Building an Integrative and Testable Hypothesis on How Edaphic Factors Ultimately Influence the Occurrence and Quality of *Ophiocordyceps Sinensis*, the Vegetable Caterpillar

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Abstract

Background

The yield of commercially harvested “vegetable caterpillar” *Ophiocordyceps sinensis* has dramatically plummeted in the last few decades, while market demand has increased. Besides controlling the obvious overexploitation of this species, understanding how edaphic factors influence this system may improve the chances of successful cultivation and thus support the conservation of *O. sinensis* in the wild. Our study investigates how the presence/absence and the quality of *O. sinensis* may be linked to a series of edaphic factors pertinent to its microhabitat, including enzyme activity, nutrients, moisture, pH and nematode population. In order to provide a preliminary hypothesis on the relationships among edaphic factors and their influence on *O. sinensis*, we performed a principal component analysis and structural equation modelling despite limited replication.

Results

Soil samples containing *O. sinensis* were more moist and contained a higher concentration of nutrients and enzyme activity than control samples collected nearby, where the species was absent. Preliminary analyses indicated that enzyme activity may be crucial and appeared to be affected by a number of other soil factors. We found that *O. sinensis* would occupy microhabitats with a relatively higher soil fertility and a more persistent enzyme activity, where the values of total nitrogen and catalase are especially important. Otherwise, with the exception of organic matter and enzyme activity, mean values did not suggest any other factors potentially corresponding to a better quality of *O. sinensis*.

Conclusions

Based on these preliminary findings and a further literature review, we formulated the first integrative hypothesis (network of interactions) on how soil factors may influence each other and *O. sinensis*. Finally, we indicate how this hypothesis may be tested in the future, in order to increase the chances for successful cultivation and thus promote the conservation and sustainable harvesting of *O. sinensis*.

Background

Ophiocordyceps sinensis (Berk.) G. H. Sung, J. M. Sung, Hywel-Jones and Spatafora (Syn. *Cordyceps sinensis*) is an endemic entomopathogenic fungus species distributed in the region of the Qinghai-Tibet Plateau. It is composed of the fruiting body of an entomophagous fungus and the larva of a host, of which several species may be colonized depending on the fungus variety [1]. There are three stages characterizing the parasitic complex between *O. sinensis* fungi and the *Hepialus* larva (Lepidoptera, Hepialidae) [2] these include; the primary infection, followed by a parasitic phase and finally a saprophytic phase [3]. Under favourable environmental conditions, the latter phase ultimately results in the fruiting body of the fungus (which germinates out of the soil from the oral cavity of the dead host), at which point commercial harvesting can occur.

For centuries, medicinal value has been conferred to *O. sinensis*, resulting in an increasingly high economic importance [4]. Among other medicinal properties, *O. sinensis* is widely used against liver, kidney and lung diseases, as well as for its anticancer effects, and even its dermatological properties [5, 6]. Nevertheless, some studies have warned about its potential toxicity [7–9]. Available harvest estimates range from 85 to 185 tons annually throughout the distribution range area of *O. sinensis* (the Himalayas, Hengduan Mountains and eastern Qinghai-Tibet Plateau) [10]. Although the exploitation of the “vegetable caterpillar” represents a significant income for local communities, harvesting practices have been deemed unsustainable leading to an obvious decline in the abundance of wild *O. sinensis* [11], even causing severe disturbances to its habitat [12, 13]. Aside from natural predators and pathogenic microorganisms, habitat alteration deriving from anthropogenic activities (e.g., herding) affects the distribution and abundance of wild *O. sinensis* [14]. Additionally, climate change is likely to have caused a vertical displacement of this species’ distribution [15, 16]: a study showed that the upper limit of the prime habitat of the caterpillar fungus shifted upwards by 200–500m (from 3900–4400 to 4400–4600) in two decades [10], resulting in a contraction of the distribution range of *O. sinensis*.

In addition to distribution and phylogenetic work [17], most studies on *O. sinensis* have focused on diverse aspects related to medicinal use or to commercial applications, including chemical composition analysis, artificial larval and mycelium cultures, among others [18–21]. Furthermore, researchers and high-tech commercial companies have been attempting to cultivate *O. sinensis* in controlled environments (in-vitro), as this could theoretically represent a rapid and effective way not only to meet the increasing demands of the market, but also to promote the conservation of this species [22–24]. After decades of effort, many achievements have been made, such as the successful artificial cultivation of fungi and host larvae separately, and the taxonomic delineation and variability of the pathogenic fungus and host larvae [25]. Other challenges that have not yet been fully overcome, include the difficulties of simulating the natural habitat in laboratories, and the technical complexity of artificially infecting the host larvae with the fungus *O. sinensis* [26]. Some of these shortcomings are clearly the result of a lack of understanding of the factors which are crucial to the natural environment of *O. sinensis* (i.e., alpine meadows), justifying more in-depth research in the ecology of the species. More specifically, soil factors are likely to be most relevant for this system, since most of the life cycle of the *Hepialus* larva, the *O. sinensis* fungus as well as the inoculation and hyphal development of the *O. sinensis* complex occur underground. Indeed, the *Hepialus* larva feeds on plant roots [27], and the *O. sinensis* fungus depends on microbial reserves in the soil [28, 29]. After being infected (in the soil) by the fungus, the caterpillar remains alive for a long period (5–12 months in laboratory, may be longer in the wild) and keeps developing until *O. sinensis* mummified [30, 31]. Upon its death, the caterpillar moves near the surface of the soil (2–5 cm depth) and places itself vertically, with the head facing upward, allowing the stromata to grow out of the soil and release the ascospores [32]. Thus, investigating the soil microhabitat around *O. sinensis* appears most relevant.

