Research Article

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The collembolan *Heteromurus nitidus* grazes the wheat fungal pathogen *Zymoseptoria tritici* on infected tissues: opportunities and limitations for bioregulation

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Abstract

BACKGROUND: Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici*, is a foliar disease affecting wheat crops against which conventional control methods are not totally effective. During inter-epidemic periods the fungus survives in wheat residues left on the ground. In this study, we tested the potential of the collembolan *Heteromurus nitidus* – a springtail species present in field soils and known to interact with different fungal species – as a potential bioregulation agent of *Z. tritici* on wheat residues through a choice and consumption experiment.

RESULTS: Springtails preferred inoculated fresh residues but did not have a preference between inoculated and uninoculated old residues. Springtails grazed on *Z. tritici* fruiting bodies and reduced pycnidiospore numbers by ten-fold compared to control inoculated fresh residues. Attraction toward fresh inoculated residues and pycnidiospore reduction support the hypothesis that *Z. tritici* is a food source for springtails. *Heteromurus nitidus* showed no preference between inoculated and uninoculated 18-month-old residues, probably because they no longer produced ascospores.

CONCLUSION: Attraction towards fresh residues and spore reduction support our hypothesis that *H. nitidus* may contribute to the bioregulation of *Z. tritici*. Perspectives for field application would be determined by the ability of *H. nitidus* and *Z. tritici* to interact at key epidemiological stages. The impact of *H. nitidus* on the quantity of pathogen primary inoculum over time should be estimated using residues of intermediate age. This would help to identify the optimal period for enhancing the effectiveness of springtails as consumers of *Z. tritici*.

Supporting information may be found in the online version of this article.

Keywords: springtails; spores; phytopathology; bioregulation; residues

1 INTRODUCTION

Agricultural ecosystems are complex and have been shaped and optimized across centuries to sustain human societies. Despite crop management practices including plant breeding and the use of plant protection products, most agrosystems still suffer from various pests, leading to yield losses.[1] Biodiversity reduction[2] caused by agricultural intensification usually leads to changes in pest communities that influence the impact of herbivory and plant pathogens.[3,4]

The ascomycete *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch (STB), is one of the most important fungal pathogens that affect wheat crops worldwide.[5,6] Populations of *Z. tritici* are very large and have extremely high genetic variability because of sexual recombination. This recombination facilitates the emergence of resistance to fungicides and to major resistance genes, leading to an erosion of their efficiency.[7–9]

The use of bioregulation agents has been successful in several crop–pest systems[10] and could be an alternative to conventional control methods. Microbial antagonist agents such as fungi and bacteria are prime candidates against fungal plant pathogens[11] including against *Z. tritici* during its epidemic phase, that is, during the growing season when the disease significantly affects plant

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growth.[12–14] However, the life cycle of *Z. tritici* presents other key steps on which biological control agents could have a significant effect. After harvest, *Z. tritici* reproduces sexually in wheat residues left on the ground and produces pseudothecia, sexual fruiting bodies that release wind-dispersed ascospores initiating STB epidemics the following season.[15,16] During this inter-epidemic period, pseudothecia on crop residues are exposed to soil communities composed of potential biological control agents such as fungivorous arthropods, including springtail (Collembola) species,[17] that can limit the quantity of pathogen primary inoculum.[18] Despite serving as a valuable ecotone bridging the interface between the 'plant' and 'soil,' crop residues have been relatively underexplored in terms of their potential for bioregulation in disease management[19,20] (Fig. 1).

Springtails are among the most widespread soil arthropods[17] and take part in several ecosystem services,[18,21,22] including the regulation of fungal communities.[17,23] As they can discriminate fungi, have food preferences,[24–27] and therefore can decrease specific fungal populations,[28–30] springtails have been evaluated as bioregulation agents against soilborne/root pathogens[31,32] such as *Fusarium* spp.[33] Furthermore, a field study by Salmon *et al.*[34] showed that wheat cultivars vulnerable to *Z. tritici* displayed a higher springtail abundance and species richness, suggesting a particular interaction with diseased plants, while Bourgeois *et al.*[28] reported promising results on the consumption of this pathogen by collembola on agar medium. However, no study has been conducted to date on the control of *Z. tritici* on wheat residues by springtails and the interactions under consideration should be examined to estimate their epidemiological impact.

