biotechnology. Biotechnology/recombinant DNA technology has equipped us with the tools necessary to mass produce, in a heterologous host, these much needed enzymes which were previously only found in plants or animals. Using recombinant DNA technology: cyanogenic plant samples are collected, their messenger ribonucleic acid mRNA is extracted, the gene coding for the hydroxynitrile lyase is located, cloned and over-expressed in a heterologous host/microbial expression system. There are several advantages to using heterologous expression, the main being cost—enzymes can now be mass produced rapidly and sold at lower prices than those previously sourced from nature. Another important advantage is sustainability—as industry has become more environmentally conscious—always researching cleaner, greener technologies and constantly seeking renewable and sustainable sources of enzymes and other valuable compounds (Terpe 2006; Yesilirmak and Sayers 2009; Chen 2012).

There are numerous examples of the cloning of the hydroxynitrile lyase gene in either a bacteria or yeast species. (Fukuta et al. 2011; Dadashipour et al. 2011; Semba et al. 2008a, b; Breithaupt et al. 1999; Hughes et al. 1994, 1997). Some researchers found that yeasts were the better hosts producing the enzyme in an active, easily accessible form.

Forster et al. (1996) reported the first recombinant hydroxynitrile lyase. The activity of their purified recombinant *Manihot esculenta* hydroxynitrile lyase (expressed in *Escherichia coli*) was 900 U/ml. Their 80 l culture, comprising of 150–200 g wet wt of cells, gave a yield of 4,000 U recombinant enzyme. They claimed that, in order to obtain the same amount of enzyme from plant material, one would have required 100–200 kg dried *M. esculenta* leaves. Hasslacher et al. (1997) reported high level intracellular expression of a recombinant *H. brasiliensis* hydroxynitrile lyase in *Pichia pastoris* which proved to be the most efficient microbial host system. An exceptional yield of 22 g pure enzyme/l of culture broth was achieved.

Semba et al. (2008a) expressed a *M. esculenta* hydroxynitrile lyase in a yeast and tested the application of this recombinant hydroxynitrile lyase in the production of (*S*)-mandelonitrile (a useful cyanohydrin) using an immobilised enzyme reactor. They also found that the hydroxynitrile lyase accepted a wider substrate range when compared with the *S. bicolor* hydroxynitrile lyase, which is exactly what industry is currently searching for. Semba et al. (2008a) reported that productivity in *Escherichia coli* was low so they used *Saccharomyces cerevisiae* as host. The yeast expressed a recombinant hydroxynitrile lyase with a specific activity of 3.5 U/mg soluble protein. They also tested (*S*)-mandelonitrile production using an immobilised recombinant *M. esculenta* hydroxynitrile lyase. Inorganic supports showed better adsorption ability than organic supports and silica gel was the best—the enzyme immobilised on silica gel was used for more than 20 batches and did not show any loss in

conversion rate or enantioselectivity. Overall, after 22 batches, 23.3 kg benzaldehyde was converted to 28.6 kg (*S*)-mandelonitrile. This shows that production can be successful on a large scale for industrial purposes.

Semba et al. (2008b) also experimented with the production of recombinant *M. esculenta* hydroxynitrile lyase using what they termed as low temperature cultivation. When the temperature was lowered, the cell yield and ratio of soluble enzyme increased significantly. The enzyme activity and yield at 17 °C was 850 times higher than those obtained at 37 °C. Their rate of hydroxynitrile lyase production was 3,000 U/h. Dadashipour et al. (2011) expressed a *B. montanum* hydroxynitrile lyase in *E. coli.* At first, the researchers experienced problems with expression at optimum temperature—having encountered a high level of inactive protein production as inclusion bodies. They therefore used a lower temperature for protein expression, as described by Semba et al. (2008b); Qing et al. (2004), which allowed them to obtain at least a small amount of the soluble or native form of the enzyme. Aromatic aldehydes and ketones were the preferred substrates for their enzyme (when compared with aliphatic substrates). The highest specific activity for their enzyme was 178 U/mg. Fukuta et al. (2011) expressed a gene coding for the hydroxynitrile lyase in *Pichia pastoris* as an active, highly glycosylated protein. An activity of 209 U/l culture supernatant was measured in a cyanohydrin synthesis reaction, which corresponded to the amount of enzyme that can be isolated from 800 g of plant material. Their new enzyme accepted a wide range of substrates including aromatic and aliphatic carbonyl compounds.

