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Impact of inoculum density of *Fusarium oxysporum* f. sp. *zingiberi* on symptomatic appearances and yield of ginger (*Zingiber officinale* Roscoe)

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Abstract

Ginger (*Zingiber officinale* Roscoe) is an important horticultural crop valued for its medicinal and culinary properties. Fusarium yellows, caused by the ascomycete fungus *Fusarium oxysporum* f. sp. *zingiberi* (*Foz*), is a devastating soil-borne disease of ginger. It has curtailed ginger production in Australia and around the world, leading to significant economic losses. An integrated approach is required to manage soil-borne diseases such as those caused by *Foz*. However, little is known about the influence of *Foz* inoculum on disease severity. This study aimed to establish a minimum threshold level of spores per gram of soil required for plant infection and to develop and evaluate a pot inoculation method for screening large numbers of plants in a controlled environment. To achieve this, the dominant Australian ginger cultivar Canton was inoculated with 10^1, 10^3, 10^5, 10^6 and 10^7 microconidia g^{-1} soil. The inoculum density was positively associated with leaf and stem yellows, and rhizome discolouration, and negatively associated with root length and rhizome weight. The lowest threshold required for plant infection was 10^1 microconidia g^{-1} soil, which may provide an important basis for outbreaks of *Foz* in the field. This finding adds significantly to our knowledge of the impact of soil health on ginger production, thereby contributing to the integrated management of *Foz*. When used at a high dose, this method can facilitate reliable and accurate screening of *Foz*-susceptible ginger genotypes in a controlled environment.

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article.

INTRODUCTION

Ginger (*Zingiber officinale* Roscoe) is an important horticultural crop valued for its medicinal and culinary properties. It has traditionally been used to reduce nausea, provide remedies for influenza and the common cold, and aid digestion [1]. In food preparations, ginger has been widely used as a cooking spice, and it is also consumed as ginger ale and tea. Although ginger is consumed all over the world, its cultivation is limited to the tropics and subtropics, with a global production of over 200000 tonnes of ginger rhizomes per annum [2]. In Australia, the ginger industry has a market value of AU $116 million per annum [3], with production primarily based in southeast Queensland.

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Keywords: crop yield; *Fusarium oxysporum* f. sp. *zingiberi*; Fusarium yellows; ginger; inoculum dose; *Zingiber officinale* Roscoe.
Abbreviations: ANOVA, analysis of variance; bp, base pairs of nucleotides; BRIP, Brisbane Plant Pathology Herbarium; °C, degree Celsius; cm, centimetre; D, depth; DNA, deoxyribose nucleic acid; FFSC, *Fusarium fujikuroi* species complex; FIESC, *Fusarium incarnatum-equiseti* species complex; FOSC, *Fusarium oxysporum* species complex; *Foz*, *Fusarium oxysporum* f. sp. *zingiberi*; f. sp., *forma specialis*; FSSC, *Fusarium solani* species complex; g, gram; GTR, general time reversible model; h, hour; INDEL, insertions and deletions; L, length; LSD, least significant difference; MAFFT, multiple alignment using fast Fourier transform; MCMC, Markov Chain Monte Carlo; mL, millilitre; mm, millimetre; µmol/m^2/sec, micromole per square metre per second; *n*, sample size; NCBI, National Center for Biotechnology Information; NRRL, Agricultural Research Service Culture Collection; PCR, polymerase chain reaction; RDI, rhizome discolouration index; rpm, revolutions per minute; SNP, single nucleotide polymorphism; spp., species; SPSS, Statistical Package for Social Science; *TEF-1*α, translation elongation factor 1-alpha; UQ, The University of Queensland; W, width.
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Impact Statement

Phenotypic screening of plants is required for the identification of host resistance against agronomically important soil-borne pathogens. In the case of *Foz*, a reliable phenotyping method needs to account for environmental variations as well as the sporadic expression of *Foz* symptoms. By establishing a consistent methodology for optimal parameters for plant growth, we intended to determine the level of inoculum dose required to induce uniform *Foz* infection. The outcomes of this study will aid future research into ginger infection by *Foz* and help us to understand the threshold level of *Foz* that is required to cause an outbreak in the field, potentially contributing to an integrated approach to manage this important disease.

Globally, ginger production is threatened primarily by rhizome rot or root rot diseases caused by seed and soil-borne pathogens such as *Fusarium* spp. and *Pythium* spp., with these being major contributing factors in yield reduction [4–6]. *Fusarium oxysporum* strains infect hundreds of plant species, producing symptoms such as wilts and yellows. However, each strain, or *formae specialis* (f. sp.), is host-specific and is generally named based on the host to which it is pathogenic [7]. *Fusarium oxysporum* f. sp. *zingiberi* (*Foz*) is pathogenic against ginger, and is known by the common names Fusarium yellows [4] or Fusarium wilt [8]. External symptoms of Fusarium yellows include stunted growth and yellowing and drooping of older leaves, leading to senescence of individual tillers [9]. However, it is the internal discolouration and rotting of the rhizomes that is associated with the reduction of ginger rhizome size and quality. Internally, this brown discolouration is observed in the water-conducting regions of the rhizome and rotting of the tissues in the rhizome cortex [9].

Once the soil is infected, *Foz* is thought to survive as chlamydopores in the absence of a susceptible ginger host and hence can persist for years in the form of these resting spores in the soil [10]. The inoculum concentrations in this study only refer to microconidia. It has not been established if macroconidia or chlamydospores would produce similar results. Chlamydospores, as long-term survival structures, may be the primary source of infection after an extended absence of ginger and it would be particularly beneficial to better understand the role of these other spores in infection cycles.

Commercial production of ginger in Australia relies predominantly on two commercial ginger cultivars, Canton and Queensland [11]. However, both cultivars are susceptible to *Foz* [9, 12]. The need to address the lack of *Foz*- and other pathogen-resistant genotypes in the Australian ginger industry [13] is an important priority for research and investment [14]. It is therefore important that an efficient method for screening ginger cultivars for resistance to pathogens such as *Foz* is developed to expedite elite cultivar release.

To study the efficacy of *Foz* infection, we performed an experiment using five different concentrations of *Foz* microconidia and analysed the effect of soil spore concentration on disease development in the ginger cultivar Canton, which is the main culinary cultivar produced in Australia [9]. The impact of *Foz* infection on ginger plants was apparent, with symptoms including leaf yellowing and reduced rhizome weight. *Foz* was detected on plants subjected to as little as 10 microconidia g^{-1} soil. Symptom severity was positively associated with the amount of spores in the inoculum and negatively associated with plant development. The outcome from this study contributes to our understanding of the threshold levels of *Foz* required to cause an outbreak of Fusarium yellows in the field as well as provide an optimized methodology for screening ginger genotypes for *Foz* resistance as new cultivars are developed in the future.

