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Quinoline Increases Ascorbate Peroxidase and Dehydroascorbate Reductase Activity in *Vicia Faba* Nodules

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Quinoline, occuring in small amounts in coal tar, is used by the chemical industry as a starting material for manufacturing different chemical products. As it is also produced during incomplete combustion of nitrogen-containing substances it can also be found together with other aza-arenes (aza-heterocyclic hydrocarbons) in urban suspended particulate matter, automobile exhausts (Dong et al. 1977) and tobacco smoke (Dong et al. 1978). The indiscriminate distribution and use of quinoline and its derivatives make them a health hazard to a large portion of the population. Microbial testing methods and animal experiments showed that quinoline can display toxic and mutagenic activities (Hirao et al. 1976; Lavoie et al. 1987; Sideropoulos and Specht 1984). However, less is known about the ecotoxicological effect in plant-soil-systems, after possible wet deposition of quinoline to soil surface and sediments (GDCh-Beratergremium 1985).

The following experiment was designed to investigate whether enzymatic activities of root systems of higher plants, respectively the root nodules of the rhizobium-legume symbiosis react to the application of quinoline and beyond that might serve as an indicator for quinoline containing soil.

Nitrogen-fixing organisms are especially vulnerable to O_2 toxicity because of the extreme sensitivity of the nitrogenase proteins to O_2 damage. Part of the sensitivity seems to arise from the production of reactive oxygen intermediates by reactions associated with nitrogen fixation. There are two main oxygen protection mechanisms besides respiratory and physical protection against reactive intermediates: superoxid dismutase catalyzes the conversion of O_2^- and H_2O_2 via a special type of peroxidase system which scavenges the hydrogen peroxide. The later system utilizes ascorbate as an antioxidant to produce dehydroascorbate and glutathione as a reductant to regenerate ascorbate. The oxidized glutathione is reduced to GSH in a NADPH-dependent reaction (Dalton et al. 1986).

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As toxic and mutagenic effects of quinoline are probably due to the generation of hazardous oxygen intermediates (Sims and Overcash 1983; Thompson et al. 1987) we wondered, whether the activity of the peroxidase system which normally prevents peroxide damage in root nodules would be increased in those plants, having been grown in quinoline treated soil. The enzymes involved are ascorbate peroxidase (EC 1.11.1.7), dehydroascorbate reductase (EC 1.8.5.1) and glutathione reductase (EC 1.6.4.2).
Supposing that metabolic changes occur before any morphological damage is noticable, the detection of these physiological adaptations or symptoms may be used for the early indication of soil contaminating chemicals.

MATERIALS AND METHODS

Growth of plants: Faba bean seeds (*Vicia faba* L.cv.Troy) were surface sterilized and planted in sterilized perlite (0/3, Dämmstoff GmbH, Dortmund). 12 days old plants were inoculated with *Rhizobium leguminosarum* strain PRE (Werner et al. 1975). Afterwards the plants were planted in 20 cm plastic pots containing sterilized soil. The soil used for this study was a humus surface sample of the botanical garden, Marburg, mixed with sand 1:1, pH 6.1. It was treated as follows:
group A = quinoline application before planting by mixing an aqueous solution of quinoline with the soil up to concentrations of 10 mg kg^{-1}; 50 mg kg^{-1}; 100 mg kg^{-1}.
group B = quinoline application after planting by syringing an aqueous solution to the soil surface. This was done once a week, for a period of 5 weeks, applying each time only a fifth of the above mentionend concentrations: 2 mg kg^{-1}; 10 mg kg^{-1}; 20 mg kg^{-1} per week.
Control group = no application of quinoline
Plants were grown in a hotbed, protecting the plants by means of glass-windows 1 m above ground against heavy rains. The pots were watered with 250 ml/pot twice or three times a week, according to the climate conditions. Samples of nodules were collected 50 days after inoculation. Assays were made from three independent preparations of the three different quinoline concentrations of the two groups.
Extraction procedures: nodules were harvested and ground at 4°C by mortar and pestle with polyvinylpolypyrrolidon (PVPP, 25 %, wt:wt) and buffer (200 % wt:wt), consisting of 50 mM KH_2PO_4/K_2HPO_4 and 0.1 mM EDTA at pH 7.0. The macerate was filtered through a 100 μm net and centrifuged (4°C, 20 min, 8000 x g) (Dalton et al. 1986). Extracts from nodule free root segments were prepared the same way.
As browning of the supernatant (henceforth called extract) occured very quickly, probably due to oxidation of phenolic compounds, e.g. melanin (Hill-Cottingham 1983), it turned out to be necessary for the stability of the extract to saturate it with N_2. Dehydroascorbate reductase activity, glutathione reductase activity and the content of leghemoglobin in the extract were determined at once. Extracts for the ascorbate peroxidase assay were stored at -80°C for later analyses.