Focusing on the microhabitat of *O. sinensis*, we investigated the relationships among edaphic factors, by measuring enzyme activity (specifically catalase, urease, sucrase, nitrate reductase (NR), acid phosphatase (ACP) and cellulase), nutrient content (including total nitrogen (TN), total phosphorus (TP), total

potassium (TK), organic matter (OM), available phosphorus (AP), rapidly available potassium (Kppm)) and nematode population in the soil. Not only soil samples need to be collected directly around *O. sinensis* and thus are of a small volume, but also such samples are difficult to come by because of the secrecy surrounding collection sites. As a result, our study ultimately suffers from limited replication. Nevertheless, in addition to a literature review, we simulate the network of soil factors using structural equation modelling (SEM). By doing so, we aim at establishing a working hypothesis in the form of a network of potential interactions among soil factors and *O. sinensis*. We then indicate how this hypothesis may be tested in future studies.

Results

In general, mean values rarely differed much among quality categories of *O. sinensis*. However, larger differences were observed between soil samples that contained *O. sinensis* and soil samples that did not. Preliminary analyses indicates that enzyme activity may be crucial, as it appears to be affected by a number of other soil factors. Our results need to be interpreted with caution; nevertheless, they allowed us to build up the first testable hypothesis integrating a large array of soil factors realistically important to *O. sinensis* (represented visually in Fig. 1).

Observed differences

Between soil samples either with or without *O. sinensis*, differences were more pronounced. For example, the moisture of the soil in which *O. sinensis* lived was higher (A, B, C: 34.85 ± 0.03 , 35.22 ± 0.05 , 37.74 ± 0.04 %) than that of the control group (28.32 ± 0.03 %). Furthermore, almost all absolute values of soil nutrients appeared to follow the same trend: samples containing *O. sinensis* had more TN (A, B, C: 6.02 ± 0.06 , 6.11 ± 0.22 , 6.54 ± 0.80 g/kg), more TP (A, B, C: 1.22 ± 0.09 , 1.23 ± 0.03 , 1.25 ± 0.04 g/kg) and for TK (A, B, C: 20.0 ± 0.72 , 18.4 ± 0.42 , 19.5 ± 1.17 g/kg), it is slightly more but overlapping with the control group (2.53 ± 0.25 , 0.87 ± 0.01 , and 17.8 ± 1.00 g/kg, respectively). For potassium, this trend was visible particularly for Kppm, which was much higher for samples that contained *O. sinensis* (465.2 ± 29.6 , 495.7 ± 29.7 , 425.4 ± 30.2 g/kg) than samples that did not (173.4 ± 16.0 g/kg). Finally, with the exception of sucrase (A, B, C: 4.18 ± 0.2 , 4.10 ± 0.3 , 5.11 ± 0.5 , CK: 4.60 ± 0.2 g glucose·(g·drysoil·d)⁻¹), all other enzymes were the lowest in the control group, including catalase (A, B, C: 13.42 ± 0.1 , 13.43 ± 0.2 , 13.08 ± 0.0 , CK: 11.50 ± 0.3 mg H₂O₂·(g·drysoil·h)⁻¹), urease (A, B, C: 6.43 ± 0.6 , 5.60 ± 0.2 , 7.53 ± 0.8 , CK: 4.33 ± 0.0 mg NH₃-N·(g·d)⁻¹), cellulase (A, B, C: 7.50 ± 2.5 , 6.88 ± 0.5 , 7.20 ± 0.7 , CK: $3.87 \pm 0.5 \times 10^{-2}$ mg glucose·(g·drysoil·d)⁻¹), and ACP (A, B, C: 2.95 ± 0.0 , 2.99 ± 0.1 , 3.73 ± 0.0 , CK: $2.66 \pm 0.1 \times 10^{-2}$ μg p-nitrophenol·(g·h)⁻¹). The nematode populations varied a lot among samples but appeared to be slightly higher in samples which did not contain *O. sinensis* (Table 1).

Table 1
Summary of soil physicochemical and nematodes effects on *O. sinensis* quality (Mean ± SD)

	A	B	C	CK	ANOVA	
					F	P
TN (g/kg)	6.02 ± 0.06	6.11 ± 0.22	6.54 ± 0.80	2.53 ± 0.25	55.06	**
TP (g/kg)	1.22 ± 0.09	1.23 ± 0.03	1.25 ± 0.04	0.87 ± 0.01	25.03	**
TK (g/kg)	20.0 ± 0.72	18.4 ± 0.42	19.5 ± 1.17	17.8 ± 1.00	3.95	0.053
OM (g/kg)	119.6 ± 7.9	139.1 ± 4.3	140.0 ± 8.1	51.0 ± 2.4	139.13	**
AP (mg/kg)	4.81 ± 0.44	3.90 ± 0.40	4.43 ± 0.28	1.90 ± 0.17	43.66	**
Kppm (mg/kg)	465.2 ± 29.6	495.7 ± 29.7	425.4 ± 30.2	173.4 ± 16.0	88.85	**
catalase	13.42 ± 0.1	13.43 ± 0.2	13.08 ± 0.0	11.50 ± 0.3	82.17	**
urease	6.43 ± 0.6	5.60 ± 0.2	7.53 ± 0.8	4.33 ± 0.0	23.17	**
sucrase	4.18 ± 0.2	4.10 ± 0.3	5.11 ± 0.5	4.60 ± 0.2	7.68	*
NR	3.50 ± 0.4	1.72 ± 0.4	2.54 ± 0.2	1.56 ± 0.3	22.92	**
cellulase	7.50 ± 2.5	6.88 ± 0.5	7.20 ± 0.7	3.87 ± 0.5	3.95	0.061
ACP	2.95 ± 0.0	2.99 ± 0.1	3.73 ± 0.0	2.66 ± 0.1	142.32	**
nematodes	199 ± 32	142 ± 90	206 ± 70	337 ± 119	2.90	0.102
pH	5.79 ± 0.01	5.89 ± 0.01	5.77 ± 0.06	5.83 ± 0.02	8.64	*
Moisture (%)	35.22 ± 0.05	37.74 ± 0.04	34.85 ± 0.03	28.32 ± 0.03	96.07	**
Catalase (mg H ₂ O ₂ ·(g·drysoil·h) ⁻¹); urease (mg NH ₃ -N·(g·d) ⁻¹); Sucrase (g glucose·(g·drysoil·d) ⁻¹); nitratase reductase (NR) (μg NO ₂ -N·(g·drysoil·d) ⁻¹); Cellulase (×10 ⁻² mg glucose·(g·drysoil·d) ⁻¹); acid phosphatase (ACP) (×10 ⁻² μg p-nitrophenol·(g·h) ⁻¹). TN: total nitrogen, TP: total phosphorus, TK: total potassium, OM: organic material, AP: organic phosphorus, Kppm: rapidly available potassium. A: high-quality <i>O. sinensis</i> groups; B: general-quality <i>O. sinensis</i> groups; C: poor-quality <i>O. sinensis</i> groups; CK: control group (without <i>O. sinensis</i>). Significance of main effect: *P < 0.05, **P < 0.01.						

