Urease Inhibitors of Agricultural Interest Inspired by Structures of Plant Phenolic Aldehydes

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The plant phenolic natural products (PNPs) protocatechuic aldehyde, syringaldehyde and vanillin were used as platforms for obtaining four urease inhibitors. Urea (urease substrate) or thiourea (urease inhibitor) core was added to the structure of newly synthesized compounds to provide inhibitors up to 230-fold more active than the PNPs they originated from. The PNP derivatives are mixed inhibitors with higher affinity to urease active site. Two compounds were as efficient as N-(butyl)thiophosphoric triamide (NBPT) toward soil. Overall, PNP derivatives are promising urease inhibitors for use as additive in urea-based fertilizers formulations.

Keywords: urea, urease inhibitor, natural product, phenolic aldehyde, fertilizer additive

Introduction

Nitrogen (N) is a key nutrient absorbed by plants mostly as nitrate (NO$_3^-$) and/or ammonium (NH$_4^+$). Despite the great abundance of N in nature, less than 2% is bioavailable to plants. Biological N fixation, soil organic matter mineralization and lightening are natural processes known to increase the input of absorbing N in soil.$^1$ However, these sources of bioavailable N are not enough to guarantee food supply for the growing world’s population predicted to reach over 9.5 billion people within the next 35 years.$^2$ Then, N fertilizers have been widely used to improve crop productivity, in particular urea, due to its high N content (46%), low price per N unit and easy management.$^3$ Urease (EC 3.5.1.5; urea amidohydrolase) catalyzes the hydrolysis of urea furnishing the gaseous products ammonia (NH$_3$) and carbon dioxide (CO$_2$).$^4$ It occurs in a variety of organisms including plants, fungi, bacteria and some vertebrates. Soil ureases play essential roles in N global cycle, also contributing to agriculture with respect to the availability of N as NH$_4^+$ to plant growth in soils supplemented with urea-based fertilizers.$^5$ On the other hand, soil ureases can also be detrimental for crop production specially when using the technique of covering fertilization. In this case, the urea applied on soil surface is rapidly hydrolyzed by soil ureases releasing NH$_3$ far away from rhizosphere allowing for N losses to the atmosphere due to the volatile nature of NH$_3$. In fact, depending on climate and soil physicochemical properties, more than 50% of the N-urea applied to soil surface can be lost mainly by NH$_3$ volatilization.$^4$ Besides negatively affecting plant N nutrition, excessive N losses to atmosphere as NH$_3$ remarkably impact natural ecosystems by contributing either directly or indirectly to acid rain, lakes and rivers eutrophication and formation of nitrous oxide, an atmospheric pollutant.$^7$

One of the strategies that have been adopted to minimize N losses as NH$_3$ is the use of urea-based fertilizers supplemented with urease inhibitors to slow down urea hydrolysis on soil surface and further increase the possibility of urea incorporation to soil by rain and irrigation.$^8$ The potential of several classes of substances to inhibit the ureolytic activity of soil ureases have been investigated. Recently, our group has described that phosphoramidate, benzothiazole and benzoylthiourea compounds are also promising urease inhibitors.$^8$ Up to now, phosphorodiamide and phosphorotriamide derivatives
are recognized as the most efficient urease inhibitors for crop production purposes.\textsuperscript{13-15} Indeed, the \textit{N-}(butyl)thiophosphoric triamide (NBPT) was found to become a very effective inhibitor when transformed to its corresponding \textit{oxo}-derivative (\textit{oxo}-NBPT) by soil microorganisms.\textsuperscript{16} The NBPT effectiveness is directly related to soil properties in which low concentration of this urease proinhibitor is needed to achieve the desired result in temperate soils while greater concentrations are required for tropical soils.\textsuperscript{17-20} Additionally, NBPT is more efficient in neutral soils with limited organic matter.\textsuperscript{17,21} Tropical soils exhibit organic matter and microbial biomass dynamics different from temperate soils.\textsuperscript{22} The amendment of soil with organic matter demanded from 2- to 4-fold NBPT to alleviate N volatilization by 20\% in comparison to soils devoid of crop residues.\textsuperscript{23} Although other works investigated the NBPT effectiveness in tropical soils,\textsuperscript{24-26} further research is needed for the development of novel and cost-effective urease inhibitors with improved efficiency in tropical soils and different environmental conditions.

