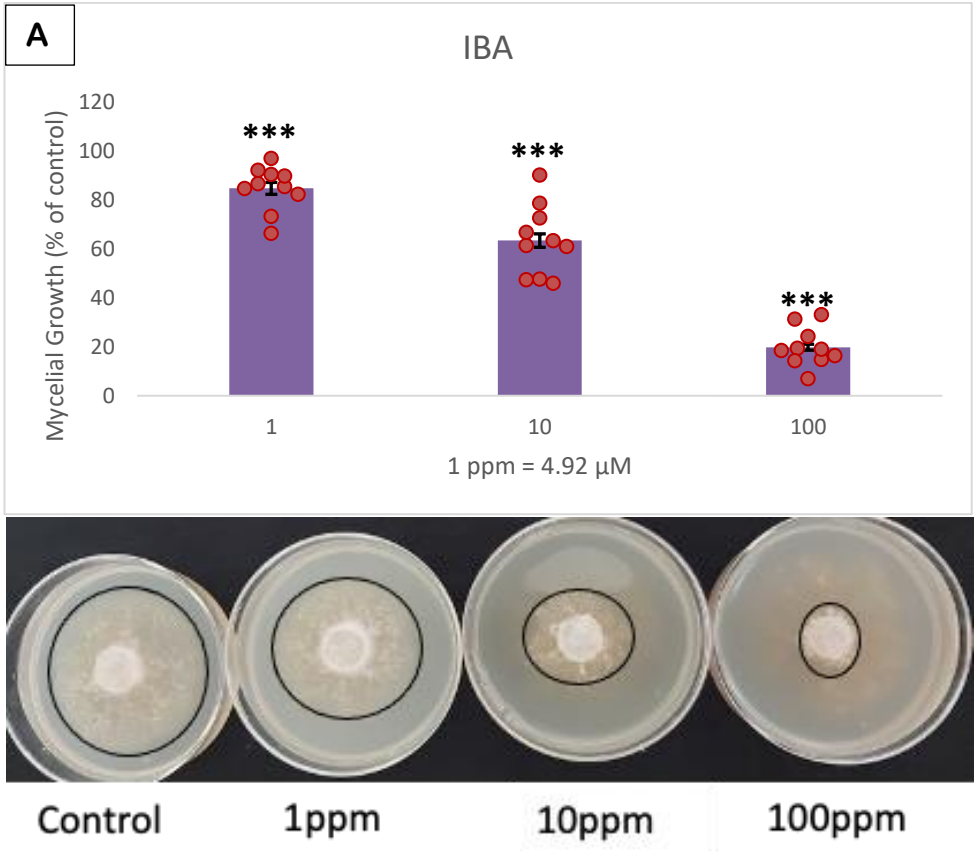
Studentship Project Interim Progress Report
Reporting period: March/2022 to September/2022

Student Name:	**Raisa Osama**	**AHDB Project Number:**	**CP 185**
Project Title:	**Role of auxin in Phytophthora root rot disease development in soft fruit**		
Lead Partner:	**The James Hutton Institute, Dundee**		
Supervisor:	**Dr Eleanor Gilroy, Dr Craig Simpson, Prof Grant Murray**		
Start Date:	**October 2019**	**End Date:**	**March 2023**

1. Project progress update including milestones:

Screening of *P. rubi* isolates with a more stable form of auxin, Indole 3-Butyric Acid (IBA)

We have been screening the mycelial growth of *Phytophthora rubi* with auxin related chemicals, benzoic acids and their derivatives. Previously, we did not see any suppression of mycelial growth with the natural form of auxin, Indole-3-Acetic Acid (IAA). Since IAA is not a stable compound, we decided to test a more stable form of auxin, IBA. We observed that IBA supressed the growth of *P. rubi* by ~20%,~60% and ~80% at 1, 10 and 100 ppm concentrations respectively (Fig. 1A). We also observed that treating agar plugs containing sporangia, with 10 ppm IBA led to darkening of sporangia and aggregation and fragmentation of mycelium (Fig. 1B).



