Belowground organism activities affect plant aboveground phenotype, inducing plant tolerance to parasites

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Abstract
Soil fauna activities are probably more important than currently acknowledged in determining individual plant response to stresses and overall plant diversity. Here we demonstrate that the positive effect of earthworms on rice could be the result of a systemic effect on plant physiology. Moreover, this effect could improve tolerance to stressors such as parasitic nematodes. In a controlled experiment, an 82% decrease in the production of infested plants was suppressed when earthworms were present. Earthworms had no direct effect on nematode population size. In their presence, however, root biomass was not affected by nematodes and the expected inhibition of photosynthesis was suppressed. In the leaves, the expression of three stress-responsive genes (coding for lipoygenase, phospholipase D and cysteine protease) was modulated by the presence of belowground invertebrate activities. We document conflicting systemic effects of parasitic nematodes and beneficial earthworms, although we cannot precisely identify the mechanism involved. These results reveal the importance of non-trophic belowground/aboveground interactions for plant health and response to stresses.

Keywords
Belowground/aboveground interactions, cysteine protease, earthworm Millionia anomala, gene expression, lipoygenase, Non-trophic interactions, phospholipase D, photosynthesis, plant parasitic nematode Heterodera sacchari.

Introduction
Plants interact with belowground communities of microorganisms and invertebrates through many different ways. While important reviews and textbooks have detailed their physiological responses to beneficial microorganisms (Smith & Read 1997; Giller 2001) and pests (Hall 1996), the relationships of plants with soil invertebrates have, in general, seldom been studied (Hooper et al. 2000). Small invertebrates like free-living nematodes and microarthropods enhance plant productivity through food-web effects on nutrient release (Coleman et al. 1984, 1989; Ingham et al. 1985; Setälä & Huhta 1991; De Ruiter et al. 1993; Chen & Ferris 2000; Schroter et al. 2003). Positive effects of earthworms and other ecosystem engineers on plant growth have also been well documented (Spain & Okello-Oloya 1985; Spain et al. 1992; Brown et al. 1999; Schu 2003). Five mechanisms have been suggested to explain earthworm positive impact on plant production: enhanced mineralization of nutrients, indirect interactions via improved availability of water and oxygen in the root zone, hormone-like effects, dispersal of growth stimulating microorganisms, pest control via the dispersal of microorganisms antagonistic to root pathogens. Plant parasitic nematodes are indeed very harmful pests that cause losses in crop production worth over 100 billion annually (Bridge et al. 1990). They seriously threaten tropical crops such as sugarcane, banana and upland rice (Bridge et al. 1990), the latter being the most widespread crop worldwide (Maclean et al. 2002). These pests are currently controlled with highly toxic chemicals which will be progressively banned because of their toxic effects on human and ecosystem health (FAO 2003). Earthworms are known to reduce plant parasitic nematode populations (Dash et al. 1980; Senapati 1992; Boyer 1998; Ilieva-Makulec & Makulec 2002) although increases in densities of Paratylenchus sp. have also been reported and

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attributed to enhanced plant production (Yeates 1981). Effects on free bacterial-feeding nematodes have not been addressed in our experiments as this functional group had been eliminated by frost sterilization. Mechanisms involved in earthworm/nematode interaction were hypothesized to be ingestion of nematodes by earthworms (Dash et al. 1980) and earthworm activation of bacteria antagonistic to plant parasitic nematodes (Boyer 1998). Reduction of plant parasitic nematodes has also been observed in soil treated with commercial vermicompost (Ribeiro et al. 1998; Arancon et al. 2003), which seems to exclude Dash’s hypothesis. In none of these studies the resulting effect of plant parasitic nematode control by earthworms on plant production has been assessed. In particular, the effects of earthworms on the physiology of nematode-infected plants have never been described.