Enzyme assays: Ascorbate peroxidase was measured by a spectrophotometric procedure based on the rate of decrease in absorbance of ascorbate at 265 nm (Dalton et al. 1986). The assay was performed in a 3 ml quartz cuvette containing 0.25 mM ascorbate as reductant (absorbance at 265 nm, ϵ = 14 mM^{-1} cm^{-1}; Asada 1984), 50 mM KH_2PO_4/K_2HPO_4 at pH 7, 1.0 mM H_2O_2 and 17.5 μl of extract. Corrections were made for the low rate of ascorbate disappearance due to nonenzymatic and H_2O_2 independent oxidation. A constant rate of ascorbate turnover was achieved by starting the reaction with the enzyme extract after a 2 min adaption of the reaction mixture inside the spectrophotometer.
Dehydroascorbate reductase was measured according to Dalton et al. (1986). The glutathione reductase activity was determined as described by Goldberg and Spooner (1983).

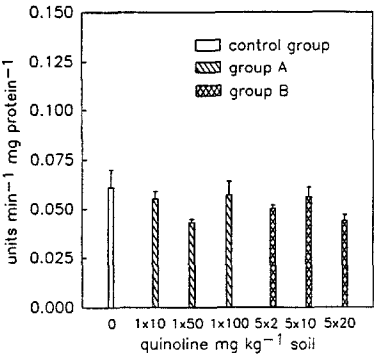
Other assays: The leghemoglobin content of the extract was determined by the pyridine hemochrome test (Appleby and Bergersen 1980). Total protein was measured by Coomassie Blue G 250 binding, using the BIO-RAD Standard assay (BIO-RAD Laboratories 1981).

Growth of *R.leguminosarum*: Erlenmeyer flasks (500 ml) with 100 ml of 20E medium (Werner et al. 1975) and 0.005 %, 0.01 %, 0.015 %, 0.02 % and 0.03 % quinoline were inoculated with 10^7 cells ml^{-1} of a pure culture of the *R. leguminosarum* strain in 20E medium (being in the log phase), for testing quinoline toxicity. The flasks were incubated at 28°C on a rotary shaker. The number of colony forming units (cfu) was determined at different periods of growth by plating appropriate dilutions on medium 20E.

Experiments with quinoline as the only carbon source were performed as described above, using a pure mineral medium instead of 20E medium with the following composition (in grams per liter): $MgSO_4 \cdot 7H_2O$, 370 mg; $CaCl_2 \cdot 2H_2O$, 73.5 mg; $Na_2MoO_4 \cdot 2H_2O$, 4.84 mg; $FeSO_4 \cdot 7H_2O$, 6.45 mg; Na_2EDTA, 4.3 mg, $NaH_2PO_4 \cdot H_2O$, 6.28 mg; K_2HPO_4, 0.78 mg. 200 μmol and 300 μmol quinoline l^{-1} were tested. At different periods of growth aliquots of the nutrient both were separated from the cells by centrifugation and analyzed for quinoline concentration at 312 nm compared to a standard curve. A possible formation of metabolites was monitored daily by following the absorption changes in the UV spectrum between 250 and 350 nm. In addition, appearence of metabolites was checked after extraction with ethyl acetate by thin-layer chromatography (TLC) according to Shukla (1987).

RESULTS AND DISCUSSION

Three enzymes of the H_2O_2 scavenging peroxidase system were determined and analyzed for changes due to the applied quinoline. The results are demonstrated in the following figures.



