



Research Article

Synergy between fungal endophytes improves fruit production in strawberry cultivar

Brian R. Murphy, Erika Soldi, Marta J. Jadwiszczak, Trevor R. Hodkinson

Abstract

Strawberries are important global soft-fruit crop, but optimal production is constrained by the diseases and chemical treatments. Grey mould disease, caused by the pathogen *Botrytis cinerea*, is a devastating pathogen of strawberry crops worldwide. In this study, we recovered fungal leaf endophytes from a wild relative of strawberry, *Fragaria vesca*, and tested the effect of inoculating the endophytes into plants of the strawberry cultivar, Elsanta grown in a glasshouse. The strawberry plants were either untreated or treated with endophytes, infected with *B. cinerea* or inoculated with both endophytes and *B. cinerea*. The endophytes were applied as a consortium of six strains (identified as strains of *Cladosporium cladosporioides*, *Colletotrichum circinans*, *Penicillium brevicompactum* and *Phoma exigua*). The plants treated with endophytes had a longer production period and produced a significantly greater quantity of fruits with increased weight. Compared with the controls, plants inoculated with the endophytes produced 48% more berries and a 22% greater weight of berries; plants inoculated with both endophytes and *B. cinerea* produced 49% more berries and a 51% greater weight of berries. While strains of each endophyte species have previously been reported to be potentially pathogenic, we have shown that the synergistic interactions between them boosted strawberry fruit production. They can be considered to act on the positive side of a mutualism-parasitism continuum. These results suggest that the endophytes have the potential to improve strawberry production in a larger scale agricultural setting and reduce the negative impact of grey mould disease.

Keywords biological control, *Botrytis cinerea*, endophytes, pathogen, strawberry, synergy, yield

Introduction

Strawberry crops

Globally, 9.2 million tonnes of strawberries (*Fragaria × ananassa* Duchesne) are produced on 402,000 hectares with a gross production value of US\$15.9 billion [1] and China is the largest grower with 3.8 MT produced on 141,000 ha in 2016 [2]. Extensive use of costly fungicides is necessary to control a broad range of strawberry diseases [3]. For example, the estimated cost of fungicides to control diseases for strawberry growers in California is US\$2,976 per hectare per cropping season, representing 3% of the total cost of production [4]. While not all the strawberry growers around the world would have similar fungicide input costs, these costs are always a significant burden to growers. The most important strawberry disease is grey mould caused by the ascomycete fungal pathogen

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Botrytis cinerea Pers. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) which can have severe impacts on strawberry yields. Global expenses for *B. cinerea* control exceed €1 billion per annum; however, the impact of product and quality loss occurring despite of all the control measures is expected to be much higher [5].

Disease control

Fungicides are the main chemical control method for grey mould and are essential to the economically viable production of strawberry crops [6], but the pathogen can develop resistance over time. Resistance to the most commonly used active principles, benzimidazoles and dicarboximides, is widespread [7-8]. Succinate dehydrogenase inhibitors (SDHIs) constitute a mainstay in the management of grey mould in strawberry. One study suggests high risks for the rapid and widespread evolution of *B. cinerea* populations resistant to SDHIs unless appropriate cultural and rotation strategies are implemented [9]. Strains of *B. cinerea* differ in their sensitivity to fungicides and broad ranges of fungicide sensitivities are present in *B. cinerea* field isolates [10]. However, there is widespread public concern about the environmental and human health impacts of extensive use of chemicals in the strawberry industry [11] and alternative methods of pest and disease control are needed.

Cultural methods of grey mould control in strawberries have limited success. As necrotic strawberry leaves are the primary source of *B. cinerea* inoculum in strawberries [12], an obvious strategy to reduce *B. cinerea* epidemics is to remove these tissues, but this practice has not slowed epidemic development [13]. Increasing aeration around the plants by single row planting has reduced the grey mould incidence [14] compared to the double and triple rows [15-16]. Plant age has also been shown to affect the severity of *B. cinerea* over growing seasons, perhaps indicating an inoculum build-up. Thus, short cropping cycles were recommended to delay the build-up of inoculum [17]. Furthermore, breeding for improved plant resistance has had limited success [18].

