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Dissipation of cyanogenic glucosides and cyanide in soil amended with white clover (*Trifolium repens* L.)

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ABSTRACT

The use of white clover (*Trifolium repens* L.) as green manure is common practice due to its high nitrogen content. White clover produces the two cyanogenic glucosides linamarin and lotaustralin, which release toxic hydrogen cyanide upon hydrolysis. The hydrolysis of cyanogenic glucosides and release of cyanide were studied in batch experiments with ground white clover added to soil at loadings of 75 g leaves per kg soil. Linamarin and lotaustralin were quickly hydrolysed with first-order rate constants of 0.026–0.0062 h^{-1} (corresponding to half lives of 11–27 h) in sandy and loamy soils at natural moisture contents and 11 °C. Experiments with addition of pure cyanogenic glucosides and with sterilized soil material as well as addition of white clover to inert quartz showed that hydrolysis by plant glucosidases is partly inhibited in the soil matrix, but also that soil glucosidases present in soil contribute to degradation of the cyanogenic glucosides, and more so for linamarin than for lotaustralin. Cyanide was produced during the hydrolysis of the cyanogenic glucosides as seen by formation of HCN(g) and WAD-CN (*Weak Acid Dissociable Cyanide*) amounting to max. 5 and 50% of total-CN in the systems with white clover added to natural soils. However, the increase in WAD-CN was transient, due to subsequent dissipation of the compound caused by abiotic and microbial CN degradation. Due to WAD-CN dissipation in the top soils studied, long term effects of cyanide on sensitive microorganisms and plants are not expected. However, knowledge on the stability of WAD-CN in subsoil is lacking and warrants further investigations.

1. Introduction

White clover (*Trifolium repens* L.) is widely used as green manure, especially in organic farming, where sustainable alternatives to chemical fertilizers are needed. Due to its nitrogen fixating abilities, it is common practice to plough white clover into soil in spring or fall to supply the following crop with nitrogen. White clover produces the two cyanogenic glucosides linamarin and lotaustralin, which release toxic hydrogen cyanide (HCN) upon hydrolysis. When the plant cells are disrupted, the cyanogenic glucosides are hydrolysed by endogenous plant β-glucosidases (Hughes, 1991). This provides an instant defence against chewing herbivores as HCN is produced immediately but may also lead to release of HCN in soil when white clover is used as green manure.

In a clover/grass field, the white clover coverage may constitute up to 1–3 t DM ha^{-1} (DM = dry matter). The content of cyanogenic glucosides and β-glucosidases varies between different cultivars; in 51 common cultivars, the content of HCN equivalents was found to vary from 0.12 to 1.1 g kg^{-1} DM (Crush and Caradus, 1995). Hence, the maximum HCN load derived from cyanogenic glucosides will be in the range 0.12–3.30 kg HCN ha^{-1}. Previous studies have shown that degradation of cyanogenic glucosides in soil is fast with estimated half lives in the order of hours to days (Johansen et al., 2006; Bjarnholt et al., 2008a). However, the rate and extent of plant-to-soil transfer of cyanogenic glucosides and the fate of the released HCN is not known. Previous studies have shown that addition of cyanogenic glucosides to agricultural soil and soil from industrial sites not previously contaminated with iron–cyanide containing waste can lead to leaching of cyanogenic glucosides and weak acid dissociable cyanide (WAD-CN) to drain water or groundwater (Balagopalan and Rajakshmy, 1998; Bjarnholt et al., 2008a).

HCN is not stable in soil, but can be removed through volatilization, complexation, sorption and degradation. HCN is a volatile

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weak acid (pKa = 9.2–9.4), which under normal environmental conditions in soil will occur as dissolved HCN(aq) or CN^- in the aqueous phase or as a gas, unless complexation or sorption takes place (Kjeldsen, 1999). The mobility of cyanide in the soil is mainly determined by the formation and precipitation of iron–cyanide complexes, but complexation with other metal ions may also take place (Theis et al., 1994; Rennert et al., 2003). When precipitation of metal cyanides is limited, sorption of cyanide complexes – in particular iron–cyanide complexes – to soil particles becomes important (Meeussen et al., 1992; Rennert et al., 2003). The degree of iron-cyanide sorption depends on soil properties such as pH, content of iron- and aluminium-oxides and soil organic matter (SOM) (Rennert and Mansfeldt, 2002). Iron–cyanide complexes are stable in darkness, whereas free cyanide undergoes abiotic as well as biotic degradation (Kjeldsen, 1999; Ebbs, 2004). Physiochemical properties of the soil as well as concentration and bioavailability of cyanide and interfering compounds affect the extent of cyanide degradation (Aronstein et al., 1994; Dubey and Holmes, 1995; Ebbs, 2004).

Cyanide is toxic in its free form (Salkowski and Penney, 1994; Kjeldsen, 1999). The toxicity of cyanide is therefore determined by speciation and can be roughly assessed by determination of two different pools by distillation: total-CN and WAD-CN (weak acid dissociable cyanide). The latter covers free cyanide (HCN and CN^-) and weak metal–cyanide complexes dissociating at pH 4.5–6 (ASTM D 6696-05, 2005). The WAD-CN pool is an approximation of bioavailable and thus potentially toxic cyanide (Shifrin et al., 1996; Kjeldsen, 1999). Under most natural conditions the difference between total-CN and WAD-CN consists of iron–cyanide complexes (Shifrin et al., 1996; Kjeldsen, 1999; Ghosh et al., 2004).

