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Microbiological Assessments in a Cut Flower Crop Polytunnel Field Trial Adopting Soil Covering and Microbiocides for *Fusarium* Wilt Suppression

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ABSTRACT

Cut flower *Matthiola incana* were raised by local commercial cultivators in a polytunnel. The field soil beds were either left uncovered as normal or covered with polythene sheets (except a hole for plant plug space). Average temperatures in the top 5 cm soil under cover dropped from 28 ºC to 18 ºC compared to its spiking up to 37 ºC in uncovered counterparts. Microbiological analyses indicated that soil covering induced two log_{10} folds reduction of the wilt causal fungi *Fusarium oxysporum* and concomitantly increased one log_{10} fold wilt antagonistic natural soil inhabiting fungi populations. Standard dip/drench mixtures of commercial and local isolates microbiocides (bacteria) applied to *M. incana* plug roots improved plant health assessed by visible scores of the level of damage or wilt symptoms under soil covered treatments. Scanning electron microscopy, cultural and 16S rRNA PCR analyses revealed potent antifungal bacteria attached to the hyphal surfaces of *F. oxysporum* as ectosymbionts that may have implications for virulence regulation and host plants' wilt disease control. Our microbiological data support the prospects of combining physiological and microbiological interventions upon covering the soil surface that offers the local horticulturists an evidence based sustainable means of *Fusarium* wilt control suppression in polytunnel crops.

Keywords: Microbiocides, *Mathiola incana*, light/temperature modulation, disease control.

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I. Introduction

Fusarium wilt of ornamental plants such as Brompton Stock (*M. incana*), Carnation (*Dianthus*) and Narcissus important in the cut-flower market in Northern Ireland and is the most frequently encountered glasshouse, polytunnel (polythene covered tunnel) raised crop disease, followed by other soil-borne phytopathogens *Pythium* and *Rhizoctania* [1] on the basis of their host specificity causing crown, root rots and vascular wilts. *Fusarium* wilt disease control measures pose a formidable challenge to the glasshouse crops industry [2] due to stringent Europe-wide pesticide usage regulations, high costs, environmental, human and soil health impact. The sustainable biological alternatives [3] for *Fusarium* wilt control include soil drenches, root dips of commercial microbiocides (Serenade Max, Prestop), mycoparasitic fungi *Clonostachys rosea* [4]. Northern Ireland cut flower growers also adopt spent compost dressings and crop rotation to mitigate the flower plants wilt, by planting wilt resistant race-specific [5] varieties lettuce (*Lactuca sativa*) providing extra farm revenue during off-flower[6]. Subsequently, the flower grower observed (personal communication) surprisingly, visible healthy looking cut flower crop in randomly polythene sheet covered polytunnel field soil plots.

Normally soil coverings in an open filed render rise in subsoil temperature, promote seed germination, prevent avian menace and increase surface saprophytic fungal populations. However, in a polytunnel, the covering of soil in local flower crops suggests light, temperature lowering environmental effects usually associated with circadian clock signal modifications that tend to regulate fungal development, in particular those of *F. oxysporum* [7] in the critical plant stem base/soil microbiome zones [8], [9]. However, despite the above valuable literature background, core microbiological supportive data in the stem wilt infection zone concomitant to the covering of soils under polytunnel field conditions are scarce. In this study, prompted by the flower grower's visible observations on healthy looking plants discovered by chance in plots with soil covering, we set out to seek the microbiological premise, and investigate the physiological on-field (soil covering) manipulation, and the scientific outlook for such practicable and grower adaptable steps in enhancement of disease management of the opportunistic soil phytopathogen *F. oxysporum*, affecting cut flower crops, such as *M. incana*.

II. Materials And Methods

A. Polytunnel field trial

Using field soil covered by the polytunnels of one of the local cut-flower growers (Greenisland Flowers Co. Armagh,

Northern Ireland, BT62 1XB), the effects of covering the soil with polythene sheets upon the suppression of the wilt phytopathogen *F. oxysporum* were investigated. The soils were either left uncovered or covered in heavy gauge polythene sheets. Plug plants of 2 cultivars of *M.incana* (as supplied to local flower growers by a stockist based in The Netherlands) commonly grown locally were included for this study. The flower grower procured and applied the normal crop production and protection measures, including commercial bacterial formulation Serenade Max (*Bacillus subtilis* QST713) as a standard fungicidal treatment (applied as per manufacturers' instructions) to plug-plants, growing either under covered or uncovered soils. Alternatively, the plug-plants were either dipped or drenched with a cocktail of wilt suppressant native bacteria (Table 1) and held in our laboratory archives as inhibitory bacteria series (IB) viz., IB6/IB12-*B. amyloliquefaciens* and IB9-*Paenibacillus polymyxa* isolated from *Fusarium* infected flower grower's soil in Northern Ireland, UK. Freeze –dried aliquots of IB cocktail mix were also applied at the same rate as Serenade Max.

