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Screening for hydroxynitrile lyase activity in non-commercialised plants

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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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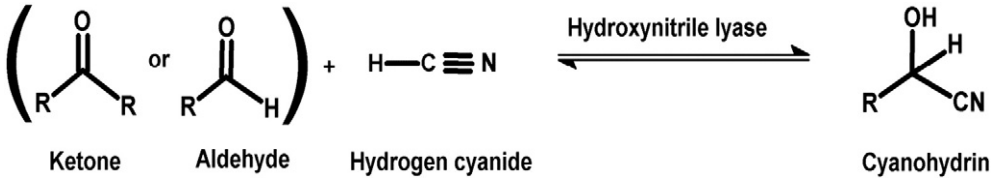

Fig. 1. General reaction catalysed by hydroxynitrile lyase.

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.

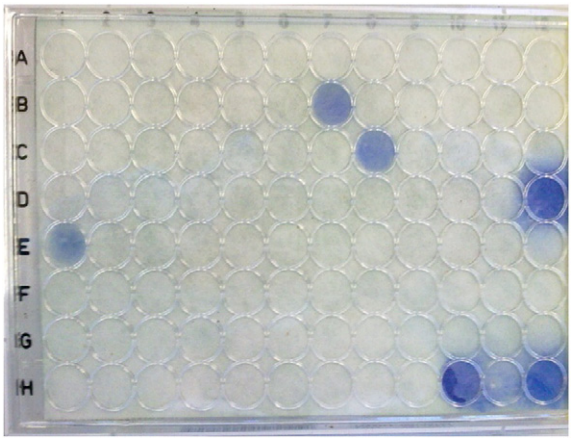

Fig. 3. Screening for cyanogenic activity — a representative Feigl–Anger detection paper exposed to samples after a 2 h incubation period. A blue spot indicates cyanogenic activity due to hydrogen cyanide detection, whilst the absence of a blue spot indicates a negative test for cyanogenesis. *Adenia* 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.

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