The aim of this investigation has been to examine the rate and extent of degradation of cyanogenic glucosides as well as formation and subsequent dissipation of cyanide from decomposed white clover plant material in soil. This was done in batch experiments by incorporating ground white clover plant material into soil and monitoring the concentration of linamarin, lotaustralin, WAD-CN and volatile HCN(g) as a function of time. Experiments with addition of pure cyanogenic glucosides, cyanide and with inert soil materials have been included to elucidate the contribution of enzymatic processes to degradation of cyanogenic glucosides in plants and soil.

2. Materials and methods

2.1. Soils

The A horizon (0.05–0.25 m) of two agricultural soils were used in the experiments, a sandy soil from Jyndevad, southern Jutland, Denmark, and a loamy soil from Sj. Odde, northern Zealand, Denmark. According to Soil Taxonomy, the Jyndevad soil is a Humic Psammentic Dystrudept and Sj. Odde soil a Typic Argiudoll (Soil Survey Staff, 1999). Both soils have been used for organic farming with application of manure, Jyndevad since 1990 and Sj. Odde since 1951 (Laegdsmand et al., 2007). At the time of sampling the Sj. Odde soil was covered with clover and grass for animal grazing, whereas the Jyndevad soil was cultivated with barley. The properties of the two soils are shown in Table 1. Note that both soils have neutral pH and almost the same content of soil organic matter. The soils were used at their natural moisture contents.

2.2. White clover plant material

Seeds of the white clover variety Klondike (DLF Trifolium, Denmark) were sown in soil and kept either outside or in green house (18 °C), depending on time of year. Above ground plant material (stems, leaves, shoots and flowers) was harvested when the plants were between 5 and 12 months and approximately 20 cm high. The harvested white clover was immediately ground manually to a size of 2–5 mm (leaves, shoots and flowers) and 1–2 cm (stems) using a parsley grinding mill (Raadvad, Denmark). Three replicates containing 2 g FW (fresh weight) ground plant material were frozen in liquid nitrogen for determination of contents of linamarin and lotaustralin.

2.3. Degradation experiments

Clover in soil: 3.75 g FW ground white clover was incorporated thoroughly into 50 g moist soil in a 300 ml gas-tight flask capped with a rubber septum. An alkali trap for HCN(g) consisting of a 15 ml glass vial containing 10 ml of 0.1 M NaOH was placed in the flask. The experiments were carried out in triplicate at 11 °C ± 1 °C in a refrigerator. Three samples were terminated at each of eight sampling times (0.5, 1.5, 3, 20, 45, 70, 95 and 165 h). The alkali traps were exchanged daily in order to prevent saturation of the NaOH with CO_2. The alkali traps were kept at 4 °C until cyanide analyses. In the three replicates for the last sampling time, the alkali traps were exchanged with 10 ml of 0.4 M NaOH (Jyndevad) and 0.5 M NaOH (Sj. Odde) to take account of the longer time between sampling. At each time of sampling, the soil/plant mixture was mixed thoroughly, and samples of 4 g FW taken for WAD-CN analysis and 5 g FW for determination of linamarin and lotaustralin. The samples were quenched in liquid nitrogen and subsequently kept at −21 °C until further treatment.

The following experiments are variations of the above described general procedure. The purpose of these experiments was to get further insight into the degradation processes.

Clover in quartz: Soil was replaced with quartz (Merck, ignited and washed, particle size 0.1–0.5 mm). Distilled water was added to gravimetric moisture content of 11% resembling the water content of the similarly textured Jyndevad soil. As degradation was expected to be slow, the experiment lasted for 240 h and with the second last sampling at 164 h. In this experiment, no CO_2 was liberated from decomposing soil organic matter and hence alkali traps filled with 0.1 M NaOH was used throughout the experiment.

Clover in sterilized Jyndevad soil: Moist Jyndevad soil was sterilized by autoclaving at 121 °C for 20 min three times with 2 days in between.

Table 1
Properties of the A-horizon material of the two investigated soils. Except from water content at field capacity, data is reproduced from Laegdsmand et al. (2007).

Location	pH[a]	C_{tot}[b] %	Bulk density[c] kg l^{-1}	Clay[d] %	Silt[d] %	Sand[d]	Water content[e]
Jyndevad	6.9	2.4	1.30–1.48 (1.41)	5	3	92	11
Sj. Odde	7.0	2.2	1.29–1.63 (1.49)	21	12	67	22

[a] pH measured in 0.01 M $CaCl_2$ (soil:solution ratio of 1:2.5).
[b] C_{tot}, was determined by dry combustion.
[c] Bulk density are displayed as the span of values measured from Laegdsmand et al. (2007) with the calculated average in brackets.
[d] Particle size distribution determined by hydrometer method plus sieving and weighing (clay < 2 μm, 2μm < silt < 20 μm, sand > 20 μm).
[e] Gravimetric water content in %, i.e. mass of water/dry weight (105 °C) × 100.