TABLE 1: LIST OF ORGANISMS USED IN THIS STUDY

Organism	Source
Fusarium species	
F. equiseti	Plant Pathology laboratory culture collection, AFBI, Newforge Lane, Belfast, UK
F. venenatum	
F. culmorum	
Wilt causal agents	
F. oxysporum 16602 (Type strain)	LGC Standards, Teddington, Middlesex, UK
F. oxysporum 16603 (Type strain)	
F. oxysporum (cured derivative)	
Other significant soil fungi	
Gliocadium spp	Plant Pathology laboratory culture collection, AFBI, Newforge Lane, Belfast, UK
Clonostachys rosea	
Eurotiomyces spp	
Trichoderma citrinoviride	
Trichoderma atroviride	
Clonostachys pseudochrolueca	
Inhibitory Bacteria (IB)	
B. amyloliquefaciens	**IB6, IB12** (AFBI, Belfast)
Paenibacillus polymyxa	**IB9** (AFBI, Belfast)
Bacillus subtilis QST713	Serenade Max (commercial)

B. Plant health scores

Scores of plant health were based on the level of damage or wilt observed: 1=(no damage), 2=(10%), 3=(11-25%), 4=(26-50%), 5=(100% dead). F. oxysporum counts were obtained for soils under both covered and uncovered conditions. The soil surface and Matthiola host-plant root systems (MHPRS) temperatures at c.~3.0 cm to 5.0 cm depth were recorded using thermal probes (Tinytag data-logger device: http://www.geminidataloggers.com/) using software compatible for data processing via a personal computer (pc). Soil moisture was maintained as per the flower growers' recommendations.

C. Microbiological investigations

1. Isolation of wilt fungi and competing antagonistic fungi from flower bed soil

The list of organisms used in this study are described in Table1. *Fusarium* genera wild type fungal colonies were normally distinguishable in Potato Dextrose agar (PDA) plates due to their distinctive colour and hyphal features (white-mild pink hypal extensions, intense carmine red pigmentation). Other fungi and bacteria co-occurring as adjacent colonies on the same agar plates exhibiting antagonistic zones against presumptive *Fusarium* colonies on PDA were carefully subcultured for further studies. Type collection cultures of *Fusarium oxysporum f. sp. mathiolii* (16602 and 16603) were obtained from LGC Standards, Teddington, Middlesex, UK for molecular comparisons with wild-type isolates.

2. Isolation of fungal hyphae surface resident bacteria, scanning electron microscopy and molecular assays

Stem sections of putatively infected *M. incana* were surface sterilized and then placed on PDA. The emergent hyphae were serially subcultured a minimum of three times onto fresh PDA. These purified *Fusarium* WT were examined to check for any co-colonising bacterial cultures adherent to the hyphae (as the presence of a glossy slime). These bacteria were carefully isolated and single-colony purified on LB agar for further molecular analyses. The presence of bacterial ectosymbionts on hyphae of WT *Fusarium* was further examined via Scanning Electron Microscopy (SEM). PCR assays for the amplification of either fungal 18S rDNA ITS regions, or 16S rRNA universal bacterial amplicons were employed to establish the identity of WT *Fusarium* morpho-types and those of the hyphal surface bacteria respectively. The resulting PCR amplicon sequences obtained for both the fungal and bacterial samples were established from chromatogram analysis [10] and the confirmed set of sequences were compared with those stored in the GenBank using the BLASTn alignment software (http://www.blast.genome.ad.jp/).

3. Effect of temperature on in vitro growth vigour of Fusarium spp.

From stock *Fusarium* species cultures 6mm diameter plugs were excised and transferred individually to the Cartesian co-ordinate centre of four directional segments marked previously using a fine-tip marker pen of fresh plates of PDA, incubated for 3 days at ambient temperature to facilitate natural contours of hyphal growth to advance. Once the culture plug establishes, the plates were incubated at temperatures viz., 4^{o}C, 10^{o}-15^{o}C, 16^{o}-19^{o}C (ambient), 20^{o}-35^{o}C) reflecting the spectrum of Poly tunnel canopies and soil thermal dynamics known in Northern Ireland. The culture plates were examined using a binocular microscope and the outline of the perimeter of the hyphae was carefully traced by marker pen. At 3-day intervals, the extent of the hyphal growth (mm) was marked up to 3 weeks. The final area covered by each culture was measured using the bio-imaging technique [11] to assess growth vigour using the Autochemisystem UVP Bioimaging system (UVP Products, Cambridge, UK), supported by LabWorks software package. Initially the instrument was set on white light and to an exposure ratio of 490:500, with a constant focus of 47% calibration. Using the "Area Density" tool, (in pixels) the

entire 'area of individual plate' was measured first, recorded, and then using freehand 'draw' tool followed on by the 'area of irregular contours' of fungal growth co-integrated within the Cartesian coordinates to give an accurate estimate of the *in vitro* growth intensity of *Fusarium* species set against the challenge of temperature regimes during their incubation.

4. Statistical analyses

The arbitrary ratio, *fungal growth*, was calculated for each treatment as the ratio of total surface area occupied by fungal growth/total surface area of the petri dish. Statistical analyses on microbiological and plant health investigations were performed employing the student t-test and values >0.05 (5%) was considered not significant.

