Eur J Plant Pathol (2016) 144:155–165
DOI 10.1007/s10658-015-0759-8

Transcriptional profiling of wheat in response to take-all disease and mechanisms involved in earthworm's biocontrol effect

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Accepted: 8 September 2015 / Published online: 11 September 2015
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Abstract Take-all disease caused by the soil-borne fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) is the most widespread and well-studied root disease of winter wheat. The absence of plant genetic resistance and efficient fungicide against this disease calls for the development of alternative management strategies such as the use of biological control agents. In a greenhouse experiment, we tested the hypothesis that the earthworm *Aporrectodea caliginosa* can control this plant pathogen by changing soil pH, inducing plant defence mechanisms or improving plant nutrition. Towards this aim, soil chemical properties, plant production, morphology and transcriptome were assessed in the different treatments to characterize the effects of *Ggt*, earthworm and the interaction between them. Sixty three days after sowing, *Ggt* was responsible for a strong reduction in fine root proportion and leaf area, and an 82 % decrease in plant total biomass. Earthworms reduced infection rate by 63 % and improved plant growth, which was not significantly different from the no-pathogen control. Neither changes in soil pH, plant defence mechanisms or plant nutrition were proved to be involved in this effect. It was concluded that *A. caliginosa* was a very efficient biocontrol agent against *Ggt* and that the mechanism responsible for this biocontrol effect could be associated with microbial community modifications or fungal consumption by earthworms.

Keywords *Aporrectodea caliginosa* · *Gaeumannomyces graminis* var. *tritici* · Defence mechanisms · Take-all · Transcriptome · *Triticum aestivum* (winter wheat)

Electronic supplementary material The online version of this article (doi:10.1007/s10658-015-0759-8) contains supplementary material, which is available to authorized users.

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Introduction

Take-all disease, caused by the soil-borne fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is the most widespread and well-studied root disease of winter wheat (*Triticum aestivum* L. subsp. *aestivum*). Besides infecting wheat, it also affects to a lesser extent barley and rye and occurs mainly in areas of intensive and continuous cereal cropping. This fungus grows as superficial mycelium on roots and produces feeder hyphae that penetrate root tissues and colonize the cortex, leading to the destruction of the root system (Cook 2003;

Freeman and Ward 2004). Between two infections, the pathogen survives saprophytically in the dead roots and stem bases that are used by the fungus as a food source before infecting the next host crop.

No plant resistance is known, and no fungicide is efficient against this fungus (Cook 2003; Freeman and Ward 2004). Chemical control (e.g., the use of soil fumigants) appears not to be affordable for an agronomic crop such as wheat (Cook 2003). As far as plant genetic resistance is concerned, the use of molecular techniques to study *G. graminis* and related fungi helped reveal the role of the enzyme avenacinase as a pathogenicity determinant in oat (*G. graminis* var. *avenae*), but not in wheat (Freeman and Ward 2004). Molecular mechanisms involved in plant response to pathogenic fungi are well documented for other fungi such as *Fusarium oxysporum* (Berrocal-Lobo and Molina 2008) or *Botrytis cinerea* (Williamson et al. 2007), but little is known about the molecular response of the plant to *Ggt* infection, except in early stages of infection (Guilleroux and Osbourn 2004). Currently, control of take-all primarily relies on cultural practices such as crop rotation with non-host plants, which allows breaking the infestation cycle. Another control strategy is ammonium fertilization to make the wheat rhizosphere pH more acidic and unfavourable to *Ggt* (Cook 2003). Some soils have been shown to be suppressive to take-all, whereas others were conducive to the disease (Freeman and Ward 2004). Efforts to identify the causes of this suppression focused primarily on isolating antagonist microorganisms. Among these microorganisms, *Pseudomonas fluorescens* has been shown to have great potential as a biological control agent (Chapon et al. 2002; Cook 2003; Freeman and Ward 2004). However, more recently it has been demonstrated that soil suppressiveness relies more on modification of the whole bacterial community, not on changes in the abundance of one single strain (Sanguin et al. 2009).

Earthworms have been shown to be effective biological control agents against *Ggt* (Clapperton et al. 2001; Stephens and Davoren 1995; Stephens et al. 1994a) and several other plant pathogens such as *Plasmodiophora brassicae*, *Fusarium oxysporum, Heterodera sacchari* or *Venturia inaequalis* (Blouin et al. 2005; Brown et al. 2004; Elmer 2009; Wurst 2010). Recently, it has been shown that earthworms can be effective against take-all disease (Hume et al. 2015; Stephens and Davoren 1995; Stephens et al. 1994a), but mechanisms responsible are not fully understood. The biocontrol of plant pathogens by soil fauna could rely on several mechanisms. Among these mechanisms are: (1) earthworms are known as ecosystem engineers, which physically modify soil structure with potential detrimental effects for the fungal pathogen (Stephens and Davoren 1995; Stephens et al. 1994a); (2) earthworms could also decrease the amount of inoculum in the soil either by eating plant detritus or the fungus living in the soil (Bonkowski et al. 2000; Friberg et al. 2005; Wolfarth et al. 2011); (3) earthworms are important drivers of nitrification, denitrification and ammonification (Parkin and Berry 1999), changing soil pH, which could lead to soil acidification harmful to *Ggt* (Cook 2003); (4) earthworms have been shown to modify the production of signal molecules such as indole acetic acid (IAA) (Muscolo et al. 1998; Puga-Freitas et al. 2012; Quaggiotti et al. 2004), signal molecules that could modify plant resistance to pathogens (Ping and Boland 2004; Van Wees et al. 2008) and could be responsible for a decrease in infection in the presence of earthworms (Blouin et al. 2005; Wurst 2010); (5) earthworms are well known for their strong impact on the N cycle. For example, in sorghum, an additional flux of 63 kg N ha^{-1} $year^{-1}$ through earthworms could account for 38 % of the total N uptake (Parmelee and Crossley 1988), this additional N could help the plant combat fungal infection (Cook 2003); (6) earthworms are responsible for pronounced modifications of microbial community structure (Bernard et al. 2012; Brown 1995; Monard et al. 2011), associated with soil suppressiveness (Sanguin et al. 2009).