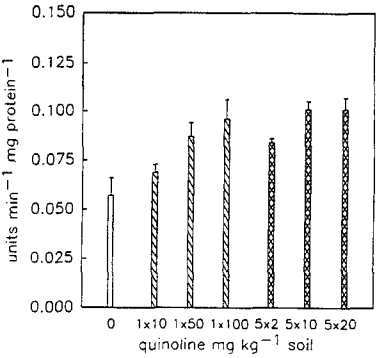
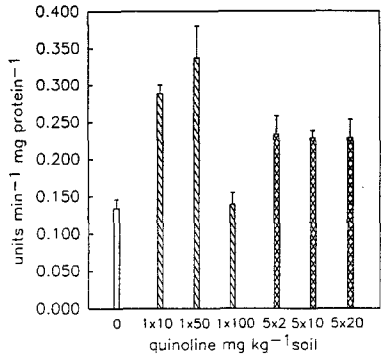


Figure 1 (left) Glutathione reductase activities in cytosol extracts of 50 days old Vicia faba nodules. Each point represents the mean (±SD) of three independent preparations, each assayed three times. Control group = no application of quinoline; group A = pre-plant application; group B = post-plant application.

Figure 2 (right) Ascorbate peroxidase activities. Statistical treatment as in figure 1.



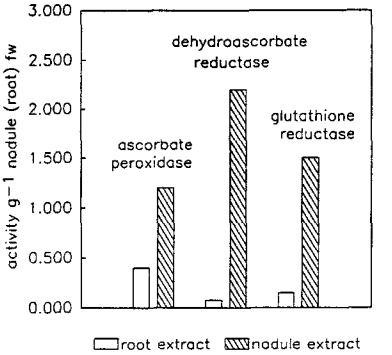


Figure 3 (left) Dehydroascorbate reductase activities. Statistical treatment as in figure 1.

Figure 4 (right) Enzyme activities in cytosol extracts of nodules and nodule free root segments 50 days after inoculation, determined as activity g^{-1} fresh weight (fw). Each point represents the mean (±SD) of three independent preparations, each assayed three times. Control group = no application of quinoline; group A = pre-plant application; group B =post-plant application.

Glutathione reductase activity (figure 1) was about the same in all samples taken from quinoline treated plants and control plants, ranging from 0.04 units min^{-1} mg^{-1} protein in samples with 50 mg quinoline kg^{-1} soil in group A (see methods) up to 0.062 units min^{-1} mg^{-1} protein in samples of the untreated control plants. No correlation between concentrations of quinoline and increase or decrease of enzyme activity was observed.
Activity of ascorbate peroxidase (figure 2) was slightly increased in group A and group B. The control plants had a minimum activity of ascorbate peroxidase of about 0.06 units min^{-1} mg^{-1} protein, whereas enzyme activity from nodules of quinoline treated plants reached a 1.5 fold higher level, rising with increased quinoline concentrations.

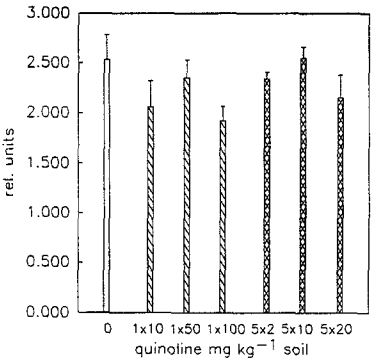
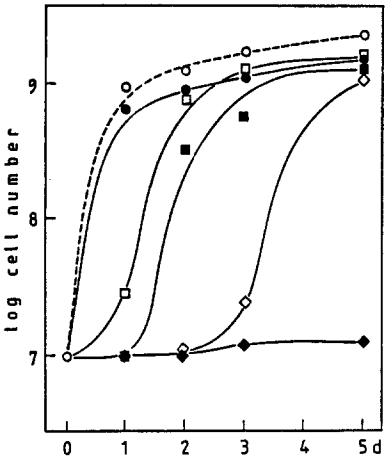


Figure 5 Leghemoglobin concentration (1 rel. unit = 1 x 10^{-2} µMol g^{-1}) of nodule extracts was determined by the pyridine hemochrome test. Statistical treatment as in figure 1.