In this study, we evaluated the potential of *Heteromurus nitidus* (Templeton, 1835), a cosmopolitan collembolan (Entomobryidae), as a bioregulation agent of *Z. tritici* infecting wheat. *Heteromurus nitidus* is a widespread springtail species living either in deep soil or upper soil horizons or litter[25,35] and is therefore considered edaphic or hemiedaphic, depending on the season and climate. It is found in crops[25,34] and can feed on fungi.[25,27] Bourgeois *et al.*[28] showed that *Z. tritici* grown on agar medium is among the preferred fungi of *H. nitidus* and that the springtail populations can increase when fed on the fungus and reduce the fungal biomass. To extend these results, we investigated the interaction between *H. nitidus* and *Z. tritici* inoculated on two types of wheat tissue residues to mimic what occurs at two different key stages of the pathogen life cycle in the field.[36] *Zymoseptoria tritici* overwinters in decaying plant tissues (leaves, sheaths and stems) left on the ground in several forms: ascospores (sexual spores) being the main form of primary inoculum, but also pycnidiospores (asexual spores) and mycelium that can persist in these tissues.[15] Because of the difficulties of producing and manipulating ascospores in controlled conditions,[37] we tested the effect of *H. nitidus* on *Z. tritici* on residues from freshly inoculated leaves from the glasshouse ('fresh residues') exhibiting abundant asexual sporulation and from older decaying tissues ('old residues') presumed to harbor *Z. tritici* in sexual forms for several months. In a first experiment we compared attraction to these types of residues in springtails and, in a second experiment, we investigated the effect of long-term consumption of host tissues, inoculated or not, on the development of a springtail population and the resulting reduction in spores. With these two experiments, we tested the following hypotheses: (i) STB infections on wheat

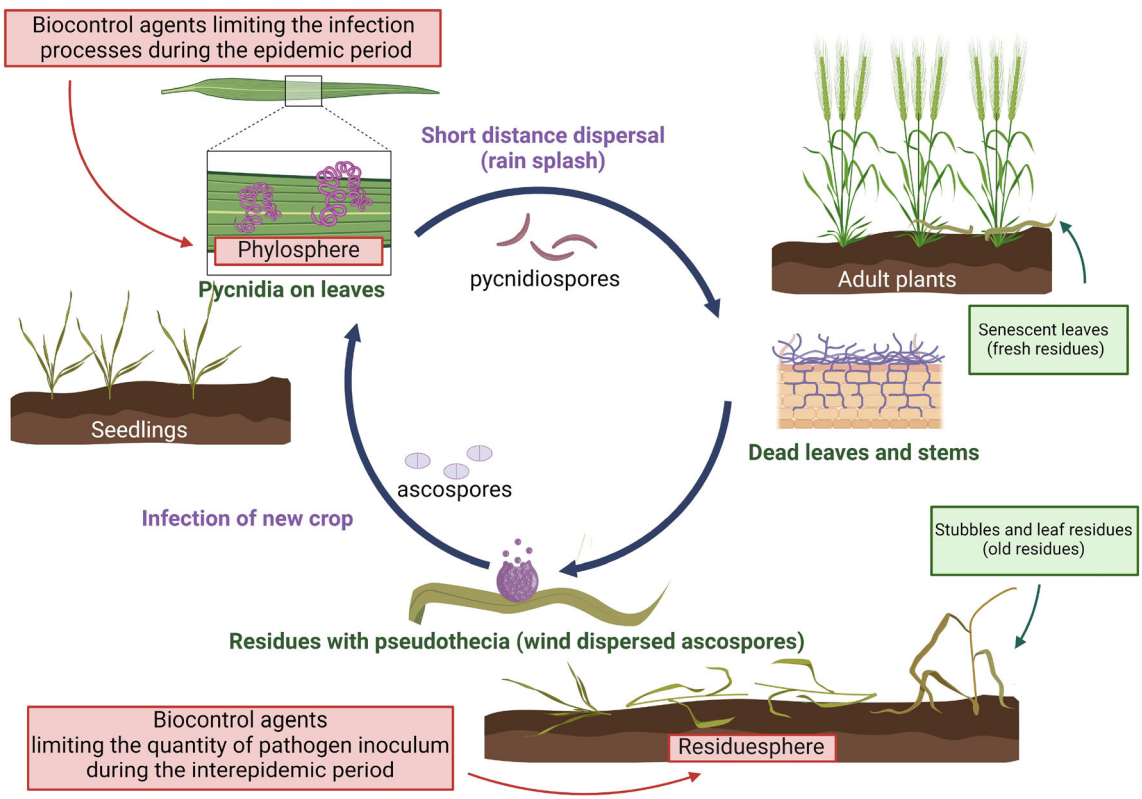

Figure 1. Life cycle of the phytopathogenic fungus *Zymoseptoria tritici*. Created with Biorender.

tissues are attractive for *H. nitius*; (ii) *H. nitidus* populations can increase while feeding on residues inoculated with *Z. tritici*; and (iii) *H. nitidus* feeding can reduce the size of the active *Z. tritici* population, that is, the quantity of infectious spores.