Nature is undoubtedly a source of metabolites with potential to interfere with the activity of ureases as determined by \textit{in vitro} assays with pure enzymes from \textit{Helicobacter pylori} or \textit{Canavalia ensiformis} (jack bean).\textsuperscript{27} Among natural products produced by plants, phenolic compounds, such as flavonoids, methyl gallate and stilbenoids have been shown to inhibit ureases.\textsuperscript{28-30} Although there is no report on the ability of natural phenolic aldehydes to inhibit ureases, it is likely that such secondary metabolites may work on ureases or be good prototypes for the design of urease inhibitors. Examples of natural phenolic aldehydes that have been explored as health promoters include protocatechuic aldehyde (PA), syringaldehyde (SA) and vanillin (VA).

The aim of the study herein presented was to use the natural products PA, SA and VA as building blocks for the development of four urease inhibitors of agricultural interest (Figure 1). Urea (urease substrate) or thiourea (urease inhibitor) core was also introduced to the structure of phenolic aldehyde derivatives synthesized (Figure 1). Then, \textit{in vitro} assays were performed with pure jack bean urease to check the potential of synthesized compounds as inhibitors of ureolytic activity and disclose the mechanism of action of promising molecules. The effect of such phenolic aldehyde derivatives on soil ureases was addressed to confirm the potential of synthesized compounds for use as additives in urea-based fertilizers.

\section*{Experimental}

\subsection*{Preparation of phenolic aldehyde derivatives}

An ethanolic mixture containing protocatechuic aldehyde (PA), syringaldehyde (SA) or vanillin (VA) individually (1 mmol; Sigma-Aldrich), ethyl acetacetate (1.5 mmol) and urea or thiourea (1.5 mmol), here referred to as (thio)urea, and \textit{p}-sulfonic acid calix[4]arene (0.5 mol\%) was maintained under reflux and vigorous stirring for 8 h. After then, the mixture was filtered and the phenolic aldehyde derivative formed was recrystallized using ethanol. The phenolic aldehyde derivatives synthesized based on urea (2A7) or thiourea (2A9, 2B10 and 2D2) structure were characterized by \textsuperscript{1}H and \textsuperscript{13}C nuclear magnetic resonance (NMR), infrared, melting point and elemental analysis and the data compared to those reported

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Design of the urease inhibitors of agricultural interest 2A7, 2A9, 2B10 and 2D2 based on protocatechuic aldehyde (PA), vanilin (VN) or syringaldehyde (SA) structures combined with (thio)urea cores.}
\end{figure}
The phenolic aldehyde derivatives were obtained in 49 to 80% yield.

**In vitro urease activity assay**

Initially, the phenolic aldehydes and derivatives 2A7, 2A9, 2B10 and 2D2 were screened for the ability to inhibit in vitro the ureolytic activity of purified *Canavalia ensiformis* (jack bean) type III urease (Sigma, St. Louis, Mo, USA). Each reaction medium containing 20, 1 and 10 mmol L\(^{-1}\) of phosphate buffer (pH 7.0), ethylene diamine tetra acetic acid (EDTA) and urea, respectively, 12.5 mU urease and compounds-test at 0 or 1.6 mM was incubated for 10 min at 25 °C. Reactions were stopped by adding 0.5 volume of 1% m/v phenol in 5 mg L\(^{-1}\) sodium nitroprusside (SNP) followed by the addition of 0.7 volume of 0.5% m/v NaOH in 0.1% v/v NaOCl solution. After samples incubation at 50 °C for 5 min, the absorbance was measured at 630 nm to determine the amount of ammonium (NH\(_4^+\)) formed.\(^{31}\) Hydroxyurea (HU) was used as a reference of urease inhibitor. Urease inhibition was determined in terms of percentage of NH\(_4^+\) formed in compounds-test reactions in relation to total urease activity in reactions without compounds. Three independent experiments were performed, each with four replicates.