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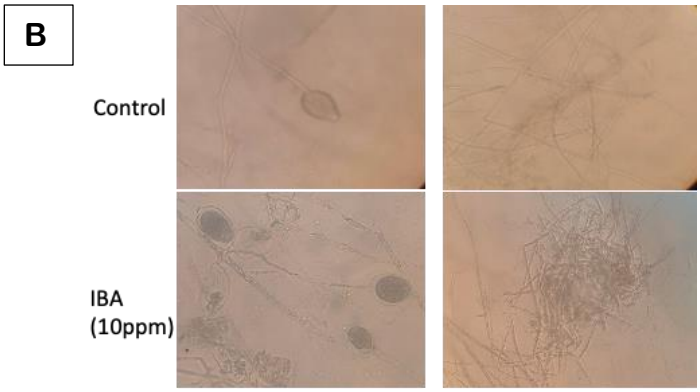


Figure 1: Auxin supress the mycelial growth of different isolates of *Phytophthora rubi*. A. Ten different isolates of *P. rubi* were grown on agar media supplemented with antibiotics and different doses of IBA. The mycelial growth was measured at 7dpi and calculated as percentage of control. The bar graph shows the average growth of all isolates at different concentrations. Each point represents the average growth of a single isolate in four replicates. Error bars are standard errors. The experiment has been repeated twice. Significance calculated by Holm-Sidak method one-way ANOVA are indicated by the asterisks. *** P= <0.001. Below is the representative growth of *P. rubi* on agar plates. **B.** Agar plugs containing mycelia and sporangia were placed in Petri dishes containing distilled water supplemented with 10 ppm IBA. The plugs were observed under a light microscope at 3dpi using x40 magnification.

Chemical screening of the model species, *Phytophthora infestans*

To check whether the chemicals causing significant inhibition of *P. rubi* growth also have similar effect on *P. infestans*, which is a well-studied oomycete pathogen, we screened it with IBA, TIBA (auxin-transport inhibitor) and salicylic acid. 100 ppm led to ~50% inhibition for IBA and TIBA and ~20% for salicylic acid at 7dpi (Fig. 2). The inhibitory effect on *P. infestans* were not as significant as *P. rubi*, which may be explained by the faster growth of *P. infestans*. At 7dpi, the control plates of *P. infestans* had reached the sides of the petridish (Fig. 2), whereas for *P. rubi,* the growth of the control did not cover the entire plate at 7 dpi (Fig. 1A)

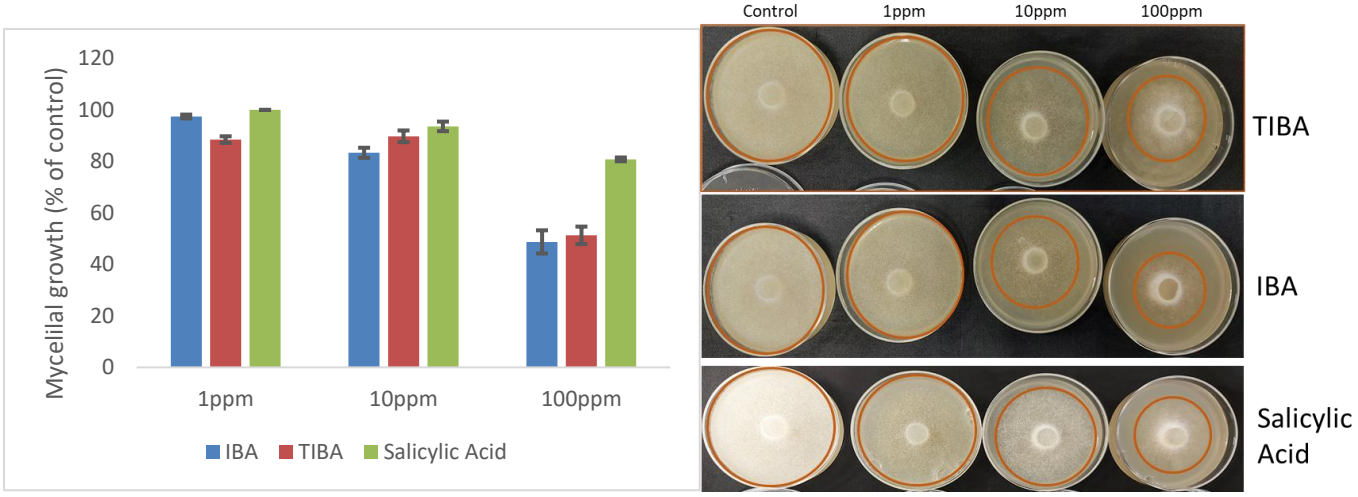


Figure 2: Auxin, auxin-transport inhibitor and salicylic acid inhibits growth of *P. infestans in vitro.*
The lab isolate of *P. infestans, 88069* was grown on agar media supplemented with antibiotics and different doses of IBA, TIBA and salicylic acid. The mycelial growth was measured at 7dpi and calculated as percentage of control. The bar graph shows the average growth of three replicates at different concentrations. Error bars are standard errors. On the right, the representative growth of *88069* on agar plates are shown. The experiment was conducted once.

