CARBON CYCLES, NITROGEN FIXATION AND THE LEGUME-RHIZOBIA SYMBIOSIS AS SOIL CONTAMINANT BIOTEST SYSTEM[1]

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ABSTRACT

The major pools and turnover rates of the global carbon (C) cycles are presented and compared to the human production of CO_2 from the burning of fossil fuels (e.g. coal and oil) and geothermal fuels (natural gases), both categorized as non-renewable energy resources which in amount reaches around 6.5 Gigatons C per year. These pools that serve as C-holding stallions are in the atmosphere, the land plant biomass, the organic soils carbon, the ocean carbon and the lithosphere. In another related case, the present focus in the area of nitrogen fixation is discussed with data on world production of grain legumes compared to cereals production and nitrogen fertilizer use. The focus to understand the molecular biology of the legume-rhizobia symbiosis as a major contributor to nitrogen fixation is in the areas of signal exchange between host plants and rhizobia in the rhizophere including the nod factor signalling, the infection and nodule compartmentation and the soils stress factors affecting the symbiosis. The use of the Legume-Rhizobia symbiosis as a biotest system for soil contaminants includes data for cadmium, arsenate, atrazine, lindane, fluoranthene, phenantrene and acenaphthene and also results on the mechanism, why the symbiotic system is more sensitive than test systems with plant growth parameters.

Keywords: Non-renewable energy source, plant biomass, CO_2, nodule

I. POOLS AND TURNOVER- RATES OF THE GLOBAL CARBON CYCLE

Our planet earth contains about 10^{17} t (10^8 Giga t) carbon (C). Most of the carbon came from meteorites (chondrites), which included up to 3.5 % carbon. Some of this carbon enters as CO_2 into the atmosphere and after cooling into the oceans, which are until today the second largest pool of carbon dioxide (38 000 Giga t of C) after the lithosphere with around 7 x 10^7 Gt (Figure 1). The pools of the biosphere are by several orders of magnitude smaller, and they will be compared here with the anthropogenic annual production of CO_2

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carbon by burning of coal, oil and gas. This ranges about 6.3-6.5 Giga t C. This moves first completely into the atmosphere, where 750 Giga t of C are present. The two other important biosphere pools are the land plant-biomass with 590 Giga t C and the organic soil carbon with 1560 Giga t C. The land plants fix about 120 Giga t C annually, from which 50% go back directly to the atmosphere by respiration, the other 50 %, equivalent to 60 Giga t C into the organic soil carbon. From this carbon pool, about 62 Giga t C go back by soil respiration into the atmosphere. This difference of 2 Giga t C seems to be small, but it is about 30% of all carbon dioxide produced by use of fossil (non-renewable) energy resources. The oceans take up about 2 Giga t C more than they release, so that for the land area and the oceans the natural turnover rates are in a remarkable equilibrium (Figure 1). When we compare the pool of all available fossil energy resources, which are about 1150 Giga t C, we can conclude, if all this would be concentrated in the atmospheric pool, the CO_2 concentration there can not increase by more than a factor of 2.5. Therefore many experiments with a tripled carbon dioxide concentration are ecologically exaggerated. What can we do, besides replacing the carbon-energy cycle of our 21 century technology gradually by a hydrogen- and electricity-energy cycle? First we need more information on the so called missing sink, since the carbon dioxide concentration in the air does not reflect the present data of the carbon pools and turnover rates.