Is the recombinant hydroxynitrile lyase as good as the native one?

A legitimate question which was rather well answered in an informative article by Hughes et al. (1997) who compared the secondary structures, specific enzyme activities, optimum temperatures, optimum pH, and isoelectric focusing of the recombinant *M. esculenta* enzyme with the native one. The recombinant and native enzymes were similar in every way except one: isoelectric focusing. The recombinant enzyme had a more basic mean isoelectric point of 5.1 whilst that of the native enzyme was 4.5.

The search for new hydroxynitrile lyases

Although recombinant DNA technology has now provided a way to mass produce these desirable enzymes for industrial applications, the ever-evolving world of pharmaceuticals and fine chemicals, the need for more environmentally friendly pesticides and herbicides, and greater environmental awareness with regard to industrial processes have now led researchers to search for hydroxynitrile lyases that have novel substrate specificity. This, hopefully, will lead to the manufacture of new types of medication, pesticides, etc. One of the main restrictions in the use of hydroxynitrile lyases today is their narrow substrate range and sometimes unstable behaviour under industrial conditions. As can be seen in the previous sections, the majority of the hydroxynitrile lyases discovered to date show activity toward mandelonitrile and acetone cyanohydrin. The search for novel or improved hydroxynitrile lyases, which will hopefully have a novel or wider substrate specificity has therefore begun once again.

Hernandez et al. (2004) screened for hydroxynitrile lyase activity in the crude extracts of several edible plants. Seeds of *Cucumis melo*, *Cydonia oblonga* and *Annona cherimolia*, as well as the leaves of *Prunus persica*, *Prunus avium*, *Prunus domestica*, *Prunus serotina* var. *Capuli* and *Pouteria sapota* exhibited activity. They were also the first to report hydroxynitrile lyase activity in the *C. melo* seed. Until 2004, no hydroxynitrile lyase activity had been reported for any member of the *Cucurbitaceae* family. Their crude extracts biocatalysed the transformation of benzaldehyde to mandelonitrile with varying degrees of enantioselectivity, some better than others. New alternative enzyme sources were found.

Asano et al. (2005) screened for new hydroxynitrile lyases from 163 plants from 74 families using their own, in-house developed HPLC method. *Rosaceae*, *Euphorbiaceae*, *Leguminosae*, *Passifloraceae*, *Araceae* and other families were tested and partially-purified (*R*)-hydroxynitrile lyases from *P. edulis* and *Eriobotrya japonica* showed activity toward benzaldehyde and an aliphatic ketone. This again confirms that hydroxynitrile lyases with varying substrate specificities do occur and this provides an incentive to search for more.

Andexer et al. (2009) suggested the following two methods or strategies: the first being a function-based

or activity-based approach, and the second a sequence-based approach. The first approach involves selecting a plant from a family that is known to contain cyanogenic species and then screening their crude/tissue extracts for the desired activity. The second involves synthesising double-stranded cDNA from the plants mRNA, sequencing the double-stranded cDNA and then scanning this sequence (using various databases) for a sequence similar to available, known hydroxynitrile lyase sequences. The similar sequence can then be synthesised and cloned into a microbial host using recombinant DNA technology for over-expression. However, a major limitation of the sequence-based approach is that an undiscovered or novel hydroxynitrile lyase could be very easily missed, especially if one is only comparing a plants cDNA sequence to those of already discovered enzymes. Wajant and Forster (1996) found that there were no obvious sequence homologies among *P. serotina* hydroxynitrile lyase, *M. esculenta* hydroxynitrile lyase or the *S. bicolor* hydroxynitrile lyase (Wajant and Mundry 1993). Therefore, trying to look for a similar sequence would not be an ideal approach.