In the present study, a greenhouse factorial experiment with wheat plants exposed to *Ggt* or earthworms alone or in combination was conducted to test the hypothesis that earthworms can control take-all disease. Changes in soil NO_3^-/NH_4^+ ratio, improvement of plant nutrition or the induction of defence mechanisms were tested as potential mechanisms to explain an earthworm effect on *Ggt* infection.

Materials and methods

Experimental design

There were four treatments in this laboratory experiment. In the control (C), wheat was grown in the absence of earthworms and *Ggt*. In condition E, wheat was grown with earthworms only. In condition *Ggt*, wheat was grown with *Ggt* pathogenic fungus only. In *Ggt*+E,

wheat was grown with both *Ggt* and earthworms. Treatments C and E were replicated eight times and treatments *Ggt* and *Ggt*+E were replicated 13 times as more variance was expected for plants exposed to pathogen. All analyses were performed on five replicates for treatments C and E, and ten replicates for treatments *Ggt* and *Ggt*+E (with the exception of transcriptomic analyses, which were performed on the last three replicates of each treatment).

Experimental conditions

Microcosms were placed in a greenhouse, at a density of 24 experimental units per square meter. Climatic conditions were set to 13/11 °C day/night temperatures during the first 5 days of the experiment. They were then set to 19/13 °C day/night temperatures from day five until the end of the experiment. The relative humidity was set to 75±5 % during the whole experiment. Each experimental unit was made of PVC cylinders (10 cm diameter, 15 cm height).

Soil

Soil was retrieved at the INRA research center of Thiverval-Grignon (France, 48°50′ N, 1°56′ E), from experimental fields conducted in monoculture of maize, then sorghum (no wheat for more than 10 years, thus low levels of *Ggt*). Soil was manually collected at a depth of 0–30 cm, randomly across the field. Then it was dried at 25 °C for a week and sieved at 2 mm mesh size in order to remove all the macrofauna. The average composition and properties of the surface (0–30 cm) horizon measured in the field were: total organic carbon content, 27.1 g kg^{-1}; total nitrogen content, 1.28 g kg^{-1}; pH (water), 8.31; CEC, 14.5 cmol kg^{-1}; texture: 28.7 % clay, 55.1 % silt, 16.2 % sand. For the experiment, each microcosm was filled with 900 g dry weight (DW) soil and maintained at 75 % field capacity. Field capacity was maintained by weighing microcosms two times a week and adding deionized water to reach 75 % field capacity (221 g kg^{-1} soil DW). No water draining was observed during the experiment. Organisms were added in the following order in microcosms: *Ggt* 21 days before sowing, earthworms 7 days before sowing and plants at day 0.

Fungal treatment

Soil inoculation was made by adding barley seeds colonized by *Gaeumannomyces graminis* var. *graminis* (Sacc.) Von Arx and Olivier (1952) in microcosms of treatments *Ggt* and *Ggt*+E. Barley seeds infected with *Ggt* were provided by the INRA IGEPP (Rennes, France). One thousand propagules corresponding to 1.60 g of barley seeds were mixed with the soil for each microcosm. In treatments without pathogen (C and E), the same amount of non-infected barley seeds was mixed with the soil.

Earthworm treatment

Earthworms *Aporrectodea caliginosa* Savigny (Annelida, Oligochaeta) were retrieved near the La Cage experimental field of the INRA research station (Versailles, France). These earthworms are endogeic and their burrows are horizontal or randomly oriented. These burrows are considered to be temporary structures because they are rarely reused (Bouché 1972). In our experiment, three earthworms (on average 0.5 g per individual) were added to each microcosm of the E and *Ggt*+E treatments 2 weeks after *Ggt* inoculation. This corresponds to 1.9 T ha^{-1}, or 380 individuals m^{-2}. the density observed in fields or pastures in France (Lavelle and Spain 2001). At the end of the experiment, earthworms were retrieved, rinsed using deionized water, slightly dried using paper towel then weighed.