2 METHODS

2.1 Biological material

Heteromurus nitidus adults used for the experiments were taken from the culture stocks of the MECADEV laboratory (Muséum national d'Histoire naturelle, Brunoy, France). Springtails of the culture stocks were collected from the Sénart forest (Brunoy, France) during multiple sampling campaigns and were reared on humid Fontainebleau sand (Sordalab, Paris, France) and fed with tree micro-algae (*Pleurococcus* spp.) and dry organic cow dung. Springtails were kept in culture boxes placed in climatic chambers (no light, 15 °C).

Fresh wheat residues consisted in inoculated and uninoculated flag (F1) and penultimate (F2) leaves from adult wheat plants (*Triticum aestivum* cv Cellule) grown in glasshouses at INRAE BIOGER (Grignon, France). Inoculated leaves presenting STB lesions were obtained after artificial inoculation with a mixture of two *Z. tritici* strains (INRA18-FS3250 and INRA18-FS2883) following the protocol described in Suffert *et al.*[38] and Orellana-Torrejon *et al.*[39] Briefly, aqueous suspensions containing blastospores of both strains in equal proportions were adjusted to 5×10^5 spores mL^{-1} and applied onto plants using an atomizer. Infection was promoted by enclosing the plants immediately after inoculation in a transparent polyethylene bag containing a small amount of water for 3 days. F1 and F2 leaves were detached from the stem 4 weeks after the appearance of the first lesions, that is, 6 weeks after inoculation, and before all wheat tissues became completely senescent. Infected leaves were used within the next 48 h for the experiments and were cut into 2 cm portions containing green parts and, for inoculated ones, visible pycnidia (asexual fruiting bodies) in the necrotic areas.

Old residues comprised wheat stems and leaves (*T. aestivum* cv Apache) from adult wheat plant artificially inoculated in a glasshouse with a mixture of the two aforementioned *Z. tritici* strains in which sexual reproduction was then induced following the protocol of Orellana-Torrejon *et al.*[39] These residues were cut into 2 cm portions and stored in porous containers under room conditions for 18 months before use in the current experiments. One week before choice and long-term consumption experiments, old residues were prepared by soaking them into tap water for 30 min. Then, the excess water was removed using absorbent paper and residues were left to dry for 24 h. The process was repeated 1 h before the experiments as soaking and drying residues stimulates ascospore production.[15]

2.2 Experiments

2.2.1 Choice experiment

Prior to the choice experiments, adult springtails were left without food for 7 days in humid culture boxes. For this set of experiments, the same choice arenas as in Bourgeois *et al.*[28] were used (Petri dishes with a diameter of 96.6 mm, a height of 13.9 mm and a substrate comprising 25 g of Fontainebleau sand and 5 mL of tap water). Twelve replicates were performed. Two tests were performed with *Z. tritici* uninoculated *versus* inoculated wheat tissues, from either fresh residues using leaf sections of 2 cm × 1 cm, and old residues using sets of 0.18 g wheat leaf and stem residues. Inoculated and uninoculated residues were placed on opposite sides at 10 mm from the edges of the dishes. Ten unsexed adult springtails were placed at the center of the Petri dishes using a funnel and left in the dark for 210 min. The position of the springtails was registered at the end of this period and each individual was recorded marked as either on residues – inoculated or not – or elsewhere in the Petri dish.