Biological control

Biological control methods offer a promising alternative or supplement to the chemical and cultural control of grey mould. In strawberries, it was shown that *Aureobasidium pullulans* is able to effectively suppress pre- and postharvest growth of *B. cinerea* [19-20]. Other biological control agents (BCAs) such as *Bacillus subtilis* and *Trichoderma harzianum* have also shown good efficacies against *B. cinerea* in strawberries [21-24].

While most of these studies have focused on a range of BCAs, there is limited evidence, with the exception of clavicipitaceous endophytes, of biocontrol using fungal endophytes [25-29], and even less information about the endophyte-associated impact on strawberry fruit yield. Cota et al., [30] showed that the inoculation with the endophytic BCA *Clonostachys rosea* improved strawberry yield, and Sinclair et al., [31] reported yield increases in salt-stressed strawberry induced by the model endophyte *Piriformospora indica*. However, positive results are not universally reported, and Dara [32], for example, concluded that treatment with the endophytic entomopathogen *Beauveria bassiana* did not significantly improve the yield in field-grown strawberries.

Experimental hypotheses

Of course, the most important factors for strawberry growers are final fruit yield and the cost of production. Very few studies have looked at the influence of fungal endophytes derived from a wild relative of strawberry on strawberry cultivar yield. In this study, we hypothesized that a consortium of fungal endophytes recovered from a wild relative of strawberry would improve cultivar strawberry yield in uninfected and *Botrytis* infected plants.



Methodology

Plant sampling

Fragaria vesca L., wild strawberry, is a perennial herbaceous plant in the rose family that grows naturally throughout the Northern Hemisphere. Leaf tissues from a plant of the wild strawberry in a natural woodland edge in Ireland were collected (location not given due to intellectual property protection issues). Whole leaves were surface-sterilized by immersing in 70% EtOH for one minute, placing in 5% NaClO for 5 minutes, immersing for another minute in 70% EtOH and then rinsing five times with sterile water (Self paper). Fifty pieces of leaf tissue of ~5 mm² were placed onto individual culture plates of potato dextrose agar (Oxoid CM0139) and incubated in the dark at 21 °C for 28 days. The powdered medium was mixed to half-strength of the manufacturers' recommendations (to avoid osmotic shock to the endophytes) using ultrapure water, then sterilized by autoclaving. From previous experience, we considered 28 days to be sufficient time to allow recovery of the slowest emerging endophytes. Dishes were inspected daily and those containing seed or husk pieces with surface fungal growth were discarded (i.e. not emerging from the cut tissue area). Emergent endophytes were removed and sub-cultured on the same medium in the dark at 21 °C for 14 days.

DNA analysis

Among 11 individual cultures recovered, we selected six for DNA sequencing and the glasshouse trials were based on our selection criteria (early sporulation and high spore yield at room temperature, 18-21 °C). For the DNA analysis, we used the hot CTAB DNA extraction method as detailed in Hodkinson et al., [33] and performed PCR using the forward primer ITS1F [34] and the reverse primer ITS4 [35] following the reaction conditions in [36]. The thermal cycling parameters were programmed to optimize primer annealing, consisting of: 3 min at 95 °C; 9 cycles of 1 min at 94 °C, 1 min at 56 °C, 2 min at 72 °C; 20 cycles of 30 sec at 94 °C, 1 min at 56 °C, 3 min at 72 °C; a final extension for 7 min at 72 °C. PCR products were cleaned up using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) and final products were sequenced using the Sanger Sequencing Service offered by Source Biosciences.

Isolated sequences were recovered and compared with NCBI GenBank (National Centre for Biotechnology Information) and UNITE (Unified system for the DNA based fungal species linked to the classification, <https://unite.ut.ee>) accessions using the Basic Local Alignment Search Tool (nBLAST) for identification. Similarity criteria for assigning taxonomic rank to the endophyte strains was allocated based on an initial survey of existing fungal taxa in UNITE and GenBank, as follows: >97% similarity was assigned to the same species, 90-96% to the same genus, 85-90% to the same order and <85% to no significant match. In all cases, genetic identity assignment was confirmed or further assessed by the examination of the morphological characters of the fungi using light microscopy and by referencing the taxonomic descriptions found in Cannon and Kirk [37].