Among the different quality categories (A, B, and C) of *O. sinensis*, we only observed a few differences. For example, a content of ca. 120 g/kg of OM appeared to be associated with the best quality (A: 119.6 ± 7.9 g/kg), whereas lower qualities had more OM (B: 139.1 ± 4.3 , C: 140.0 ± 8.1 g/kg), and the control group (D) was characterized by less OM (51.0 ± 2.4 g/kg). A similar situation was found for AP, for which the best quality had the highest content (A: 4.81 ± 0.44 mg/kg), lower qualities containing slightly less (B: 3.90 ± 0.40 , C: 4.43 ± 0.28) and the control containing only little (CK: 1.90 ± 0.17). Finally, samples showing

a higher activity of the NR were also the group with the best quality of *O. sinensis* (A: 3.50 ± 0.4 [$\mu\text{g NO}_2\text{-N} \cdot (\text{g} \cdot \text{drysoil} \cdot \text{d})^{-1}$], less for those groups with lower quality *O. sinensis* (B: 1.72 ± 0.4 , C: 2.54 ± 0.2), the control group displaying the least activity (CK: 1.56 ± 0.3).

Finally, some soil characteristics did not appear to vary among samples. This was the case of the pH, which was typically between 5.77 and 5.90 (A: 5.79 ± 0.01 , B: 5.89 ± 0.01 , C: 5.77 ± 0.06 , CK: 5.83 ± 0.02), and the sucrase activity (A: 4.18 ± 0.2 , B: 4.10 ± 0.3 , C: 5.11 ± 0.5 , CK: 4.60 ± 0.2 g glucose $\cdot(\text{g} \cdot \text{drysoil} \cdot \text{d})$).

Pearson correlation between environmental variables and eigenvalue of *O. sinensis*

In order to build up our hypothesis, we needed to identify potential correlations. Hence, we tentatively performed statistical analyses despite pseudo-replication. These results need to be cautiously evaluated and the p-values provided are only indicative. Pearson correlation was applied to analyse interrelation of each environmental variable and eigenvalue of *O. sinensis* (Table 2). We found that the polypide, stroma and the fresh weight (FW) of *O. sinensis* were likely to be strongly correlated with each other (indicative $p < 0.01$), and that some environmental variables may be correlated to all three of them. These environmental variables may include catalase, TN, TP, OM, AP, Kppm, and moisture. Additional, yet probably weaker correlations (indicative $p < 0.05$), may exist between the eigenvalue of *O. sinensis* and other edaphic factors, such as cellulase, NR, TK, and nematode populations. As expected, based upon the mean values themselves (see above), the eigenvalue of *O. sinensis* had no distinct relationship with pH, but a positive correlation may be visible with some soil enzyme activity, moisture, and all measured soil nutrients. Among these factors, some may be correlated. For example, the respective correlations between catalase and urease with other enzymes appeared significant (indicative $p < 0.01$). Also, each type of soil nutrient appeared to have a significant correlation with each other, and correlations between enzymes and nutrients are also mostly significant, soil moisture did not show any significant correlations with most of the enzyme activity, but did with most of the nutrients ($p < 0.01$). In addition, the pH appeared to be positively correlated only to sucrase and TK, two factors that did not vary much among groups. Nematodes population showed opposite correlations as those observed for moisture (indicative $p < 0.05$), and nematode populations were negatively correlated ($p < 0.01$) with moisture. In conclusion, the quality of *O. sinensis* showed relationships with most of the soil enzyme activity, all soil nutrients, as well as moisture and nematode populations.

Table 2
Correlations between environmental variables and eigenvalue of *O. sinensis*