2.2.2 Long-term consumption experiment

The long-term consumption experiments were conducted in culture boxes with humid sand substrate (97 g of Fontainebleau sand and 21 mL of tap water) adapting an experimental setup from Bourgeois *et al.*[28]

For the long-term consumption experiment with fresh residues, four treatments were prepared in culture boxes with ten replicates each. Thirty unsexed adult springtails were placed with (i) three uninoculated leaf sections of 2 cm × 1 cm or (ii) *Z. tritici* inoculated leaf sections bearing pycnidia. (iii) Boxes containing three *Z. tritici* inoculated leaf sections pycnidia without springtails were used as reference to estimate the number of pycnidiospores in the absence of consumption. (iv) In the fourth treatment, 30 adult springtails were placed in culture boxes without leaf sections as a control without food. The number of pycnidia were counted on each inoculated leaf section from (ii) and (iii) before the deposit of springtails [mean ± standard error (SE) = 505.0 ± 38.6 pycnidia per box, Supporting Information, Fig. S1]. This count allowed us to ensure that there was the same magnitude order of pycnidia in each treatment, that is, the same amount of potential food offered to the springtails assuming that each pycnidia releases a similar quantity of pycnidiospores.[38] Experimental boxes were then placed in climatic chambers (no light, 15 °C). The experiment was stopped after 2 weeks before the degradation of pycnidiospores in controls without springtails or the leaf decay that could provide an alternate food source to springtail populations. *Heteromurus nitidus* adults and juveniles were counted in each replicate. Inoculated fresh residues were placed in plastic vials containing 4 mL (for leaves exposed to springtails) or 8 mL (for leaves not exposed to springtails) of sterile water to evaluate the average amount of remaining pycnidiospores. Spore concentration from each aqueous suspension was calculated by sampling each replicate three times and counting pycnidiospores in a Malassez cell.

For the long-term consumption experiment with old residues, four treatments were prepared in culture boxes: two treatments with 30 unsexed adult springtails placed with (i) 2.5 grams of uninoculated residues or with (ii) *Z. tritici* inoculated residues on which sexual reproduction has occurred. (iii) A control comprised inoculated residues without springtails. The fourth treatment (iv) was used as a control and consisted of 30 springtails on the humid sandy substrate only. After 10 weeks, adults and juveniles were counted. Residues were soaked in water for 30 min and left to dry under Petri dishes filled with potato dextrose agar (PDA) medium for 2 days to collect ascospores ejected from pseudothecia following the method described in Morais *et al.*[40] and Orellana-Torrejon *et al.*[39] After this, PDA boxes were left to incubate for 7 days at 18 °C in the dark, and the resulting *Z. tritici* ascospore-derived colonies were then counted.

2.3 Statistical analysis

For the choice experiments, the matrix of the number of individuals choosing one side (inoculated tissues) and the number of individuals not choosing this side (uninoculated tissues) at the end of the experiment (210 min) was used as the response variable (i.e., a matrix [individuals on a side,; (individuals on the other

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side$_i$ + individuals not on the residues$_i$)] with i = number of replicates). The condition of the wheat tissue (inoculated or uninoculated fresh/old residues) was used as a fixed effect categorical variable. The identification number of the Petri dish was included in the model as a random factor. Generalized linear mixed effect models (GLMMs) were fitted as data were not normal following Bolker *et al.*[41] with the package 'lme4'.[42] Proportion data were analyzed assuming that the response variable followed a binomial distribution with a logit link function.[41]

For the long-term consumption experiments, the numbers of living springtails, adults and juveniles were compared after 2 weeks (springtails on fresh residues) or 10 weeks (springtails on old residues) depending on the condition (uninoculated residues, inoculated residues and sandy substrate alone) by analysis of variance (ANOVA). If data were not normal, generalized linear models (GLMs) were used with a Poisson distribution for count data. In case of overdispersion, GLMMs with a negative binomial distribution and a log link function were fitted with the package 'MASS'.[43] Pairwise comparisons between the different conditions were performed using estimated marginal means (EMMs) with the package 'emmeans'.[44]

For the long-term consumption experiment on leaves, the number of pycnidiospores at the end of the experiment was analyzed using a GLMM with a Poisson distribution for count data with the package 'lme4'.[42] The presence or absence of springtails was used as a fixed factor and the number of pycnidia at the beginning of the experiment was added as a random factor. No analysis was conducted on spores coming from old residues as no colony developed from inoculated residues (even without collembola) on the Petri dishes.

All statistical analyses were conducted on the software R version 4.1.1[45] and all models were checked with the package 'DHARMa'[46] to simulate scaled residuals and test for correct distribution, overdispersion and outliers.