Effect of phenolic aldehyde derivatives on the kinetic parameters of jack bean urease

The inhibition profile exhibited by the natural product derivatives 2A7, 2A9, 2B10 and 2D2, synthesized in this study, was determined by incubating inhibitors at concentrations necessary to inhibit jack bean urease activity between 30 and 40% (from 0.3 to 1.6 mM) in reaction medium containing 20 mmol L\(^{-1}\) phosphate buffer (pH 7.0), 1 mmol L\(^{-1}\) EDTA, urea (ranging from 1 to 32 mmol L\(^{-1}\)) and 12.5 mU urease. The stoppage of reactions, NH\(_4^+\) quantification and urease inhibition calculation were done as described previously. Jack bean urease kinetic parameters such as initial velocity (V\(_i\)), K\(_m\) (Michaelian constant) and maximum velocity (V\(_{max}\)) were obtained using Hyper32 software.\(^{34}\) The OriginPro8 (Origin Lab, Northampton, MA) software was used to pursue Michaelis-Menten hyperbolas and Lineweaver-Burk plots. The equilibrium dissociation constants for urease-inhibitor complex (K\(_i\)) and for urease-urea-inhibitor complex (K\(_i^*\)) were determined from the \(\alpha\) and the \(\alpha^*\) values.\(^{35}\)

**Soil ureases activity assay**

The effect of phenolic aldehyde derivatives on the activity of soil ureases was assessed by the salicylate method as described elsewhere,\(^{36}\) with some modifications. Clayey dystrophic Red Latosol (oxisol) soil was collected from Brazilian Cerrado (19°28'01.2''S, 44°10'24.5''W). The physical features of the collected soil were 6, 4, 12 and 78% of coarse sand, fine sand, silt and clay, respectively, and chemical analyses showed pH 6.3, 10 mg L\(^{-1}\) P\(_{miclich-1}\), 129 mg L\(^{-1}\) K, 4.4, 0.9, 0.1 and 2.6 cmol L\(^{-1}\) of Ca\(^{2+}\), Mg\(^{2+}\), Al\(^{3+}\) and H + Al, respectively, sum of bases of 5.6 cmol L\(^{-1}\), 68% base saturation, organic matter of 2.5 dag kg\(^{-1}\).

Sieved soil samples (0.5 g; particles smaller than 2 mm) were incubated with 72 mmol L\(^{-1}\) urea in the absence or 3.2 mmol L\(^{-1}\) of 2A7, 2A9, 2B10, 2D2 or NBPT (used as a reference of soil ureases inhibitor) at 37 °C for 1 h. Ureases activity was stopped by incubating the systems with 5 mL of 1 mol L\(^{-1}\) KCl in 10 mmol L\(^{-1}\) HCl for 30 min at 25 °C. A supernatant aliquot was taken after soil decantation and added to a solution containing 3.4, 2.5 and 2.5% of sodium salicylate, sodium citrate and sodium tartrate, respectively, and 120 mg L\(^{-1}\) SNP. After 15 min incubation at 25 °C (under darkness), 0.1 volume of 3.0% NaOH in 1.0% sodium hypochlorite was added to each reaction system following incubation under darkness for 1 h at 25 °C and 600 rpm. The NH\(_4^+\) formed was detected by spectrophotometric measurements at 660 nm.

Then, assays using different concentrations (from 0.05 to 3.2 mM) of phenolic aldehyde derivatives or NBPT were performed to determine the concentration of compound-test that causes 50% inhibition of soil ureases (IC\(_{50}\)). Independent experiments were performed, each with at least five replicates.

**Statistical analysis**

Data were subjected to analysis of variance (ANOVA) by general linear model (GLM) procedure and contrast analysis at 5% significance level using the software R (Software Foundation, Boston, MA, USA).

**Results**

Inhibition of ureolytic activity of jack bean urease

The in vitro assay with purified jack bean type III urease showed that, among the natural products tested, only protocatechuic aldehyde (PA; at 1.6 mM) effectively inhibited the enzyme activity (68% inhibition) while vanillin (VN) and syringaldehyde (SA) marginally reduced the production of NH\(_4^+\) (Figure 2). The derivatives 2A7 and 2B10, originated from PA, were the most potent urease inhibitors showing results (94% urease inhibition) comparable to that observed for the standard inhibitor.
hydroxyurea (HU; Figure 2). Compounds 2A9 and 2D2, derived from VN and SA, respectively, caused enzyme inhibition that averaged 58.6% (Figure 2).