As part of this renewed interest, two papers offering alternative screening methods, one novel, and the other a modern take on a classical method are of particular interest and worth noting.

Krammer et al. (2007) developed a novel hydroxynitrile lyase screening assay, concentrating specifically on its application in high-throughput screening of *E. coli* mutant libraries. This method can also be used to discover improved hydroxynitrile lyase variants and makes use of a key substrate. Only colonies carrying the gene coding for the desired enzyme will be able to break down the substrate, resulting in a visible colorimetric reaction when a by-product is detected, for example, when HCN is detected, the test paper will turn blue, as in the case of the hydroxynitrile lyase.

Takos et al. (2010) developed a cyanogenic screening test for plants. This method is based on the method by Feigl and Anger (1966) and makes use of a single freeze-thaw tissue cycle. A visible colorimetric reaction is produced on a specially prepared filter paper upon detection of HCN (a concise blue spot), as used by Krammer et al. (2007) as well. This method allows for the screening of plant samples for cyanogenic activity on a rapid and large scale (up to 96 samples or more at one time).

Conclusion and perspectives

Hydroxynitrile lyases, a concept or technology which was thought, by some, to be outdated, having had its hey-day in the 1980s and 1990s, is slowly gaining new interest. Recombinant DNA technology has solved the initial concerns regarding lack of availability and insufficient amounts of this enzyme for industrial application. Pharmaceutical companies have realised how essential this enzyme is to produce numerous novel medicines and pesticides. But, the search is on again, this time not for a way to produce more hydroxynitrile lyase for industrial applications, but to find a previously undiscovered hydroxynitrile lyase and, more importantly, to find a hydroxynitrile lyase that has novel substrate specificity.

Acknowledgments We would like to thank Mr. Kevin Slaney for his assistance with the chemical structures used in this review.

Conflict of interest The authors report no conflict of interest.

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South African Journal of Botany 93 (2014) 9–13

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Screening for hydroxynitrile lyase activity in non-commercialised plants

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ARTICLE INFO

Article history:
Received 22 January 2014
Received in revised form 26 February 2014
Accepted 5 March 2014
Available online xxxx

Edited by JJ Nair

Keywords:
Adenia
Cyanogenesis
Biocatalysis
Hydroxynitrile lyase

ABSTRACT

Hydroxynitrile lyases are used for the synthesis of enantiomerically pure cyanohydrins which are of great importance in the pharmaceutical and fine chemical industries. In this study, the hydroxynitrile lyase activity of 100 plants from 40 families was investigated, first by screening for cyanogenic activity, followed by a hydroxynitrile lyase activity assay. Of the 100 plants, four were found to be cyanogenic and exhibited specific hydroxynitrile lyase activity: *Adenia* sp. (0.44 U/mg), *Adenia firingalavensis* (2.88 U/mg), *Adenia fruticosa* (1.99 U/mg) and, *Adenia pechuelii* (2.35 U/mg), all from the family Passifloraceae. This is the first report of hydroxynitrile lyase activity in these plants.

1. Introduction

In plants, a cyanohydrin is broken down by a hydroxynitrile lyase into hydrogen cyanide and an aldehyde or ketone. This reaction, known as cyanogenesis, occurs in over 3000 plant species and serves as an ingenious self defence mechanism — the hydrogen cyanide released by these cyanogenic plants protects them from further microbial, fungal and animal attacks (Conn, 1981; Erdman, 2003; Jones, 1998; Sharma et al., 2005; Ueatrongchit et al., 2010; Zagrobelny et al., 2008). However, in principle, each enzymatic reaction is completely reversible, and it is this reversible reaction (Fig. 1) which is of interest.

The chiral cyanohydrins produced using this reversible reaction — the condensation of hydrogen cyanide with an aldehyde or ketone is of great importance to the pharmaceutical and fine chemical industries where they are used as building blocks in the production of various essentials such as pesticides, medicines, agrochemicals, etc. (Dadashipour et al., 2011). Hydroxynitrile lyase can also be used to detoxify cyanogenic food plants (Fokunang et al., 2001; Hasslacher et al., 1996).