Possible target of TIBA on Phytophthora species

We have shown that both here and in previous reports, TIBA inhibits the mycelial growth of *P. rubi* and *P. infestans in vitro*. This led to the deformation of sporangia, aggregation and fragmentation of mycelia. Screening *P. rubi* isolates with another auxin-transport inhibitor, Naphthylphthalamic acid (NPA) did not show any effect on the mycelial growth. This led to the screening of different benzoic acid and their derivatives, as TIBA is a benzoic acid with three iodine attached to its benzene ring. Although we observed the suppression of mycelial growth with benzoic acid, the effect was not as strong as TIBA. We then searched the literature for possible targets of TIBA and found that TIBA has shown to bind to Villin protein and interfere with actin dynamics in Arabidopsis roots (Zou et al., 2019). To understand whether TIBA had any effect on the actin

dynamics of phytophthora, we treated actin-labelled transformant of *P. infestans* with 100 ppm TIBA and 100 ppm IBA. Actin plaques were clearly observed on the mycelium treated with TIBA and were absent on the IBA treated mycelium (Fig. 3). This may imply that TIBA is stabilizing the actin movement and retarding its growth. On the other hand, IBA is inhibiting growth through other mechanism.

Searching the genome database of *P. infestans* with *AtVillin*, gave us one significant match at both the nucleotide and amino acid levels. We are currently silencing regions of *PiVillin* and plan to use it for Host Induced Gene Silencing experiments. We have also extracted RNA from TIBA and IBA treated mycelium for RNA-sequencing, which will provide us valuable information at transcriptomic level.

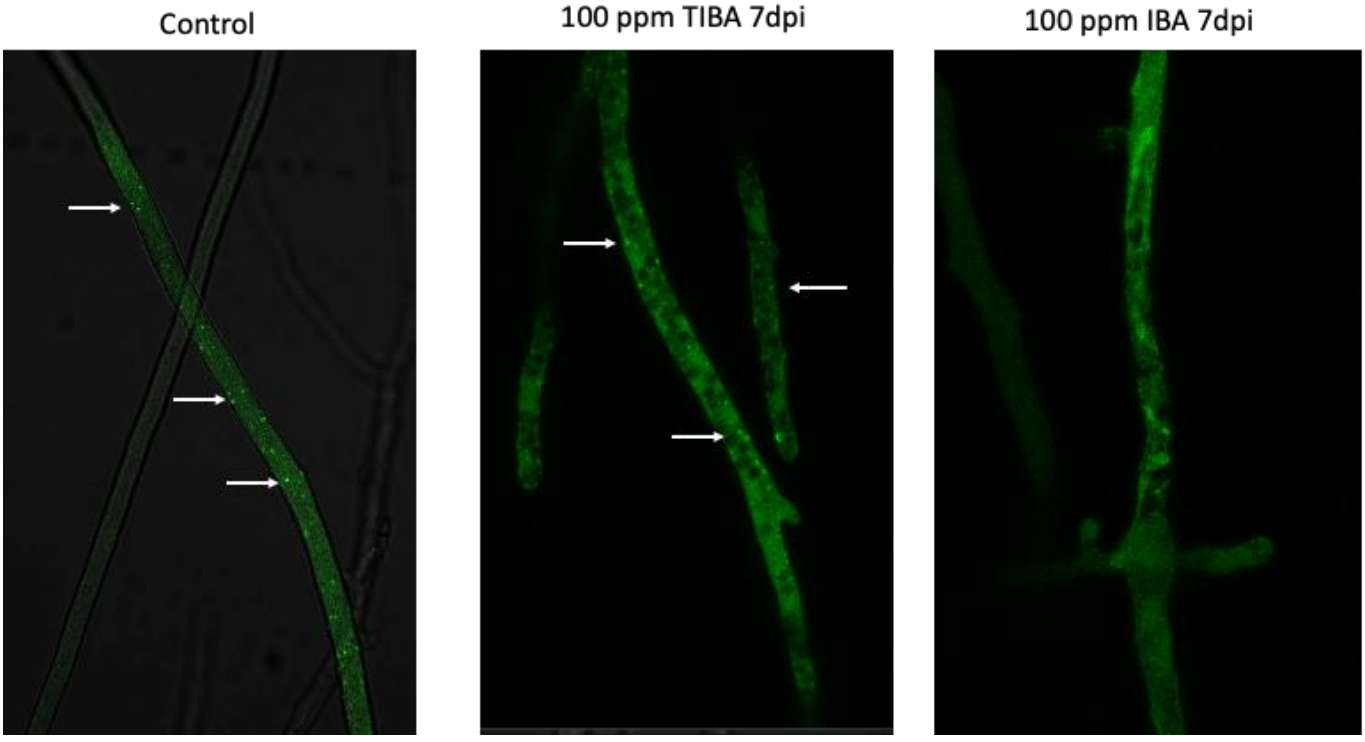


Figure 3. TIBA stabilizes actin plaques of *P. infestans* mycelia. The actin-labelled transformant of *P. infestans*, Life-Act Neon-6, were grown on agar plates supplemented with geneticin and ampicillin. Agar plugs were then transferred to Petri dishes containing Lima bean broth and 100 ppm TIBA or IBA. For control the liquid media was supplemented with DMSO. After seven days mycelia floating on the media were observed under confocal microscope. The white arrows indicate the visible actin plaques which is absent in the IBA treated mycelium.