![Figure 2. Inhibition of jack bean urease by phenolic aldehydes and its derivatives. The compounds hydroxyurea (HU), protocatechuic aldehyde (PA), syringaldehyde (SA), vanillin (VA), 2A7 and 2B10 (PA derivatives), 2A9 (SA derivative) and 2D2 (VN derivative) were employed at 1.6 mM in reactions containing 10 mmol L\(^{-1}\) urea and 12.5 μU urease. Results are representative of three independent experiments, each with four replicates. Different letters indicate significant difference (\(p < 0.05\) by contrast analysis) among the compounds.](image)

**Mechanism of action of phenolic aldehyde derivatives toward jack bean urease**

Urease is categorized as a Michaelian enzyme since the graph of initial velocity (\(V_0\)) versus urea concentration exhibits a typical hyperbolic behavior (Figure 3). The average urea \(K_{M}^{\text{a}}\) (Michaelian constant) and urease maximum velocity (\(V_{\text{max}}^{\text{a}}\)) in reactions free of urease inhibitor were, respectively, 3.4 ± 0.4 mM and 8.1 ± 0.4 μmol NH\(_4\)\(^+\) min\(^{-1}\) mg\(^{-1}\) protein. The addition of 2A7, 2A9, 2B10 or 2D2 to the reaction medium caused a concentration-dependent increment of urea \(K_{M}^{\text{d}}\) (apparent Michaelis constant: \(K_{M}^{\text{d(app)}}\)) and decrease of urease \(V_{\text{max}}^{\text{d(app)}}\) (Figure 3). All the phenolic aldehyde derivatives tested behaved as mixed inhibitors, as attested by the lines intersection in the second quadrant of Lineweaver-Burk plots (Figure 3; right column).

The derivative 2A7 was the most potent mixed inhibitor since the \(K_{i}\) and \(K'_{i}\) values for complexes formed with this compound were the lowest in comparison with the others inhibitors (Table 1). In general, \(K_{i}\) values were lower than the \(K'_{i}\) values for complexes related to the same inhibitor by 2.4- to 15.5-fold.

**Soil ureases activity assay**

When tested at 3.2 mM, all phenolic aldehyde derivatives were able to inhibit soil ureases at different extents; 2A7 (PA derivative) and 2D2 (SA derivative) were found to be as efficient as NBPT (commercial inhibitor; 40% enzyme inhibition) while the VN-derived 2A9 and the PA-derived 2B10 inhibited soil ureases by up to 30% (Figure 4).

The concentration of 2A7 and 2D2 necessary to cause the inhibition of soil ureases by 50% (IC\(_{50}\)) were, in average, 3.25 mM. The derivative 2A9 exhibited a maximum inhibitory activity of 16% when used at 0.05 mM or higher concentrations. There was not a pattern in the behavior of results observed for the replicates of independent experiments performed with 2B10 and NBPT, which did not allowed for determining the IC\(_{50}\) values for such inhibitors.

**Thermal stability of natural phenolic-derived urease inhibitors**

The thermal stability of the natural phenolic aldehyde-derived urease inhibitors, assessed by mass changes of compounds as a function of fast increments in temperature, revealed that the first event of mass loss (decrease by 5%) for derivatives 2A7, 2D2, 2A9 and 2B10 occurred at 254, 253, 244 and 226 °C, respectively (Figure S1, Supplementary Information section). This same event was observed when NBPT was subjected to 151 °C (Figure S1, Supplementary Information section). The second event, characterized by 20% mass loss, took place at 200.5 °C for NBPT while similar percentage of mass loss for the phenolic aldehyde derivatives was registered at 241, 269.1, 271.2 and 276.4 °C, respectively (Figure S1, Supplementary Information section). The second event was visible when enzyme inhibitors assessed by mass changes of compounds as a function of fast increments in temperature, revealed that the first event of mass loss (decrease by 5%) for derivatives 2A7, 2D2, 2A9 and 2B10 occurred at 254, 253, 244 and 226 °C, respectively (Figure S1, Supplementary Information section). The second event, characterized by 20% mass loss, took place at 200.5 °C for NBPT while similar percentage of mass loss for the phenolic aldehyde derivatives was registered at 241, 269.1, 271.2 and 276.4 °C, respectively (Figure S1, Supplementary Information section).