III. Results And Discussion

A. Soil covering and its influence on subsoil temperature flux

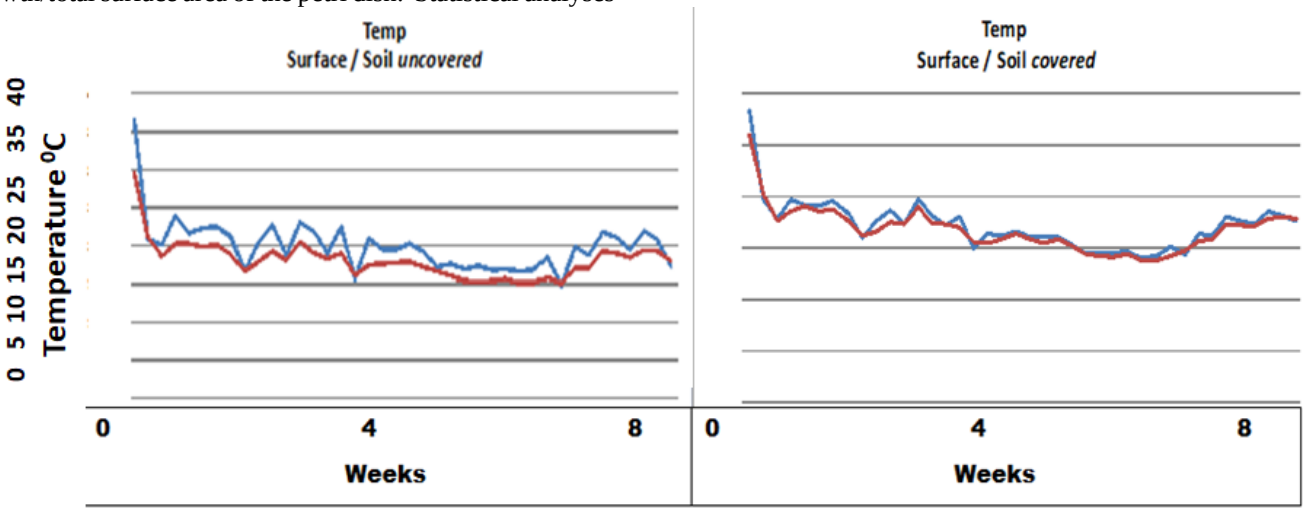

Fig. 1. Temperature gradient recorded over 8 weeks after *Mathiola* plug transplant into covered and uncovered poly tunnel field soils. Thermal ramp recordings of surface and subsurface (3.0-5.0 cm deep) soils just closest to *Mathiola incana* plant plug stem base / emerging root zone (MPHRZ) in uncovered or polythene sheets covered regimes in poly tunnel field cultivation system.

The temperature ramps recorded over a 8-week period when the soil surface was covered with the polythene sheets showed (Fig. 1) a dramatic drop from 28 ºC to 18 ºC within the first week of plant growth, and after flux of ± 2 ºC for the next weeks, the temperature dipped down to 14ºC for the ensuing 4 weeks before normalising at 18ºC for surface to root depth layer up to 5 cm deep. The relative sharp dip in the temperature, by a margin of over 10-14 ºC during the crucial phase of *M. incana* host plug plant establishment, is also important for the growth and proliferation of the saprophyte wilt causal agent *F. oxysporium*. As such, it is a critical time window for host-pathogen interaction. On the other hand, in the uncovered soils, while the surface ambience also had initial fall in temperature from a maximum of ±37 ºC down to 28 ºC, the soil bed beneath had an average of 24ºC; the surface ambient temperature and the MHPRZ (5 cm deep) soil in covered state were divergent throughout the first 8-week period recorded and lacked any normalisation of the two thermal ramps compared to the synchronisation and harmony found in temperature gradient in the surface versus underground 5 cm deep covered soil. Increased symptoms of wilt in *Chrysanthemum morifolium* when cultivated in higher temperature (> 24 ºC) in greenhouses in the USA [12] during summer seasons have been a well-known phenomenon; symptoms became more severe when temperatures ≥27 ºC, such as those recorded in our studies on *M. incana* cut flower plantlets raised in polytunnels in Northern Ireland. The apparent divergent patterns of thermal flux between the covered and uncovered soil environment could have impacted the population dynamics of the emergent fungi colonising the plug plant host (*M. incana*) roots.

B. Plant health evaluations: effects of soil covering and application of microbiocides

Two cultivars CV1 and CV2 of *Matthiola incana* (supplied to the flower grower as plug plants by a stockist based in The Netherlands). Scores of plant health were based on the level of damage or wilt observed: 1=(no damage), 2=(10%), 3=(11-25%), 4=(26-50%), 5=(100% dead). Error bars indicate the standard deviations of the mean.