The first discovery of hydroxynitrile lyase occurred in 1837, by two German chemists — Justus von Liebig and Friedrich Wohler. This was found in an *Amygdalus communis* (bitter almond) extract (Hosel, 1981). According to literature, the two main families containing cyanogenic plants and therefore contributing hydroxynitrile lyases are the Euphorbiaceae and Rosaceae. Despite the numerous discoveries of other hydroxynitrile lyases since, the application of these naturally occurring enzymes in enantioselective biocatalytic synthesis was limited due to the difficulty of obtaining a sufficient amount and most importantly, lack of novel substrate specificity (Asano et al., 2005; Dadashipour et al., 2011; Hernandez et al., 2004; Hughes et al., 1994; Sharma et al., 2005; Wajant et al., 1995; Xu et al., 1988).

Although recombinant DNA technology has now provided a way to mass produce these enzymes for industrial applications, there is still a lack of novel substrate specificity.

One solution to this is to identify multiple plants exhibiting hydroxynitrile lyase activity as there is the potential that each such plant may contain a hydroxynitrile lyase with novel substrate specificity, partially addressing the second issue. The aim of this study is to identify such new plants.

2. Materials and methods

2.1. Plant identification and collection

Apical buds from 100 plant species, comprising 40 families (Table 1) were collected in triplicate from the Pretoria National Botanical Gardens (National Herbarium, South African National Biodiversity Institute).

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http://dx.doi.org/10.1016/j.sajb.2014.03.004
0254-6299/

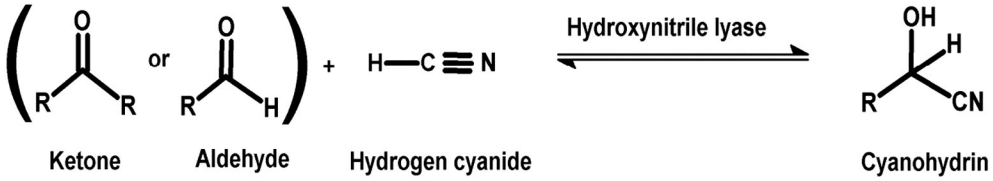

Fig. 1. General reaction catalysed by hydroxynitrile lyase.

These plants were specifically selected from families in which cyanogenesis had been reported previously.

The microtitre plates containing the samples were kept on ice at all times during sample collection to prevent wilting and were stored at −80 °C within 4 h.

2.2. Cyanogenic activity

Cyanogenic activity was tested using the Feigl–Anger test (Feigl and Anger, 1966; Takos et al., 2010) which relies on the oxidation of a tetrabase in the presence of hydrogen cyanide (a by-product of cyanogenesis) to create a distinct blue spot on a specially prepared detection paper after tissue disruption by a single freeze–thaw cycle. A Whatman 3MM filter paper, cut to the dimensions of 8 cm × 11 cm to fit the microtitre plate was used. The solution was then prepared by separately dissolving 75 mg of copper ethylacetoacetate (Sigma-Aldrich) and 75 mg of the tetrabase 4,4-methylenebis (N,N-dimethylalanine) (Sigma-Aldrich) in 7.5 ml of chloroform (Merck) each and then combining both solutions. The filter paper was then saturated with this combined solution and allowed to dry. After drying, the resultant

Table 1
Plants used in this study.