Wheat plants

Seeds of *Triticum aestivum* cv Soissons were purchased from the breeder Florimond-Desprez (Cappelle-en-Pévèle, France). Seeds were soaked in water for 48 h and vernalized for 2 weeks at 5 °C. One week after the introduction of earthworms, three seeds of wheat were sown per microcosm and the resulting seedlings were kept until the end of the experiment. Twenty six days after sowing, plants were supplied weekly with a nutritive solution (33.5 % NH_4NO_3) diluted 100 times. Plants were harvested for analysis 63 days after sowing (DAS).

Assessment of disease severity

At the end of the experiment, the root systems of the three plants were retrieved from each microcosm, the number of roots with necrosis was counted and total root biomass was weighed. The infection rate (average number of necrotic roots per unit of root biomass) was then calculated.

Soil chemical analyses

Total carbon and nitrogen were analysed by elemental analysis after dry combustion (NF ISO 10694 and NF ISO 13878). Soil nitrate and ammonium contents were determined by KCl extraction and quantified by spectrocolorimetry. Phosphorus was determined by spectrometry (NF ISO 11263). All these analyses were performed at the INRA "Laboratoire d'Analyse des Sols" (Arras, France) on two soil samples of 80 g each, taken from the centre of each microcosm.

Root system and leaf morphological analysis

The distribution of dry root biomass between classes of diameter was established according to the method of Blouin et al. (2007). Briefly, shredded dry roots were passed through a column of sieves with decreasing mesh sizes. Root biomass in each sieve was then weighed with the aim of obtaining the distribution of root biomass in each diameter classes. The relative root biomass proportion per class of diameter was calculated by dividing absolute biomass per diameter class by total root biomass. These ratios gave a qualitative descriptor of root system structure which can be used to compare root systems whatever differences in size (Blouin et al. 2007). The leaf surface area of each plant was determined using an LI-3100 Area Meter (Li-Cor Inc, U.S.A). Plant height was measured at 13, 28, 38, 50 and 63 days after sowing.

Transcriptome analysis

In order to obtain a global analysis of gene expression in *Triticum aestivum* in response to soil organisms, microarray analyses were performed on a pool of one leaf cut on each of the three plants of a given microcosm. This was done for three replicates from the four treatments (n=12 in total), harvested at day 52 after sowing. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, France) with an on-column DNase digestion using DNase I (Qiagen, France). The quality of the RNAs was assessed by capillary electrophoresis using the Agilent Bioanalyser (Agilent, Santa Clara, U.S.A.) and concentration was measured by absorbance at 260 nm with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, U.S.A).

For microarray analysis, total RNA was processed by PartnerChip (Evry, France). This analysis was performed on Affymetrix GeneChip Wheat Genome arrays containing 61 127 probes sets corresponding to more than 55 052 Unigene clusters, according to the two-cycle amplification protocol from the manufacturer (Affymetrix, U.S.A). Total RNAs (200 ng) were reverse transcribed in the presence of T7 oligo (dT) primer and Superscript II Reverse Transcriptase to generate first-strand cDNA. Second-strand was synthesized using DNA polymerase I and RNaseH. After second-strand synthesis, in vitro transcription was carried out using T7 RNA polymerase and biotinylated nucleotides analog/ribonucleotide mix for cRNA labeling (GeneChip IVT Labeling Kit, U.S.A) then purified using the GeneChip Sample Cleanup Module and quantified by absorbance measurement at 260 nm. The resulting biotinylated cRNA was fragmented by incubation in a buffer at 94 °C for 35 min to reduce fragment size to approximately 100–120 nucleotides. Fragmented cRNA was hybridized on Affymetrix GeneChip Wheat Genome array for 16 h at 45 °C along with internal hybridization controls. Washing and staining procedures were performed in an Affymetrix Fluidics Station 450. Probe arrays were exposed to ten washes in non-stringent wash buffer A (6× SSPE, 0.01 % Tween20) at 30 °C, followed by six washes in stringent buffer B (100 mM MES, 0.1 M [Na^+] and 0.01 % Tween20) at 50 °C. Biotinylated cRNA were stained with a streptavidin–phycoerythrin conjugate (SAPE, 10 μg ml^{-1}) and washed again ten times with non-stringent buffer A. Finally, arrays were scanned in an Affymetrix GeneChip Scanner 3000.

Probe-level expression data (CEL files) were produced using GeneChip® Operation Software (GCOS) version 1.4 and data were normalized using the MAS5 algorithm. Quality-control and statistical analyses (background adjustment, normalization, and probe-level summarization of data) used the GC-Robust Multi-Array average algorithm (GC-RMA) from the GeneSpring GX11 Software. Data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE47479 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47479).

Annotation and database analysis

Partial wheat genome annotation given by Affymetrix (http://www.affymetrix.com/analysis/index.affx) was completed by a transcript characterization following the recommendations of the Wheat Genome Database

(http://jcvi.org/wheat/annotate_methods.shtml). A putative function was attributed to each modulated gene on the basis of *Arabidopsis thaliana* genome by comparing the RefSeq proteins of the cluster transcripts of wheat UnigeneID with those of *Arabidopsis thaliana*. A Gene Ontology function was attributed to each gene according to the functional genomics tool DAVID (Huang et al. 2009) (http://david.abcc.ncifcrf.gov/summary.jsp). Finally, a pathway analysis for all genes with modified transcript abundance was performed using the KEGG pathway mapping tool (Kanehisa et al. 2012) (http://www.genome.jp/kegg/pathway.html).