	Catalase	Urease	Sucrase	NR	Cellulase	ACP	TN	TP	TK	OM	AP	Kppm	pH
Catalase													
Urease	<i>0.649*</i>												
Sucrase	-0.255	0.238											
NR	0.549	<i>0.608*</i>	-0.136										
Cellulase	<i>0.795**</i>	<i>0.708*</i>	0.009	0.439									
ACP	0.469	<i>0.864**</i>	0.570	0.304	0.495								
TN	<i>0.929**</i>	<i>0.748**</i>	0.064	0.487	<i>0.768**</i>	<i>0.682*</i>							
TP	<i>0.903**</i>	<i>0.713**</i>	-0.027	0.507	<i>0.652*</i>	<i>0.615*</i>	<i>0.968**</i>						
TK	<i>0.600*</i>	0.506	0.301	<i>0.623*</i>	<i>0.688*</i>	0.430	<i>0.681*</i>	<i>0.595*</i>					
OM	<i>0.909**</i>	<i>0.734**</i>	-0.012	0.377	<i>0.697*</i>	<i>0.681*</i>	<i>0.971**</i>	<i>0.966**</i>	0.488				
AP	<i>0.895**</i>	<i>0.770**</i>	-0.050	<i>0.724**</i>	<i>0.711*</i>	<i>0.567</i>	<i>0.928**</i>	<i>0.949**</i>	<i>0.724**</i>	<i>0.876**</i>			
Kppm	<i>0.969**</i>	<i>0.611*</i>	-0.241	0.466	<i>0.711*</i>	0.452	<i>0.944**</i>	<i>0.959**</i>	0.545	<i>0.948**</i>	<i>0.911**</i>		
pH	-0.063	-0.404	- <i>0.630*</i>	-0.573	-0.287	- 0.438	-0.267	-0.199	- <i>0.741**</i>	-0.078	-0.366	-0.020	
Moisture	<i>0.952**</i>	0.571	-0.295	0.325	<i>0.730*</i>	0.440	<i>0.900**</i>	<i>0.890**</i>	0.395	<i>0.939**</i>	<i>0.802**</i>	<i>0.969**</i>	0.152
Nematode	<i>-0.652*</i>	-0.391	0.292	-0.146	-0.527	- 0.244	- <i>0.608*</i>	- <i>0.616*</i>	-0.117	- <i>0.684*</i>	-0.561	- <i>0.683*</i>	- 0.264
Polypide	<i>0.981**</i>	<i>0.705*</i>	-0.234	<i>0.591*</i>	<i>0.784**</i>	0.496	<i>0.941**</i>	<i>0.928**</i>	<i>0.586*</i>	<i>0.927**</i>	<i>0.933**</i>	<i>0.974**</i>	- 0.108
Stroma	<i>0.970**</i>	<i>0.659*</i>	-0.305	<i>0.652*</i>	<i>0.772**</i>	0.402	<i>0.901**</i>	<i>0.894**</i>	<i>0.607*</i>	<i>0.871**</i>	<i>0.930**</i>	<i>0.955**</i>	- 0.126
FW	<i>0.934**</i>	0.548	-0.425	<i>0.673*</i>	<i>0.728*</i>	0.239	<i>0.818**</i>	<i>0.821**</i>	<i>0.587*</i>	<i>0.777**</i>	<i>0.884**</i>	<i>0.914**</i>	- 0.090
Significant correlations ($p < 0.05$) are given in italics. * represents significant correlation ($p < 0.05$), ** represents highly significant correlation ($p < 0.01$). Abbre phosphatase, TN: total nitrogen, TP: total phosphorus, TK: total potassium, OM: organic material, AP: organic phosphorus, Kppm: rapidly available potassium													

Network of multiple environmental variables on *O. sinensis* in SEM

The Kaiser-Meyer-Olkin (KMO) value and Bartlett's test of sphericity both revealed the rationality of analysis (enzyme activity: KMO = 0.736, Bartlett Sig.<0.01; nutrient: KMO = 0.787, Bartlett Sig.<0.01; *O. sinensis*: KMO = 0.504, Bartlett Sig.<0.01). Likewise, the accumulated variance of the factor analysis satisfied the

standard requirements (enzyme activity: 72.912; nutrient: 95.361; *O. sinensis*: 98.358).

Based on previous studies [33–35] and our analysis, we developed an initial model by assuming that all the variables were directly correlated with the quality of *O. sinensis*, and that there were connections between nutrients and enzyme activity, as well as causal relationships between soil moisture, pH and soil nutrients, enzyme activity and nematodes. After fitting the model, we made progressive modifications to the original design with the remainders of the parameters until an optimized model fit was achieved ($\chi^2 = 4.249$, $p = 0.643$). The final model fitting is presented in Fig. 1 and Supplementary Table S4 [see Additional file 1], and constitute the testable hypothesis we provide for future research.

In this path analysis, soil moisture, nutrients and enzyme activity were associated with the higher quality of *O. sinensis*. First, we assumed that soil nutrients would have a strong direct effect, yet it appeared that soil nutrients possibly affected the quality of *O. sinensis* by altering enzyme activity. The path coefficient from nutrients to *O. sinensis* was only 0.10 (indicative $p = 0.719$), and the path coefficient from nutrients to enzyme activity was 0.92 (indicative $p < 0.001$). Soil moisture was also positively associated with soil nutrients (path coefficient = 0.93, $p < 0.001$), in addition to having a direct effect on the quality of *O. sinensis*. Moreover, lower pH values were associated with higher nutrient content, which in turn was associated with larger nematode populations. The values of pH did not appear to have any noticeable relationships with nematode populations.

Finally, in order to investigate the relationship between soil nutrients and *O. sinensis* in more details, we disassembled the one-dimensional nutrient data and estimated the contribution of the different nutrient components (which included TN, TP, OM, AP and Kppm) to *O. sinensis*. A similar procedure was conducted with data on soil enzyme activity (which included catalase, urease, NR and cellulase). The results indicated that TN made a large contribution to the quality of *O. sinensis* (92.59%) and that the remaining nutrient variables only accounted for small fractions (Kppm = 6.20%, AP = 0.90%, OM = 0.31%, TP < 0.01%). Enzyme activity, catalase and NR were found to potentially have a major contribution to the quality of *O. sinensis* (i.e., catalase = 74.57%, NR = 22.78%), with the remaining enzymes contributed less than 3.0% altogether (i.e., urease = 1.73%, cellulase = 0.92%). These seemingly strong results, as all of our results, are however only indicative.