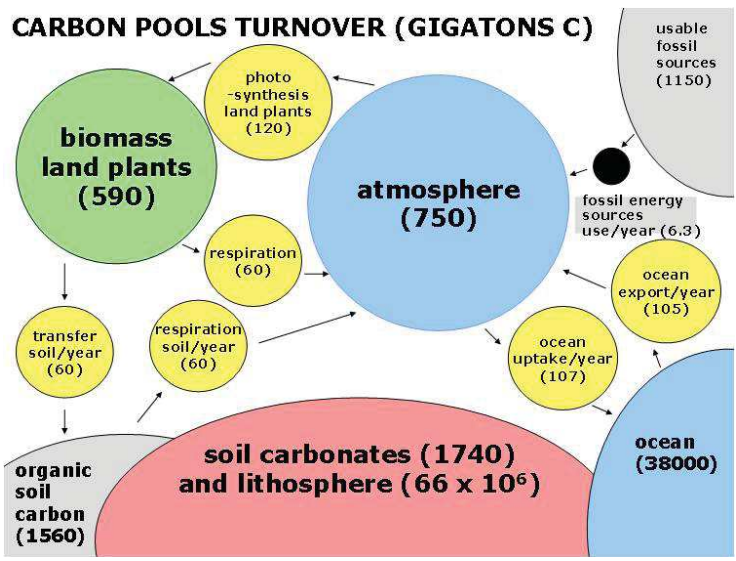

Figure 1. Global carbon cycle: pools and turnover rates

When we look to the geographic regions from the south pole to the equator and further to the north pole including land and oceans, we observe two large zones as CO_2 sinks at 30 °S and 60 °N , whereas regions around the equator are major CO_2 sources (Enting and Mansbridge, 1991). A major limitation for these data is however, the very limited number of stations, located more by historical reasons than by geographic arguments on islands scattered in the oceans of the world. Deforestations and land clearance in developing countries alone contribute about 1.6 G t C annually, about a quarter of the global carbon dioxide emission (Murdiyarso, 2007)

II. THE NITROGEN CYCLE: LEGUME NITROGEN FIXATION

More than 60% of the worlds legume grain production is occupied by soybeans (Table 1). Therefore, this crop has been covered in this article by several chapters as a model for several other grain legumes in which our knowledge is much less developed due to the limited areas of production and therefore lower economical impact. However for the people in large areas, especially in South America and Africa, other grain legumes are decisive for the diets of the population and for the production of the small farmers. For sustainable agriculture, also fodder legumes and legumes used as green manure are equally important. As examples of recent progress in understanding production and BNF (biological nitrogen fixation) in these legumes, the following species will be covered: Beans (*Phaseolus* sp. and *Vigna*), chickpea (*Cicer arietinum*), pigeon pea (*Cajanus cajan*), peanuts (*Arachis* sp.), *Mucuna* and other tropical legumes. Further, in order to acquire brief information on nutrition value of grain legumes, in Table 2 are summarized their protein, lipid, and carborhydrate contents.

Table 1. World Production (FAO, 2002)

	Million tons
Soybeans	176.6
Groundnuts	35.1
Dry beans (*Phaseolus* and *Vigna*)	16.8
Dry peas	10.5
Chickpeas	6.1
Dry faba beans	3.7
Lentils	3.1
Green beans	4.7
Green peas	7.1

Table 2. Protein, lipid and carbohydrate content of dry seeds of grain legumes *).

Grain-producing -species	*Grains* **)		
	Protein %	*Lipid* %	*Carbohydrate* %
Arachis hypogaea	**25.3**	**48.1**	8.3
Cajanus cajan	20.2	1.4	47.0
Canavalia ensiformis	25.5	2.5	50.0
Cicer arietinum	19.8	3.4	41.2
Dolichos lablab	22.0	1.5	50.0
Glycine max	**33.7**	**18.1**	6.3
Lens culinaris	23.5	1.4	52.0
Phaseolus acutifolius	24.5	1.5	65.5
Phaseolus lunatus	20.6	1.4	45.0
Phaseolus vulgaris	21.3	1.6	40.1
Pisum sativum	22.9	1.4	41.2
Psophocarpus tetragonolobus	**33.1**	**16.2**	30.8
Vicia faba	23.0	2.0	55.0
Vigna aconitifolia	23.6	1.1	56.5
Vigna angularis	20.7	1.4	56.4
Vigna mungo	23.1	1.2	41.5
Vigna radiata	24.0	1.1	43.6
Vigna subterranea	19.0	7.0	54.0
Vigna umbellata	21.5	0.3	60.9
Vigna unguiculata	23.5	1.4	41.7