3 RESULTS

3.1 Choice experiments

For the choice experiments, 81.4% of the individuals placed in the choice arenas were responsive (i.e., they chose one of the sides) when exposed to old residues and 65.8% when exposed to fresh residues. Springtails made different choices depending on the tissue hosting *Z. tritici*. Regarding fresh residues consisting of sections of wheat leaves, *H. nitidus* preferred inoculated leaves on which *Z. tritici* was abundant as asexually sporulating pycnidia (mean ± SE: 5.7 ± 0.5 individuals) to uninoculated ones (0.6 ± 0.3 individuals; GLMM with binomial distribution, Z-value = −7.18, $P < 0.001$; Fig. 2(a)). *Heteromurus nitidus* showed no preference between inoculated (4.1 ± 0.5 individuals) and uninoculated old residues (3.6 ± 06 individuals; GLMM with binomial distribution, Z-value = −0.812, $P = 0.417$; Fig. 2(b)).

3.2 Long-term consumption experiments

3.2.1 *Impact on springtail population size*

During the long-term consumption experiments, *H. nitidus* populations developed differently depending on the wheat tissue offered as the food source. The population size of springtails exposed to fresh residues was not significantly different between the three food sources after 2 weeks (ANOVA, $F_{2,27} = 2.99$, $P = 0.067$; Fig. 3(a)). Populations exposed to uninoculated leaves (mean ± SE: 31.7±0.7 individuals; 30.0 ± 0.1 adults and 1.7 ± 0.6 juveniles) or inoculated leaves (31.1 ± 0.6 individuals; 29.9 ± 0.3 adults and 1.2 ± 0.4 juveniles) were not significantly different from those on sand substrate alone (29.9 ± 0.3 individuals; 29.9 ± 0.3 adults and 0 juveniles). For the experiment on old residues, population sizes were different depending on the food source after 10 weeks (negative binomial GLMM, Z-value = 56.5, $P < 0.001$, Fig. 3(b)–(d)). The population size exposed to inoculated residues (441.8 ± 52.0 individuals) was not significantly different from the population size exposed to uninoculated residues (324.5 ± 34.3 individuals). Both populations were markedly larger than populations reared on sand substrate (12.7 ± 1.5 individuals). The number of adults varied significantly depending on the food source (Poisson GLMM, Z-value = 37.1, $P < 0.001$; Fig. 3(c)): there were more adults in populations exposed to inoculated residues (23.7 ± 1.4 adults) than to uninoculated ones (17.1 ± 1.6 adults) and the size of both adult cohorts was significantly higher than those exposed to the sand substrate alone (12.3 ± 1.3 adults). The comparison of the number of juveniles after 10 weeks gave a similar result to that of the overall population, in which they represented 90%, and differed depending on the food source (negative binomial GLMM, Z-value = 50.1, $P < 0.001$) with significantly fewer juveniles in populations exposed to sand substrate (0.4 ± 0.4 juveniles; Fig. 3(b)). No significant difference was observed in the mean number of juveniles exposed to inoculated (418.1 ± 51.3 juveniles) and uninoculated residues (307.4 ± 33.3 juveniles).

3.2.2 *Impact on the residual sporulation capacity*

The number of pycnidia at the beginning of the experiment (mean ± SE: 505.0 ± 38.6 pycnidia per box) did not differ significantly between sets of fresh residues used in the two treatments (presence *versus* absence of springtails; Fig. S1). The impact of the presence of *H. nitidus* on the number of pycnidiospores from fresh residues was significant (GLMM with Poisson distribution, Z-value=112, $P < 0.001$; Fig. 4): after 2 weeks pycnidiospores were ten-fold less numerous when springtails were present.

4 DISCUSSION

Inoculated fresh residues were significantly more attractive to *H. nitidus* than uninoculated ones (Fig. 2(a)). Moreover, the presence of springtails resulted in a substantial reduction of the residual sporulation capacity of the pycnidia after 2 weeks: pycnidiospores were ten times less numerous in presence of springtails (Fig. 4). The 90% reduction of pycnidiospores presence on the leaves exposed to *H. nitidus* is congruent with previous studies showing that springtails influence fungal growth[47–49] including *Z. tritici* growth on agar medium[28] and negatively impact fungal networks.[50–52] The combination of these two results allows us to conclude that *H. nitidus* is attracted to STB lesions and feeds on pycnidiospores exuded from pycnidia, highlighting their inclination towards food choices that maximize their fitness.[53,54]

The consumption of pycnidiospores on fresh residues had no effect on the springtail population growth after 2 weeks: pycnidiospores were consumed but no differences were observed in the size of populations between groups that fed on uninoculated and inoculated fresh residues (Fig. 3(a)). The duration of the experiment is a likely explanation for this lack of difference as *H. nitidus* can survive for 2 weeks without food while eggs hatch about 9 days after being laid[55] and most eggs hatched after 3 weeks in a previous experiment.[28] In our study it was not possible to make the consumption experiment last longer as freshly cut leaves were used and their decay in a humid environment could