Discussion

Ophiocordyceps sinensis is a notorious component of traditional medicine, and is widely used across the region of the Qinghai-Tibet Plateau. Its commercial harvest has plummeted in the last few decades partly due to over-exploitation. In order to preserve the natural populations of *O. sinensis*, as well as to bypass problems related to soil contamination by heavy metals (rendering *O. sinensis* improper to consumption), researchers have mainly focused on improving artificial cultivation of the species [25, 36, 37]. Until now, such *in vitro* studies have made a series of major breakthroughs for the production of mycelia and the cultivation of the fungus, as well as for rearing host larvae and even the cultivation of the complex post-infection. However, challenges still persist for large-scale cultivation, the most prominent of them being the initial inoculation of the host larvae by *O. sinensis* under controlled environment [25]. This crucial step may strongly depend on edaphic conditions in alpine meadows, the natural habitat of *O. sinensis* [38]. Admittedly, collecting *O. sinensis* in phases prior to its fructification is challenging because this organism is so elusive. Investigating soil conditions around the fructification is, for now, the best possible proxy.

Because integrative studies on soil properties of the native habitat of *O. sinensis* are lacking, a working and testable hypothesis on their relationship is needed. To develop such a hypothesis, we performed an experiment targeting the effect of soil factors on the commercial quality of that species. Since the statistical power of our experiment is limited, our aim is only to provide a hypothesis for future studies to test. Generally speaking, we find that soil moisture, soil nutrients and soil enzyme activity are likely to be crucial factors for the quality of *O. sinensis*. In the following, we will discuss the potential network of relationships among soil factors and their effect on the quality of the “fungus caterpillar”. We finally cross-check and combine our findings with the available literature.

Edaphic factors and their effects on *O. sinensis*

Soil enzymes are crucial to soil ecology, and to the development of a variety of organisms [39], which appears to be also the case for *O. sinensis*. We find that catalase, cellulase, urease and NR (simplified as one factor in a PCA) are likely to affect the quality of this species positively, with catalase having possibly the strongest effect, followed by NR. Hence, we believe that *O. sinensis* requires a dynamic soil micro-habitat, with a high ability for redox and denitrification, as expected by the functions of the catalase and the NR, respectively [37, 40]. The interaction network (our hypothesis, see Fig. 1) also suggests that most environmental parameters (among others, nutrient and soil pH; see Fig. 1) may ultimately influence *O. sinensis* by indirectly affecting soil enzymes. These results potentially confirm that soil enzyme activity could act as an indicator for soil functions in this ecosystem. Our results corroborate other studies showing that soil enzyme activity is an important biochemical indicator of soil quality, and an indicator of nutrient dynamics in general [41, 42]. For example, soil enzymes participate in the formation and evolution of components leading to soil fertility [43]. Furthermore, soil enzyme activity appears to be influenced by a series of other soil parameters acting in concert. In the following, we will discuss the different indirect mechanisms by which these factors may affect soil enzyme activity, and ultimately the quality of *O. sinensis*.

Although soil nutrients do not appear to be associated in a direct manner with the quality of *O. sinensis* in the SEM analysis (path coefficients = 0.10, $p = 0.719$), they should not be neglected in this network (Fig. 1): nutrients act indirectly on the vegetable caterpillar by modifying enzyme activity. More specifically, TN may be instrumental, and the relationship between *O. sinensis* and NR is probably strong. Together, these results suggest that *O. sinensis* develops best in relatively nitrogen-rich habitat. Our study thus aligns with the results of Wu et al. [44], who observed a direct effect of soil nutrients (incl. hydrolysable nitrogen) on the spatial distribution of *O. sinensis* (although that study did not investigate enzyme activity). On the contrary, our results may, at least at first sight, contrast with a series of other studies which revealed an inhibitory effect of nutrients on enzyme activity [45–47]. However, these papers only investigated the direct effect of a given nutrient on a particular enzyme *in vitro*, while our approach was designed to include simultaneously a range of nutrients as well as a range of enzymes. This constitutes the strength of our hypothesis, as the dynamics between nutrients and enzymes should be, if possible, investigated

together [42] within a near natural setting. The positive effect of nutrients on enzyme activity may in fact only be apparent when a substantial portion of this system is investigated at once.

Moreover, our results tend to show that nutrients availability was directly associated with pH values, as expected. We found that pH values (for the soil samples containing *O. sinensis*) corresponded to previous investigations as 5.5 to 7.5 [48, 49], and were more often slightly acidic. The possible association between pH and nutrients availability obviously depends on the nutrient considered, yet in our samples, slightly lower pH values always corresponded to overall greater nutrient availability (considering all four nutrients investigated). Although soil pH is not the primary influence on *O. sinensis*, a suitable range of pH is important to the quality of *O. sinensis* and steady pH over time is beneficial [19, 48, 49]. Furthermore, our study may indicate that nutrient availability might have an effect on the nematode population, with different trophic groups of nematodes (e.g., bacterivores, fungivores) responding differently back to front. For example, bacterivores are known to be positively affected by a nitrogen increase, whereas fungivores populations would decline [50]. However, in general, unbalanced amounts of soil nutrients are expected to negatively impact the nematode community, which has repercussions at the ecosystem level [51]. Moreover, the destabilization of soil nutrients content could lead to changes in the microflora, which then reflect back into the nematode community [52, 53]. Overall, our study suggests that increased nutrient content in the soil negatively affects nematodes populations, which matches the results of Li et al. [51]. Even though the nematode population appears to occupy a marginal location in our network of interactions, it may still indirectly influence *O. sinensis*, because of its strong correlation with soil nutrients and the microflora in general. Clearly, trophic groups of nematodes should be further investigated both individually and in concert to grasp the mechanisms dominating their interaction with soil nutrients. Yet, overall, our study does find trends which do align with other studies even despite a limited dataset.