Experimental protocol

Individual endophyte inoculants were prepared by washing mature culture plates of the endophyte strains with 10 ml ultrapure water to dislodge the spores. The spore solution was transferred to individual 50 ml plastic tubes and further diluted with ultrapure water to give a final concentration of 1×10^6 spores/ml (measured with a haemocytometer). For the inoculant treatment of all six endophyte strains, equal aliquots from each of the individual spore solution preparations were combined.

Young nursery plants of the strawberry cultivar Elsanta (English's Fruit Nursery Ltd., County Wexford, Ireland) were planted into John Innes No. 2 compost (Westland Garden Health) in 1.5 litre plastic pots. Ten pots per treatment were sown and the pots were labelled with a reference number (reference numbers were anonymous to the plant grower). Roots were inoculated using a root dip containing either the endophyte spore solution at 10^6 spores/ml or ultrapure water for the controls. Pots were randomly distributed in two blocks in the glasshouse and repositioned every week during the experimental period. A freeze-dried culture of *Botrytis cinerea* Pers. (CBS 120092) was obtained from CBS-KNAW, Netherlands (<http://www.westerdijkinstituut.nl/Collections/>) and inoculated onto oat agar in 90 mm Petri dishes. The



culture dishes were incubated in the dark at 21°C for 28 days. Spores were collected from the surface by washing the mature culture plates with 10 ml ultrapure water to dislodge the spores. The spore solution was further diluted with ultrapure water to give a final concentration of 1×10^6 spores/ml (measured with a haemocytometer). The plants were inoculated at 11 days after planting with either 1 ml of the *B. cinerea* spore solution or 1 ml pure water by making a small cut ($\sim 1 \text{ mm}^2$) on the upper side of the base on one leaf of each plant and running the inoculant down the leaf mid-vein into the cut. No supplemental nutrients were added during the experimental period and all plants were watered as necessary. Flowers were manually pollinated every day by randomly moving the pollen between all open flowers with a small paint brush. Mature strawberry fruits were harvested and weighed as they were produced. Final number of fruits and weight of total fruit production per plant were calculated and plotted.

Post-harvest tests

To test for the presence of the endophyte strains in strawberry root tissue, we prepared 1 cm root pieces from a random selection of 5 endophyte-inoculated plants from *Botrytis*-infected and uninfected plants, which were surface-sterilized and plated out as for the leaf tissue. The root tissue was sampled from mature plants that had finished fruiting.

To test for antagonism interactions, a 1 cm agar plug of each of the endophyte strain cultures was plated out on half-strength PDA with an equal sized plug of the *B. cinerea* culture, replicated 3 times. After 14 days of co-culture in the dark at 21°C, the plate coverage for each organism was measured using the ImageJ-plugin “ColonyArea” software program [38]. In the same way, we also plated out all the endophytes together, without *B. cinerea*, to assess the interactions between the strains.

Data analysis was carried out using single factor ANOVA with Bonferroni correction and Pearson’s Product Moment correlation statistical analyses supplied with the Data Analysis module within Microsoft Excel© and Datadesk 7.01. In addition, overall total for each measured trait and treatment were calculated and compared.

Results

Endophyte identification

The six endophyte isolates were compared with GenBank and UNITE database accessions, revealing close matches (99-100% pairwise similarity) with 4 different fungal species (Table 1). Sequence length ranged from 517 to 558 characters. The isolate sequences were deposited in NCBI GenBank under accession numbers MH714547 – MH714552.

Table 1. Identity of experimental fungal endophyte strains inferred from GenBank and UNITE database searches using the ITS gene

Endophyte Strain id	GenBank Accession	Nearest BLAST Match	% pairwise similarity
FvL18DB11	MH714547	<i>Phoma exigua</i>	99
FvL18DB14	MH714548	<i>Penicillium brevicompactum</i>	99
FvL18DB19	MH714549	<i>Colletotrichum circinans</i>	100
FvL18DB20	MH714550	<i>Colletotrichum circinans</i>	99
FvL18DB35	MH714551	<i>Cladosporium cladosporoides</i>	99
FvL18DB36	MH714552	<i>Cladosporium cladosporoides</i>	99

Harvest results

Strawberry fruit was harvested over a 5-6 week period from mid-May to late June. Overall, the plants not inoculated with *Botrytis* produced more fruit over a longer period than *Botrytis*-inoculated plants, with a longer period of production associated with endophyte inoculation. Fruit production was greatest during the first 3 weeks of this period (Figure 1).