Statistical analysis

All statistical analyses were performed using the R software (Team 2008). Output variables (soil properties, plant growth and morphological parameters and infection rate) were analysed using a two-way ANOVA testing *Ggt* and earthworm effects and the interaction between these two factors, with a Tukey honestly significant difference (HSD) multiple mean comparison *post hoc* test. Non-linear regressions for infection rate and total biomass were used to determine the equation of a regression curve. The variation explained by the model was calculated by comparing the residual variation with the total variation.

Results

Plant growth

Experimental conditions were favourable to earthworms, with only 7 % of dead/missing individuals, and an increase by 32 % of their biomass at the end of the experiment. No significant effect of treatments on earthworm survival was observed. At the beginning of the experiment, *Aporrectodea caliginosa* significantly promoted wheat growth in treatment E (Fig. 1a): plant height was increased by 36, 31 and 28 % respectively at 13, 28 and 38 days after sowing (DAS) when compared to the control. Toward the end of the experiment (38 to 63 DAS), this positive effect of earthworms disappeared: no more significant difference was observed between C and E treatments. A negative effect of *Ggt* on plant heights was observed along the whole experiment (comparison between the *Ggt* and C treatments). At the beginning of the experiment (day 13), earthworms had no effect on the height of plants inoculated with *Ggt* (comparison between E and *Ggt*+E treatments). However, the positive effect of earthworms was significant (days 28–63); at 50 and 63 DAS, plant height was increased by 47 and 39 % respectively, in treatment *Ggt*+E as compared with treatment *Ggt*. At the end of the experiment (63 DAS), there was no significant difference in plant height from treatments *Ggt*+E, E and C, whereas wheat growth was reduced by 37 % in the *Ggt* treatment. The negative impact of *Ggt* was also observed on above-ground, below-ground and total plant biomass (Fig. 1b), with a reduction of 83, 81 and 82 % respectively, as compared to control plants (C). In the *Ggt*+E treatment, total plant biomass was not significantly different from the control (−41 %, $P>0.05$) and 2.3 times the *Ggt* treatment ($P<0.05$). Nitrogen and carbon contents in plant leaves and roots were measured, but no significant differences were found (data not shown).

Plant infection

The infection rate was very low in the C and E treatments (0. 8 necrosis per plant in average), whereas inoculated treatments exhibited a high number of necrosis, with an average of 231 for *Ggt* and 85 for Ggt+E, showing that *Ggt* inoculation was efficient. Plant infection rates comparing *Ggt* and *Ggt*+E treatments was reduced by 63 % (Fig. 2a) in the presence of earthworms ($P=0.004$). The infection rate was correlated with total biomass following a reciprocal (or multiplicative inverse) function (Fig. 2b). The infection rate varied widely between replicates of the *Ggt* treatment, from 76 to 483 necrotic roots per gram of dry root, with an associated total biomass of 0.06–0.24 g DW. Between-replicate variance was smaller in the *Ggt*+E treatment. The infection rate in the *Ggt*+E treatment was around 85 necrotic roots per gram of dry root, with a total biomass of 0.25–0.50 g DW.

Plant morphology

Ggt negatively impacted above and belowground plant morphology. The presence of *Ggt* reduced leaf area by 78 % (Fig. 3a) and decreased the proportion of fine roots (up to 200 μm) when compared to control plants (Fig. 3b). Earthworm treatment had a positive impact on above-ground morphology of plants infected with *Ggt* with a 3-fold enhancement of leaf area in *Ggt*+E

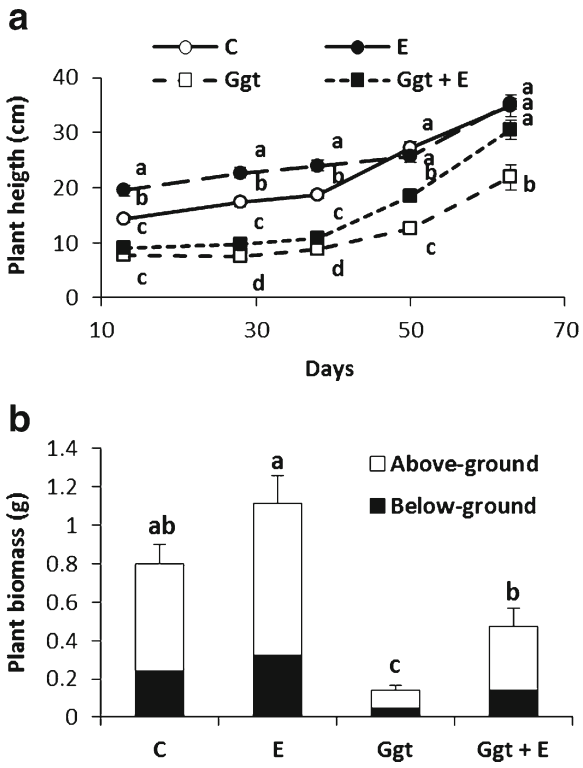


Fig. 1 Effect of the earthworm *Aporrectodea caliginosa* and the pathogenic fungus *Gaeumannomyces graminis* var. *tritici* on: **a** wheat height during the experiment and **b** wheat root, shoot and total biomasses at the end of the experiment (63 days). C: control treatment; E: treatment with earthworms; *Ggt*: treatment with the pathogenic fungus; *Ggt*+E: treatment with fungus and earthworms. Means±s.e., n=5 for C and E treatment and n=10 for *Ggt* and *Ggt*+E treatments, different letters indicate a significant difference, Tukey HSD, P<0.05