Family	Species
Acanthaceae	*Barleria obtusisepala* C.B. Clarke
Aizoaceae	*Ruschia* sp. C.f. *indurata*, L.c. *Trichodiadema* sp.
Anacardiaceae	*Searsia lancea* (L.F) F.A. Barkley
Apocynaceae	*Orbea melanantha* (Schltr.) Bruyns, *Pachypodium namaquensis* (Wyley ex Harv.) Welw., *Pachypodium lamerei* Drake, *Strophanthus amboensis* (Schinz) Engl. & Pax, *Acokanthera oblongifolia* (Hochst.) Benth. & Hook.f. ex B.D. Jacks., *Catharanthus roseus* (L.) G. Don, *Rauvolfia caffra* Sond.
Asclepiadaceae	*Huernia zebrina* (Phillips) L.C. Leach
Asparagaceae	*Asparagus densiflorus* (Kunth) Jessop
Asteraceae	*Kleinia stapeliiformis* (E. Phillips) Stapf, *Senecio barbertonicus* Klatt
Boraginaceae	*Ehretia rigida* (Thunb.) Druce
Cactaceae	*Rhipsalis baccifera* (J.S. Muell.) Stearn
Cannabaceae	*Celtis africana* Burm. f.
Celastraceae	*Elaeodendron croceum* (Thunb.) DC., *Gymnosporia tenuispina* (Sond.) Szyszyl., *Putterlickia verrucosa* (E. Mey. Ex Sond.) Szyszyl.
Crassulaceae	*Kalanchoe beharensis* Drake, *Adromischus* sp., *Adromischus filicaulis* (Eckl. & Zeyh.) C.A. Sm. subsp. *filicaulis*, L.c *Adromischus diabolicus* Toelken
Cucurbitaceae	*Xerosicyos danguyi* Humbert
Cycadaceae	*Cycas thouarsii* R. Br.
Dichapetalaceae	*Dichapetalum cymosum* (Hook.) Engl.
Didiereaceae	*Alluaudiopsis fiherenensis* Humbert & Choux, *Alluaudia procera* (Drake) Drake, *Alluaudia dumosa* (Drake) Drake, *Alluaudia humbertii* Choux, *Decarya madagascariensis* Choux
Dioscoreaceae	*Dioscorea dregeana* (Kunth) T. Durand & Schinz
Ebenaceae	*Euclea* sp. (Pretoria National Botanical Gardens 18236/73)
Euphorbiaceae	*Euphorbia tortirama* R.A. Dyer, *Euphorbia fruticosa* Forssk, *Euphorbia platyclada* Rauh, *Euphorbia stellispina* Haw., *Euphorbia leistneri* R.H. Archer, *Euphorbia pseudocactus* A. Berger, *Euphorbia cylindrica* Marloth ex A.C. White, R.A. Dyer & B. Sloane, *Euphorbia clivicola* R.A. Dyer, *Euphorbia buruana* Pax, *Euphorbia jansenvillensis* Nel, *Euphorbia fusca* Marloth, *Euphorbia gummifera* Boiss., *Euphorbia* C.f. *aeruginosa*, *Euphorbia bupleurifolia* Jacq., *Euphorbia lignosa* Marloth, *Euphorbia pulvinata* Marloth, *Euphorbia aeruginosa* Schweick, *Euphorbia monteiroi* Hook., *Euphorbia antso* Denis., *Euphorbia invenusta* (N.E.Br.) Bruyns, *Euphorbia schubei* Pax, *Euphorbia lugardae* (N.E.Br.) Bruyns, *Euphorbia ritchiei* (P.R.O Bally) Bruyns, *Euphorbia guentheri* (Pax) Bruyns, *Euphorbia virosa* Willd., *Euphorbia dregeana* E. Mey. Ex Boiss., *Euphorbia cupularis* Boiss., *Spirostachys africana* Sond., *Sclerocroton ellipticus* Hochst., *Croton sylvaticus* Hochst., *Croton gratissimus* subsp. *Gratissimus*, L.c. *Jatropha curcus*
Fabaceae	*Burkea africana* Hook., *Philenoptera violaceae* (Klotzch) Schrire, *Peltophorum africanum* Sond., *Bauhinia galpinii* N.E.Br., *Colophospermum mopane* (Benth.) Leonard,
Geraniaceae	*Pelargonium ceratophyllum* L'Her., *Pelargonium klinghardtense* R. Knuth, *Pelargonium crassicaule* L'Her.
Gesneriaceae	*Streptocarpus* sp. Pink cultivar
Hernandiaceae	*Gyrocarpus americanus* Jacq.
Icacinaceae	*Pyrenacantha cordata* Villiers.
Juncaceae	*Juncus effusa* L.
Lamiaceae	*Tetradenia fruticosa* Benth.
Malvaceae	*Grewia flavescens* Juss.
Meliaceae	*Turraea obtusifolia* Hochst.
Menispermaceae	*Tinospora fragosa* Verdoorn & Troupin
Moringaceae	*Moringa drouhardii* Jum.
Pandanaceae	*Pandanus epiphyticus* Martelli
Passifloraceae	*Adenia* sp. (Pretoria National Botanical Gardens 14638/69), *Adenia* sp., *Adenia firingalavensis* (Drake ex Jum.) Harms, *Adenia fruticosa* Burtt Davy, *Adenia pechuelii* (Engl.) Harms, *Adenia gummifera* (Harv.) Harms
Pedaliaceae	*Ceratotheca triloba* (Bernh.) Hook.f.
Phyllanthaceae	*Bridelia catharthica* subsp. *Carthartica*
Polygalaceae	*Polygala myrtifolia* L.
Pteridaceae	*Adianthum* sp.
Rosaceae	*Leucosidea sericea* Eckl. & Zeyh.
Rubiaceae	*Vangueria infausta* Burch., *Xeromphis obovata* (Hochst.) Keay
Solanaceae	*Solanum tomentosum* L., *Solanum seaforthianum* Andrews
Zamiaceae	*Encephalartos friderici-guilielmi* Lehm.