METHODS

Four *Fusarium oxysporum* strains (UQ6848, UQ6851, UQ6852 and UQ6853) were isolated from ginger plants symptomatic for yellows from the following locations in southern Queensland, Australia: Gympie (one), Bundaberg (one) and Nambour (two). *Formae specialis* of these isolates were confirmed to be *Fusarium oxysporum* f. sp. *zingiberi* based on their ability to cause Fusarium yellows in ginger pot trials and by phylogenetic analysis based on the translation elongation factor 1-alpha (*TEF-1α*) gene of the isolates [15]. Monoconidial isolates were subsequently reisolated using hyphal tips of single germinated conidia. Spore suspensions were prepared for the four isolates according to a previously published method [9] and then mixed in equal ratios of spore concentrations prior to plant inoculation. The isolates were mixed in equal proportions to allow for differences in virulence between isolates. To generate the spore suspensions, flasks of potato dextrose broth were inoculated with each of the isolates and were incubated for 7 days at 26 °C with shaking at 120 r.p.m. on a rotating platform (Ratek, Victoria, Australia). The spores were then extracted, counted and adjusted using sterile water to 10^2, 10^4, 10^6, 10^7 and 10^8 microconidia ml^{-1} for inoculation on ginger plants, as per a previous study [9].

DNA was extracted from the mycelia of the four isolates using a microwave method [16]. *TEF-1α* was PCR-amplified using published primers (5′-ATGGGTAAGGARGACAAGAC) and (5′-GGARGTACCAGTSATCATGTT) [15] and DreamTaq (Thermo Fisher Scientific, Waltham, MA, USA). A single product of 656 bp was visualized on a 1% agarose gel and then purified using a

GeneJET PCR purification kit (Thermo Fisher Scientific, Waltham, MA, USA), and Sanger-sequenced at the Australian Genome Research Facility, Melbourne, Australia.

TEF-1α sequences representing 156 isolates belonging to four *Fusarium* species complexes were retrieved from previous studies [9, 15, 17, 18]. Geneious Prime v 2023.0.1 (Biomatter Pty Ltd, Auckland, New Zealand) was used for the phylogenetic reconstruction of the isolates. Firstly, multiple sequence alignment was performed using MAFFT v 7.490 [19]. The subsequent alignment was used to pick regions with minimized gaps and derive a consensus alignment sequence of 543 bp, which was then used as an input in MrBayes v 3.2.6 to reconstruct the phylogeny using the Bayesian inference method [20]. The running parameters used the GTR-G-I model of substitution with two independent analyses on four Markov chain Monte Carlo (MCMC) chains for 2000000 generations. A burn-in rate of 25% was used to sample every 1000 generations. *Fusarium delphinoides* (NRRL36160) was used as an outgroup to anchor the phylogenetic tree. The tree branches were transformed into a cladogram and visualized in Geneious Prime.

The ginger plantlets of cultivar Canton were produced by *in vitro* tissue culture [21, 22] and then grown in a glasshouse to maintain a disease-free state at the Maroochy Research Facility, Queensland, Australia. A quantity of approximately 400 g of a premium potting mix (Searles, Kilcoy, Queensland, Australia) was used per 100 mm (L) × 100 mm (W) × 120 mm (D) square pot. In total, 53 plants of the susceptible ginger cultivar Canton at a stem height of 15–20 cm carrying 8–12 leaves each were transferred to a Conviron GEN1000 TA plant growth chamber (Winnipeg, MB, Canada) at the University of Queensland, St Lucia, Australia. The growth parameters include a 14 h light/10 h night photoperiod regime with lights set to 100% intensity at a photon irradiance rate of 700 µmol m^{-2} s^{-1}, and day and night temperatures kept at 27 and 24 °C, respectively. Watering was done three times a week. Plants were climatized in the chamber for 1 week. For each pot, 40 ml of a concentrated spore suspension was mixed with 400 g of soil and the plant was potted into the inoculated soil. The final spore concentrations were 10^1, 10^3, 10^5 and 10^7 microconidia g^{-1} of soil, respectively. Control plants were mock inoculated with sterile water. All but the 10^7 microconidia g^{-1} of the soil treatment group had 10 plants each. For the 10^7 microconidia g^{-1} of the soil treatment group, due to a limited availability of plants, only three plants were used. Each pot was self-contained in a zip-lock bag and grouped with replicates. External and internal symptoms were assessed 8 weeks post-inoculation. Interval symptom was scored using a 1–6 severity scale based on the visual identification of discoloured regions in the rhizome (Fig. 1). Reisolation of *Foz* in the rhizome, roots and tillers was performed according to a previous study [9].

The statistical software SPSS v28.0.1.0 (142) (IBM Corp., Armonk, NY, USA) was used to perform one-way analysis of variance (ANOVA) in a pair-wise manner, with measurements for the various traits set as dependent variables and the treatments as the categorical variable. Waller–Duncan's multiple range testing was performed as a post-hoc test to separate the means into subsets by least significant difference (LSD). The harmonic mean sample size of 7.2 was estimated and used to account for the unequal variance associated with the uneven sample size (n) of the treatments. The type 1/type 2 error seriousness ratio (k-ratio) was set to 100 (α=0.05).

RESULTS AND DISCUSSION

TEF-1α sequencing showed that UQ6848 (NCBI GenBank: OR425153), UQ6851 (OR425154), UQ6852 (OR425155) and UQ6853 (OR425156) shared 100% nucleotide identity with each other, as well as with three other known *Foz* isolates (*Foz* Goomboorian, *Foz* Eumundi, *Foz* BRIP44986). Their sequences differ from that of *Foz* BRIP39299 by three SNPs and a single base INDEL. The phylogenetic tree reconstructed using Bayesian inference produced a topology that separated the four species complexes of *Fusarium* (Fig. 2). This phylogeny was presented, and its topology described in a previous study [18]. Within the *Fusarium oxysporum* species complex (FOSC), all four isolates used in this study are clustered together in a subclade with known *Foz* isolates, including *Foz* Goomboorian, *Foz* Eumundi, *Foz* BRIP44986 and *Foz* BRIP39299 (Fig. 2). The sequences and the phylogenetic positions of these four isolates within FOSC confirmed their *Foz* identity.

Inoculum concentration had a discernible effect on the severity of Fusarium yellows in ginger cultivar Canton (Fig. 3). *Foz*-inoculated plants could be easily differentiated from uninoculated controls by the development of yellowing symptoms typical of *Foz* infection (Fig. 3a). The degree of senescing leaves and tillers was positively associated with the inoculum dosage (Fig. 3b), with sporadic yellowing of stems and leaves observed at the low-to-medium inoculum concentration (10^1, 10^3, 10^5 microconidia g^{-1} soil) in comparison to the uninoculated controls which showed little-to-no yellows. *Foz* susceptibility was exacerbated at the high inoculum levels (10^6, 10^7 microconidia g^{-1} soil), where tiller abortions due to Fusarium yellows occurred at a relatively high rate and the whole root systems appeared discoloured (Fig. 3b).