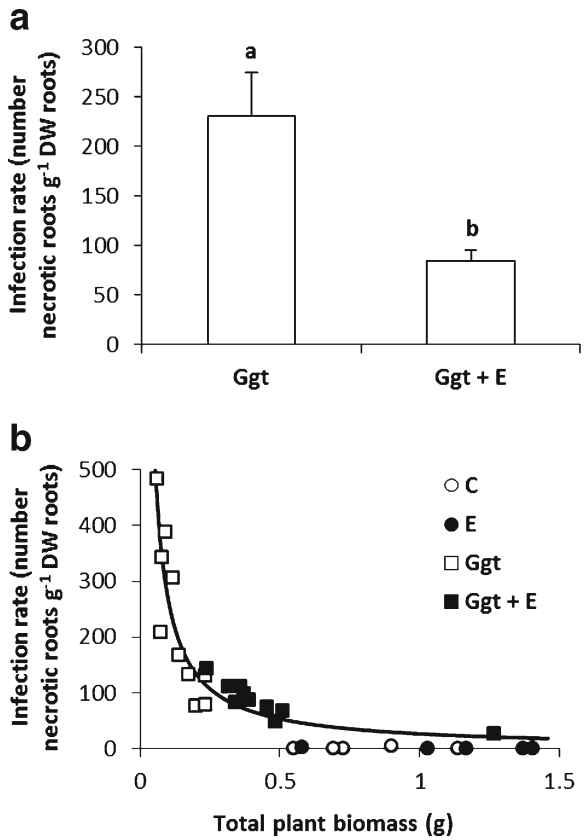


Fig. 2 **a** Effect of the earthworm *Aporrectodea caliginosa* on the infection rate of the pathogenic fungus *Gaeumannomyces graminis* var. *tritici* on *Triticum aestivum*. **b** Relationship between the infection rate and plant total biomass. The model ($y=a/x$, with a=26.78±1.41) explained 87.17 % of the total variation in infection rate (n=30). C: control treatment; E: treatment with earthworms; *Ggt*: treatment with the pathogenic fungus; *Ggt*+E: treatment with fungus and earthworms. Means±s.e., n=5 for C and E treatment and n=10 for *Ggt* and *Ggt*+E treatments, different letters indicates a significant difference, Tukey HSD, P<0.05

treatments as compared to *Ggt* (Fig. 3a). There were no significant difference on root distribution between E and C treatments, but there was a significant effect of earthworms in the presence of *Ggt*, with an increase in the proportion of medium roots (400–630 μm) in *Ggt*+E as compared to *Ggt* (Fig. 3b).

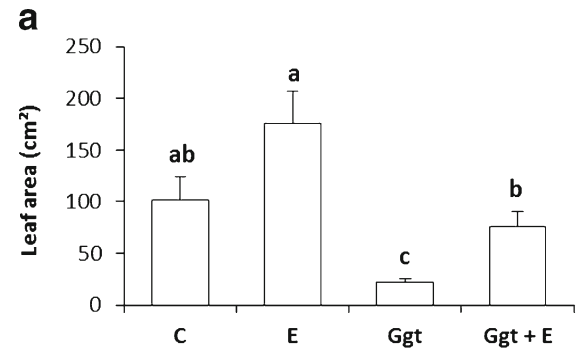
Soil properties

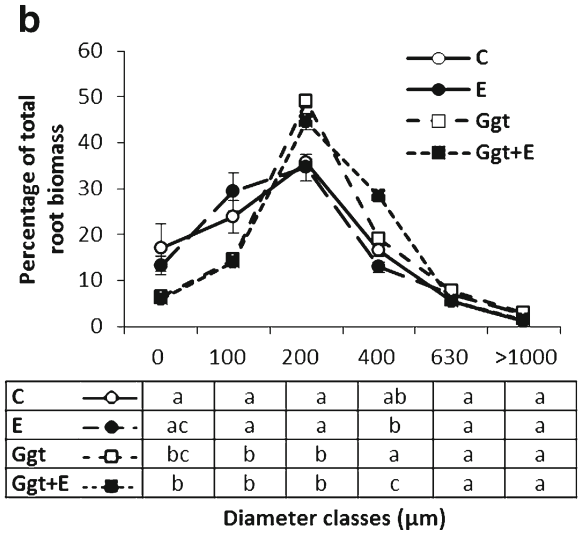
Most soil properties were not affected by the different treatments. No significant differences were observed for total carbon (19.3±0.9 g kg^{-1}), total nitrogen (1.96± 0.9 g kg^{-1}), phosphate (0.25±0.06 g kg^{-1}) and ammonium (10.4±1.5 mg kg^{-1}). However, soil nitrate content was significantly modified by *Ggt* (P=3.65 10^{-5}) and by earthworms (P=0.02). Nitrate content was 1.2 times and 1.6 times that of the control in *Ggt* and *Ggt*+E treatments respectively (Fig. 4). Since no change in ammonium content was observed, the ammonium:nitrate ratio (Fig. 4) showed an opposite pattern to nitrate content.