pale green detection paper was stored in a dark, dry place at 4 °C until required.

The trays containing the samples were removed from the −80 °C freezer and the detection paper was immediately overlaid on the plate. The plate was then covered, and the lid weighed down to create a tight fit between plate, detection paper and lid to prevent diffusion of hydrogen cyanide from individual wells. The tissue was then allowed to thaw and disrupt on the laboratory bench at room temperature. Results were recorded within 3 h (assessed after 1, 2 and 3 h) in order to detect any hydrogen cyanide released.

Apical buds from *Manihot esculenta* Crantz (cassava), were used as a positive control whilst distilled water was used as a negative control. A positive result was indicated by a change in colour of the detection paper from white to blue. No change in colour indicated a negative result. An image of each result was captured immediately as the colour is known to fade with time.

2.3. Hydroxynitrile lyase activity

2.3.1. Crude enzyme extraction from cyanogenic plants

Extraction of the crude enzyme was performed according to Ueatrongchit et al. (2010) with slight modifications. Young leaves (1 g) were frozen in liquid nitrogen and homogenized by mortar and pestle to form a fine powder. The powder was re-suspended in 1 ml of 50 mM sodium citrate buffer (pH 5.0) and vortexed vigorously for 5 min. The resultant slurry was centrifuged at 20000 ×*g* for 10 min. The supernatant was used as the crude enzyme extract.

2.3.2. Protein assay

Total protein was quantified in a Qubit Fluorometer (Life Technologies) using a Qubit protein assay kit (Life Technologies) according to the manufacturer's instructions.

2.3.3. Hydroxynitrile lyase activity assay

Hydroxynitrile lyase specific activity was measured spectrophotometrically as described by Krammer et al. (2007) and Zhao et al. (2011) by following the formation of benzaldehyde from a racemic mixture of mandelonitrile (Fig. 2).

The reaction mixture comprised 50 mM sodium citrate buffer (pH 5), 150 μl of the crude enzyme extract and 10 mM mandelonitrile (Sigma-Aldrich) to a final volume of 3 ml in a Quartz cell. The substrate was always added last. Twenty seconds after the substrate was added, the spectrophotometer (Jenway 6305 UV/Vis, Staffordshire, United Kingdom) was blanked using the reaction mixture and the formation of benzaldehyde was tracked by monitoring the increase in absorbance at 280 nm for 10 min at room temperature.

Due to the fact that mandelonitrile breaks down spontaneously at a pH > 5, a control reaction was run in parallel with the samples and comprised 50 mM sodium citrate buffer (pH 5) and 10 mM mandelonitrile in a final volume of 3 ml. The linear slope of absorbance resulting from the spontaneous decomposition of mandelonitrile (control) was subsequently subtracted from the slope of absorbance obtained for the crude enzyme assays in order to avoid false indications of hydroxynitrile lyase activity in the plant macerates. All assays were carried out in triplicate.