Endophyte inoculated plants produced 41% greater mean number of production days per plant (18.2 vs 12.9) for the *Botrytis*-inoculated plants and 25% greater mean number of production days per

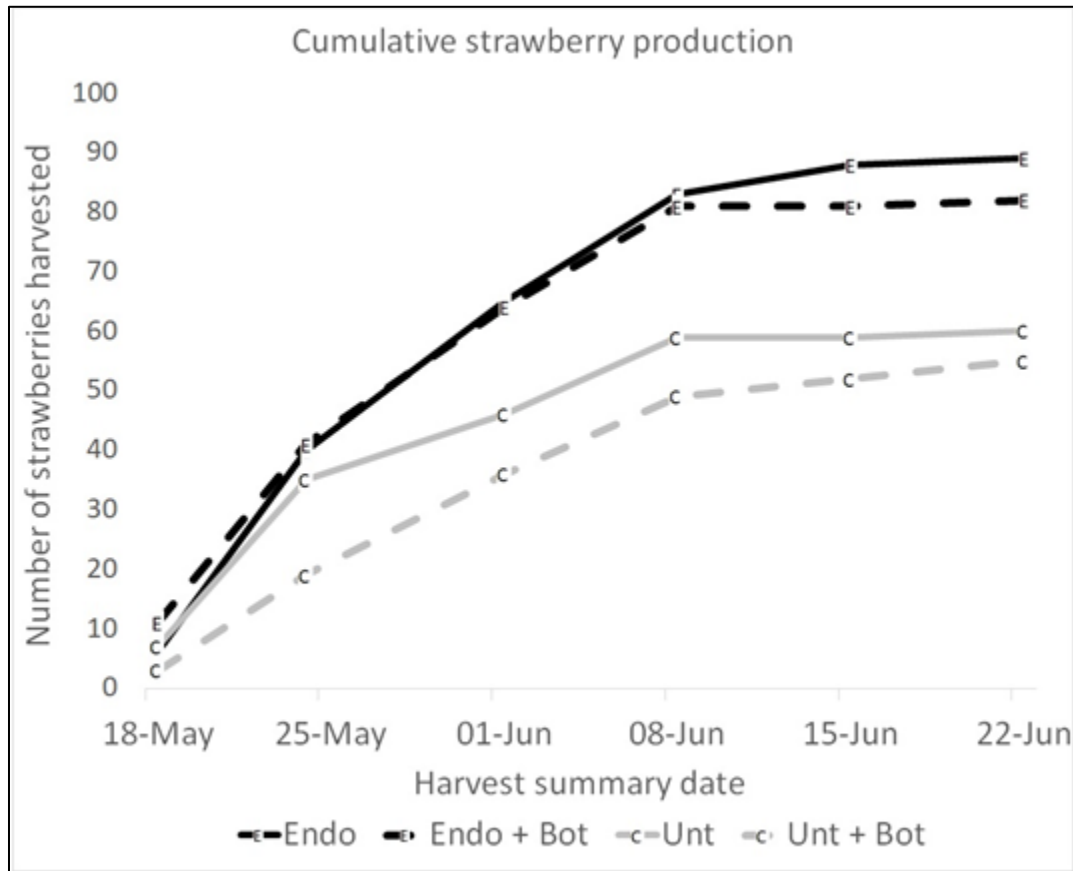


Figure 1. Cumulative number of strawberries produced. E signifies the endophyte infected and C signifies the control plants. Dashed lines are for *Botrytis* inoculated plants

plant (15.7 vs 12.6) for uninfected plants. Five of the control plants produced no berries compared with only one of the endophyte-inoculated plants. Though relatively large, these differences were not statistically significant due to the very high standard deviations, ranging from 57 - 89% of the mean production period values.

Compared with the controls, plants inoculated with just endophytes produced 48% more berries and 22% greater weight of berries ($P < 0.01$) (Figure 1) while plants inoculated with both endophytes and *Botrytis* produced 49% more berries and 51% greater weight of berries ($P < 0.01$) (Figure 2). Additionally, a significant correlation ($P < 0.01$) was found between the number and weight of berries produced.

For both endophyte and control treatments, we found that for the *Botrytis*-inoculated plants, 2% of fruits were misshapen or otherwise not of saleable quality compared with the 1% for plants not inoculated with *Botrytis*.