Transcriptome profiling

Results of the annotation with their respective gene ontology function are available in Online Resource 1. The expression profile in response to the E, *Ggt* and *Ggt*+E treatments was compared to that of the control; in addition, *Ggt*+E was compared to *Ggt*.

There were 1461 genes differentially expressed when comparing the *Ggt* treatment with the control (*Ggt vs* C) (Table 1). The majority of genes differentially regulated by





	0	100	200	400	630	>1000
C	a	a	a	ab	a	a
E	ac	a	a	b	a	a
Ggt	bc	b	b	a	a	a
Ggt+E	b	b	b	c	a	a

Fig. 3 Effect of the earthworm *Aporrectodea caliginosa* and the pathogenic fungus *Gaeumannomyces graminis* var. *tritici* on: **a** wheat leaf area and **b** wheat root biomass distribution in diameter classes. C: control treatment; E: treatment with earthworms; *Ggt*: treatment with the pathogenic fungus; *Ggt*+E: treatment with fungus and earthworms. Means±s.e., n=5 for C and E treatment and n=10 for *Ggt* and *Ggt*+E treatments, different letters indicates a significant difference, Tukey HSD, $P<0.05$

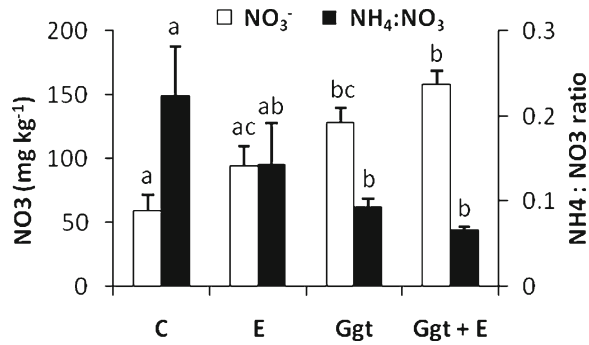


Fig. 4 Effect of the earthworm *Aporrectodea caliginosa* and the pathogenic fungus *Gaeumannomyces graminis* var. *tritici* on soil nitrate content and ammonium:nitrate ratio. C: control treatment; E: treatment with earthworms; *Ggt*: treatment with the pathogenic fungus; *Ggt*+E: treatment with fungus and earthworms. Means± s.e., n=5 for C and E treatment and n=10 for *Ggt* and *Ggt*+E treatments, different letters indicates a significant difference, Tukey HSD, $P<0.05$

Ggt infection belonged to the following functional categories (Fig. 5a, b): oxidation-reduction processes, response to abiotic stimulus, metabolism (including phosphorus and lipid metabolism), proteolysis, transcription/translation and unclassified genes (including unknown functions). Behind these coarse function categories, many of these genes are associated with two physiological changes induced by the fungus in the host plant: the induction of hypersensitive responses and hormone signalling. Genes potentially involved in the hypersensitive responses are: *CPK7* (Ta.4580), coding for a Ca^{2+}-dependent protein kinase (CDPK), *RBOHF* (Ta.7051), coding for a putative NADPH oxidase RBOH (Respiratory burst oxidase homolog) and a gene (Ta.13803) coding for a putative calcium-binding protein CML25 (CaMCML) (Ma and Berkowitz 2011). Genes potentially involved in hormone signalling are related to different plant hormones such as: (1) jasmonate, with the down-regulation of *COI1* (Ta.9471), coding a putative Coronatine-insensitive protein 1, (2) gibberellins, with the down-regulation of the gene *GID1* (Ta.5616), coding a putative Gibberellin receptor, (3) auxin, through the up-regulation of *AUX1* (Ta.49842) and *IAA3* (Ta.41052), coding respectively for a putative auxin transporter protein and an auxin-responsive protein, and (4) abscisic acid, with the down-regulation of *ABI2* (Ta.26201), *HAI1* (Ta.60923) and *HAI3* (Ta.10207) coding for putative protein phosphatase 2C (PP2C); *OST1* (Ta.5236), *SRK2A* (Ta.57483) and *SRK2C* (Ta.2551) coding for a putative serine/threonine-protein kinase (Pieterse et al. 2009; Puga-Freitas and Blouin 2015; Robert-Seilaniantz et al. 2011).

In contrast to the *Ggt* treatment, 212 genes differentially expressed out of 55 052 analysed when comparing the earthworm treatment with the control (Table 1). This relatively small impact of earthworms on plant gene expression was also observed in plants infected with *Ggt* since only 28 genes were differentially expressed when comparing *Ggt*+E *vs Ggt*. These genes were mainly involved in transcription or unknown functions (results not shown).