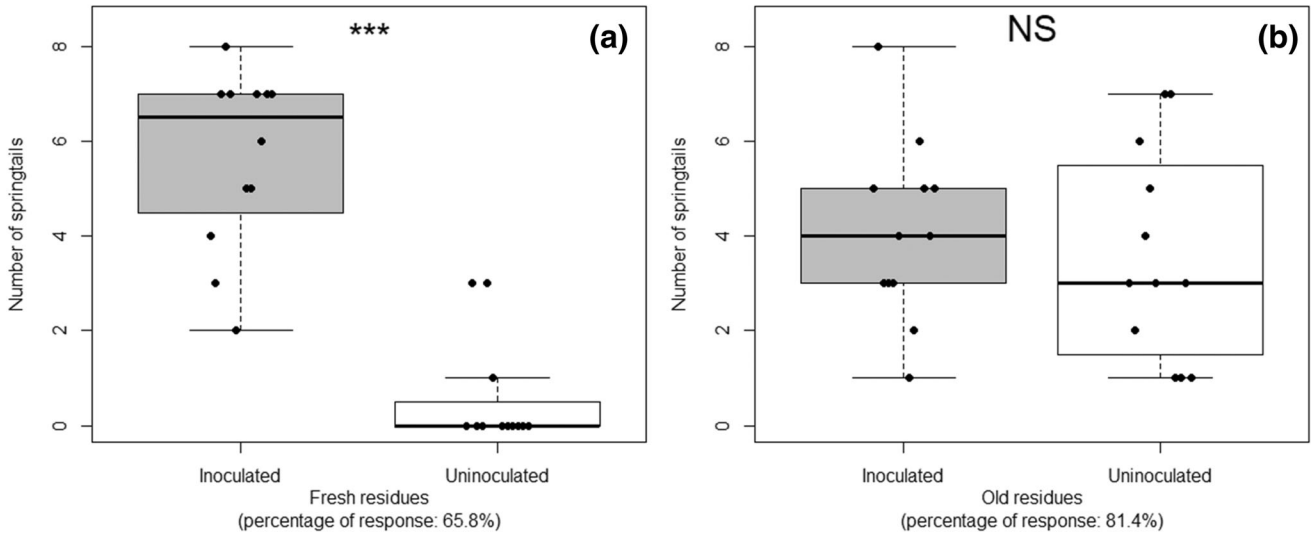

Figure 2. *Heteromurus nitidus* choices 210 min after the beginning of the experiment between: (a) fresh residues consisting of sections of leaves inoculated with *Zymoseptoria tritici* (gray box) and uninoculated leaves (white box); (b) old residues inoculated with *Z. tritici* (gray box) and uninoculated residues (white box). Boxes represent the first and third quartile, the black line the median number of springtails per box with ten individuals. Whiskers represent maximum and minimum values within 1.5 times the interquartile value. Dots represent individual values. Asterisks represent the level of significance and NS stands for non-significant.