Endophyte inoculation was also associated with greater daily fruit production rate. Endophyte inoculated plants produced 47% greater mean number of berries per day (3.45 vs 1.99) for the *Botrytis*-inoculated plants and 74% greater mean number of berries per day (2.18 vs 1.48) for uninfected plants.

Post-harvest test results

We were able to recover four of the endophyte strains, 2 x *Colletotrichum circinans*, *Penicillium brevicompactum* and *Phoma exigua* from the mature root tissue of both *Botrytis*-infected and uninfected plants. The *Cladosporium cladosporioides* strains were not recovered from the root tissue.

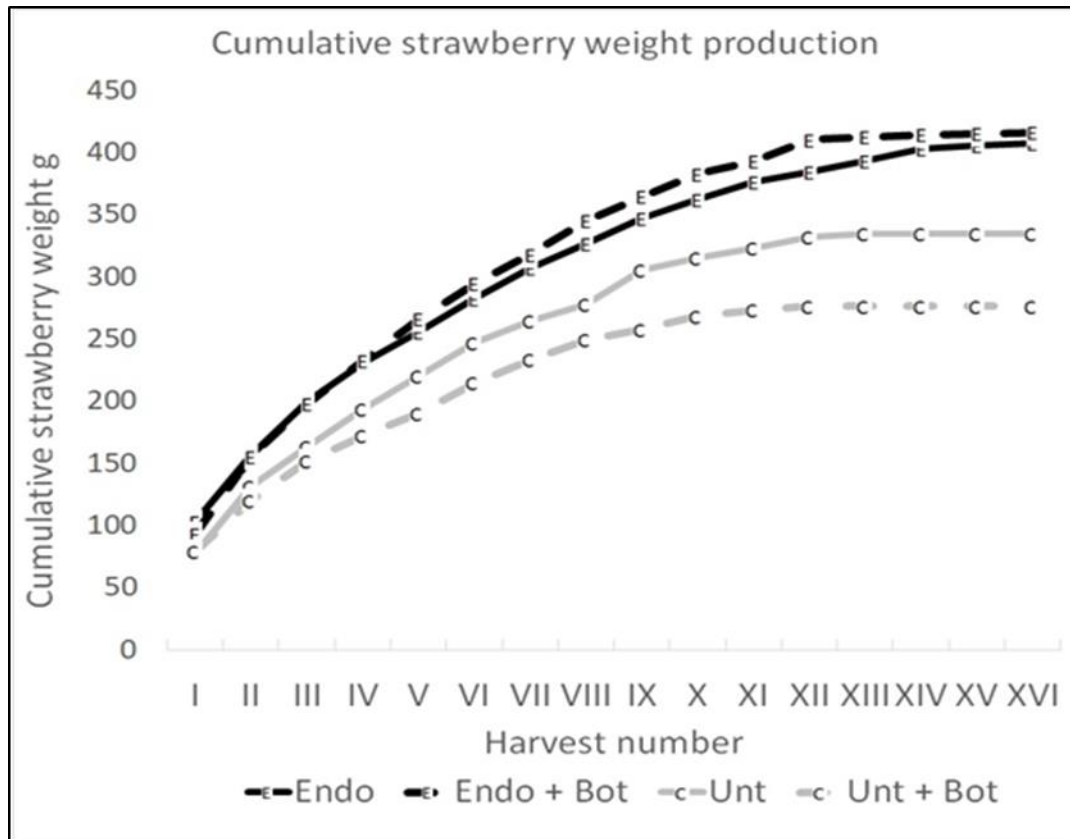


Figure 2. Cumulative weight of strawberries produced. E signifies endophyte infected C signifies control plants. Dashed lines are for *Botrytis* inoculated plants

For the *in vitro* antagonism tests, we found that each of the endophyte strains differentially inhibited the spread of *B. cinerea* and each other (Table 2). The strains FvL18DB14 (*Penicillium brevicompactum*) and FvL18DB11 (*Phoma exigua*) were most aggressive in suppressing *B. cinerea*, while the other endophyte strains also controlled *B. cinerea*, but had less distinct zones of inhibition. The early sporulating strain FvL18DB14 completely prevented the lateral spread of and actively colonized the surface of the *B. cinerea* culture, forming the multiple spore-initiated colonies causing clear holes in the mycelial mat. Strain FvL18DB14 also showed active antagonism to all of the other strains, exhibiting similar interactions as it did with *B. cinerea*. However, all of the other strains did not interact as strongly with each other and had regular and stable interaction zones between strain cultures (data not shown).