Internally, rhizome discolouration was positively and quantitatively associated with the inoculum level (Fig. 4a). While the uninoculated controls were completely clean, the 10^1 microconidia g^{-1} of soil dose produced a low but significant level discolouration with a mean score of 2 (<10%). This difference is also associated with a significant reduction in rhizome size but not with root length or number of tillers alive (Fig. 4b-d). This suggests that a low dosage of *Foz* spores can still infect the rhizome, potentially reducing the crop yield despite plants inoculated at low spore concentrations having a similar number of tillers or length of root systems as uninoculated

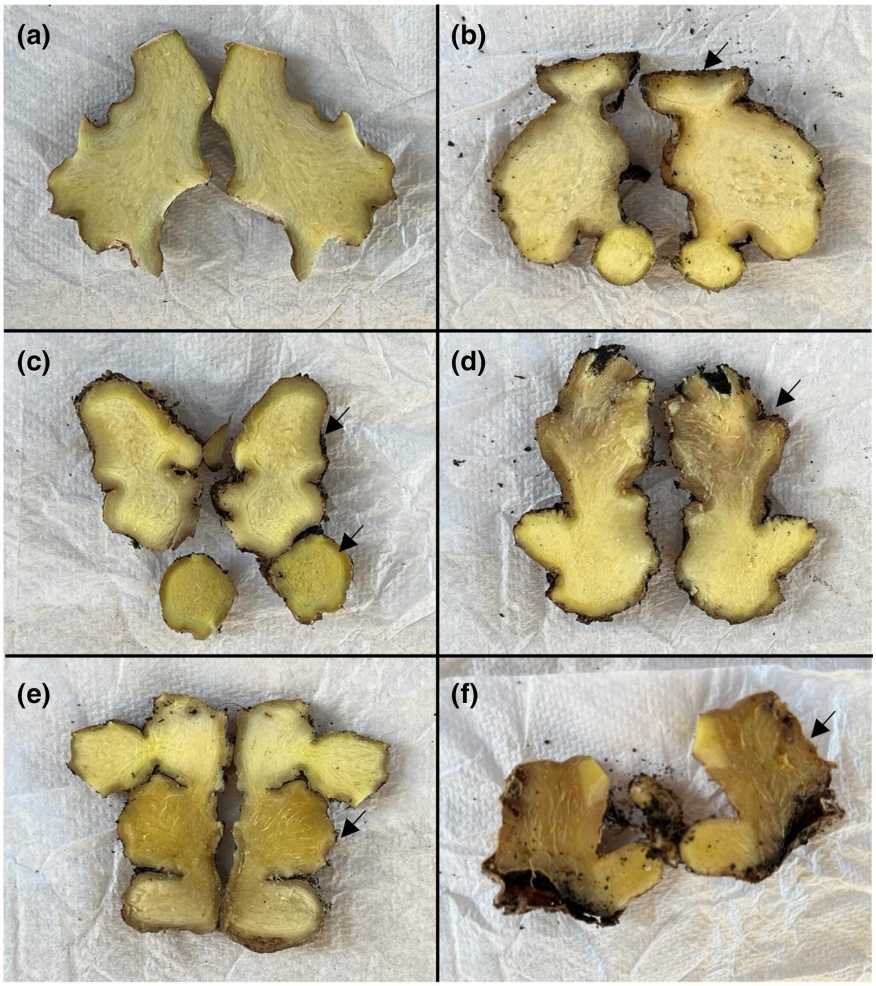

Fig. 1. Ginger rhizome discolouration index used to score the internal symptoms of the diseased plants in this study. The scale was (a) completely clean, no discolouration observed; (b) less than 10% discolouration including edge effects or small patches, where it is not completely clean; (c) brown discoloured areas occupying over 10% and up to 25% of the rhizome; (d) brown discoloured areas occupying over 25% and up to 50% of the rhizome; (e) brown discoloured areas occupying over 50% and up to 75% of the rhizome; (f) brown discoloured areas occupying over 75% and up to 100% of the rhizome. Arrows indicate the visible regions of discolouration in the rhizomes.

controls. The rhizome discolouration index (RDI) provided the best sensitivity for differentiating the dose levels amongst the four traits scored (Fig. 4a). RDI differentiated the low dose 10^1 microconidia g^{-1} soil from that of control, as well as differentiating medium doses of $10^3/10^5$ microconidia g^{-1} soil from the high doses $10^6/10^7$ microconidia g^{-1} soil. This quantitative effect of discolouration is negatively associated with a reduction in root length and rhizome weight (Fig. 4b). However, rhizome weight seems to be most sensitive to *Foz* infection, with 10^3, 10^5, 10^6, 10^7 microconidia g^{-1} soil treatments all suffering similar weight reductions (Fig. 4c).

Furthermore, reisolation of *Foz* showed that it was present in the rhizome of all treatment groups and in the roots of all except the plants subjected to the spore concentration of 10^1 microconidia g^{-1} soil (Table 1). The alive tillers count was the least affected by *Foz*, with a significant reduction only observed in 10^6 microconidia g^{-1} soil or higher dosage (Fig. 4d). This is consistent with the presence of *Foz* detected in the tillers of treatment groups 10^6 and 10^7 microconidia g^{-1} soil but not in the other treatments or the control (Table 1). These observations demonstrate that the external symptoms above ground may not necessarily reflect the reduction in rhizomes and roots below ground at low *Foz* doses. At the high doses of 10^6 and 10^7 microconidia g^{-1} soil, the saturation of spores in the soil allowed plants to be uniformly infected, leading to the wilting of whole plants or even plant death. Evaluation methods for screening *Fusarium* wilt resistance have been available for other plant species such as cotton and lettuce [3, 23], but the absence of a *Fusarium* screening method for ginger has hampered disease research. A previous study also demonstrated that the spore concentrations of fungal pathogens have an effect on the wilt symptoms of ginger [8]. In comparison, the data generated in this study show that ginger can respond to *Foz* at a lower or higher dose than 10^3–10^5 microconidia g^{-1} soil, allowing sensitivity in host responses against *Foz* to be refined.