Clearly, some additional data are required to fully understand the dynamic interactions between *O. sinensis* and its micro-habitat, and not only in terms of replication for the factors that we investigated. First, there are feedbacks between vegetation (plant coverage, abundance, species richness, community, above- and below-ground biomass etc.), nutrients, enzyme activity and even the nematode community [42, 44, 50]. Thus, in order to fully unravel interactions among soil nutrients, enzyme activity, and vegetation, as well as their effect on the quality of *O. sinensis*, biomass data would need to be included in future research. Some studies have revealed that below-ground plant biomass can directly affect both the host larvae (as food source) and the fungus [37, 54], indicating that above- or below-ground plant biomass may play an important role in this network. Another component of this dynamic system would be the population of microorganisms, which is not only important for the entire ecosystem (as decomposers, producers of soil enzymes and components of soil nutrients), but also for *O. sinensis* specifically. Indeed, in the study of Xia et al. (2016), communities of microorganisms have been associated with the development and metabolic process of *O. sinensis* [55]. Microorganisms also play an important role in soil food webs, for example as a food source for nematodes [34], which we investigated here. By considering the potential role of biomass and microorganisms, our hypothesis is the most integrative to date, and represents a necessary first step towards incorporating more parameters and gathering a better understanding of the interactions between *O. sinensis* and its immediate environment.

Limitations of the study design in time and space: perspectives

Our study was performed in only one locality involved in the harvesting of *O. sinensis*, thus limiting its power for generalization. Although we believe our study site is highly representative and bears some potential for broader conclusions, we cannot exclude that the results may vary if study sites were replicated throughout the distribution range of the vegetable caterpillar. Hence, investigating a variety of geographically distant sites would be necessary in future studies. In addition, conducting a series of control experiments either in-situ or in an artificial environment would allow identifying thresholds (maximum and minimum), beyond which several factors of the soil would become detrimental to *O. sinensis*. Among others, nitrogen enrichment may restructure plant and nematode communities and exacerbate the toxic effect of ammonium and aluminium [50]. Therefore, nitrogen intake beyond such threshold would modify the equilibrium of available nutrients, probably affecting the quality of *O. sinensis* in an indirect manner (via enzyme activity), as shown in our study. Identifying such thresholds would be instrumental in establishing locally adapted strategies for sustainable harvesting in concert with strategies aiming at preventing overexploitation [13]. The network of interaction we produced as a testable hypothesis can thus serve as a base to investigate how the role of edaphic factors may vary spatially.

Furthermore, our study only represents a snapshot in time of the relationship between *O. sinensis* and edaphic factors, which corresponds to the sporulation phase and the optimal harvest time. Although our study depicts conditions under which high commercial values may be reached, gaining an understanding of the interaction between the vegetable caterpillar and soil components at earlier phases of the development of this complex (including the primary infection and the parasitic phase) would be necessary. So far, our study can only assume that the effect of soil factors remains stable throughout the growing season. In controlled conditions, the failure to infect the host larvae with *O. sinensis* (whereas infected young larvae captured in the wild would develop normally in controlled conditions) has only been attributed to missing cryptic environmental factors [26]. However, no-one can exclude that soil conditions included in our hypothesis may vary through the life cycle of *O. sinensis* and may favor or prevent inoculation depending on the season. Our research, providing the first integrative framework of edaphic factors on the sporulation phase of this parasitic complex, thus represents a base to which other phases of the lifecycle of *O. sinensis* can be compared. Hence, future studies should investigate a more comprehensive set of soil parameters (e.g., also including plant biomass and microorganisms) at different developmental phases of *O. sinensis*, and from replicated populations in time and space.

Edaphic conditions, climate change and pasture management: towards assembling the big picture

We found that a higher quantity of nutrients may be favorable to *O. sinensis*, but we could not identify an upper threshold beyond which the quantity of nutrients in the soil would become detrimental to *O. sinensis*. Identifying this threshold would be crucial, because it may be attained when pastoral practices are causing an excess of grazing or fertilizing, both of which being critical for nutrient cycling and soil fauna biodiversity [56]. Our hypothesis could represent a good base to investigate differences in pastoral practices and their impact on *O. sinensis* and more generally, its habitat. We argue that further studies

should investigate in detail the complex interactions between grazing intensity (such as from nomadic to static herding), edaphic factors (especially soil enzyme activity), soil biomass, and the abundance of *O. sinensis*, using a geographically and temporally replicated design. In addition, climate change not only has a direct impact on edaphic conditions by affecting the temperature and precipitation [57, 58], but also leads to an upward displacement of *O. sinensis* indirectly [10, 59–61]. Therefore, such studies should obviously project their findings into the future, using available climate scenarios.

Conclusions

In this study, we investigated the relationships among enzyme activity, nutrient content, moisture, pH, nematode population and the quality of *O. sinensis* in the soil community in southeastern edge of the Qinghai–Tibet Plateau. Despite a limited statistical power due to the difficulty to gather sufficient samples, we developed an integrative and testable hypothesis (see. Figure 1) combining our preliminary results with earlier findings in the literature. Among others, our study suggests that soil moisture, enzymes activity and nutrients content may be positively associated with the quality of *O. sinensis*, and most edaphic factors may ultimately influence the quality of *O. sinensis* by modifying the values of soil enzyme activity. Our hypothesis on the relative role of all involved factors sets the base for further research, ideally throughout the distribution range and the life cycle of *O. sinensis*, thus possibly contributing to the conservation of this old Chinese medicinal organism and the fragile environment in Qinghai-Tibet Plateau.