We first checked earthworm effects on a plant exposed to nematodes in an experiment using a combination of earthworm, plant and parasitic nematode that had not been tested so far. The infection rate (number of cysts reported to root biomass) would allow determining if infected plants were effectively suffering a lower stress in presence of earthworms. We would then determine if earthworms had effectively decreased the total number of nematodes, or if they had rather improved the ability of plants to respond by physiological mechanisms. Measurement of such physiological indicators as photosynthesis parameters (gas exchanges, fluorescence and chlorophyll concentrations) and expression of selected stress responsive genes [coding respectively for lipoxygenase, phospholipase D (pld) and cysteine protease (cp)] would allow to test this hypothesis. Lipoxygenase (lox) is the first enzyme involved in the production of jasmonate which triggers defence mechanisms against phytophagous and pathogenic organisms in plants (Farmer et al. 2003). pld and cp are stress-responsive genes encoding enzymes involved in cell signalling pathways and in membrane lipid and protein degradation (Roy Macauley et al. 1992; Wang 2002). These genes are considered reliable indicators of plant health (El-Maarouf et al. 1999; Cruz de Carvalho et al. 2001; Matos et al. 2001).

**MATERIALS AND METHODS**

**Microcosms and organisms**

Experimental units were 10 cm diameter and 15 cm height PVC pots, filled with 1 kgs (dry weight) of a sandy ultisol from the Ivory Coast. Original soil fauna was eliminated by frost. Soil at 80% of the field capacity was frozen at −20 °C for 2 days. Encysted nematodes hatched after a 3-day incubation at 30 °C were further eliminated by another freezing period (Boyer 1998). This treatment does not affect microbial communities, a necessary condition to earthworm survival (Gilot-Villenave 1994). After germination, rice plants (*Oryza sativa* L., cv. Moroberekan) were submitted to one of the following four treatments (each with six replicates): control treatment (C) without fauna, earthworm treatment (E), with 1 g fresh biomass per pot (4–6 earthworms), nematode treatment (N) with an initial inoculum of 2300 nematodes per pot, and earthworm/nematode treatment (EN) with both earthworms and nematodes in the same amounts as in earthworm and nematode. Earthworms (*Millonina anomala* Omodeo) are indigenous to this soil and belong to a functional group dominant in humid tropical soils (mesohumic endogeic) (Lavelle 1983; Blanchart et al. 1999).

Plant parasitic nematodes (*Heterodera sacchari*, Luc & Merny 1963) form external cysts on rice roots, leading to serious damage in upland rice fields all over Africa and particularly in the Ivory Coast (Coyne & Plowright 2000).

At the end of the experiment, earthworms were weighed to verify that nutrients released by earthworm weight loss would not affect plant production (data not shown). Count of full and empty cysts was chosen as an indicator of nematode activity accumulated over the experimental period. Full cysts are *H. sacchari* gravid females; empty cysts are still recognizable envelopes of hatched cysts. Three generations are expected to have occurred in our 90-day experiment as life cycle extends over 24–30 days (Bridge et al. 1990). We did not find any significant differences between treatments, whatever the generation (data not shown). No other nematode species, either parasitic or free living, was found showing that they had been efficiently eliminated by freezing.

**Plant growth and physiology**

Young rice seedlings were grown for 90 days under artificial light (200 μmol photons m⁻² s⁻¹) at 29 °C/day and 24 °C/night temperatures. Air moisture was kept at 75% ± 5%. Plants received 40 mL of demineralized water daily and 30 mL of one-tenth diluted Hoagland nutritive solution weekly.

At the end of the experiment, gas exchanges were measured under cold light from 36 red Light Emitting Diodes (630 nm) using the analyzer ADC LCA2 (ADC®, Hoddesdon, UK) set up in an open circuit. Maximal photochemical quantum yield of PSII (Fv/Fm) was measured with the mobile Teaching PAM (Walz®, Effeltrich, Germany) fluorimeter, after 1 h of darkness. Chlorophyll concentrations were measured by spectroscopy.