Table 2. Mean culture plate coverage \pm S.D. of co-cultivated endophytes and *Botrytis cinerea* cultures incubated together for 14 days

Co-culture organisms	Endophyte Plate coverage %	<i>B. cinerea</i> plate coverage %
FvL18DB11 + <i>B. cinerea</i>	40 \pm 3.1	27 \pm 7.8
FvL18DB14 + <i>B. cinerea</i>	62 \pm 4.5	7 \pm 3.1
FvL18DB19/20 + <i>B. cinerea</i>	20 \pm 3.2	36 \pm 2.6
FvL18DB35/36 + <i>B. cinerea</i>	8 \pm 2.3	55 \pm 3.9
All Endophytes + <i>B. cinerea</i>	62 \pm 2.5	17 \pm 6.3

Discussion

This study has clearly demonstrated that a consortium of fungal endophytes recovered from the wild strawberry *Fragaria vesca* can increase fruit production in plants of the strawberry cultivar Elsanta that was infected with the destructive pathogen *Botrytis cinerea*. This pathogen causes major losses in the strawberry production industry due both to losses associated with the resultant grey mould disease and due to the cost of chemical control efforts. Fruit production was also increased in plants that were not inoculated with *B. cinerea*, a result that may offer organic farmers an alternative pre-emptive non-chemical means of protecting plants and increasing production.

The large endophyte-induced increases in strawberry production that was recorded in this study (a mean of 36% greater fruit weight) would have significant positive implications for commercial strawberry growers. The overall mean yield increase associated with endophyte inoculations would represent a massive boost to grower incomes if the results translate to commerce. All other things being equal, the current production value of US\$15.9 billion could increase to US\$21.6 billion, and that is without taking into account the reduced cost of fungicides. Industry insiders comment that strawberries remain a growth category and fit nicely with consumer trends toward healthy eating (personal communication), so new biotechnologies using endophytes will have much more impact in the future.

While we tested the endophyte strains for *in vitro* antagonism towards *B. cinerea*, we did not determine the underlying mechanisms involved in the endophyte-induced trait improvements, though some inferences can be made. The virulence and behaviour of different strains of *B. cinerea* is probably related to environmental conditions. Van Kan et al., [39] showed that *B. cinerea* is capable of colonizing plants internally, presumably as an endophyte, without causing any disease or stress symptoms. They suggest that the extent of the facultative endophytic behaviour of *B. cinerea* and its relevance in the ecology and disease epidemiology may be vastly underestimated. Interestingly, they also discuss the recent discovery of a novel *Botrytis* species, *B. deweyae*, which normally grows as an endophyte in ornamental daylilies (*Hemerocallis*), but displays facultative pathogenic behaviour, and is increasingly causing economic damage. This behaviour will be familiar to plant pathologists, many of whom consider that there is no such thing as a pathogen, rather merely a ‘patho-system’ where all parts of the necessary causal factors of disease need to be in place – the pathogen, a compatible host and suitable environmental conditions [40]. We sourced a strawberry-specific virulent strain of *B. cinerea* from a reputable source to ensure that we obtained disease development in the strawberry plants, which were grown in conditions amenable to grey mould development [41].

Inoculation by washing strawberry roots in an endophyte solution is an effective means to achieve plant colonisation by the endophyte. Using this method, another *Penicillium* species, *P. pinophilum*, was found to increase biomass, N and P content and the photosynthetic rate of strawberry plants [42], and this could be the case for the endophytic species used in the current experiment. While the recovery of four of the original endophyte inoculants from the mature root tissue suggest a long-term residence and effect on the plants, there may also have been an early beneficial effect due to a ‘priming’ of the plant defences by endophyte colonisation, in essence vaccination [43-44]. Further work is needed to fully elucidate the mechanisms involved.



Bacterial species can also suppress the development of *B. cinerea* in strawberry. *Bacillus brevis* and *B. polymyxa* produce peptide antibiotics that strongly inhibit germination of *Botrytis cinerea* on strawberry fruit *in vitro* and field tests [45]. Some bacterial species have the potential to increase the yield, growth and nutritional content of strawberry plants [46] and it is possible that the fungal endophytes used in the current study also possess such antibiotic and yield enhancing properties.