**Discussion**

The potential of a series of plant natural products as urease inhibitors of clinical and/or agricultural interest has been documented.\(^{27}\) Among them, methyl gallate (phenolic ester) and its glycosylated derivative isolated from *Paeonia lactiflora* roots were shown to be promising with respect to the inhibition of *H. pylori* urease.\(^{37}\) The promising effect of these phenolic esters prompted us to investigate the potential of phenolic aldehyde derivatives as urease inhibitors of agricultural interest. Thus, the plant natural products protocatechuic aldehyde (PA), vanillin (VN) and syringaldehyde (SA) were selected as prototypes for the design of new urease inhibitors based on urea or thiourea scaffolds, urease substrate and inhibitor respectively.

*In vitro* assays revealed that PA per se decreased the activity of jack bean type III urease, while VN and SA were...
found to be inactive as they caused less than 1% enzyme inhibition (Figure 2). Besides methyl gallate and related derivatives, other plant phenolic compounds, such as (iso) quercitrin, avicularin, guaijaverin, flavonoid glucosides and shoreaphenol, were also reported to inhibit jack bean urease. Interestingly, structural modifications on PA, VN and SA dramatically improved their ability to inhibit the ureolytic activity of jack bean urease (Figure 2). Indeed, the conversion of phenolic aldehydes to derivatives bearing urea or thiourea core (Figure 1) yielded the VN derivative

Figure 3. Representative Michaelis-Menten hyperbola and Lineweaver-Burk plots for jack bean urease in the presence of phenolic aldehyde derivatives. The compounds 2A7 and 2B10 (PA derivatives), 2A9 (SA derivative) and 2D2 (VN derivative) were employed at different concentrations (0.3 to 1.6 mM) in reactions containing urea ranging from 1 to 32 mmol L⁻¹ and 12.5 mU urease. A $V_o$ versus urea concentration plot obtained from data of assays with 2A7 is shown to exemplify the Michaelian behavior of urea catalysis.
2D2 that is 230-fold more potent than VN by itself, the SA derivative 2A9 of potency 66-fold higher than that of SA by itself and the PA derivatives 2A7 and 2B10 were about 40% more potent in comparison with PA. Under our experimental conditions, the novel urease inhibitors 2A7 and 2B10 were as potent as hydroxyurea (HU; known urease inhibitor), while 2D2 (also novel) and 2A9 was less effective than HU (Figure 2). The potential of the derivative 2A9 as an inhibitor of one of the jack bean ureases was recently reported, although the experimental conditions were different from the one reported herein. Thus, the outstanding performance of these phenolic derivatives, compared to the natural products they originated from, might be attributed to the combination of a catechol skeleton with urea or thiourea core.

We performed kinetic experiments varying urea concentration in urease-catalyzed reactions containing each inhibitor at fixed concentrations (Figure 3). The urea Kₘ value obtained from reactions carried out in 20 mmol L⁻¹ phosphate buffer (pH 7.0) was, in average, 3.4 mM and urease Vₘₐₓ 8.1 μmol NH₄⁺ min⁻¹ mg⁻¹ prot. Other studies with jack bean urease, under experimental conditions distinct from the one used here, reported urea Kₘ values ranging from 1.0 to 4.0 mM.

The kinetic behavior of jack bean urease in the presence of the phenolic aldehyde derivatives is consistent with the one expected for an enzyme in the presence of a mixed inhibitor. Mixed inhibitors are known to be capable of binding both the free enzyme (forming an enzyme-inhibitor complex) and the enzyme-substrate complex (forming an enzyme-inhibitor-substrate complex). The values obtained for the dissociation constants for both urease-inhibitor and urease-urea-inhibitor complexes indicate that the phenolic aldehyde derivatives synthesized bind more efficiently to the urease active site in comparison to allosteric ones (Table 1). The potency of compounds with respect to the binding to urease active site is 2A7 > 2B10 > 2D2 > 2A9. As for the binding to allosteric site(s) the order of potency is 2A7 >> 2B10 = 2D2 = 2A9.