Discussion

Our study reveals that earthworms can control the infection of wheat by *Ggt* in a spectacular manner, and alleviated the negative effect of this disease. This biocontrol

Table 1 Comparison of the number of genes differentially expressed between treatments

	E *vs* C	*Ggt vs* C	*Ggt*+E *vs* C	*Ggt*+*E vs E*	*Ggt*+E *vs Ggt*
Up-regulated	110	790	1016	602	14
Down-regulated	102	671	701	436	14

C: control treatment; E: treatment with earthworms; *Ggt*: treatment with the pathogenic fungus *Gaeumannomyces graminis* var. *tritici*; *Ggt*+E: treatment with *Gaeumannomyces graminis* var. *tritici* and earthworms. For the comparison *Ggt vs* C, *Ggt*+E *vs* C and *Ggt*+E *vs* E, genes with a fold-change >3 and a P-value<0.05 were considered. For the comparison E *vs* C and *Ggt*+E *vs Ggt,* genes with a fold-change >1.5 and a P-value<0.05 were considered

effect was achieved in a situation where plants were almost killed by the pathogen. A high number of different mechanisms could be responsible for this biocontrol. Identifying the relevant ones is an important issue to progress in developing new disease management strategies.

Effects of *Ggt* on wheat

Ggt was responsible for an 82 % decrease in total plant biomass as compared with control treatment (Fig. 1). Fine root proportion in the 0–100 μm diameter class was reduced by 63 % in *Ggt* as compared with C (Fig. 3b) which may have prevented the plant from uptake of soil NO_3^- (Fig. 4). This was associated with leaf area decrease by 78 % (Fig. 3a). At the end of the experiment, plant height was also affected by *Ggt* but in a lesser extent.

Gene expression of wheat in response to *Ggt* has already been studied during early steps of take-all disease (4-day-old infected seedlings) (Guilleroux and Osbourn 2004), but not in late steps. We found that at day 52 after sowing, transcript accumulation was strongly influenced by *Ggt* attack. Wheat plants appeared to be responding to the presence of *Ggt* as shown by the accumulation of transcripts for several genes involved in the signal transduction following the perception of pathogen-associated molecular patterns (PAMPs). We observed a transcript accumulation of a putative gene coding for a RBOHF protein, which is required for full reactive oxygen intermediate production (Torres et al. 2002) and genes involved in oxidation–reduction processes and proteolysis. Although it was not demonstrated in our study, we speculate that *Ggt* could induce a hypersensitive response (HR), a defence mechanism which relies on the production of Reactive Oxygen Species (ROS) (Ma and Berkowitz 2011; Mittler 2006; Rejeb et al. 2014). However this defence mechanism is not sufficient to overcome the infection, as we observed a significant reduction in wheat growth in response to *Ggt*.

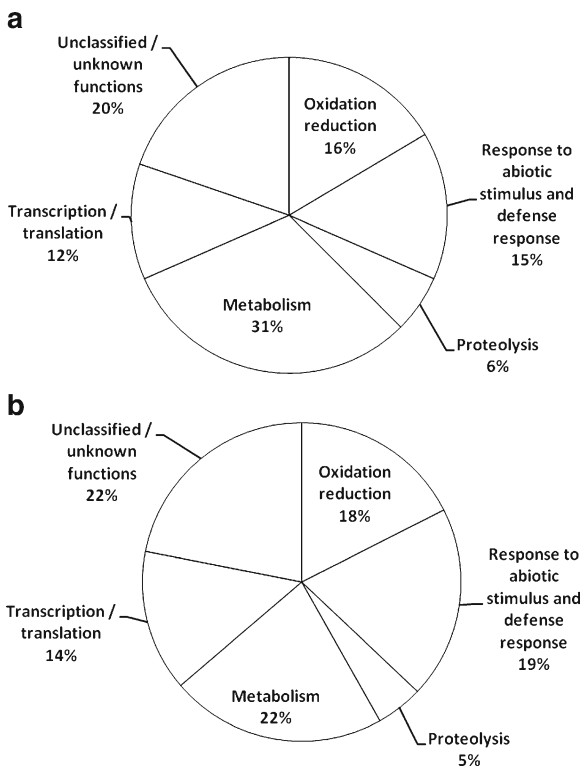


Fig. 5 Function of differentially expressed genes of *Triticum aestivum* in the presence/absence of *Gaeumannomyces graminis* var *tritici*. Pie charts show the percentage of **a** up-regulated and **b** down-regulated genes in each of the functional categories. Functional classification was established according to the DAVID Bioinformatics Resources 6.7

Earthworms are an efficient biocontrol agent against *Ggt* in wheat

Earthworms had a significant effect in the presence of *Ggt*. At the end of the experiment, there were no significant differences between *Ggt*+E and C treatments for biomass and shoot morphology. At a density of 380 individuals m^{-2}, earthworms were responsible for a

huge reduction in infection rate (−63 %). Our results are in accordance with the 69 % decrease in *Ggt* lesions observed with the earthworms *Aporrectodea rosea* and *Aporrectodea trapezoides* at the maximum earthworm density of 471 individuals m^{-2} (Stephens et al. 1994a). In the present study, control of *Ggt* lesions by earthworms for plant production parameters were even stronger (Stephens and Davoren 1995; Stephens et al. 1994a): biomass was three times higher in *Ggt*+E than in *Ggt*. As the earthworm positive effect on plants exposed to the fungal pathogen was associated with an increase in root biomass (Fig. 1b) without strong modifications in root system structure (Fig. 3b), it was concluded that additional biomass investment in the root system in the presence of earthworms (Fig. 1b) was sufficient to compensate for the negative effect of *Ggt* on fine roots.