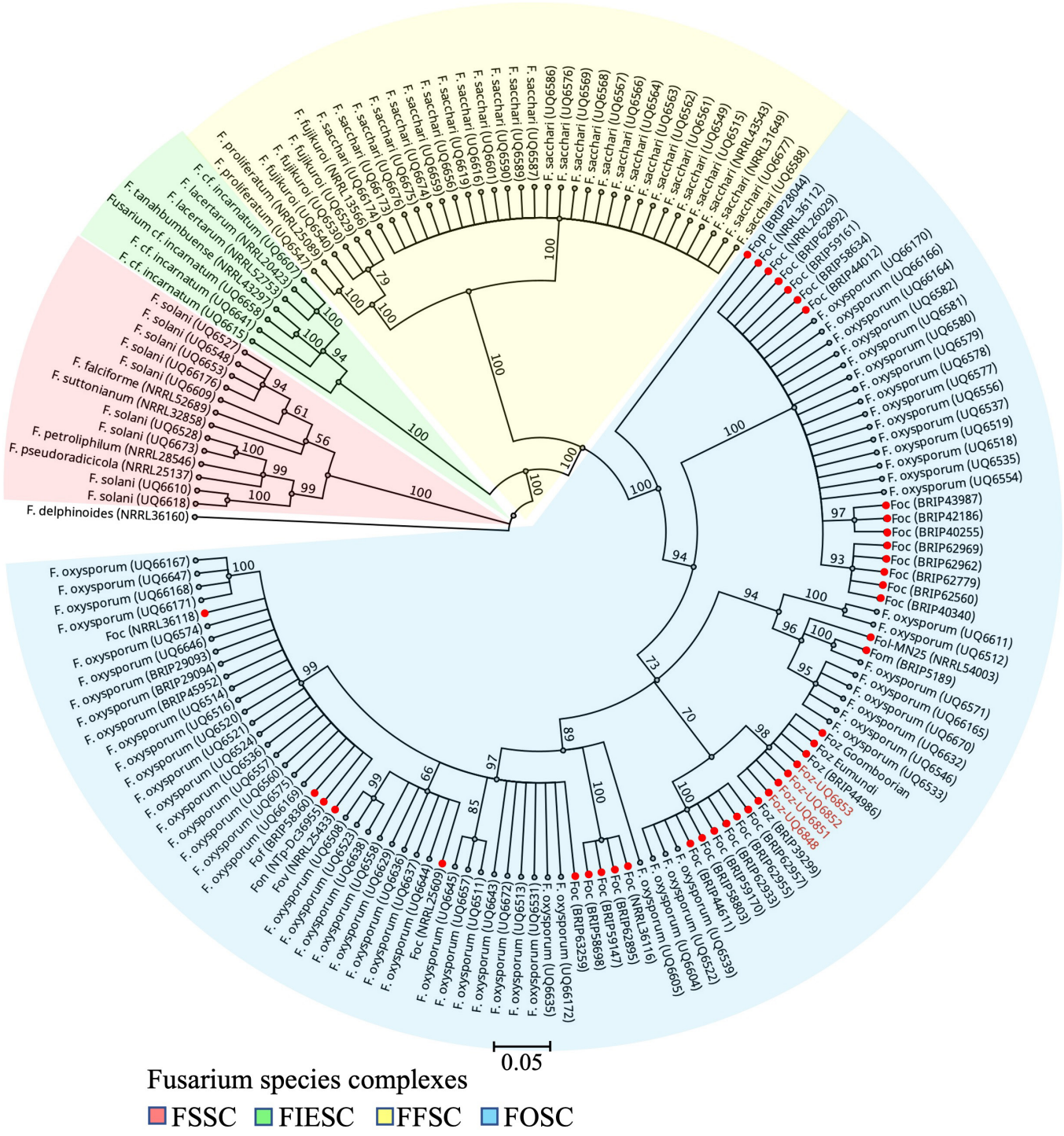

Fig. 2. Determination of the phylogenetic positions of *Foz* isolates UQ6848, UQ6851, UQ6852 and UQ6853 within the *Fusarium oxysporum* species complex using translation elongation factor 1-alpha (TEF-1α). The four isolates are highlighted in red. The four *Fusarium* species complexes presented in this Bayesian inference phylogeny include the *Fusarium incarnatum-equiseti* species complex (FIESC); the *Fusarium fujikuroi* species complex (FFSC); the *Fusarium oxysporum* species complex (FOSC); and the *Fusarium solani* species complex (FSSC). Where it is known, the *formae speciales* of pathogenic isolates within FOSC are indicated with a red circle. Branch labels indicate the posterior probability as a percentage. A scale range of 0.05 is indicated below the tree.

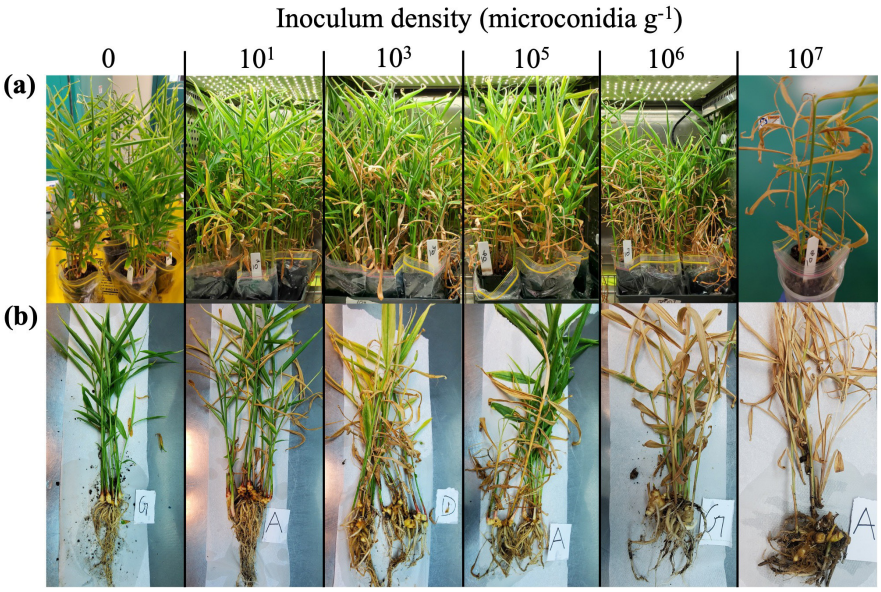

Fig. 3. A qualitative assessment of external symptoms of Fusarium yellows on *Foz*-susceptible ginger cultivar Canton. Plants were examined at 8 weeks post-inoculation. (a) Potted ginger cultivar Canton plants at the time of harvest. (b) Individual representative plants from each treatment with above and below ground level portions cleaned and then visualized.