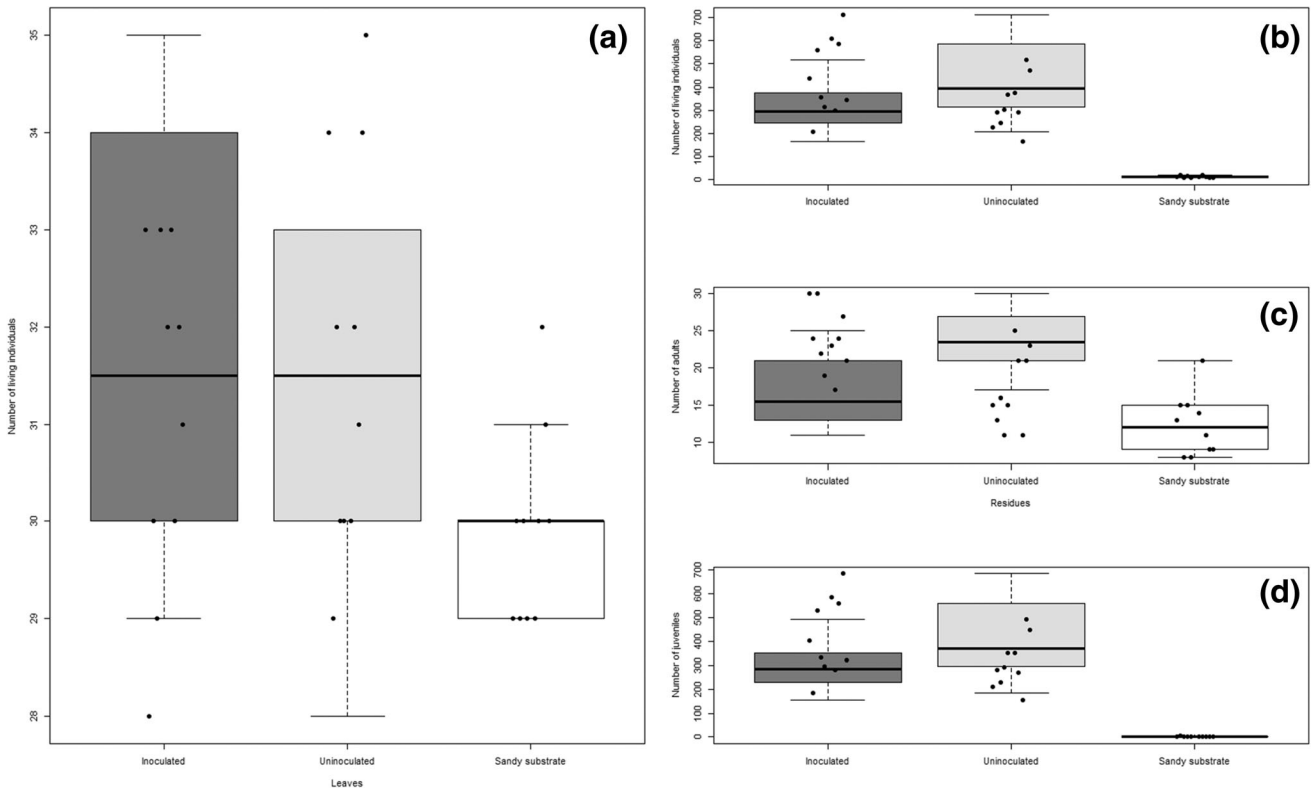

Figure 3. Number of living springtails after 2 weeks (a) depending on fresh residues consisting of sections of wheat leaves inoculated with *Zymoseptoria tritici* (dark gray box) and uninoculated ones (light gray box). Number of living springtails (b), adults (c) and juveniles (d) after 10 weeks depending on old wheat residues inoculated with *Z. tritici* (dark gray boxes) and uninoculated ones (light gray boxes). White boxes show a control with no food. Boxes represent the first and third quartile, the black line the median number of springtails among ten. Whiskers represent maximum and minimum values within 1.5 times the interquartile value. Dots represent individual values. Letters indicate significant differences and NS stands for non-significant.

have led to the development of opportunistic fungi that could have provided an alternative food source to *H. nitidus*. We hypothesize that in a longer experiment *H. nitidus* populations would grow in the presence of inoculated fresh residues as they were attractive and pycnidiospores were consumed. However, springtail grazing can also increase fungal growth[28,47] and