Hydroxynitrile lyase activity was then calculated using the equation:

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

$$\Delta\text{OD} = \Delta A_{280}/\min_{\textit{crude enzyme}} - \Delta A_{280}/\min_{\textit{spontaneous}}$$

where ΔOD represents the difference in the maximum linear rate between the crude enzyme extract and the spontaneous decomposition reaction, ε is the absorption coefficiency (1.3761 ml/μmol/cm) at 280 nm, V_t and V_e are the total volume of the reaction and enzyme volumes used respectively. 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.

3. Results

3.1. Cyanogenic activity

Of the 100 plants tested, four were found to be cyanogenic: *Adenia* sp., *Adenia firingalavensis*, *Adenia fruticosa*, and *Adenia pechuelii*, all from the family Passifloraceae (Fig. 3).

3.2. Hydroxynitrile lyase activity

Hydroxynitrile lyase specific activity (Table 2) was calculated for the crude enzyme extracts of *Adenia* sp., *A. firingalavensis*, *A. fruticosa* and *A. pechuelii* which were identified as cyanogenic. The total activity represents the yield of enzyme at each step, whereas the specific activity is a measure of the purity of the enzyme. *A. firingalavensis*, *A. fruticosa* and *A. pechuelii* exhibited the highest hydroxynitrile lyase specific activities.

4. Discussion

To our knowledge, cyanogenic properties and hydroxynitrile lyase activity have not been reported for any of the selected plants, until now, and no study of this nature has been performed previously in South Africa.

Of the four new cyanogenic plants discovered in this study, all were of the genus *Adenia*, from the Passifloraceae family. Certain species within the Passifloraceae are known to be cyanogenic (Asano et al., 2005; Hernandez et al., 2004; Ueatrongchit et al., 2010) however, no studies are known to the authors that report cyanogenic activity in *Adenia*.

In 2005, Asano and co-workers were the first to report (*R*)-hydroxynitrile lyase activity in *Passiflora edulis* Sims. Thereafter, in 2010, Ueatrongchit and co-workers characterised and purified a hydroxynitrile lyase from *P. edulis*, a member of the Passifloraceae family, for the first time. They reported a specific activity of 2.47 U/mg in the

Fig. 2. Two products of hydroxynitrile lyase mediated catalysis.

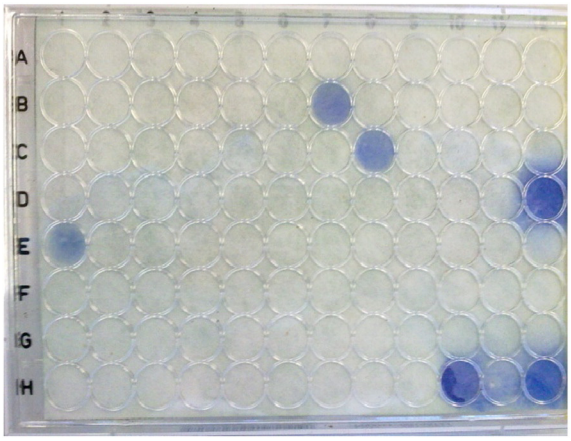

Fig. 3. Screening for cyanogenic activity — a representative Feigl–Anger detection paper exposed to samples after a 2 h incubation period. A blue spot indicates cyanogenic activity due to hydrogen cyanide detection, whilst the absence of a blue spot indicates a negative test for cyanogenesis. *Adenia* sp. was in well B7, *Adenia firingalavensis* in C8, *Adenia fruticosa* in D12, and *Adenia pechuelii* in E1. The positive control *Manihot esculenta* was in wells H10 and H12 and negative control (distilled water) in wells F10 and F12.

crude extract from the leaves of *P. edulis*. This specific activity is not dissimilar from three of the findings in the current study, indicating that the enzymes present in the currently studied plants have similar levels of activity to those found in other plants from the same family. However, it may also indicate that these enzymes have the same characteristics as those found in *P. edulis*. Further study is thus needed to exclude this possibility and confirm the presence of novel substrate specificity.