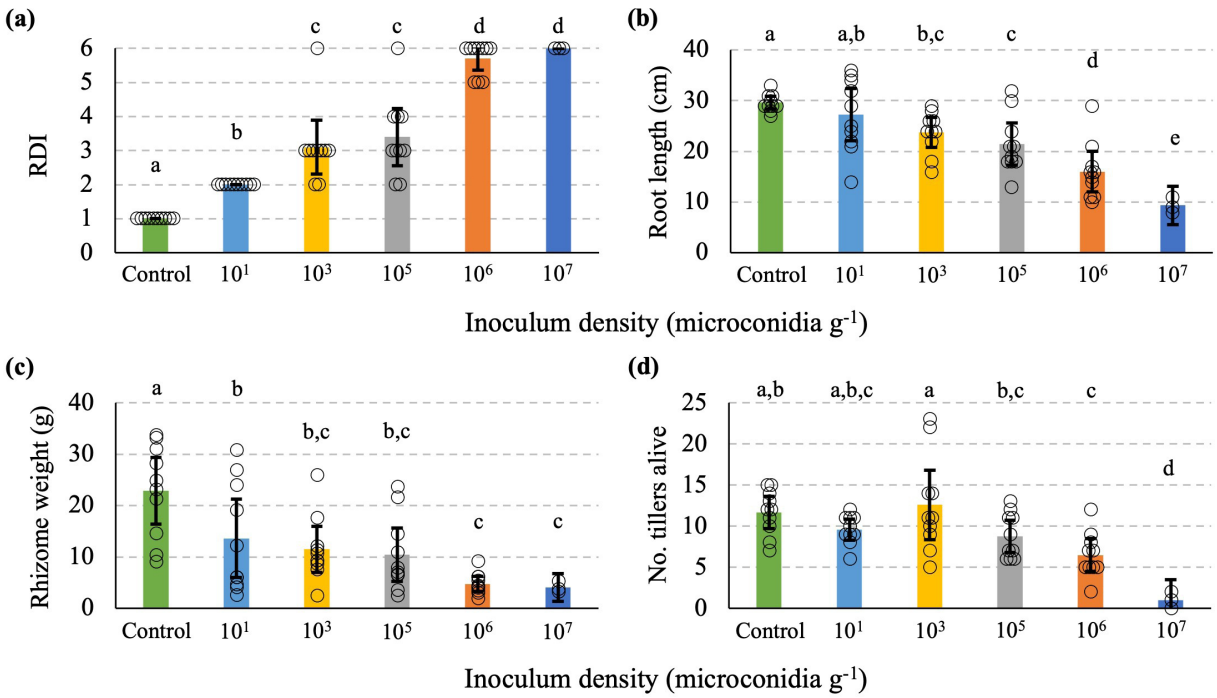

Fig. 4. Characterization of *Foz*-infected ginger cultivar Canton plants at 8 weeks post-inoculation. (a) Rhizome discolouration index (RDI) scored using a 1–6 scale: 1, clean rhizome; 2, >0 and <=10%; 3, >10% and <=25%; 4, >25% and <=50%; 5, >50% and <=75%; 6, >75% and <=100% discolouration. (b) Root length in centimetres of the longest adventitious root of a rhizome node. (c) Weight of the entire rhizome of the ginger plants. (d) Number of tillers that were still green or alive at the time of harvest. Control, uninoculated plants. The treatments were 10^1, 10^3, 10^5, 10^6 and 10^7 of microconidia g^{-1} soil. n=10 for each treatment from 10^1 to 10^6, n=3 for 10^7. The errors bars indicate 95% confidence intervals of the means. Means for each treatment group were pairwisely seperated by Duncan's multiple range test at α=0.05. Matching superscript letters above data columns (Duncan grouping) indicate that their means are not significantly different from one another at $p \leq 0.05$.

Table 1. Three plants randomly selected from each treatment group (n=10, labelled A to J for each treatment from 10^1 to 10^6, n=3 for 10^7) were used to reisolate *Foz* from the rhizome, roots and tillers. '+' indicates a positive colony that conformed to a typical *F. oxysporum* f. sp. *zingiberi* morphology [9, 32] and '–' indicates the absence of any *F. oxysporum*-like colonies

Treatment	Plant	Rhizome	Roots	Tillers
Control	B	–	–	–
Control	D	–	–	–
Control	F	–	–	–
10	C	+	–	–
10	E	+	–	–
10	H	+	–	–
10^3	F	+	+	–
10^3	I	+	–	–
10^3	J	+	–	–
10^5	A	+	+	–
10^5	E	+	+	–
10^5	F	+	–	–
10^6	A	+	–	+
10^6	B	+	+	–
10^6	G	+	+	–
10^7	A	+	+	+
10^7	B	+	–	+
10^7	C	+	+	–

Management of soil-borne pathogens is a significant challenge for ginger production in Australia, with diseases such as Fusarium yellows, Pythium rot caused by *Pythium myriotylum* [6] and root-knot nematodes (*Meloidogyne incognita* and *M. javanica*) [24, 25] requiring a combination of management techniques, including fallowing land, improving soil health, managing drainage and the use of biological control agents and clean rhizomes for planting [26]. The application of biocontrol control agents such as *Trichoderma* has the potential to control and reduce *Fusarium* spp. and other soil phytopathogens [27]. One of the key aspects of controlling Fusarium yellows is the improvements in minimizing the inoculum pressure for diseases in the field by using clean planting materials and schemes through tissue culture [28] or using material produced from clean mother blocks. In Australia, ginger is produced mainly on red ferrosol soils and is managed intensively with inputs and tillage during multiple annual production cycles before the field is rotated to fallow crops [3, 29, 30]. When disease outbreaks occur, it is important to understand the infection process and disease threshold so that *Foz* can be diagnosed and monitored through immunological, phylogenetic and pathogenicity-related approaches [31].

This study has developed a rigorous methodology to test the virulence of *Foz* isolates on ginger plants by establishing a minimum threshold level of microconidia per gram of soil required for infection in a controlled environment. This screening method can be used to assess *Foz* resistance levels in wild relatives and cultivars of ginger. Rhizome discolouration offers a reliable trait marker for screening ginger's susceptibility to *Foz*. Therefore, it can be used to rapidly fast track elite resistance phenotypes for introduction into commercial ginger cultivars. Further optimizations can potentially allow this method to be adapted to screen for other plant–soil phytopathogen interactions.

Plant breeding for disease resistance is a part of plant disease management. Integrated management practices, including improvements in soil health, addition of organic amendments such as poultry manure and sawdust, annual rotations of suitable cover crops and prolonged fallow between ginger cultivations will help reduce but not completely eliminate *Foz* in the soil [29, 30]. Even with management practices applied, re-emergence of the disease can potentially override biological suppression developed through improved soil health [3]. It is therefore important to ensure, where possible, that cropping soil used for ginger is not contaminated with soil-borne pathogens such as *Foz*, as it has been demonstrated that even low numbers of microconidia per gram of soil can still result in significant loss of rhizome quality and quantity, and therefore crop yield.