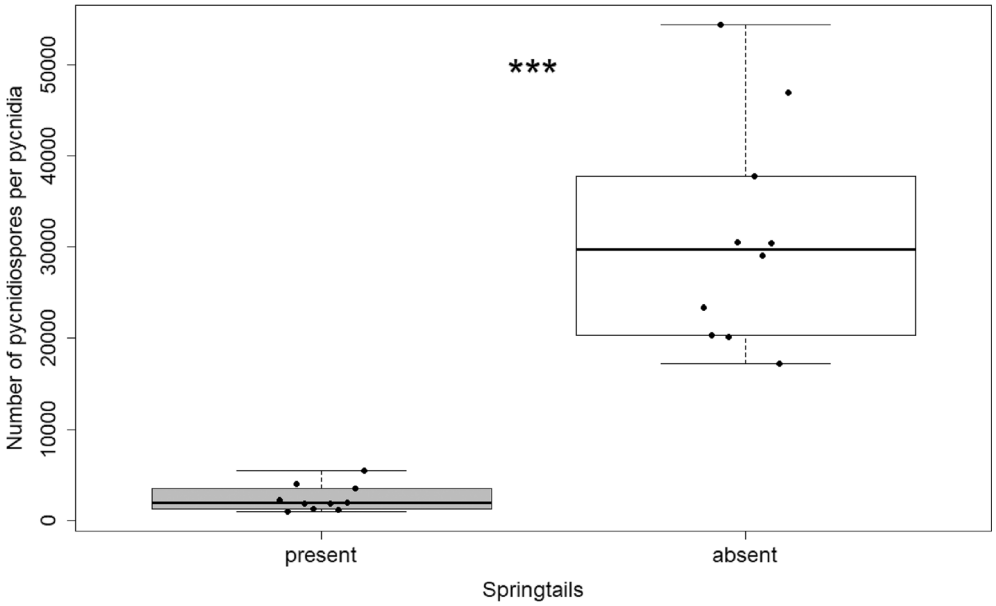

Figure 4. Ratio of the average number of pycnidiospores collected per box at the end of the experiment and the number of pycnidia per box at the beginning of the experiment depending on the presence (gray box) or absence (white box) of springtails. Boxes represent the first and third quartile, the black line the median per leaf. Whiskers represent maximum and minimum values within 1.5 times the interquartile value. Dots represent individual values. Asterisks represent the level of significance.

stimulate microbial activity[48] and the long-term impact of the springtails on *Z. tritici* should be assessed to ensure that they do not stimulate spore production.

Regarding old residues, inoculated tissues were not more attractive for *H. nitidus* than uninoculated ones in the choice experiment (Fig. 2(b)), and springtail populations exposed to both residues grew over 10 weeks, although adults were more numerous on inoculated residues (Fig. 3(c)).

On old residues, springtails populations consisted of 90% juveniles and were not significantly different after 10 weeks of exposure to inoculated residues and uninoculated ones (Fig. 3(b),(d)). This lack of preference could be explained by a low presence of 'active' forms of *Z. tritici* as highlighted by the absence of ascospores collected during the ejection test at the end of the experiment (data not shown). The pseudothecia were probably empty 18 months after sexual reproduction had occurred.[39] This is consistent with the dynamics of pseudothecia maturation in other phytopathogenic ascomycete fungi, such as an absence of sexual sporulation in *Leptosphaeria maculans* after 2 years.[56] Although *Z. tritici* was not detected on old inoculated residues by the ascospore ejection test, its presence in dry wheat tissues and its potential consumption by the springtails should not be excluded. Indeed, after 10 weeks of incubation, adult *H. nitidus* representing 10% of the total population had better survival on inoculated residues than on uninoculated ones (Fig. 3(c)). The two diets with residues allowed population growth with juveniles and eggs observed in every culture box as all populations were significantly larger than control populations reared on the sand substrate. Moreover, mycelium and fruiting bodies from unidentified fungal species were observed on the residues, inoculated or not, and could have acted as a food source for collembolans. These different fungal structures, combined with decaying plant tissues that *H. nitidus* can also consume in small quantities,[57] is known to provide a mixed diet that increased springtails growth and reproduction[54] suggesting that wheat residues – infected or not by *Z. tritici* – are a favorable diet for springtail populations.

The attractivity of *Z. tritici* and its nutritional value highlight the potential of *H. nitidus* as a bioregulation agent of this pathogen that shares the same ecosystems for a part of its life cycle (see Fig. 1). In wheat fields, infected leaves bearing *Z. tritici* pycnidia are usually not in contact with the soil except when they belong to lower plant layers. It is only after harvest that they end up on the ground and contribute to the pool of primary inoculum with pathogen fruiting bodies (mainly pseudothecia, but also pynidia) exposed to springtails living in the soil litter.[58] *Heteromurus nitidus* could contribute to the management of STB by regulating the populations of *Z. tritici* in different forms and places during the inter-epidemic period, complementary to the action of other biocontrol agents, notably beneficial microorganisms from, or applied to, the phylosphere during the epidemic phase (Fig. 1).

Differences of interactions between springtails and fresh and old residues could be the result of differences in the nutritional value of the fungal tissues, differences in the quantities available or a combination of both. Further studies using residues of different ages and quantities are required to assess whether springtails can be exploited in field conditions to control STB epidemics, taking their lifestyle into account (ecological niche, population dynamics, etc.). Identifying the most attractive fungal forms and the optimal period under field conditions is necessary to assess the effectiveness of springtails as potential bioregulation agents. This is even more important for STB control, as the early incidence and severity of epidemics are positively correlated with the amount of inoculated residues acting as primary inoculum, that is, active fungal biomass.[37] This study paves the way toward more complex experiments in microcosms to better understand the role that springtails could play in future integrated pest management targeted at *Z. tritici*.

ACKNOWLEDGEMENTS

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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