Many fungal endophytes produce polyphenols which can suppress pathogen development. Jersch et al., [47] suggest that proanthocyanadin content is positively related to *B. cinerea* quiescence. Peng and Sutton [48] found that isolates of *Penicillium* and *Colletotrichum* species recovered from strawberry cultivars suppressed the incidence of *B. cinerea* on fruits by 48-76%, respectively, the lower range of which reflects the same range of fruit production benefits that we found in our study.

So, mechanisms involved in endophyte-associated reduction in *B. cinerea* pathogenicity may include mycoparasitism, the production of antimicrobial compounds and enzymes (collectively called antagonism), competition for nutrients and space, the activation of downstream signal transduction pathways and ultimately the defence response resulting in induced systemic resistance (ISR) (summarised in Vos et al., [49]).

All four species of endophytic microorganism used in this study have previously been reported as plant pathogens (*Colletotrichum circinans*, *Cladosporium cladosporioides*, *Penicillium brevicompactum* and *Phoma exigua*). While we were able to recover four of the endophyte strains, *Colletotrichum circinans* (x 2), *Penicillium brevicompactum* and *Phoma exigua*, from the mature root tissue of both *Botrytis*-infected and uninfected plants, the *Cladosporium cladosporioides* strain did not appear to be present in the mature root tissue, suggesting either an earlier benefit or no beneficial contribution from this strain. The presence of these microorganisms as endophytes recovered from *Fragaria vesca*, and which we used in the inoculant consortium, may seem counter-intuitive but we will discuss the species individually to assess their possible role in enhancing strawberry yield.

Cladosporium cladosporioides infects the anthers, sepals, petals and pistils of the strawberry blossom [50] and has been shown to be involved in the rotting of strawberry blossoms. It is typically observed on older flowers with dehisced anthers and signs of senescence [51]. Gubler et al., [50] found that infection of strawberry blossoms by *C. cladosporioides* was associated with simultaneous infections by *Xanthomonas fragariae* (in California), and more recently *C. tenuissimum* (in Korea) [52]. In contrast, Barrera Necha and Bautista-Baños [53] reported that isolates of *C. cladosporioides* (as well as *C. oxysporum* and *Bacillus subtilis*) were the most effective microorganisms against *B. cinerea* in flower buds, where they reduced the number of lesions in the range of 42-65%, compared with 59-89% for a standard fungicide (vinclozolin). Our previous work has demonstrated an endophyte-associated increase in barley yield for strains of *C. cladosporioides* [54]. So while *C. cladosporioides* is a relatively weak plant pathogen it may have competitively excluded the more virulent *B. cinerea* in our infected plants and other potential environmental pathogens in uninfected plants, with an overall benefit to the plants in both cases.

Colletotrichum circinans is an ascomycete parasite of *Allium* species including leeks, onions, garlic, and shallots [55-58]. While the genus *Colletotrichum* is generally regarded as a pathogen of cucurbits [59], Morin et al., [60] report that a biocontrol strain of *Colletotrichum orbiculare* behaved as a hemibiotrophic parasite on rust-infected tissue of Noogoora burr (*Xanthium strumarium*). Following infection, *C. orbiculare* spread necrotrophically in the rust lesions, thus destroying the living plant cells which are essential for the growth of rust. Such a mechanism may have been the case in our experiment, with *C. circinans* mitigating the destructive potential of *B. cinerea*. Hiruma et al., [61] found that *C. tofieldiae*, an endemic endophyte in natural *Arabidopsis thaliana* populations in central Spain, promoted plant growth under phosphorus-deficient conditions. Colonization by *C. tofieldiae* initiates in roots but can also spread systemically, where it transfers the macronutrient phosphorus to shoots, promotes plant growth, and increases fertility, a nutrient status dependent relationship that might have facilitated the transition from pathogenic to beneficial lifestyles. As we provided no supplemental nutrients to the strawberry plants there may have been a deficiency of phosphorous thus triggering a similar behavioral response in the strain of *C. circinans* used here.