Note : *) Modified from Souci *et al*. (1994)
**) Percentage is based on dry weight of gram seeds

The protein contents in most species range 20 to 30% protein in dry seeds. Only *Psophocarpus tetragonolobus* with 33% protein, *Glycine max* with about 34% and *Lupinus mutabilis* with 48% are significantly beyond this range. Very low is the lipid content with 1 to 2% in *Vigna*, *Phaseolus*, *Pisum*, *Cajanus*, *Lens* and *Dolichos* species. Three genera with a protein content beyond 25%, have high lipid concentrations with 16% in *Psophocarpus tetragonolobus*, 18% in *Glycine max* and up to 48% in *Arachis hypogaea* (peanuts) (Werner, 2005).

III. SOIL STRESS FACTORS AFFECTING THE SYMBIOSIS

Our knowledge about the symbiosis development described so far is to a large extent based on laboratory experiments and observations. The real world of legumes and rhizobia are of course featuring the various ecosystems such as arable agricultural land, pastures, savannahs and forests, where the “standard development” is affected by a large number of environmental factors such as :

- soil water stress (osmotic stress)
- nutrient stress
- pH stress and competition stress (Werner, 2007).

The best studied system for osmotic stress in rhizobia is *Sinorhizobium meliloti*, which accumulates very different compatible solutes such as glutamic acid, glycine betaine, proline betaine, trehalose and N-acetylglutaminylglutamine amide (Botsford, 1984; Boscari *et al*., 2002). The basic understanding of osmoregulation is even more advanced in *Escherichia coli* and *Bacillus subtilis* (Schiefner *et al*., 2004; Holtmann and Bremer, 2004). In *Bacillus subtilis* there are two classes of osmotically regulated genes:

1. genes, which are permanently switched on at high osmolarity, as the Opu Transporters and genes for the proline biosynthesis. Altogether more than 100 genes belong to this group, most of them with unknown functions. A central role has the two component regulatory system called DegS/DegU (Steil *et al*., 2003)
2. genes, which are only transiently up-regulated, such as the *yhaSTU* genes , responsible for K^+ /Na^+ export. Mechanosensitive channel proteins play hereby an important role.

Besides osmotic adaptation, the tolerance of those genes to desiccation is another important factor to water supply and shortage. But the genetics and biochemistry of desiccation in rhizobia is poorly studied (Sadowsky, 2005). A decrease in unsaturated fatty acids under these conditions has been reported (Boumahdi *et al*., 2001). When we include the plant partner it is remarkable that N-2 fixation is more sensitive to dessication than photosynthesis and nitrate assimilation (Purcell and King, 1996). Nutrient stress and limitation has a fundamental impact on growth and development of the host plants as well as the microsymbionts. The limitation of a single essential element of the around 20 essential soil elements limits also finally the symbiosis. In the keynote lecture of the International Nitrogen Fixation Conference in Peking (Werner *et al*., 2005) it was remembered, that already 20 years ago (Werner *et al*., 1985) it was demonstrated, that root hairs of soybeans contain almost 10 times more calcium, cobalt and iron that than soybean roots and 3 to 8 times more than wheat root hairs. Soybean roots contain also about 10 times more molybdenum than wheat roots (Table 3). For all these essential elements, rhizobia have an unusual high requirement for growth and differentiation. The central hypothesis from these (limiting) data is that the symbiotic interaction does not start with a carbon/ nitrogen exchange, which takes place only in already developed nodules, but with the supply of essential trace elements by the host plants to the rhizobia in a competition limiting environment (Werner *et al*., 1985) In the last years it has been established, that calcium spiking in root hairs of legume plays a central role

in the signalling pathway for nod factor reception (Wais *et al*., 2002). In many soils, the limitation of available phosphate is a major constrain and also for this element, the symbiotic bacteria and the legume host plants compete for this element. The phosphorus concentration in fix^+ nodules is about 60 % higher than in root tissue, but the same as in fix^- nodules (Kuhlmann *et al*., 1982). On the other side, the chloride concentration in both types of nodules is reduced by about 80 % compared to that in roots (both 20 days old).