Methods

Soil and *O. sinensis* samples

Soil samples were collected in Hongyuan County of northern Sichuan Province (China), which is located on the south-eastern edge of the Qinghai-Tibet Plateau (31°50'N–33°22'N, 101°51'E–103°23'E). Hongyuan county, at an elevation of 3210–4857 m, is located in the frigid temperate zone and alpine monsoon climate characterized by an annual average temperature of 10.1°C and annual precipitation reaching 753 mm [62]. Because these climatic conditions are very favorable to *O. sinensis*, this region stands as a major production center in Sichuan. The *in-situ* vegetation was characterized by numerous Cyperaceae and *Polygonum viviparum* as dominant taxa, as well as (among others) by *Elymus spp*, the Chinese cinquefoil herb (*Potentilla chinensis* Ser.), *Deschampsia caespitosa* (Linn.) Beauv, *Cremanthodium reniforme* (DC.) Benth, *Geranium spp* and various bryophytes.

Soil samples containing *O. sinensis* were collected in April 2014, which corresponds to the early fruiting season of the fungus and to the optimal collection time associated with the highest commercial value [10]. Samples of *O. sinensis* were harvested randomly throughout the study site, in the natural habitat of this species on the private land of local expert collectors. In total, 40 samples were collected, each of them containing one individual of *O. sinensis*. Samples were immediately handed over to us, in compliance with local customs, including the secrecy of collection spots, which ultimately limited the number of samples we could obtain. Each soil sample containing *O. sinensis* was approximately 160 cubic centimetres and 100 grams, and the distance from the head of the polypide to the surface of the soil ranged from 0 cm to 4.6 cm. As control group, additional soil samples (N = 10) which did not contain *O. sinensis* were also collected randomly from the same alpine meadow, and were characterized by the same surrounding floristic composition.

Quality estimation of *O. sinensis* samples

After measuring the physical characteristics of each sample (i.e., length, width, height, and weight), we separated *O. sinensis* specimens from the soil. Meanwhile, we measured the growth depth, the length of the polypide and stroma, the FW of the entire parasitic complex. We then used a cluster analysis in IBM SPSS statistics to separate *O. sinensis* specimens into three groups corresponding to three different levels of quality (i.e., high-quality (A), general-quality (B) and poor-quality (C)). Estimation of the quality of *O. sinensis* specimens was based on the length of polypide, stroma and FW of the entire parasitic complex, as well as on additional visual examination (robustness and shape) (Supplementary Tables S1, S2). We categorized the 40 *O. sinensis* specimens as follows: high-quality (i.e., larger and heavier specimens, group A, 13 specimens), general-quality (group B, 14 specimens), and poor-quality (group C, 13 specimens) (Supplementary Table S1, S2 in Additional file 1). We followed a well-established categorizing strategy for the quality of *O. sinensis* that is commonly used for commercial purposes [63, 64]. We then carefully separated *O. sinensis* specimens from the soil, and pooled (mixed) the different soil samples according to the quality of the *O. sinensis* they contained (e.g., all soil samples that contained a high-quality *O. sinensis* specimen were pooled together), resulting in three experimental composite samples and one control composite sample. Thus, the four different composite samples contained each between 10 and 14 soil samples, which we believe covers the natural variation of the soil at the study site. Since collecting soil samples makes sense only in the immediate surrounding of *O. sinensis* specimens, but the soil samples per *O. sinensis* specimen would have been too small to measure all variables of interest, hence pooling (mixing soil samples) was necessary. Finally, we divided each of these composite sample into three equal parts to act as replicates to account for measurement error for the different factors we investigated. The use of composite samples is often applied in rhizosphere research, in order to determine the effect of diverse factors on communities or target organisms, as for example the relative effects of soil nutrients, plant species, microbial activity and rhizosphere bacterial community on strawberries (*Fragaria ananassa* Duch.), oilseed rapes (*Brassica napus* L.), and cucumbers (*Cucumis sativus* L.) seedlings [65, 66]. For each of the 12 sub-samples, we first used approximately 100 grams of soil and conducted the nematode population experiment immediately. Next, we divided the remaining soil samples into two parts, one part of which was first used for soil moisture measurement and the rest was stored at 4°C to measure enzyme activity, the second part was dried under natural air in a cool and ventilated place for the pH and nutrient tests.

Soil enzyme activity, physicochemical, and nematode population analysis

Soil moisture was calculated as percentage of weight loss by using the fresh soil samples immediately, after the samples were dried in an oven for 18h at 105°C (until constant mass was achieved). Catalase, urease, sucrase, NR, ACP and cellulase were determined by different methods as shortly outlined hereafter. We used titanous sulphate-colorimetry for catalase's activity, expressed as the mass ratio (mg/g) of hydrogen peroxide hydrolyse in desiccated soil after one hour [67]. Urease activity was measured via phenol sodium-sodium hypochlorite-colorimetry, expressed as the mass ratio (mg/g) of ammonium

nitrogen ($\text{NH}_4^+\text{-N}$) in desiccated soil (after 24 hours drying). Invertase activity was estimated with the 3,5-dinitrosalicylic acid-colorimetry and expressed as the mass ratio (mg/g) of glucose in desiccated soil (after 24 hours). Then, 2,4-dinitrophenol-colorimetry was used for nitratase, with the mass ratio (mg/g) of nitrate nitrogen ($\text{NO}_2^-\text{-N}$) in desiccated soil after 24 hours [68]. The disodium phenylphosphate-colorimetry was used for ACP, expressed as the mass ratio (mg/g) of phenol released from soil after one hour. Finally, the 3,5-dinitrosalicylic acid-colorimetry was performed for cellulase, expressed as the mass ratio (mg/g) of glucose in desiccated soil (after 72 hours).