Penicillium brevicompactum is commonly isolated from a wide range of fruit, where it is a weak pathogen [62-63]. Some of the most potent microbial effectors have been derived from fungal products, such as compactin produced by *P. brevicompactum* [64]. This species also produces Mycophenolic Acid, a mycotoxin further reported to be produced by many strains of *P. roqueforti*. A cell free culture filtrate of *P. chrysogenum* has been shown to be a potential biological control agent against *Botrytis fabae* on faba beans [65] and our own previous work has demonstrated that an endophyte strain related to *P. brevicompactum* was able to suppress disease development caused by several important cereal crop fungal pathogens [66].

Phoma exigua (= *Boeremia exigua* (Desm.) Aveskamp, Gruyter and Verkley) is a facultative saprophytic fungus [67] and is used as a bioherbicide [68]. The genus includes many plant pathogenic fungi responsible for severe diseases on many plant species. In some cases the pathogenic nature of *Phoma* is regarded as helpful, acting as a biocontrol agent of plant pathogens. Other studies have shown the antagonistic effect of *P. glomerata* and *P. etheridgei* against *Microsphaera penicillata* and *Phellinus tremulae*.

The *in vitro* antagonism tests in our study support the evidence for antifungal activity previously reported for related fungal strains and may be the primary mode of action that increases strawberry resistance to *B. cinerea* pathogenicity. However, this behavior may not necessarily be the same *in vivo* [69-70].

Despite of the previously reported plant pathogenicity for the fungal species we recovered as endophytes, our strains in nearly all cases were only 99% similar to known accessions so may have different characteristics than pathogenic strains. Redman et al., [71] showed that a change in the expression of a single gene was responsible for the switch between pathogenic and plant mutualistic behavior for *Colletotrichum* spp.

As well as the possible mechanisms discussed above for each individual endophyte strain, there may also have been a beneficial combinatorial effect of using all the strains together as a single inoculant. Recent rapid progress in rhizosphere microbiome research has revived the understanding that plants may benefit more from association with interacting, diverse microbial communities (the microbiome) than from individual members in a community [72]; a view with which we concur. Murphy et al., [73] reasoned that the different modes of action associated with different endophytes, for endophytes obtained from the same plant species, would allow a degree of compatibility when used as a consortium, with each endophyte bringing different functional mechanisms to the target plant in a particular ecological niche space. The endophytes we used as a consortium in the current study were recovered as a co-occurring group in a single host plant which was healthy and growing strongly, and which were likely existing as members of a compatible group within the plant.

This synergistic effect is difficult to predict when assessing each individual strain based on previous studies of similar organisms. While each of the fungal endophyte strains used in our experiment have previously been reported as plant pathogens, their behaviour is dependent on many interacting factors, including the strain and host identity, environmental conditions, plant age, nutrient availability, and the presence and identity of other microorganisms. Murphy et al., (unpublished) suggest new terms to define endophytes based on their behavior. One of these terms is 'endosympaths', where the endophytes are 'sympathetic' to the environmental conditions and also either behaves as symbionts or pathogens existing on the mutualism-parasitism continuum. The endophytes in the current study can therefore be considered to act on the positive side of a mutualism-parasitism continuum.

We propose that the poised tension within the complex experimental interaction system resulted in a beneficial plant stimulation that enabled an increase in yield and a more successful vaccine-like defence against *B. cinerea*. Indeed, all of the endophyte strains used in this study (which were recovered from a single host), with the exception of the aggressive FvL18DB14 (*P. brevicompactum*), were compatible in a co-culture and grew together *in vitro* without any strain dominating, eventually forming stable colonies in distinct non-overlapping zones. However, the behaviour of interacting microorganisms *in vitro* does not necessarily mean that this same behavior will be apparent *in vivo*, and must be regarded only a probable indicator at best [69-70].



While the results from this study are very encouraging, more work needs to be done. As in any promising new agricultural technology, full validation of beneficial results must be carried out in a field situation over several growing seasons, which will provide robustness and confidence in the results. In particular, independent professionally run trials need to be conducted using standard commercial strawberry growing protocols with varying levels of supplemental nutrients, fungicides and watering. We are in the process of testing these endophyte strains in a production environment under glass with an industry partner, and results from these trials will determine the future possibilities for commercialization of the strains.

Successful transition of this research to commercially grown crops would represent both a significant advance in the battle against one of the most devastating diseases of strawberry and a general biological method of increasing the yield in this important global crop.

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