Table 3. Calcium, iron and cobalt accumulation in root hairs of soybean (*Glycine max*)

	Ppm (dry matter)			
Element	Soybean root hair	Soybean root	Wheat root hair	Wheat root
K	11740 ± 2450	12840 ± 2640	4670 ± 1010	4780 ± 990
S	530 ± 165	560 ± 170	180 ± 55	190 ± 60
Fe	414 ± 138	31 ± 5	120 ± 35	44 ± 26
Co	7.9 ± 3.8	0.88 ± 0.4	2.6 ± 0.8	1.3 ± 1.1
Ca	2200 ± 460	287 ± 70	246 ± 60	288 ± 70
Mo	3.1 ± 0.5	5.4 ± 0.7	0.6 ± 0.12	0.5 ± 0.3

Source: Werner *et al.* (1985)

The acquisition of phosphate is in many cases a limiting factor of legume development (Vance, 2001). Therefore the tripartite symbiosis of legumes with arbuscular mycorrhizal (AM) fungi and rhizobia is of special interest, since the AM-fungi reduce the phosphate limitation and the rhizobia reduce the nitrogen limitation (Barea *et al*., 2005). Besides the nutrient acquisition, the AM-fungal symbiosis also protects the host plants against drought stress (Ruiz-Lozano *et al*., 2001) and also salinity stress (Augé, 2001). Also PGPR (Plant Growth Promoting Rhizobacteria) can assist AM fungi in solubilizing phosphate for the host plants (Barea *et al*., 2002a; Barea *et al*., 2002b). The complexity of the interactions has further increased by the observations that endosymbiotic bacteria are present within certain species of AM-fungi, such as *Gigaspora* and have been identified as the new species *Candidatus Glomeribacter gigasporum* (Bianciotto *et al*., 2002). The bacteria are stable cytoplasmatic components, transmitted over vegetative spore generations (Bianciotto *et al*., 2004) and considered as obligate endosymbionts (Bonfante, 2005). Bacterial responses to pH in general have been summarized in detail by Poole (1999). The sensor/regulator pair ActS/ActR has been studied in most details in *Sinorhizobium meliloti* (Glenn *et al*., 1999).

The most interesting special feature of rhizobia compared to other rhizosphere bacteria is that they inhabit in both parts of their life cycle an acid environment: as free living bacteria in the rhizosphere before infection and as bacteroids in the symbiosome. This has been used to identify additional new genes responsible for the later stages of nodule colonization, by producing acid sensitive mutants in the free living stage and to test them in competition experiments during nodule compartmentation. With the acid tolerant strain *Rhizobium* CIAT 899, the gene *atvA* ("acid tolerance and virulence") was identified, with a strong homology to the *acvB* gene in *Agrobacterium tumefaciens* (Vinuesa *et al*., 2003). A serine/alanine exchange in the lipase motive of the protein leads to an acid sensitive phenotype with a significantly reduced competitiveness for nodule occupancy. Mutation in the upstream localized *lpiA* gene also leads to much reduced competitiveness. Also mutants with an increased acid tolerance have been created. A leucine biosynthesis mutant was able to survive at pH 3.5, where the wild type strain could not (Steele *et al*., 2003). The wild type could survive and grow down to pH 4.0. The mutant was able to raise the pH of the medium from 3.5 to 3.8 as a leucine auxotroph, where the wild type did not raise the pH of the medium. It is known that catabolisation of amino acids leads to an alkalinisation of the medium. This mechanism may explain that in the rhizosphere of many legumes amino acid auxotrophs can be found. Two other genes involved in acid tolerance have been most recently also identified in *Rhizobium tropici*: the gene *sycA* has strong identity with a CIC chloride channel and the second gene in the same operon, *olsC* is homologous to aspartyl-asparginyl ß hydroxylase (Rojas-Jimenez *et al*., 2005). This hydroxylase modifies two ornithine containing membrane lipids of the microsymbionts. Both genes are involved in acid tolerance in the free-living state as well as in colonisation of the infected cells by affecting competitiveness. The organization of these genes is shown in Figure 2.