Plant health scores (Fig. 2) showed the percentage of plants compared to their score damage per treatment and cultivar (CV1, CV2) variations against covered and uncovered regimes. Plants with covered roots generally exhibited higher number of plants with less damage when the bacterial treatments were administered as a plug drench, while in uncovered plants, a wider range of symptoms were apparent on visible scores basis. Both covered and uncovered plants secured better and higher scores when a drench treatment followed a dipped mode of treatment before planting the plug plants in the pots. For example, when plants were uncovered regardless of the mode of bacterial pre-treatment (dipped or drenched) or post treatment, over 60% of CV2 plants had only 10% damage, whereas over 30% of CV1 had 10% damage. However, in the case of soil covered plants, drenched method of pre-treatment of plugs showed CV2 plants had only succumbed to 10% of wilt damage, whereas over 60% of CV1 had 10% damage. Control treatments in general which received no bacterial treatment had a higher number of plants (≥10%) with wilt damaged appearance. Overall visible scores indicated that plug plants grown under covered soil seemed to be healthier plants and showed more vigour. The covered treatment scores were generally lower (less damage to plants)

therefore the differences between treatments and control (no microbiocides) was less. However, in the uncovered treatments, plants were either dead or dying (≥5) and there were more overall variations in scores (≥2). From the visual observations it can be seen the covered plants look healthier and bigger than the uncovered.

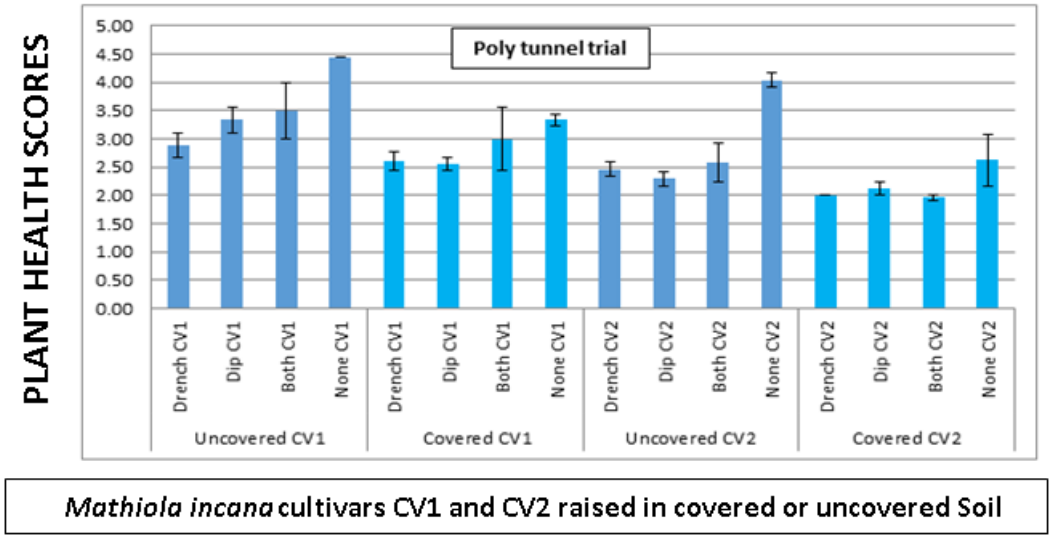

Fig. 2. Plant Health (wilt damage) assessments on *Mathiola incana* plants in polytunnel field trial.

Our preliminary visible scores data upon plant health (Fig. 2) also heralds the importance of circadian influence on fungal pathogenesis which corroborates those of several researchers who have highlighted the regulation of growth, metabolism, and biotic and abiotic stress resistance responses to light upon a number of fungal pathogens including *Fusarium* spp [13], *Aspergillus* spp [14], *Phytophthora* spp [15]. Our results also heightens the need for utilizing natural physiological tools (e.g. covering the soils around the stem base and roots) as a practical supplementary means for enhanced suppression of the wilt pathogens in tandem with current trends of biological control measures of dipping or drenching treatments (e.g. microbiocides) of plant plugs in field conditions. The systemic toxin theory [16] considers that toxins, (such as fusaric acid produced by *F. oxysporum*) disturb the metabolism of the infected host plant, resulting in leaf wilt with a consequent reduction in root growth, leading to apoptosis, necrosis, and even death. However, in our experiment, our primary goal insofar as heat and light effects are concerned was to evaluate the overbearing influences that the soil covering manipulation has, on the saprophytic soil inhabiting *F. oxysporum* in the soil surface and *Matthiola* host-plant root systems (MHPRS) before it penetrates the host plant cells.

C. Effect of temperature on in vitro growth of Fusarium species

Our preliminary experiment (Fig. 3) to gauge the effect of temperature on *in vitro* growth vigour of *Fusarium* spp. in PDA plates incubated in temperature gradients viz., (4 ºC, 10 ºC -16 ºC (ambient), 19 ºC (mean annual polytunnel temperature in local flower growers yards), 25 ºC-35 ºC (polytunnel in this study)) indicated that local native *Fusarium* spp. were heat sensitive and their growth vigour significantly (P= 0.005) varied sharply, based on the temperature of their environment. In our study, we covered the soils with polythene to simulate a physiological effect of induced light, temperature as compensates of environmental (hidden) factors in air or immediate underground beneath the soil [17].

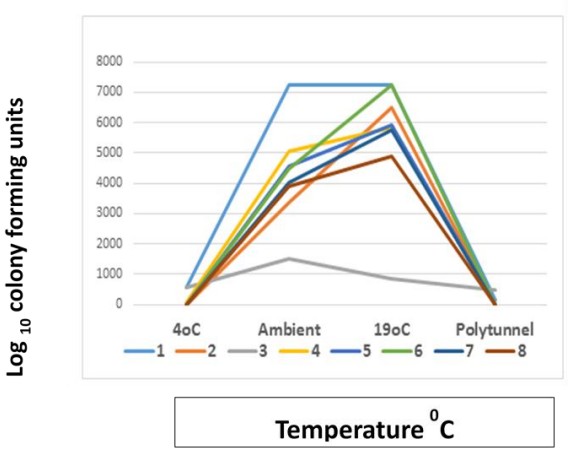

Fig. 3. Effect of temperature on *in vitro* growth vigour of *Fusarium* spp.