Chemical assays on *P. infestans* leaf infection

Previously we observed that treating the roots with TIBA at the same time as infecting with *P. rubi* improved root volume and surface area and reduced incidence of disease symptoms. We wanted to check the effect of TIBA and IBA on the infection of *P. infestans* on *N. benthamiana* leaves, as this is a more rapid model system for infection assays. We sprayed leaves with different concentrations of TIBA and IBA and then infected them with *P. infestans* spores. We found that the number of inoculations developing signs of sporulation were highest on leaves treated with 100 ppm IBA and 100 ppm TIBA (Fig 4A). The average lesion diameter was significantly higher in chemically treated leaves (Fig 4B).

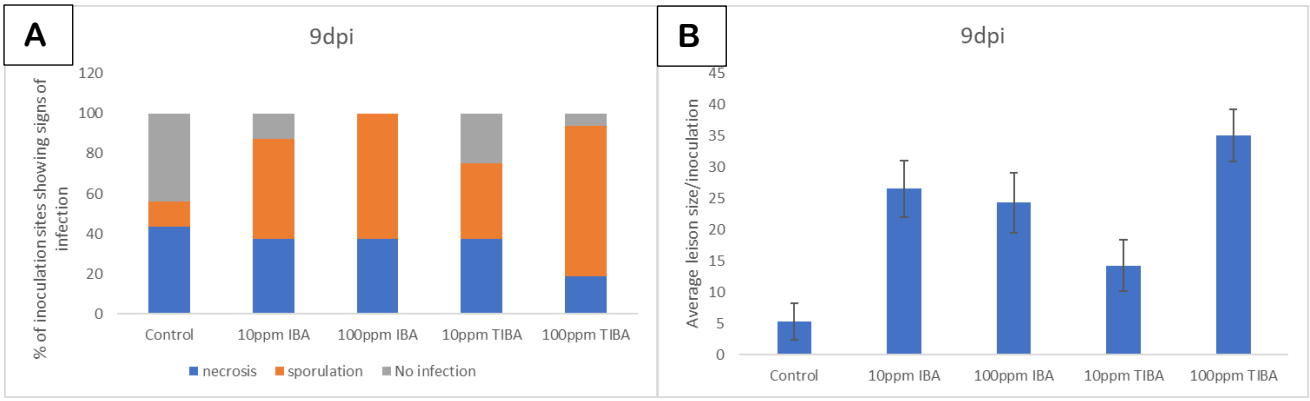
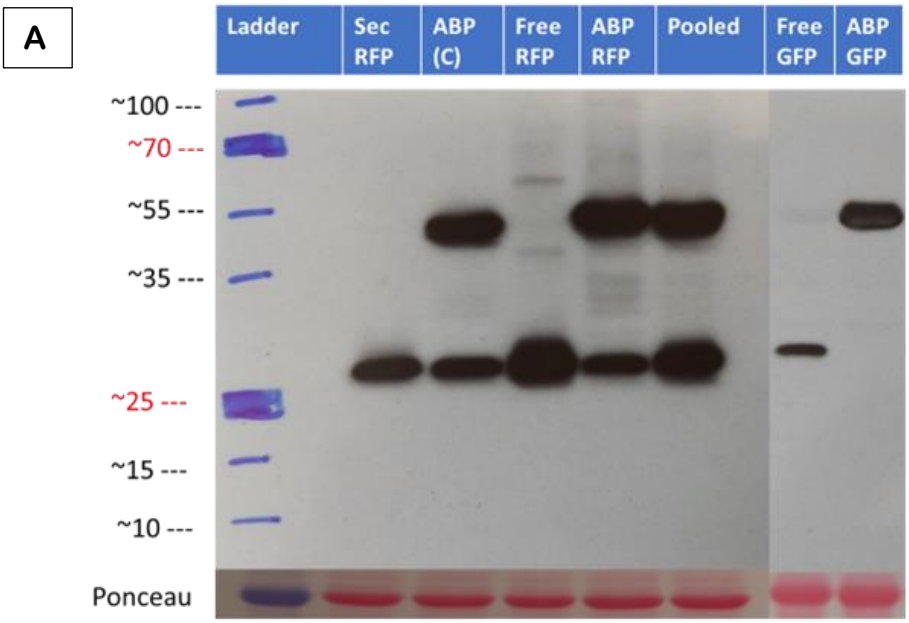