After photosynthesis measurements, plants were cut at ground level. Soil particles were removed from the root system by washing on a sieve. Leaves and roots were oven dried for 2 days at 60 °C and weighed, taking into account the pieces collected for molecular assessments.
Plant gene expression

We tested the expression of three stress-responsive genes coding for phospholipase D α (PLDα) (accession number: AB001920), lipoxygenase-1 (LOX-1) (accession number: AJ270938), and cysteine protease (CP) (accession number: AY062178). In 30-day old plants, the youngest developed leaves of each plant were frozen in liquid nitrogen and stored at -80 °C. Total RNA’s were extracted with the RNeasy Midi kit (Qiagen®, Valencia, California, USA) following the manufacturer’s instructions. For each gene, semi-quantification of mRNAs from each treatment was performed by RT-PCR with the One Step RT-PCR kit from Qiagen® in the thermocycler Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). Specific primers, adequate annealing temperatures and number of PCR cycles were adapted to each gene. After separation of RT-PCR products on agarose gels, band intensity was determined under UV light using the Gene Tools system (Syngene, Cambridge, UK).

RESULTS

Ninety days after the onset of the experiment, we investigated the effect of earthworm inoculation on plant biomass production, nematode infection rate, nematode absolute number and plant photosynthesis activity and gene expression.

Plant growth was very limited in N as compared with C and E treatments. In the EN treatment, shoot and root dry biomasses were not statistically different from the control (Fig. 1a,b), showing the efficiency of earthworm control on the negative effect of nematodes.

Infection rate, that is the average number of nematodes per unit of root biomass, was 82% lower in EN than in N (Fig. 2a). Nevertheless, the total cyst number per pot was not statistically different in the EN and N treatments as root biomass was much higher in the EN than N treatment (Fig. 2b). Thus, the lower infection rate in EN was not explained by an earthworm effect on nematode population but rather by a direct effect on root biomass (Fig. 1c).

To better understand the observed suppression of nematode effect, we tested the hypothesis that earthworms enhance plant tolerance through the improvement of photosynthetic activity, and/or the regulation of stress gene expression. Ninety days after the onset of the experiment, photosynthetic activity was close to zero in the N treatment (Fig. 4a) and chlorophyll concentration in leaves was significantly lower than in the other treatments (Fig. 4b). In E, chlorophyll concentration was significantly higher than in C (+43%) (Fig. 4b). In EN, photosynthesis was not significantly different from control, indicating that earthworms counteracted the negative effect of nematodes alone (N). Moreover, chlorophyll concentration was intermediate between control and E treatment levels, showing a positive effect of earthworms on this parameter in spite of nematode presence (Fig. 4b). Maximum photochemical quantum yield of photosystem II (Fv/Fm) (Fig. 4c), measured by leaf fluorescence (Maxwell & Johnson 2000), did not differ between C and E. A 15% decrease in N (P < 0.01) indicated an irreversible alteration of photosynthetic mechanisms (Epron & Cornic 1993). Conversely, the nematode effect on PSI was not observed in EN, as EN maximal photochemical quantum yield was equivalent to that of control plants.

Figure 1 Rice biomass in different treatments. (a) Total dry biomass; (b) shoot dry biomass; (c) root dry biomass. C, control; E, earthworms; N, nematodes, EN, earthworms and nematodes. Significant differences between means (± 1 SE) are marked by different letters (least square means, Tukey–Kramer test, P < 0.05).
In the leaves, we determined expression levels of genes encoding proteins involved in cell signalling pathways, senescence and plant stress response: the lipoxygenase (\textit{lox}) (Porta & Rocha-Sosa 2002), PLD\(\alpha\) isoform (\textit{pld}) (El-Maarouf et al. 1999), and cysteine protease (\textit{cp}) (Turk et al. 1997). In N, transcript accumulation was increased, when compared with C (Fig. 3a–d). Earthworms alone (E) triggered an increase in lipoxygenase gene expression (Fig. 3a, b) and an important decrease in cysteine protease gene expression (\textasciitilde 58\%; Fig. 3a, d) when compared with C. However, no modification of PLD gene expression was observed (Fig. 3a, c). In the presence of both earthworms and nematodes (EN), expression of the three genes was higher than in the control treatment, although lipoxygenase and cysteine protease mRNAs remained slightly less abundant than in N.