It is thus perceivable from our generic experimental results presented in this study, the 'light' perception of host (*M. incana*) canopy above the ground would be the same for covered or uncovered soils, However, for sessile saprophytic fungi such as *Fusarium* spp, it is critical for the saprophytic mycota to especially evade the substrate in the harsher temperatures prevalent in top surface soil and either head beneath in the competitive substrate-rich root rhizosphere, or produce spores for spreading in the soil/plant interface (e.g emerging stem area near top soil) environment for expressing a temperature modulated opportunistic pathogenicity, as it is apparently the case in *M. incana* stem wilt disease development. Thus, lowering of temperature at this critical soil-plant interface (MHPRZ) will limit the progress of fungal growth and development and besides the ensuing physiological (light, temperature modulated) molecular plant-pathogen interactions.

D. Microbiological assays

The soil samples were obtained from a local flower grower's farm (Greenisland Flowers, Co Armagh, Northern

Ireland) and *Fusarium* spp. amongst other fungi were isolated from soil collected from polytunnel which had a history of *Fusarium* infestation of *M. incana*. The soil sampling was carried out closest to the plug plant roots and the stem base where wilt damage was most evident, when the plug-plant transplants were 3-4 weeks old. Concomitantly, a piece of infected stem tissue of *M. incana* which had been surface sterilised and then placed on PDA yielded emergent presumptive *Fusarium* hyphae from infected tissue. Soil samples taken from closest to the base of the stem and emerging *Matthiola* host plant root zone (MHPRS) yielded a mixed population of bacterial and fungal colonies on PDA plates (Fig. 4 a). On certain of these plates there was evidence of inhibition of fungal growth, due to bacterial antagonism of the expansion of the hyphae (data not shown]. Single purified *Fusarium* colonies were incubated and plates with fungal hyphae swarmed by bacterial satellite colonies were retained.

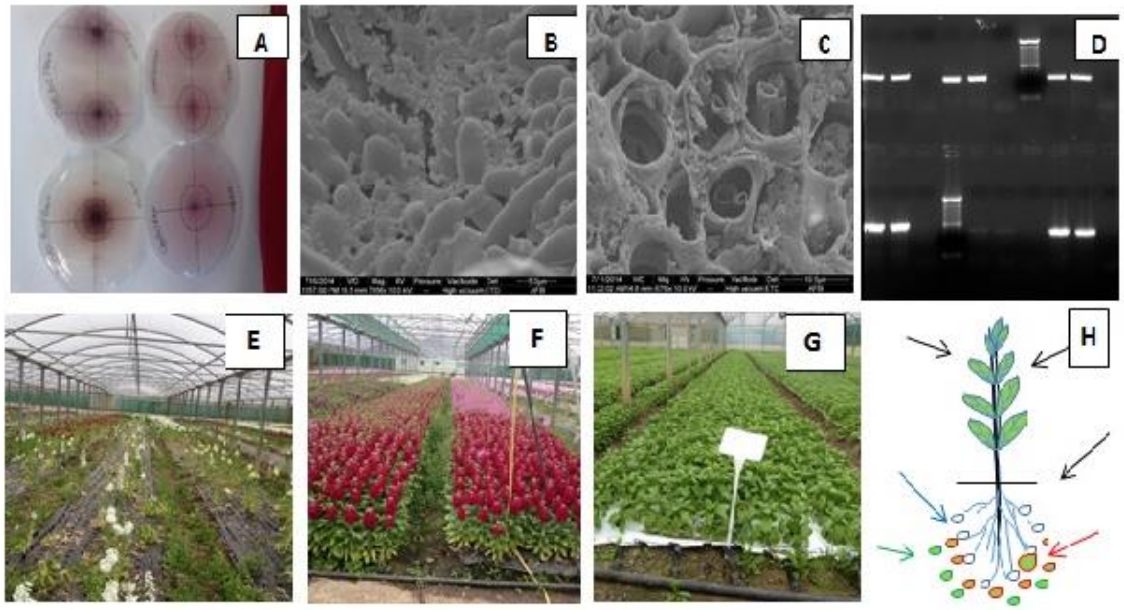

Fig. 4. Isolation and identification of bacterial ectosymbionts on *Fusarium oxysporum* hyphae.

Ectosymbiont bacteria colonising *Fusarium* hyphae and their influence on disease expression:

Top Row: Swarms of bacteria emerge from *Fusarium oxysporum* hyphae isolated from infected stem (A). Scanning Electron Micrograph (SEM) (B). Wilt causal *Fusarium oxysporum* hyphae seen proliferating in Xylem structures (C) characteristic of wilt pathogenesis. PCR analyses demonstrating the *Fusarium oxysporum* hyphae carrying ectosymbiont *P. polymyxa* (D).