Figure 4: Spraying leaves with IBA and TIBA makes it more susceptible to *P. infestans* infection.
4-5 weeks old *N. benthamiana* plants were sprayed with 10 or 100 ppm TIBA or IBA. Control plants were sprayed with DMSO. After 2 hours when the chemicals dried off, leaves were detached from the plants and the abaxial sides were inoculated with *P. infestans* spores. Number of inoculations showing signs of infection (**A**) and the diameter of lesions (**B**) were measured. Error bars are standard errors. The experiment has been repeated twice.

<u>Cloning and sub-cellular localization of *ABP19.5*</u>:
We have previously reported that a putative germin-like protein, *ABP19* is significantly upregulated in infected resistant raspberry cultivar, Latham. This gene is closely located to Rub118b, the genetic marker for root rot resistance (Graham et al., 2011). When studying the genomic region in more detail it was found that there were nine copies of *ABP19* in close proximity to each other. Among these, the fifth copy, *ABP19.5*, showed upregulated expression in infected Latham by qRT-PCR analysis. We cloned *ABP19.5* into two different expression vectors, N-term GFP and N-term RFP. We did Western blot to check the integrity of the proteins. Expected sized bands (~50 kDa) were observed in all lanes but a second smaller band was observed below *ABP19.5:RFP* similar to the size of free RFP (~27 kDa) (Fig. 5A, lanes 3 & 5). This indicates the protein may have been cleaved. The *ABP19.5:GFP* showed intact protein with no cleavage (Fig 5A, lane 8). Overexpressing the proteins in *N. benthamiana* leaves showed that both *ABP19.5:GFP* and *ABP19.5:RFP* were mainly localized to the ER with some *ABP19.5:GFP* being secreted to the plasma membrane (PM) (Fig 5B & C).



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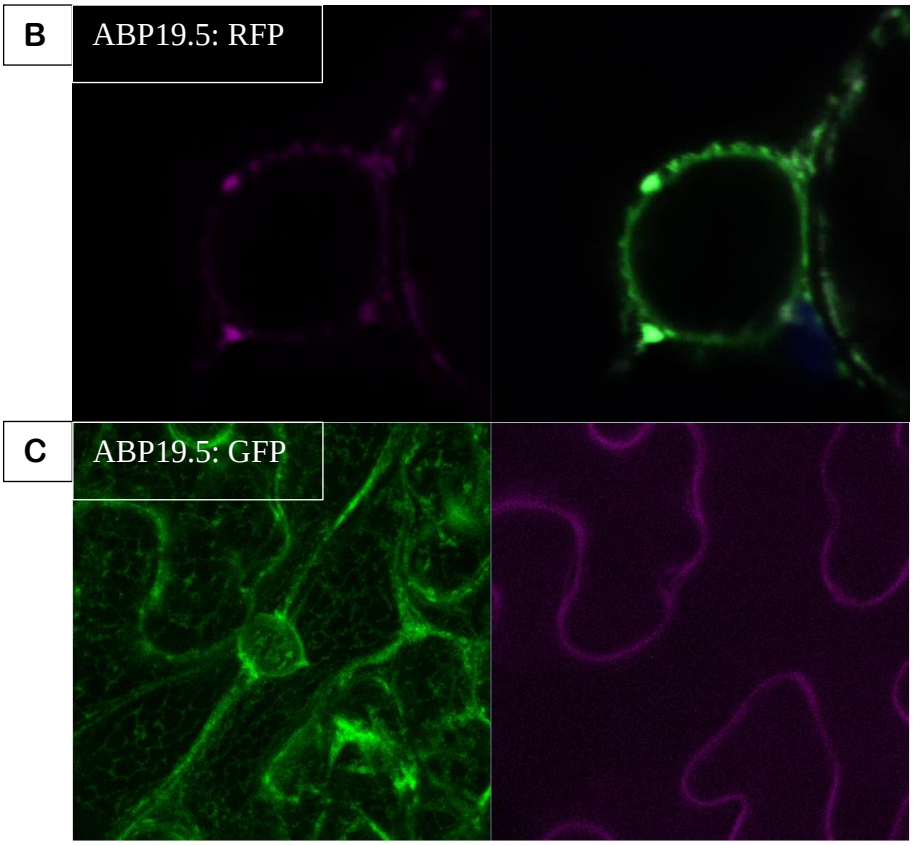