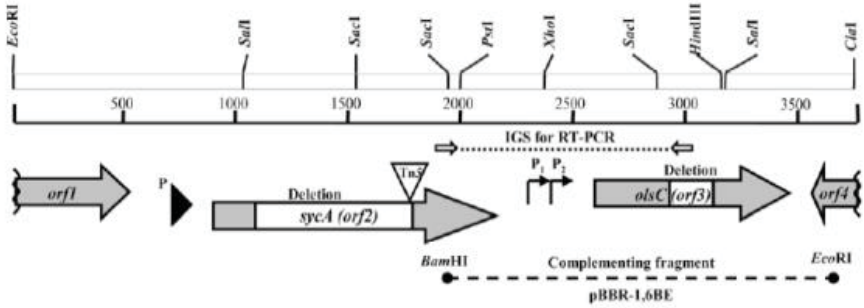

Figure 1. Genetic and physical maps of the 3,761-bp *Eco*RI-*Cla*I region from *Rhizobium tropici* CIAT899 analyzed in this study (accession number AY954450). Selected restriction sites are shown. Four open reading frames (ORF) (represented by arrows) were detected. The site of the Tn*5* insertion located in *sycA* between nucleotides C1763 and T1764 is indicated by an open triangle. Nonpolar deletion mutants lacking the regions shown in white were generated in *sycA* and *olsC*. Predicted promoters are shown as thin arrows. The dotted line represents the intergenic spacer between *sycA* and *olsC* subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (shown in D). The dashed line shows the location of the 1.66-kb *Bam*HI-EcoRI fragment cloned into pBBR-MCS5 and used to complement strain 899-olsCΔ1. (Rojas-Jimenez *et al*., 2005).

The two soybean nodulating rhizobia also differ in their acid tolerance: *Bradyrhizobium japonicum* can still grow at pH 4.5 but not under alkaline conditions of pH 9.0, whereas *Sinorhizobium fredii* will not grow at pH 4.5 but at 9.0 (Sadowsky *et al*., 1983). From genistoid legumes the new acid tolerant species *Bradyrhizobium canariense* has been isolated and characterized (Vinuesa *et al*., 2005a), which can nodulate *Chamaecytisus proliferus*, *Teline stenopetala* and *Lupinus luteus*, but not *Glycine max* and *Glycine soja*. This new species has been characterized in relation to population genetics and the phylogeny with all other *Bradyrhizobium* species, such as *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense*, *Bradyrhizobium yuanningese* and *Bradyrhizobium betae* (Vinuesa *et al*., 2005b).

IV. THE LEGUME-RHIZOBIA SYMBIOSIS AS BIOTEST SYSTEMS FOR SOIL CONTAMINANTS

A. Soil Biotests

Compared to biotests for groundwater and surface water quality, the number of accepted and sensitive biotests for soil contamination is rather limited. Growth effects on a number of agricultural plants such as *Avena sativa, Brassica rapa* or *Phaseolus aureus* are used or effects on root growth. In general, plants are suitable as bioindicators of soil health, due to their intensive root system and the large contact area between the soil and the biomonitoring organisms. The basis is a long tradition of using plants to indicate specific soil conditions such as *Salicornia* species for salt affected soils, *Teesdalia nudicaulis* for acid soils and *Mercurialis annua* for alkaline soils. Well studied are the use of sensitive plant species against heavy metal contamination and the colonization of these areas by metal tolerant populations. As specific biochemical markers, phytochelatins, specific enzyme activities such as peroxidise activity and the polyamines have been used. Changes in microbial populations and activities are another area of bioindicators of soil health. Carbon transformations are widely used, but not very specific, for they react against any other environmental stress on the microorganisms such as temperature, soil pH and water conditions.

Other bioindicator systems use extrapolation in ecosystems as the factor method. The ratio of PEC (Predicted Environmental Concentration) over by PNEC (Predicted No Effect Concentration) larger than 1 is considered as an environmental risk. Also for other methods the no observed effect concentration is of central importance. Therefore the development of a more sensitive biotest than the established systems is very important for risk assessment.