The derivatives 2A7 and 2D2 were the most efficient compounds to inhibit soil ureases, clustering together with the reference inhibitor NPBT (Figure 4). These synthesized compounds are able to inhibit soil urease activity by 50% when used at 3.25 mM. Notably, the maximum inhibition of soil ureases exhibited by the SA derivative 2A9 was 16% when employed at 0.05 mM, no matter higher concentrations would be applied on soil. In fact, in the case of soil ureases, (a)biotic conditions such as temperature, pH, moisture and the presence of different types of clays, organic matter and viable microorganisms particularly are known to affect ureases performance. Moreover, soil matrix comprises complex physicochemical features and biological processes that may culminate in the chemical modifications of xenobiotic substances, as is the case of synthetic urease inhibitors. Such chemical transformations triggered by soil microbiota may result in loss or increment of the function of a certain compound. Alternatively, the complex nature of soil matrix may affect the bioavailability of the xenobiotic for interaction with the target enzymes. Taking these into account it is likely that the PA derivative 2B10 undergoes some structural transformation caused by soil microbiota as it is known to occur with NBPT.

It is well known that NBPT is sensitive to heat. For the most active derivatives on soil (2A7 and 2D2), thermogravimetric analysis shows no decomposition of such compounds up to 170 °C (Figure S1; Supplementary Information section). In addition to the thermal stability, the derivatives 2A7 and 2D2 are obtained in a single synthesis step (78% average yield) after a simple purification procedure (recrystallization) that furnishes compounds as

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Kᵢ / mM</th>
<th>Kᵢ' / mM</th>
</tr>
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<tbody>
<tr>
<td>2A7</td>
<td>0.23 ± 0.01</td>
<td>1.19 ± 0.07</td>
</tr>
<tr>
<td>2A9</td>
<td>3.83 ± 0.84</td>
<td>9.29 ± 3.78</td>
</tr>
<tr>
<td>2B10</td>
<td>0.69 ± 0.02</td>
<td>10.71 ± 3.78</td>
</tr>
<tr>
<td>2D2</td>
<td>1.26 ± 0.06</td>
<td>11.22 ± 4.57</td>
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Kᵢ: equilibrium dissociation constant for urease-phenolic aldehyde derivative complex; Kᵢ': equilibrium dissociation constant for urease-urea-phenolic aldehyde derivative complex; values are the mean ± standard deviation of triplicate determinations from a representative experiment.
solid materials. These are desirable features for obtaining urease inhibitors to be used as additive in urea-based fertilizers.

Conclusions

Overall, the hybridization of structures of the natural products PA, SA and VN with (thio)urea core furnished derivatives with inhibitory effect on ureases activity displaying mechanisms of action typical of mixed inhibitors. The interesting physicochemical features of phenolic aldehyde derivatives herein studied, together with their ability to inhibit soil ureases, make these compounds, especially 2A7 and 2D2, promising candidates for further studies as additive in urea-based fertilizers.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

The development of Novel Urease Inhibitors which is financially supported by the Brazilian agencies National Counsel of Technological and Scientific Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES) and the Minas Gerais State Research Foundation (FAPEMIG). LVM and AF are recipients of research fellowships from CNPq.

References

Carvalho, J. E.; Sabino, A. A.; Modolo, L. V.; de Fátima, A.; 
34. Easterby, J. S.; Hyper32: Version of Hyper for 32 Bit Operating 
Systems: Hyperbolic Regression Analysis of Enzyme Kinetic 
35. Voet, D.; Voet, J.; Biochemistry, 4th ed.; John Wiley & Sons: 
New York, 2011.
Food Chem. 2012, 60, 9062.
Res. 2011, 13, 799.
Chaudhary, M. I.; Ansari, F.; J. Mol. Graphics Modell. 2013, 
43, 47.
99, 15.
42. Segel, I. H.; Enzyme Kinetics: Behavior and Analysis of Rapid 
Equilibrium and Steady-State Enzyme Systems; John Wiley and 
43. Fetzner, S. In Biotechnology, Environmental Biotechnology- 
Socio-Economic Strategies for Sustainability, Encyclopedia 
of Life Support Systems (EOLSS), vol. X; Doelle, H. W.; 

Submitted: April 26, 2016
Published online: July 13, 2016