Figure 5: Fusion of *ABP19.5:GFP* mainly localized to the ER with some secreted to the apoplast. A. Four weeks old WT *N. bentahmiana* leaves were inoculated with *ABP19.5:GFP* and *ABP19.5:RFP*. Free RFP, Free GFP and secreted RFP was used as controls. Leaf discs were collected after two days and used for Western blot analysis. The lanes 1 to 8 indicates the following; protein ladder, secreted RFP (sec RFP), *RiABP19* cloned by a previous student (ABP C), Free RFP, *ABP19.5:RFP* (ABP RFP), pooled samples from all leaf discs (pooled), Free GFP and *ABP19:GFP* (ABP GFP). The upper bands showing ~ 50 kDs shows *ABP19.5* with GFP or RFP fusion. The lower band ~27 kDa is the size of GFP or RFP proteins. Below is the Ponceau staining of the samples as a loading control. This has been repeated on two biological replicates and repeated at least three times. **B.** *ABP19.5:RFP* expressed on leaves with GFP-labelled ER. **C.** *ABP19.5:GFP* expressed on leaves with RFP-labelled PM.

Cloning of *ABP19.10*:

To understand the role of TIBA in root rot infection at the transcriptomic level, we set up experiments, where raspberry plants rooted via the hydroponic system, were infected with *P. rubi* and treated with TIBA at the same time. After seven days, roots were collected from the samples and used to extract RNA which were then sent for RNA-sequencing. After receiving our data from RNA-seq experiment, we were able to identify the specific copy of *ABP19* gene that is significantly upregulated in infected roots of Latham. To our surprise, this gene was not located with the cluster of *ABP19* genes we found previously. Rather it was found in a different scaffold, and we named it *ABP19.10*. We confirmed the upregulation of *ABP19.10* in infected Latham by qRT-PCR (Fig 6A).

To understand whether the tagging GFP at the C-terminal was responsible for the cleavage of *ABP9.5:RFP* this time, we cloned *ABP19.10* into N-term GFP and C-term GFP. Western blots showed that *GFP:ABP19.10* was cleaved but *ABP19.10:GFP* was intact (Fig 6B). Next, we will express these proteins in transgenic plants with RFP-tagged PM and RFP-tagged ER to confirm the sub-cellular localization.

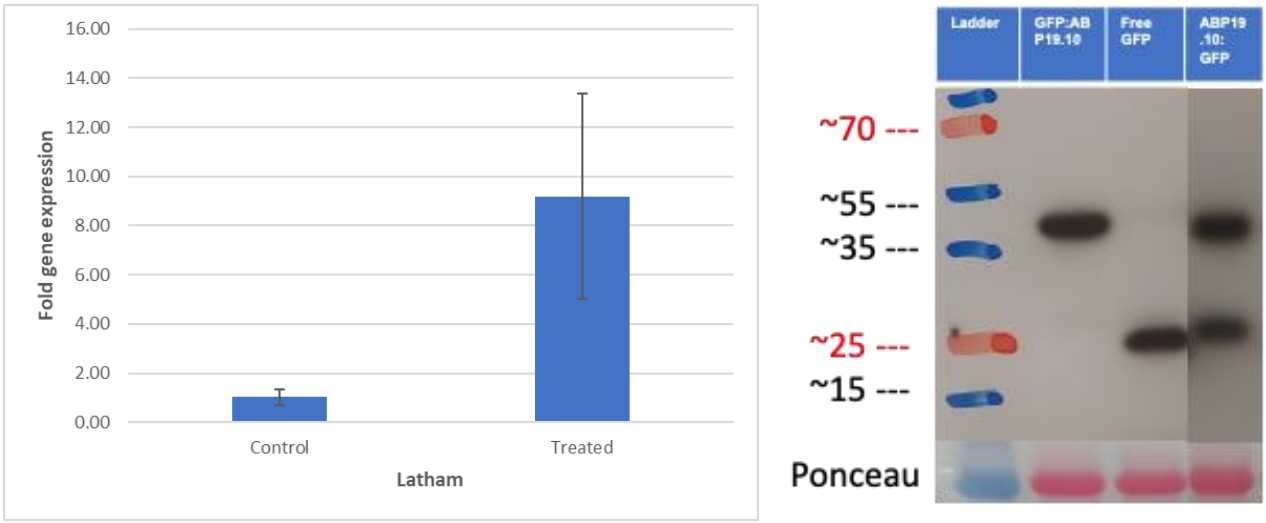

Figure 6: *ABP19.10* is significantly upregulated in infected roots of resistant cultivar. The C-terminal GFP fusion of *ABP19.10* showed intact protein. A. qRT- PCR showing the expression of *ABP19.10* being significantly upregulated in infected roots of Latham at 7dpi. This is the average of three biological replicates. Error bars are standard errors. The experiment was repeated three times. **B.** Western blot showing the intact protein of *ABP19.10:GFP* fusion (Lane 2) and the cleaved protein of *GFP:ABP19.10* fusion (Lane 3). Below is the Ponceau staining of the samples as a loading control. This has been repeated on two biological replicates and repeated two times.