Bottom row: Effects of soil covering on the cut flower *Mathiola incana* cultivated in a polytunnel: Two cultivars (CV1 and CV2raised in uncovered or covered soil beds (E) and received no crop protection treatment (control). The emerging healthy looking *Mathiola* plant (F) under covered soils (G). The encircled red zone highlights an example of bacterial ectosymbionts on fungal hyphae in mycosphere. Such mycosphere fungal-bacterial interactions are present near the stem base (right) - left arrow pointing towards horizontal black line (H).

E. Scanning Electron Microscopy (SEM)

The *Fusarium* hyphae obtained from the infected tissue were transferred on to fresh PDA plates, following which bacterial colonies were visible surrounding the emergent mycelial hyphae. Scanning electron microscopy (SEM) of the fungal hyphae from these culture plates revealed the presence of ectosymbiotic rod-shaped bacteria (Fig. 4 b, c) attached to the wilt pathogen *Fusarium oxysporum* hyphae (obtained via the wilt disease prone *Mathiola incana* infected tissue). Interestingly *Paenibacillus polymyxa*, was amongst potent antifungal native bacteria isolated from our previous study alongside *Bacillus subtilis* and *B. amyloliquefaciens*; all three of which were demonstrated *in vitro* [18] for their efficacy to act as natural alternative microbial source for wilt control in floriculture. The generic physiological mechanisms of the pathological wilting of higher plants [19] are generally attributed to xylem or phloem vessel plugging and/or systemic toxicity. The plugging theory indicates that the vascular vessels of infected plants are obstructed by fungal hyphae, thus limiting water transport in the xylem.

F. Molecular analyses

The bacterial colonies associated with the fungal hyphae in PDA plates were carefully isolated, single-colony purified and individual colonies identified using PCR. Molecular analyses (Fig. 4 d) indicated three specific bacteria viz., *Pseudomonas fluorescens, Stenotrophomonas maltophila,* and *Paenibacillus polymyxa* to be the ectosymbionts found on the hyphae of wilt causal *Fusarium oxysporum* wild types in healthy looking plants (Fig 4 F, G) but these ectosymbionts were absent in *Fusarium* hyphae of wilt disease affected field zone (Fig. 4 e). The ectosymbiont bacterium attached to the fungal hyphal surface may have also its own circadian clock mechanisms, in which bacterial cell signalling systems that detect blue photons within the visible light spectrum via a class of flavin-binding photosensors known as **L**ight, **O**xygen, **V**oltage (LOV) domains [20]. In covered soils, particularly just beneath the proximal top surface, such circadian factors can be expected to regulate the growth, reproduction cycles in a different manner to those of

uncovered soils. In our study, the inoculant bacteria (*Paenibacillus* spp) agent appears to have acted as a biological control agent [21] and unsuspectingly via apparent selective attachment to the hyphae as ectosymbionts may have possibly promoted "virulence silencing mechanisms" in *F. oxysporum* [e.g. 22].

We have summarised an overview of our present study (Fig. 4 e-h) and its visual impacts. Light manipulation in horticulture crops using variable wavelength lighting is increasing interest within the horticulture business and industry [23]. The light regulation of metabolic pathways of fungi is now a major carrier of adaptation signalling information [24] for their interactions with host cells. To this end, light sensing is crucial to host plant and the fungi it interacts with as well as the bacteria that reside on the fungal hyphae, on account of the hyphal resident ectosymbiont bacterial secretion factors that are well documented to contribute to regulation of fungal pathogenicity and virulence mechanisms on host plants. Although not by any means complete, and requiring further studies employing *in vivo* molecular expression analyses, our soil covering operation manipulate light and temperature regimes and visibly (Fig. 4) influenced the outcome of pathogen-plant interactions. Plant stem / emerging roots interface establish holistic relationships with diverse microbial community in soil [25], nowadays known as "plant microbiome" i.e. total microbiota of plants [26] immensely impact the overall pathogen and disease control strategies. Given that, our results indicating that the soil covering promoted pathogen suppression via the healthy looking plants (Fig. 4 f, g) offers a practicable tool to the challenge of transposing the science of physiological molecular plant pathology to a tangible biotechnological solution on the ground from a farmer's perspective.