B. The Rhizobium-Legume Symbiosis as a New Sensitive Biotest

During the last years the methodology and application of the *Rhizobium*-legume symbiosis as a new sensitive biotest has been developed towards heavy metals and organic contaminants (Wetzel *et al*., 1991; Wetzel *et al*., 1994; Wetzel *et al*., 1995, Wetzel and Werner, 1995; Wetzel, 1998, Neumann *et al*., 1998). Table 4 summarizes data of the significantly high sensitivity of nodulation, compared to shoot growth and root growth for most of the substances tested.

Table 4. EC50 for nodulation, shoot growth and root growth in the system *Medicago sativa/Sinorhizobium meliloti*

Test compound	Nodulation	Shoot growth	Root growth
	EC50 mg l^{-1} and µg		cm^{-2}(*)
Cadmium iodile	0.5	10.4	72.9
Cadmium chloride	1.1	19.6	4
Cadmium acetate	2.5	17.4	22.6
Sodium (meta) arsenate	0.3	93.9	82.4
Disodium hydrogen arsenate (V)	1.7	248.7	218.1
Arsenic pentoxide	4.2	157.6	143.2
Atrazine	0.13	0.15	0.09
Lindane	6.7	20	7.7
Fluoranthene*	2.5	>35	>35
Phenanthrene*	5.4	28	27
Fluorene*	7.1	21	22
Acenaphthene*	20.0	52.5	50.8

Note : For cadmium and arsenic compounds the nodulation biotest is more sensitive by a factor af 10 to 100 compared to shoot growth and root growth. Against organic contaminants such as fluoranthene and acenaphthen the nodulation biotest is more sensitive by a factor of 2 to 10. A factor of 10 of higher sensitivity is a very significant progress in evaluation the potential risk in the food chain from the soil to the plant and to humans and animals.

C. Gene Expression in the Symbiotic System Modulated by the Xenobiotics Cadmium and Fluoranthene

Using mRNA differential display techniques in the symbiotic test system affected by cadmium and fluoranthene, 37 differentially displayed transcripts were detected. Two of them called DDMs1 and DDMs2 were confirmed by northern hybridization, regulated by the presence of the two xenobiotics (Neumann and Werner, 2000). Transcription of DDMs1 was highly affected by the cadmium concentrations with an EC50 of 5.9 μM. This is almost identical to the EC50 found for nodulation. Sequence analysis of DDMs1 gave a significant identity to a hypothetical protein from *Arabidopsis thaliana* with a high similarity to a copper transporting ATPase. DDMs2 has a strong similarity (82% identity) to the cytoplasmatic 60S ribosomal protein L18 from *Arabidopsis thaliana*.

D. Phytoremediation

The concept of phytoremediation has gained increasing general interest in the last years. Phytoremediation is the use of plants to reduce pollutants in the soil by enrichment in plant material and removal of these plant parts. The application of phytoremediation is especially attractive when hyperaccumulating species for different heavy metals are used, such as *Psychotria douarrei* for nickel, *Thlaspi caerulescens* for cadmium, *Ipomoea alpine* for copper or *Macadamia neurophylla* for manganese. From a joint study at the Semipalatinks Test Site, very interesting results have been found for radionuclide accumulation in specific plant species from this region. In the legume *Glycyrrhiza uralensis* a 10 to 100 fold increase of ß-radionuclides in the plant ash of above ground material compared to the soil has been found. For root ash the ratio was between 20 to 160 even higher. For cesium^{137} the ratio was between 2 to 40 for ash from different plants compared to the soil. The use of this and other endemic species from this region is very promissing aspect for phytoremediation of this unique contaminated area where more than 20% of all atomic bombs have exploded on this planet. For many countries in Asia and Europe it is especially important to realize that from unvegetated soils there is a significant transfer of radionuclides via the air into the food chain of many other countries of this planet which could continue over long periods of time if we do nothing (Werner *et al*., 2000).

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