AvrPto Cell Death Assay on *NbABP19* VIGS plants:

Previously we reported that cell deaths (CD) triggered by the virulence protein secreted by *Cladosporium fulvum*, *Avr4*, were significantly delayed in *ABP19* silenced plants. *AvrPto* is another virulence protein secreted by *Pseudomonas syringae* and shares the same signalling cascade as *Avr4* i.e MAP3Kε signalling cascade (Stulemeijer et al., 2007, King et al., 2014). We wanted to test whether *ABP19* silenced plants shows similar response to *AvrPto* CDs. We observed that the hypersensitive response (HR) to *AvrPto* was also significantly lower in *ABP19* silenced plants (Fig. 7) implying *ABP19* may be directly or indirectly involved in host immune response via MAP3Kε signalling cascade.

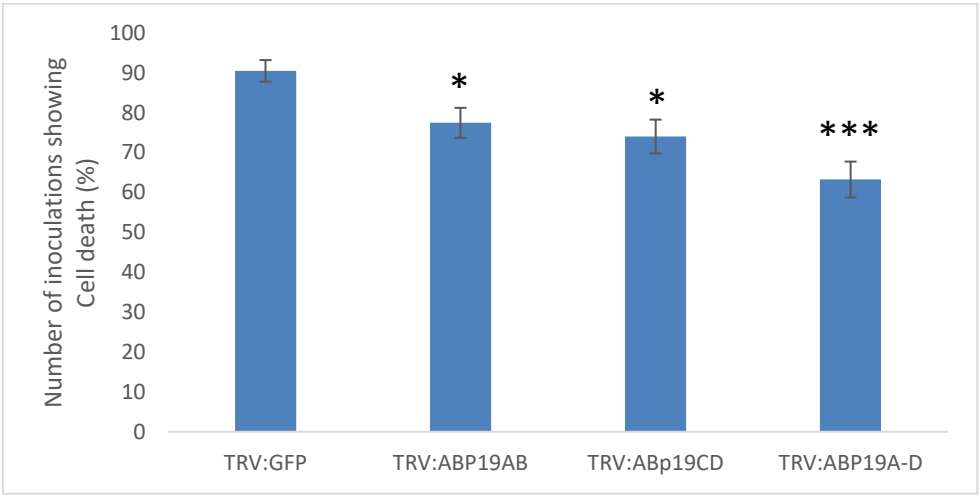

Figure 7: The HR response to AvrPto infection is significantly lower in *ABP19* VIGS plants. The bar graphs depict the average percentage of infiltrated sites that developed CD at 7 dpi. Error bars are standard errors, and the graph represents the combined data from three biological replicates. Significant differences to the GFP control by Holm-Sidak method one-way ANOVA are indicated by the asterisks. * P=<0.05 and *** P= <0.001

Infection of VIGS leaves with *P. infestans*:

We also infected *NbABP19* VIGS leaves with spores of *P. infesans* to check whether silencing of *ABP19* made the leaves more susceptible to infection. Even after 13 dpi, we hardly observed any infection. TRV makes the leaves very hard and crunchy which may make it harder for the oomycete to penetrate. However, counting the number of leaves at 13 dpi showed higher number of *ABP19* silenced leaves are being infected

compared to control (Fig. 8). More replicates will be needed to establish significance. Using tender and younger leaves may improve the rate of infection.

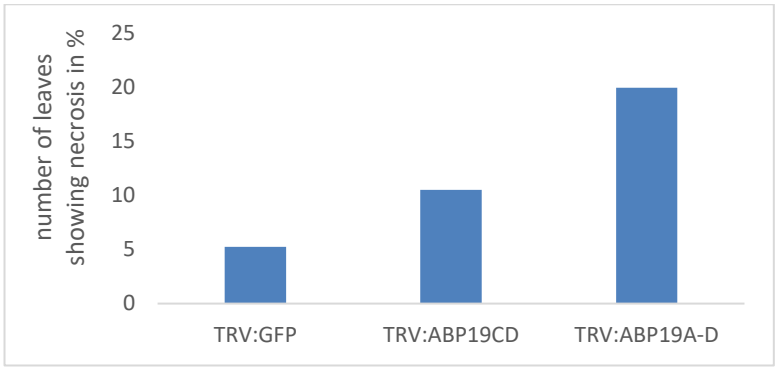

Figure 8: Silencing *ABP19* makes the plants more susceptible to *P. infestans* infection. Leaves were collected from *NbABP19* silenced plants and inoculated with *P. infestans* spores. The number of leaves showing signs of necrosis was counted at 13 dpi.