G. Impact of physiological factors (light, temperature) and microbial interventions

Our combinatorial data upon microbiological, SEM and preliminary molecular analyses suggest that *Paenibacillus polymyxa* followed by *Pseudomonas* spp., to be the bacteria most frequently found attached to the *Fusarium* hyphae that penetrated the *M incana* roots. In order to ascertain if these consortia of bacteria attached to fungal hyphae imposed any influence on the pathogenicity of *Fusarium*, the hyphae of bacteria were cured by repeated subculture on PDA supplemented with the antibiotic oxytetracycline (250µg /ml) and SEM confirmed that they were rendered virulent by virtue of penetrating plant root cell structures (Fig. 4 b, c). Our data obtained on bacterial ectosymbionts of *Fusarium oxysporum* hyphae from the rhizosphere of the host plant *M. incana* concurs with those of previous research [e.g. 27] on alternative horticultural crops such as lettuce. This is in agreement with current knowledge that diverse bacteria inhabit as fungal hyphal symbionts over a diverse range of phylogenetic variations [28]. Complex mechanisms of antibiosis, circadian clock phenomena, overarching cell signal proteins, Type III secretions, exudates, metabolite exchange, chemotaxis, microbial cross-talking (quorum sensing), symbiotic, host pathogenicity/virulence determination [29] including *Fusarium oxysporum*, these fungi-bacteria symbiosis are not exclusive to specific hosts. This microbial warfare happen as a rule in the overall development of host-pathogenic relationships in horticulture crops. Our microbiological data suggests that when wilt *Fusarium* hyphae are predisposed to inoculant biocontrol agent (e.g. *Paenibacillus polumyxa*) via the dip and drench treatments, the apparent virulence regulation effects expected may well lead to enhanced biological control of wilt disease. Further studies on light dependent gene expression analyses using latest real-time molecular tools may be required to monitor this environmental sensory perception of MHPRZ on both pathogenicity (wilt causal agent) and the virulence regulation (imposed by ectosymbiont on phytopathogenic fungal hyphae) in covered / uncovered regimes.

H. Effects of soil covering, thermal variations and influence on soil fungal populations

Results based on microbiological observations such as colony colour and hyphal features in the PDA plate and subsequent molecular confirmatory assays of a doctoral thesis in our laboratory [30] indicated that both covered and uncovered flower growers' soil bed samples harbour *Fusarium oxysporum, F. venenatum F. culmorum* and *F. equiseti* as the main Fusaria (Table 2). The types of soil saprophytic fungi we identified corroborated other molecular investigations [31] to discriminate *Fusarium* spp. that are common soil inhabiting phytopathogens of small grain cereals, wheat and barley crops in Ireland soils [32]. The generic population density and their dynamics may also be attributed to the reduction of ambient temperature at both the surface and the top 2-5 cm soil depth following covering of the soil, compared to the uncovered treatments.

TABLE 2: PREVALENCE AND DISTRIBUTION OF FUSARIUM SPP AND HYPHAL ECTOSYMBIOTIC BACTERIAL COLONISERS IN *MATHIOLA INCANA* CULTIVATED POLY TUNNEL FIELD SOIL BEDS

Fungi isolated and identified from cut flower-bed soil	***In vitro Fusarium oxysporum* inhibition** (% of inhibitory zone)			
	Control	*F. oxysporum*[2]	*F. oxysporum*[3]	*F. oxysporum*[4]
	[1]*Fusarium* spp			
F. equiseti	88.2	62.5	82.3	68.9
F. venenatum	105.1	66.1	69.6	58.1
F. culmorum	98.6	79.9	89.6	86.7
	Other native soil fungi[5]			
Gliocadium spp	101.6	43.3	54.0	47.0
Clonostachys rosea	104.6	67.9	58.2	38.2
Eurotiomyces spp	103.2	38.5	60.7	43.7
Trichoderma citrinoviride	101.0	35.0	52.8	56.4
Trichoderma atroviride	101.0	35.0	52.8	56.4
Clonostachys pseudochrolueca	98.6	27.9	29.6	36.7

The native *Fusarium* spp. populations exhibited ca. ~2.0 x 10^4 colony forming units (CFUs) g^{-1} of either covered or uncovered soils that were widely prevalent in the Northern Ireland polytunnel floriculture soil-bed. The wilt causal fungi *Fusarium oxysporum* (wild types) occurred at a higher population density of 2.4 x 10^5 CFUs g^{-1} in uncovered soils, and there was a marked reduction of two $\log_{10}$ folds to 3.4 x 10^3 CFUs g^{-1} when the soils were covered by polythene. Non-pathogenic *F. oxysporum* isolates commonly occur in the soil as saprophytes, while some have been identified as biocontrol agents and endophytes [33].

I. Soil as a natural reservoir for non-pathogenic fungal antagonists against wilt Fusarium

In this present study, it was intriguing to note the widespread prevalence of a natural reservoir of other fungal antagonists (Table 2) such as *Trichoderma atroviride* formerly *T. harzianum* [34], *T. citrinoviride, Eurotiomyces* spp., *Clonostachys pseudochrolueca* [35] which possess inhibitory potential towards *Fusarium* populations. Some of the above fungal-fungal antagonists are registered as commercial formulations in Europe [36] as wilt fungal disease suppressants. *In vitro* inhibitory assay (Table 3) revealed that the *Fusarium* spp achieved a moderate (~20%) suppression of local wilt causal isolates of *F. oxysporum*, while other fellow native soil fungi (*Gliocadium*, *Chlonostachys* and *Trichoderma* species) in the soils were significantly stronger (> 60%) inhibitors of the local flower wilt Fusaria. Wilt antagonistic fungi [*Trichoderma atroviride, T. citrinoviride, Eurotiomyces* spp., *Clonostachys pseudochrolueca*] increased by at least 1.0 x $\log_{10}$ fold from ca <1.0 x 10^2 CFUs g^{-1} in uncovered soils to ~3.2 x 10^3 CFUs g^{-1} when covered with polythene sheets. Such small but significant (p=0.01) shifts in fungal population densities were noticeable after physiological manipulation of covering the soil than their counterpart uncovered soil plots which exhibited lower abundance of co-colonising wilt antagonistic fungal populations. The soil covering operation in our study is quite encouraging in respect of the same serving as a natural means of supplementing the biological agents commonly used for suppression of wilt disease and offers low cost biosecurity options for the cut-flower growing rural entrepreneurs in challenging competitive farming markets for Ireland within Europe. Our findings that varying environmental conditions and thereby enhancing the prospects of non-pathogenic natural biological control fungal agents inhabitants in soil themselves for suppression of *Fusarium* wilt diseases concurs in some respects with previous attempts to manipulate cropping conditions, soil type and *Fusarium oxysporum* inhibitory fungi [37], and as alternative disease management strategy in greenhouses for control of *Fusarium* wilt of tomato [38].