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Author contributions
Conceptualization, S.H., E.A.B.A., A.M.; methodology, S.H., A.M., A.C.; validation, S.P.T.M., A.C.; investigation, S.P.T.M., A.M., A.C.; formal analysis, S.P.T.M., A.C.; data curation, A.C., S.P.T.M.; resources, S.H., A.M.; writing – original draft preparation, A.C.; writing – review and editing, A.C., A.M., S.H., E.A.B.A.; supervision, E.A.B.A., A.C., S.H., A.M.; project administration, E.A.B.A.; funding acquisition, E.A.B.A. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000605.v2.3

Justine Rudkin; University of Oxford, Nuffield Department of Population Health, UNITED KINGDOM, Oxford

Date report received: 17 August 2023
Recommendation: Accept

Comments: Thank you for so thoroughly addressing the reviewers comments and giving a point by point rebuttal to each one to make my job easier. I am satisfied that each point raised by the reviewers has been addressed, including the addition of extra MLST data and photographs of the scoring system used for disease manifestation. Thank you to both reviewers for such thorough and constructive reviewers. They have certainly improved the manuscript.

SciScore report

https://doi.org/10.1099/acmi.0.000605.v2.1

iThenticate report

https://doi.org/10.1099/acmi.0.000605.v2.2

Author response to reviewers to Version 1

Manuscript number: ACMI-D-23-00057

We would like to thank both reviewers and the handling editor for their time in reviewing this manuscript.

Reviewers' comments and responses to custom questions:

Reviewer 1 Comments to Author: Lines 1-3: Lowest may refer that 10 cfu/gm soil be the least and there may be a need to run logistic or other growth models to showcase the clearcut point where the threshold is significant. Anything <10 cfu? This reviewer suggests modification of a title to "Impact of inoculum density of F.o.z. on symptomatic appearances and yield of ginger." or modified version of it.

Response: The title has been modified to 'Impact of inoculum density of *Fusarium oxysporum f. sp. zingiberi*on symptomatic appearances and yield of ginger (*Zingiber officinale*Roscoe)'.

Line 32: Horticultural

Response: corrected.

Line 35: Remove 'dosage'

Response: corrected.

Lines 36-38: Two objectives set and readers may anticipate outcomes for each of them.

Response: We removed the reference to *Foz*resistance as we have not tested any *Foz*resistance plants in this study. Instead, we modified the sentence to reflect the development of a screening methodology.

'This study aimed to establish a minimum threshold level of spores per gram of soil required for plant infection and to develop and evaluate a pot inoculation method for suitability in screening large number of plants in a controlled environment.'

Line 39: Remove 'pre-determined amounts of colony-forming unit (cfu), ranging 40 from 0 in control, to treatments with'

Response: corrected.

Line 40: 10(1) better to add the superscript to imply 10 to the power 1. Add colony-forming unit (cfu) after 107

Response: corrected.

Line 41: Change dosage to density

Response: corrected.

Line 45: Change to 'impact of soil health to ginger production thereby contributing' to the integrated…

Response: corrected.

Line 70: Globally, ginger production is threatened primarily by rhizome rot or root rot diseases primarily caused by seed and soil-borne pathogens caused by Fusarium spp. and Phytium spp.

Response: corrected.

'Globally, ginger production is threatened primarily by rhizome rot or root rot diseases primarily caused by seed and soil-borne pathogens such as Fusarium spp. and Phytium spp.'

Response: corrected.

Line 71: Remove fungal

Response: corrected.

Line 75: stunted growth and yellowing ultimately leading to dropping and untimely senescence of old leaves. Authors may have to check if dropping of leaves is common.

Response: We changed 'dropping' to 'drooping'.

Line 84: Since the central theme of the study focuses on density/dosage, authors need to clarify with the use of the terms and preferrably pinpoint if only micro-conidia (what about macro-conidia?) are used and across the manuscript terms need to be standardized to conidial concentration etc.d

Response: We added the following sentences to clarify the use of the terms in describing the inoculum.

'The inoculum concentrations in this study only refer to microconidia. It has not been established if macroconidia or chlamydospores would produce similar results. Chlamydospores, as long-term survival structures, may be the primary source of infection after an extended absence of ginger and it would be particularly beneficial to better understand the role of these other spores in infection cycles.'

Line 87: symptoms? extent of symptom development or severities ... please rewrite. The intent I believe was to correlate inoculum density vs. symptom or plant development

Response: This sentence was rewritten as the following:

'Symptom severities positively associated with the amount of spores in the inoculum and negatively associated with plant development.'

Line 95: Remove 'Fusarium'

Response: removed.

Line 135: 'significant' - discernible (I wasnt sure if the term is used to show any statistical significance or not). Authors may pay attention to usage of similar contextual inferences

Response: changed to discernible.

Line 136: This reviewer wishes to see a high resolution 1-6 scaling as referenced by the authors. Next, assigning the average visual scoring of each picture (in Fig. 1) would give the reader a better understanding.

Response: As per reviewer's request, we've presented high-resolution images of symptoms scaled 1-6 as Figure 1. The corresponding text in the methods have also been changed accordingly (original document line 124).

Line 170: 'A previous study' - Doesnt seem to be a reference to the same pathogen, please clarify

Response: We changed that to the following:

'A previous study also demonstrated that the spore concentration of fungal pathogens have an effect on the wilt symptoms of ginger.'

Line 179: 'Reference 15' - Recent studies with positive outcomes related to use of Trichoderma-based BCAs in temperate areas can be added here.

Check reference 7

Response: Added a sentence to discuss the potential use of biological control agents against soil phytopathogens such as Fusarium.

'The application of a biocontrol control agent such as *Trichoderma*has the potential to control and reduce *Fusarium*spp. and other soil phytopathogens [27].'

27Tyśkiewicz R, Nowak A, Ozimek E, Jaroszuk-Ściseł J. *Trichoderma*: The current status of its application in agriculture for the biocontrol of fungal phytopathogens and stimulation of plant growth. *Int J Mol Sci*2022;23:2329. https://doi.org/10.3390/ijms23042329

Please rate the manuscript for methodological rigour

Reviewer 2: Satisfactory

Please rate the quality of the presentation and structure of the manuscript

Reviewer 2: Good

To what extent are the conclusions supported by the data?