Potentiometry (GB 7859 – 1987) was used to measure soil pH with 1:5 (w/v) soil-to-water ratios [69]. The semi-micro Kjeldahl method (GB 7173 – 1987) was used to determine soil TN content, which included both nitrate-nitrogen and nitrite-nitrogen [70]. We used the Mo-Sb colorimetric method to determine TP after high-temperature melting in sodium hydroxide (GB 7852 – 1987) [71]. The flame photometry method was performed to determine TK after high-temperature melting in sodium hydroxide (GB 7854 – 1987) [72]. The determination of Kppm was also measured with the flame photometry method after the extraction of samples using ammonium acetate (GB 7856 – 1987) [73]. Colorimetry was used to determine AP after acid leaching by 0.05 mol/L HCl-0.025 mol/L H_2SO_4 (GB 7853 – 1987), which was suited to acidic soil [74]. The soil OM was determined by potassium dichromate titrimetric (GB 9834 – 1988) [75]. Finally, we used the Baermann funnel method for 24 hours to separate nematodes, which were stored in a 5% formaldehyde solution until counting under the anatomical lens (Leica DM4000 B) was completed [76].

Statistical Analysis

Limitations in sampling resulted in a lack of replication in our experiment, thus strongly diminishing the power of statistical analyses. However, our goal is to provide a first network of interactions as a working hypothesis for future studies to test. Thus, although questionable, we believe our approach represents a first step in describing how edaphic factors are intertwined and affect *O. sinensis*.

We first identified the soil variables that showed a significant difference among the four groups based on the result of one-way analysis of variance (ANOVA) and Pearson correlation analysis. The ANOVA was used for testing the variation among each group and the Pearson correlation analysis was applied for investigating the correlation relationship among each soil variable and the three *O. sinensis* characters (Table 1 and Table 2), both of them were calculated using SPSS. Then we used a principal component analysis (PCA) [77] to reduce the dimension on multiple targets with characteristic indexes for these selected soil variables and *O. sinensis* containing the three characters, which means, multiple nutrient variables were aggregated into one, the same for soil enzyme activity and *O. sinensis*. Meanwhile, we applied Bartlett's test of sphericity on these variables. The results indicated that the variables were suitable for factor analysis. Therefore, the enzyme indicators that contained catalase, urease, NR and ACP were simplified as one factor, as were the nutrients that contained TN, TP, OM, AP, Kppm and the characteristic targets of *O. sinensis* that contained the length of polypide, stroma and FW.

The variable indicators including soil enzymes, nutrients, moisture, pH, nematode populations and the characteristic targets of *O. sinensis* were used in the following SEM analysis, all the four groups (including control group) are included. Before the SEM, we conducted an ANOVA analysis to check the relationship between these indicators, especially the effects of environmental indicators on *O. sinensis*. According to the ANOVA calculated for each variable beforehand (Supplementary Table S3, see Additional file 1), only the nematode populations showed a non-significant effect, but in consideration of the theoretical relationship among nematode, enzyme activity and *O. sinensis*, we still retained all the data points in the analysis. Structural equation modelling [78, 79] was conducted to quantify direct and indirect effects between environmental variables and the quality of *O. sinensis*. The initial SEM was built on the basis of prior theoretical knowledge, which means the relationship between variables needed to be set up in advance. Structural equation modelling was propitious to show the theoretical causal relationships between inter-correlated variables; thus, it was used to evaluate potential multivariate relationships [80]. Generally, the χ^2 test indicated whether the model was suitable for fitting [80]. We used IBM SPSS Amos 22.0 (Amos Development Corporation, Crawfordville, FL, USA) to operate the SEM model. According to the modification indices, we added new paths to improve the model adaptability, which could also be legitimately explained. The modified model adequately fit these data ($\chi^2 = 4.249$, $p = 0.643$), and the other model fit information are shown in Supplementary Table S4 [see Additional file 1]. The contribution values of soil nutrients and enzyme observational variables to *O. sinensis* were calculated by aggregation tree analysis in R with the package gbmplus [81, 82].

Abbreviations

NR: nitrate reductase

ACP: acid phosphatase

TN: total nitrogen

TP: total phosphorus

TK: total potassium

OM: organic matter

AP: available phosphorus

Kppm: rapidly available potassium

SEM: structural equation modeling

CK: control group

FW: fresh weight of the *O. sinensis*

PCA: principal component analysis

KMO: kaiser meyer olkin

ANOVA: analysis of variance

Declarations

Ethics approval and consent to participate

The samples of *O. sinensis* and soil were collected in the wild or in the natural habitat of this species on private land, the vegetation was investigated in the wild, all complying with institutional and national guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

KY conceived this project and formulated the idea. XLD performed the experimental work, data analysis, XLD and AF prepared the manuscript. All authors discussed the interpretation of the results and provided editorial advice.

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Additional files

Additional file 1.docx: Supplementary Table S1, S2: Detailed data relating to the statistical analysis, including: Clusters in eigenvalue of *O. sinensis* before and after adjusting by eye; Supplementary Table S3: ANOVA table on the effects of the environmental variables on *O. sinensis*; Supplementary Table S4: results of structural equation modelling of the environmental variables on *O. sinensis* illustrated in Fig.1.

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Figures

Figure 1

SEM results for the network among enzymes, nutrients, nematodes, pH, moisture and *O. sinensis*. The khaki-coloured boxes display the environmental variable indicators, and the blue box displays the indicator of *O. sinensis*. Solid-lined arrows represent significant positive relationships ($P < 0.05$); dotted-lined arrows represent nonsignificant positive relationships (black means positive, red means negative). The grey boxes represent hypothetical variables not measured in this

study, and the dashed grey arrows show the proposed mechanisms that explain the relationships. The pie charts represent the contributions of enzymes and nutrient variables to the quality of *O. sinensis* and were respectively calculated with aggregation tree analysis. NR: nitrate reductase, TN: total nitrogen, TP: total phosphorus, OM: organic material, AP: organic phosphorus, Kppm: rapidly available potassium

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