TABLE 3: FUNGAL – FUNGAL ANTAGONISM BETWEEN NATIVE SOIL FUNGI VERSUS WILD TYPE *F. OXYSPORUM* ISOLATES

Fungi isolated and identified from cut flower-bed soil	***Fusarium* spp population (gm^{-1} soil)**		**Bacterial Hyphae colonisation (EM, culture/PCR)**
	Uncovered	**Covered**	
	Start of trial	End of trial	
Fusarium species *F. equiseti* *F. venenatum* *F. culmorum*	2.1 x 10^4	2.2 x10^4	Non-specific, variant bacteria (e.g. *Bacillus* spp; *Serratia* spp)
Wilt causal agent *F. oxysporum* (wild types)	2.4 x 10^5	3.4 x 10^3	Specific, native bacteria: *Pseudomonas flourescens, Stenotrophomonas maltophila, Paenibacillus polymyxa*
Significant other fungi *Trichoderma atroviride*	<1.0x 10^2	3.2 x 10^2	Not tested
Clonostachys pseudochrolueca	<1.0x 10^2	1.8 x 10^2	Not tested

J. Rotational lettuce cropping, phytosanitation and their impact on Fusarium Wilt

The flower growers' field soils in Northern Ireland have a planting history involving both ornamental and lettuce crops and provided us with the opportunity to investigate wilt causing *Fusarium* populations involving both hosts regardless of the soil covering operations. Interestingly we found that local growers who were alternating crops of *M.incana* with winter lettuce *Lactuca sativa*, did not find visible symptoms of lettuce wilt caused by *F. oxysporum* f.sp. *lactucae*, This observation was in sharp contrast to the recent findings of lettuce field crop trials [39,40] on severe *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *lactucae* in England and Europe. This phytopathogen was not detected by either plating on PDA or by PCR assays for genetic marker [41], sequence alignment comparisons of the same in our soils (unpublished data). This indicates that the wilt causal *Fusarium* populations surrounding the *M. incana* hosts may have been quite distinctive pathovars from those of the virulent, notorious *F. oxysporum* f. sp race 4 [42], and thus most likely to be of native in origin, confined to the local Northern Ireland soils within the island of Ireland. Our molecular analyses (Fig 4 D) concurred with the conclusions of another study in Northern Italy in that, the Italian researchers seminal work [43] and later using molecular characterization [44] through IGS sequencing demonstrated that the forma specialis *matthioli* (ATCC16602 and ATCC16603) were not pathogenic on lamb's lettuce and isolates of different origins differed genetically from isolates that were poorly, moderately or highly virulent.

Due to the fact that both lettuce and cut flower picking involves phytosanitory considerations, transfer of infested soil through trays, pallets and the foot wear of farm workers has been confirmed as the main route that the disease lettuce *Fusarium* wilt as well those encountered in cut flower wilts are spread. In another study in Florida, researchers [45] found that due to the limitations of land availability for fallowing and rotation, cut flower growers have to contend with a number of other soil-borne wilt causal agents viz., *Pythium* and *Rhizoctonia* apart from *Fusarium* menace. These phytopathogenic species are present throughout their

cultivation cycles in the year and are unresponsive to routine soil sterilisation. However, given that in our study, the grower had imposed stringent soil disinfection measures, as well as covering the soil with polythene sheets, the impact of the intercropping and human foot spread of *Fusarium* wilt may be subdued. Alternative biologically sustainable methods are increasingly becoming popular with farmers such as corn crop liquor [46] for lettuce root rot, new range of biostimulants also known as 'bioeffectors' [47], [48] comprising plant growth promoting, pathogen controlling bacteria, fungi and processed macroalgae extracts for crop management to reduce increasing climate change driven abiotic & biotic stresses [47], [48].

IV. Conclusion

Fundamental molecular mechanisms of saprophytic fungal spore adaptation, growth physiology and host pathogenicity are innate fungal behaviours. To translate this science and transfer the knowledge into 'practicable biotechnological solutions' for ornamental (cut flower) crops disease management by farmers is a challenge for agricultural scientists. To this end, our present work demonstrated simple agricultural practices such as physiological manipulation of temperature and light via covering of soil and interactive use of an enrichment compendium of native microbe as biocontrol agents themselves isolated from the disease prone soils for poly tunnel farming offer plausible solution to horticulturists. Our results also indicate the sustainable potential interventions to increase the resilience of horticulture and floriculture crops against recurrent persistent soil inhabiting pathogen opportunism and infection control.

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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