Reviewer 2: Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

Reviewer 2: No:

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Reviewer 2: Yes:

Reviewer 2 Comments to Author: This manuscript determines the infection threshold and dose-associated symptoms of Fusarium oxysporum f. sp. Zingiberi in the ginger cultivar "Canton", which is of importance in Australia. The research objectives are clearly stated, although to be completely met further cultivars would be included in the experimental design. Notwithstanding, the provided methods and results could be of help for applying in other cultivars. Methods are adequately described to reproduce the experiments once the minor suggestions below are implemented. Identification of Foz isolates by molecular methods would be required, at least to confirm the species. Statistical analysis was appropriate. In general, the article is well written and conclusions are sound by the presented results. Below, my suggestions for improvement of the manuscript are found:

1. Methodological rigour, reproducibility and availability of underlying data

- In the abstract, authors declare that their research objectives were:

i) "to establish a minimum threshold level of spores per gram of soil required for plant infection" (lines 36-37)

ii) "to develop and evaluate a pot inoculation method for suitability in genetic studies on Foz resistance" (lines 37-38)

In my opinion, i) has been met, but ii) was only partially covered by the experiment, since for that aim, different cultivars with variable resistance to Foz should be included and compared.

Response: We would like to clarify that the objective ii) was meant to develop a suitable screening methodology in a controlled environment that can be used to screen for varietal differences in resistance in the future. We have not performed any resistance screen in this study so therefore we have reworded the second objective to:

'..to develop and evaluate a pot inoculation method for suitability in screening large number of plants in a controlled environment.'

- Line 83: authors state: "To study the efficacy of Foz infection, we performed a replicated experiment using five different concentrations of Foz microconidia", but indeed it is not indicated if the experiment was replicated and results reproducible. Please, add clarification.

Response: Apologies for the confusion. It is a single experiment to determine the Foz dosage response. We have removed the word 'replicated'.

However, this is an established method has worked well in disease assays and gene expression analysis on ginger plants. See our publication [9].

- Lines 96-98: authors state that "Formae specialis of these isolates were confirmed to be Fusarium oxysporum f. sp. zingiberi based on their ability to cause Fusarium yellows in ginger pot trials". Why didn't authors complement the identification with a more accurate method to determine whether those isolates are classified as F. oxysporum f. sp. Zingiberi, for example, using molecular markers? It would be required to identify molecularly the isolates at species level.

Response: We have since performed *TEF-1α* sequencing on these 4 isolates and created a Bayesian inference phylogenetic analysis using isolates belonging to different Fusarium species complexes to show that they are most likely Foz.

The corresponding changes (revised version) are as follow:

Line 124-126 (methods)

'…. by phylogenetic analysis based on the translation elongation factor 1-alpha (*TEF-1α*) gene of the isolates [15].'

Line 136-153 (methods section)

'DNA was extracted from the mycelia of the four isolates using a microwave method [16]. *TEF-1α*was PCR-amplified using published primers (5′-ATGGGTAAGGARGACAAGAC) and (5′-GGARGTACCAGTSATCATGTT) [15] and Dreamtaq (Thermo Fisher Scientific, Waltham, MA, USA). A single product of 656 bp was visualised on a 1% agarose gel and then purified using a GeneJET PCR purification kit (Thermo Fisher Scientific, Waltham, MA, USA), and Sanger-sequenced at the Australian Genome Research Facility, Melbourne, Australia.

TEF-1α sequences representing 156 isolates belonging to four *Fusarium*species complexes were retrieved from previous studies [9, 15, 17, 18]. Geneious Prime v 2023.0.1 (Biomatter Pty. Ltd., Auckland, New Zealand) was used for the phylogenetic reconstruction of the isolates. Firstly, multiple sequence alignment was performed using MAFFT v 7.490 [19]. The subsequent alignment was used to pick regions with minimised gaps and derive a consensus alignment sequence of 543 bp which was then used as an input in MrBayes v 3.2.6 to reconstruct the phylogeny using the Bayesian inference method [20]. The running parameters used GTR-G-I model of substitution with two independent analyses on four Markov Chain Monte Carlo (MCMC) chains for 2,000,000 generations. A burn-in rate of 25% was used to sample every 1000 generations. *F. delphinoides*(NRRL36160) was used as an outgroup to anchor the phylogenetic tree. The tree branches were transformed into a cladogram and visualised in Geneious Prime.'

Line 192-202 (results)

'*TEF-1α*sequencing showed that UQ6848 (NCBI-GenBank:OR425153), UQ6851 (OR425154), UQ6852 (OR425155) and UQ6853 (OR425156) shared a 100% nucleotide identity with each other, as well as with three other known *Foz*isolates (*Foz*Goomboorian, *Foz*Eumundi, *Foz*BRIP44986). Their sequences differ to that of *Foz*BRIP39299 by 3 SNPs and a single base INDEL. The phylogenetic tree reconstructed using Bayesian inference produced a topology that separated the four species complexes of Fusarium (Figure 2). This phylogeny was presented, and its topology described in a previous study [18]. Within the *Fusarium oxysporum*species complex (FOSC), all four isolates used in this study are clustered together in a subclade with known *Foz*isolates including *Foz*Goomboorian, *Foz*Eumundi, *Foz*BRIP44986 and *Foz*BRIP39299 (Figure 2). The sequence and the phylogenetic positions of these four isolates within FOSC confirmed their *Foz*identity.'

- Lines 100-101: Could authors explain the reason why they mixed 4 four isolates for the inoculum?

Response: We added the following sentence to explain this.

'The isolates were mixed in equal proportions to allow for differences in virulence between isolates.'

- Lines 104-105 and rest of manuscript: My suggestion is to use the unit microconidia/mL or microconidia/g of soil, instead of cfu/mL or cfu/g. For the second, it would be required having a probe that the microconidia had the ability to form colonies (for example, by plating diluted inoculum and counting colonies). Please, strict to this unit in the whole text.

Response: We have changed the unit to microconidia/mL for this and the rest of the document.

- Lines 116-120: Please, rewrite these sentences to clarify how soil inoculation was conducted. As it is explained, it seems that pots with soil were inoculated and then mixed with extra 400 g of soil.

Response: We have rewritten this section as the following:

'For each pot, 40mL of a concentrated spore suspension was mixed with 400g of soil and the plant was potted into the inoculated soil. The final spore concentrations were 10^1, 10^3, $10^5$and 10^7microconidia/g of soil, respectively.'

- Lines 120-122: Please, explain the reason why treatment 107 microconidia/g of soil only had three plants.

Response: Some of the tissue culture plants did not survive the process so we ended up running short in numbers. We added the following:

'For the 10^7microconidia/g of soil treatment, due to a limited availability of plants, only three plants were used.'

- Please, clarify if plants were grouped as replicates.

Response: we added the following.

'Each pot was self-contained in a zip-lock bag and grouped with replicates.'

2. Presentation of results

- General: use of the term "correlation" (lines 41, 87, 88, 138, 156). Authors use the term "correlation" to reflect association of results and trends for different variables. However, I would suggest avoiding this term, since it could lead to the thought that statistical correlation analyses have been conducted, when it is not the case. Please, rephrase those sentences.