**DISCUSSION**

Improvement of nutrient availability by earthworms (Lavelle \textit{et al}. 1992; Subler \textit{et al}. 1997; Araujo \textit{et al}. 2004) may have reduced the need to mobilize nitrogen through autophagic processes in growing and/or stressed plants. This might explain the increase in chlorophyll concentration and the decrease in cysteine protease gene expression in the E treatment when compared with the control. Other processes, such as an improved availability of oxygen and/or water to the roots (Brown \textit{et al}. 1999) or the release of hormone-
like chemicals from earthworm casts (Muscolo et al. 1999; Quaggiotti et al. 2004) may also have contributed to improve plant health and decrease the need for cysteine protease. Finally, the overexpression of lox gene, involved in the initiation of jasmonate signalling pathway, may result from physical damage caused by earthworms on some roots. This could prepare the plant to better tolerate stress.

In leaves, nematodes induced increases in the three stress-responsive gene expressions and irreversible damage to the photosynthetic machinery. This suggests that leaf cells underwent uncontrolled degradation processes leading to leaf senescence. In the presence of both nematodes and earthworms, stress gene expression remained high, even if it seemed to be slightly alleviated. Thus, changes in gene expression observed in our experiments do not alone account for the net improvement in plant biomass and photosynthetic activity brought by the worms. In the EN treatment, enhanced biosynthesis reactions because of earthworm activity balanced catabolic reactions triggered by nematodes.

Chlorophyll concentration in EN was significantly higher than in the N treatment and intermediate between the C and E values. Plants may derive part of the nutrients necessary to produce chlorophyll from ammonium-rich earthworm casts deposited in the rhizosphere. The maintenance of root biomass at control level (Fig. 1c) suggests that sufficient water and nutrient uptake allowed to maintain a high chlorophyll concentration in leaves (Fig. 4b). Consequently, irreversible damage of photosystem II observed in N was prevented (Fig. 4c) and total biomass was similar to control in the EN treatment (Fig. 1a).

In this study, we have demonstrated that decrease in rice growth because of the parasitic nematode *H. sacchari* was suppressed in the presence of earthworms. This is the first time earthworms have been shown to obviate nematode effects in infested plants. In our experiment, the absolute number of cysts did not decrease in the presence of earthworms, whereas number per unit of root biomass decreased by 82%. These results, in accordance with a previous work (Yeates 1981), differ from other reported studies (Dash et al. 1980; Senapati 1992; Boyer 1998; Ilieva-Makulec & Makulec 2002). Earthworms enhanced plant tolerance to nematodes in a systemic manner. Their presence in the rhizosphere induced changes in gene expression, PSII maximal photochemical quantum yield and chlorophyll concentration in the leaves.

These results emphasize the importance of belowground interactions in plant ecophysiology which certainly have profound effects in plant community dynamics. Recent papers have emphasized the role of multitrophic interactions in plant community composition and production (Van der Putten et al. 2001; De Deyn et al. 2003; Wardle et al. 2004). While effects of food-web regulations have been largely investigated in a number of field and laboratory experiments (Coleman et al. 1984, 1989; Ingham et al. 1985; Setälä & Huhta 1991; De Ruiter et al. 1993; Chen & Ferris 2000; Schrotter et al. 2003), current models generally ignore non-trophic effects of ecosystem engineers.

![Figure 4](https://example.com/figure4.png)

**Figure 4** Photosynthesis. (a) Net assimilated CO₂ (µmol of CO₂ m⁻² s⁻¹); (b) chlorophyll concentration (µg mm⁻²); (c) maximal photochemical quantum yield (Fv/Fm). C, control; E: earthworms; N, nematodes, EN, earthworms and nematodes. Significant differences between mean values (± 1 SE) are marked by different letters (least square means, Tukey–Kramer test, *P* < 0.05).
and their mechanisms. Given the huge effects of these organisms in their respective functional domains in most ecosystems, it is likely that food-web models that do not take them into account will remain very limited in their capacity to explain dynamics of the plant–soil system. One way to solve the problem may be to consider that food-web interactions are constrained in the limits of physical domains of engineers (Lavelle 2002) or to allow ecosystem engineers to influence consumption rates in existing food-web models.

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