The specific activities measured in this study were not unexpected as hydroxynitrile lyase activity is usually low in the crude homogenate of leaves. This can be attributed to the low concentration of hydroxynitrile lyase present relative to the higher concentrations of other contaminating proteins in the crude extract. Hydroxynitrile lyase activity also largely depends on the pH of the plant macerates requiring an acidic pH to be active (Dadashipour et al., 2011). Ueatrongchit et al. (2008) isolated and purified to homogeneity a hydroxynitrile lyase from the seeds of *Eriobotrya japonica* (Thunb). They reported a specific activity of 0.8 U/mg in the crude extract. However, after affinity column Concanavalin A Sepharose 4B purification, the specific activity had increased to 40.9 U/mg.

Although the remaining 39 families studied are known to contain cyanogenic species, none of the selected plants from these families exhibited cyanogenic activity in this study. It is therefore important to note that because a plant family contains cyanogenic plant species, it cannot be presumed that every member of that family will be cyanogenic. This cyanogenic variation amongst species may be attributed to a cyanogenic polymorphism (Goodger and Woodrow, 2002).

However, it should be noted that a negative test for cyanogenic activity is not necessarily evidence that a plant is not cyanogenic. It is possible that the age or stage of growth of the plant, the part of the plant that was tested, environmental conditions, climate and seasonal variation could have very well contributed to the results of this study. In this study, it was only the apical bud of each plant that was tested as the apical bud and younger leaf tissues are known to contain the highest concentration of cyanogenic glycosides (Gleadow and Woodrow, 2000).

Gleadow and Woodrow (2000) found that the concentration of cyanogenic glycosides decreases as the leaves mature. They also found that the cyanogenic glycoside concentration in the young leaf tips varied seasonally, and propose that this seasonal variation in cyanogenic glycoside concentration may be due to the availability of nitrogen in the soil, climate, temperature, etc.

Using field and greenhouse studies in 2001, Gebrehiwot and Beuselinck confirmed seasonal variation in hydrogen cyanide concentrations. They found that plants in Spring and Summer had a 50% greater concentration of hydrogen cyanide than the same plants in Autumn or Winter, with the lowest concentrations observed in Winter. The presence and concentrations of various compounds in a plant are known to vary with regard to season and age (Pichersky and Lewinsohn, 2011).

Hernandez et al. (2004) found that the different parts (leaves, seeds, etc.) of the same plant gave a different result. They found in some plants that the seeds were cyanogenic, but the leaves were not, and vice-versa.

Table 2
Hydroxynitrile lyase activity in the crude extracts of cyanogenic plants.

Plant species	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
Adenia sp.	0.304 ± 0.169	0.692	0.440 ± 0.190
Adenia firingalavensis	0.559 ± 0.265	0.194	2.884 ± 0.240
Adenia fruticosa	0.878 ± 0.094	0.440	1.998 ± 0.214
Adenia pechuelii	1.917 ± 0.630	0.815	2.353 ± 0.200

5. Conclusion

In this study, four new cyanogenic plants exhibiting hydroxynitrile lyase activity have been reported. The crude extracts of these plants exhibited specific activities similar to that found in the crude extract of *P. edulis*. These findings expand the number of plants known to exhibit hydroxynitrile lyase activity and there is the potential that these plants may contain an enzyme with novel substrate specificity.

The remaining 96 plants which tested negative cannot however be confirmed as non cyanogenic due to the effects of seasonal variation.

Future work will involve the expression of the hydroxynitrile lyases from these four plants in a microbial host (heterologous expression), followed by purification and characterisation (including stereochemistry and substrate specificity studies). Industrial applications of the current study's findings will be better understood after this further study.

Acknowledgements

From the School of Molecular and Cell Biology, University of the Witwatersrand, we would like to thank Ms. Sarah Rogans for providing the *Manihot esculenta* used as the positive control and Dr. Derryn Legg-E'silva for her expert assistance with the preparation of this manuscript. We would also like to thank Mr. Kevin Slaney for his expert assistance with the chemical structures used and preparation of this manuscript.

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