Dehydroascorbate reductase activities (figure 3) also increased evidently in quinoline treated samples compared to the control group. Where quinoline had been added by mixing with the soil, the effect was most obvious after treatment with 50 mg quinoline kg^{-1} soil. Here 0.34 units min^{-1} mg^{-1} protein were determined compared to 0.13 units min^{-1} mg^{-1} protein in the untreated samples. Only the application of 100 mg quinoline kg^{-1} soil seemed to have no effect. The application of the highest dose of quinoline to the young plants might have had a damaging effect to the peroxidase system, so that it could not adapt during growth. The enzyme activity in all samples, where quinoline had been given weekly in small amounts, was about 0.23 units min^{-1} mg^{-1} protein, a 85% increase of activity compared to the control group. Activity of these three enzymes was also studied in root tissue of unnodulated root segments. Comparing the enzyme activities g^{-1} root fresh weight with the corresponding activities g^{-1} nodule fresh weight of the control group, we found the values of the the roots to be on a lower level (figure 4). Ascorbate peroxidase activity was about 32 % of the enzyme activity in the nodule extract, dehydroascorbate reductase only had 2.2 % activity, glutathione reductase activity was at about 12.5 % related to enzyme activities per gram fresh weight. No significant increases of enzyme activities with rising concentrations of applied quinoline could be detected in the root extracts. Protein and leghemoglobin content are known to decrease under stress conditions, such as senescence (Rabe 1981; Thompson 1987; Dalton et al. 1986) and might therefore serve as indicator for loss of vitality. Application of quinoline, however, did not lead to a significant change neither in protein nor in leghemoglobin content of nodules compared to the untreated plants.

Our results indicate, that quinoline contaminated soil affects the peroxidase system in *Vicia faba* root nodules. Since this enzyme system is involved in the detoxification of reactive oxygen intermediates, it can be assumed that an increase of activity of the ascorbate consuming and regenerating system is a defensive mechanism against directly or indirectly generated oxygen radicals, which are probably responsible for the toxicity and mutagenicity of polynuclear aromatics (Sims and Overcash 1983; Zander 1982). The content of ascorbate in nodules under stress conditions and its possible role in defensive mechanisms will be an interesting and important topic for further studies .



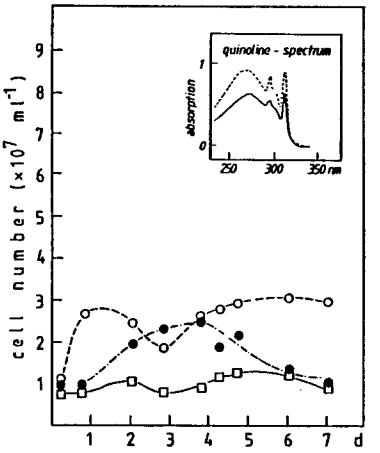


Figure 6 (left) Growth of R. leguminosarum PRE in 20E medium with different quinoline concentrations: ○ no quinoline; ● 0.005 % quinoline; □ 0.01 % quinoline; ■ 0.015 % quinoline; ◇ 0.02 % quinoline; ◆ 0.03 % quinoline.

Figure 7 (right) Growth of R. leguminosarum PRE in mineral medium with different quinoline concentrations: no quinoline; ○ 200 µmol/l quinoline; ● 300 µmol/l quinoline
The UV-Spectrum of quinoline (—200 µmol and - -300 µmol/l) in time of inoculation and after 7 days incubation time is demonstrated as insert. During the 7 days lasting experiment, no differences in the UV-spectrum could be observed.

Apart from the possible use of root symbiosis systems for indicating soil contaminating chemicals, we were also interested in examining whether these special root organs might attribute to the detoxification of quinoline in soil. Growth experiments with free living *R.leguminosarum* were designed to determine the toxic concentration of quinoline in batch cultures and to test the bacteria's ability of metabolizing quinoline.

As can be seen in figure 6 growth of *R.leguminosarum* in 20 E medium with quinoline occured rapidly at 0.005 % and 0.01 % quinoline; growth occured with a lag phase at 0.015 % and 0.02 % quinoline. Higher concentrations were toxic and inhibited growth completely. Growth in the log phase seemed to be comparable to the untreated culture, but after incubation with 0.02 % quinoline cell numbers in the stationary phase were reduced to 50 % in comparison to the control cultures.

Rhizobia which are related to *Pseudomonas* are known to be capable of metabolizing aromatic compounds via the α-ketoadipate pathway (Glenn and Dilworth 1981; Röhm and Werner 1985). Testing the ability of metabolizing quinoline we were not able to show that *R.leguminosarum* strain PRE can use quinoline as the only carbon source (figure 7), as it is known for some *Pseudomonas* species (Shukla 1987). Neither did the amount of quinoline decrease nor did any metabolites appear.
However, the chromosomal backround of various effective plasmid harbouring strains of *R.leguminosarum* can be very different and other strains may be able to metabolize quinoline.

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