Mechanisms involved in earthworm biocontrol of *Ggt*

Mechanisms responsible for the biocontrol effect of earthworms can be very diverse. First, it has been proposed that reduction in the severity of the disease could be explained by the disturbance of soil profile resulting in the disruption of fungal hyphae (Stephens and Davoren 1995); however, this has not been demonstrated.

Secondly, the ingestion of fungi by earthworms could explain the reduction in the number of lesions. Earthworms feed preferentially on fungi over bacteria or soil organic matter (Bonkowski et al. 2000; Friberg et al. 2005; Shan et al. 2013; Wolfarth et al. 2011).

Thirdly, changes in soil pH could be detrimental to *Ggt*. Changes in the ratio of the different N forms (a decrease in the NH_4^+ pool at the benefit of the NO_3^- pool) could decrease the pH through a release of H^+ in the soil, with negative consequences on *Ggt* (Christensen et al. 1987; Cook 2003). We did not measure the pH in the present study. However, the pool of NH_4^+ was the same in all treatments, suggesting that pH was probably not affected by changes in N forms in our experiment.

Fourthly, the stimulation of plant defences is another mechanism potentially responsible for the positive effect of earthworms on plants exposed to pathogens and parasites (Blouin et al. 2005; Puga-Freitas et al. 2012; Wurst 2010). Plant Growth Promoting Bacteria (PGPB) are known for their positive effect on plant defence mechanisms. They can induce systemic resistance (ISR), characterized by a change in the expression of genes involved in the signalling pathway of plant hormones such as salicylic acid and jasmonate (Van der Ent et al. 2009; Van Wees et al. 2008). In this way, they confer a broad-spectrum resistance to plant pathogens and insect herbivores (Van Wees et al. 2008). These ISR-inducing PGPB can be stimulated by soil animals such as springtails (Endlweber et al. 2011) and earthworms (Puga-Freitas et al. 2012). However, in this experiment, earthworms were only responsible for a differential expression of 28 genes when comparing E and *Ggt*+E treatments (Table 1), none of which are associated with defence mechanisms such as ISR. Hence, the improvement of plant defence by earthworms was not demonstrated, but a transient effect on plant defence cannot be excluded.

Fifthly, an increase in nutrient availability in the presence of earthworms has been proposed to explain the biocontrol effect of earthworms on *Ggt* (Stephens et al. 1994a). *A. rosea* can, under certain conditions, increase wheat foliar concentration of Ca, Cu, K, Mn, N, Na and P and *A. trapezoides* can increase the foliar concentration of Al, Ca, Fe, K, Mn, N and Na (Stephens et al. 1994b). This improved nutrient availability could benefit the plant by compensating for a deficient root system. In the present study, an increase in NO_3^- concentration in soil was observed in the *Ggt*+E treatment. As NO_3^- concentration in soil was higher in the *Ggt* treatment than in the E treatment, the increased concentration in the *Ggt*+E treatment was mainly due to *Ggt*, which could impair plant uptake. So, improved plant nutrition in the presence of earthworms was probably not relevant to explain these results.

Finally, a stimulation of microbial antagonism of *Ggt* by *A. caliginosa* could be responsible for the biocontrol effect. Earthworms are able to reduce several soil-borne diseases through triggering changes in microbial communities (Clapperton et al. 2001; Elmer 2009). For example, the amount of root lesions caused by *Fusarium oxysporum* on *Asparagus* was halved in the presence of earthworms. This was not due to significant changes in *Fusarium* density but was correlated with an increase in PGPB populations (Elmer 2009). It has been previously show that PGPB can inhibit *Ggt* by the production of several antibiotics (Cook 2003; Freeman and Ward 2004). The hypothesis of changes in microbial communities has previously been used to explain *Ggt* soil suppressiveness (Sanguin et al. 2009).

The present study confirms the potential effect of using earthworms as a biocontrol agent against wheat infection by *Ggt*. It also provides evidence for the

beneficial biocontrol effect of earthworms on plant growth. Further investigations, on the relationship between soil bacterial communities and the *Ggt* infection rate conducted using next-generation sequencing technologies could provide more definitive answers.

Acknowledgments This work was supported by AgroParisTech (France). We are very grateful to Damien Marchand for technical assistance and to Germain Meulemans for English language editing. We also thank the INRA IGEPP laboratory (Rennes, France) for providing fungus inoculums.

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