Response: These phrases are now changed to '.. are associated' in their respective sentences.

- Line 141: Authors use the term "accelerated", when results do not reflect an earlier appearance of symptoms, but an exacerbation of those. Please, change "accelerated" by "exacerbated".

Response: Changed to 'exacerbated' as requested.

- Line 162: The significant reduction in comparison to control is actually observed in 106 cfu/g of soil or higher. Please, correct it.

Response: Thank you. It is corrected.

- Figure 2: Please add in caption the number of plants for each treatment, as done in Table 1.

Response: added the following:

'n = 10 for each treatment from $10^1$to 10^6,n= 3 for 10^7'

3. Literature analysis or discussion

- Line 85: Please, add a reference.

Response: reference added.

- Discussion: Much information about agricultural practises related to ginger are explained, but I miss further discussion on their relation with this study, its importance, and conclusions; mainly focused on the improvements on disease control and management of crop productivity.

Also, it would be interesting to discuss limitations of the study, for example: plant variety selection, number of plants, isolate virulence, etc.

Response: We've re-written parts of the discussion to highlight the importance of this work in resistance screening and virulence detection.

Line 266-274:

'This research has developed a rigorous methodology to test the virulence of *Foz*isolates on ginger plants by establishing a minimum threshold level of microconidia per gram of soil required for infection in a controlled environment. This screening method can be used to assess *Foz*resistance levels in wild relatives and cultivars of ginger. Rhizome discolouration offers a reliable trait marker for screening ginger's susceptibility to *Foz*. Therefore, it can be used to rapidly fast track elite resistance phenotypes for introduction into commercial ginger cultivars. Further optimisations can potentially allow this method to be adapted to screen for other plant-soil phytopathogen interactions.'

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000605.v1.5

Justine Rudkin; University of Oxford, Nuffield Department of Population Health, UNITED KINGDOM, Oxford

Date report received: 12 July 2023
Recommendation: Major Revision

Comments: The majority of the points raised seem to be easy fixes, but I have selected major revisions as both reviewers highlight that the stated aims have not been fully met by the experimental set up, and this may require either a good rewrite or further experimental work (should the authors wish to go down that road). One point has also been raised about the identification of isolates as Fusarium oxysporum f. sp. zingiberi using disease manifestation rather than accurate molecular techniques, and I am concerned about how robust this method of identification is. Please respond to all reviewers comments below and revise the manuscript accordingly.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000605.v1.3

Sandra Díaz-González; Universidad Politecnica de Madrid Escuela Tecnica Superior de Ingenieria Agronomica Alimentaria y de Biosistemas, Centro de Biotecnología y Genómica de Plantas, Campus UPM - Montegancedo, Lab 235, Pozuelo de Alarcón, SPAIN
https://orcid.org/0000-0003-4061-072X

Date report received: 12 July 2023
Recommendation: Minor Amendment

Comments: This manuscript determines the infection threshold and dose-associated symptoms of Fusarium oxysporum f. sp. Zingiberi in the ginger cultivar "Canton", which is of importance in Australia. The research objectives are clearly stated, although to be completely met further cultivars would be included in the experimental design. Notwithstanding, the provided methods and results could be of help for applying in other cultivars. Methods are adequately described to reproduce the experiments once the minor suggestions below are implemented. Identification of Foz isolates by molecular methods would be required, at least to confirm the species. Statistical analysis was appropriate. In general, the article is well written and conclusions are sound by the presented results. Below, my suggestions for improvement of the manuscript are found: 1. Methodological rigour, reproducibility and availability of underlying data - In the abstract, authors declare that their research objectives were: i) "to establish a minimum threshold level of spores per gram of soil required for plant infection" (lines 36-37) ii) "to develop and evaluate a pot inoculation method for suitability in genetic studies on Foz resistance" (lines 37-38) In my opinion, i) has been met, but ii) was only partially covered by the experiment, since for that aim, different cultivars with variable resistance to Foz should be included and compared. - Line 83: authors state: "To study the efficacy of Foz infection, we performed a replicated experiment using five different concentrations of Foz microconidia", but indeed it is not indicated if the experiment was replicated and results reproducible. Please, add clarification. - Lines 96-98: authors state that "Formae specialis of these isolates were confirmed to be Fusarium oxysporum f. sp. zingiberi based on their ability to cause Fusarium yellows in ginger pot trials". Why didn't authors complement the identification with a more accurate method to determine whether those isolates are classified as F. oxysporum f. sp. Zingiberi, for example, using molecular markers? It would be required to identify molecularly the isolates at species level. - Lines 100-101: Could authors explain the reason why they mixed 4 four isolates for the inoculum? - Lines 104-105 and rest of manuscript: My suggestion is to use the unit microconidia/mL or microconidia/g of soil, instead of cfu/mL or cfu/g. For the second, it would be required having a probe that the microconidia had the ability to form colonies (for example, by plating diluted inoculum and counting colonies). Please, strict to this unit in the whole text. - Lines 116-120: Please, rewrite these sentences to clarify how soil inoculation was conducted. As it is explained, it seems that pots with soil were inoculated and then mixed with extra 400 g of soil. - Lines 120-122: Please, explain the reason why treatment 107 microconidia/g of soil only had three plants. - Please, clarify if plants were grouped as replicates. 2. Presentation of results - General: use of the term "correlation" (lines 41, 87, 88, 138, 156). Authors use the term "correlation" to reflect association of results and trends for different variables. However, I would suggest avoiding this term, since it could lead to the thought that statistical correlation analyses have been conducted, when it is not the case. Please, rephrase those sentences. - Line 141: Authors use the term "accelerated", when results do not reflect an earlier appearance of symptoms, but an exacerbation of those. Please, change "accelerated" by "exacerbated". - Line 162: The significant reduction in comparison to control is actually observed in 106 cfu/g of soil or higher. Please, correct it. - Figure 2: Please add in caption the number of plants for each treatment, as done in Table 1. 3. Literature analysis or

discussion - Line 85: Please, add a reference. - Discussion: Much information about agricultural practises related to ginger are explained, but I miss further discussion on their relation with this study, its importance, and conclusions; mainly focused on the improvements on disease control and management of crop productivity. Also, it would be interesting to discuss limitations of the study, for example: plant variety selection, number of plants, isolate virulence, etc.

Please rate the manuscript for methodological rigour
Satisfactory

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000605.v1.4

Anonymous.

Date report received: 30 April 2023
Recommendation: Major Revision

Comments: Lines 1-3: Lowest may refer that 10 cfu/gm soil be the least and there may be a need to run logistic or other growth models to showcase the clearcut point where the threshold is significant. Anything

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000605.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000605.v1.2