Resistance in Latham cultivar:

The raspberry cultivar, Latham is resistant to root rot. This cultivar has vigorous root system and contains the root rot resistant marker, Rub118. To understand its mechanism of resistance we first wanted to confirm whether the pathogen is able to enter the roots. We infected Latham root segments and roots attached to the plant with GFP-tagged transformant on *P. rubi*. We observed the infection peg forming to penetrate the root, followed by intercellular development of the mycelia in the root at 9dpi (Fig 9). This confirmed that Latham roots are being infected by *P.rubi* but they are combating the disease through other mechanism.

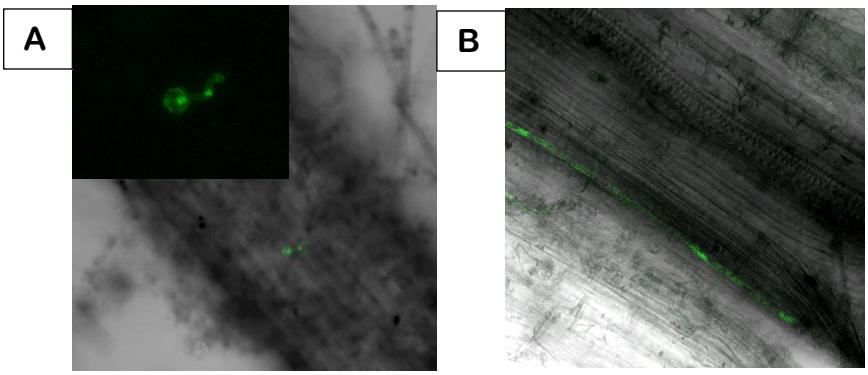

Figure 9. Latham roots are infected by *P. rubi.* A. Infection peg forming to penetrate the roots. **B.** Intracellular development of *P. rubi* in Latham root segments at 9 dpi.

To test whether Latham roots were responding to pathogen attack by early cell deaths, we attempted to stain cell deaths in infected roots with trypan blue, but we could not see any clear cell deaths. Since the upregulated ABP19 in Latham roots belong to the germin family of proteins, many of which have oxalate oxidase or superoxide dismutase activity, we hypothesized that Latham roots may be defending itself through reactive oxygen species (ROS) burst. To test this hypothesis, we attempted to stain infected raspberry roots with DAB. This experiment was also not successful as the roots were too dark to differentiate between the control and infected. We will reattempt these root staining experiments with optimised protocols.

Milestone	Target date	Achieved date
Analysis of transcriptomics data from roots	December 2022	
qPCR to confirm the upregulation of important genes	December 2022	
Treatment of raspberry roots with IBA/TIBA +/- infection in hydroponic system	March 2023	

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Treatment of raspberry roots with IBA/TIBA in hydroponic system to study the effect on roots	March 2023	
Expression of ABP19 in infection time course	December 2022	
Pull down to study the ABP19 protein interaction	December 2022	
Overexpression of ABP19 and Cf4/Avr4, AvrPto CD assay	Ongoing	
Overexpression of ABP19 and infection with P. infestans	March 2023	
Infection of VIGS leaves with P. infestans	Ongoing	
Host induced gene silencing of PiViiin	December 2022	
Sub-cellular localization of ABP19.10	December 2022	
Re attempt to stain roots with DAB/Trypan blue to see ROS burst/Cell death in Latham roots	March 2023	
Imaging of Actin labelled *P. infestans* after treating with TIBA/IBA	March 2023	
Transcriptomics Analysis of RNA-seq data from P. rubi	March 2023	

2. Issues arising / Risk management:

3. Knowledge transfer (KT) activities/resources delivered and future KT opportunities:

(please attach any KT outputs relating to the project e.g. presentations, articles)

- I gave five-minute presentation at the JHI Postgraduate Event 2022.
- I will be giving a 15-minute talk at the ISRR Dundee Medal Lecture and Root Research Workshop 2022
- I will be presenting at the CMS seminar at JHI

4. Placement requirements (if applicable)

Where applicable as part of contract, indicate industry/AHDB placement dates and any activity or outputs from the placement

Start Date:		**End date:**	

References:

1. Graham, J., Hackett, C. A., Smith, K., Woodhead, M., MacKenzie, K., Tierney, I., Cooke, D., Bayer, M. & Jennings, N. (2011). Towards an understanding of the nature of resistance to Phytophthora root rot in red raspberry. *Theoretical and applied genetics*, *123*(4), 585-601.
2. King, S. R., McLellan, H., Boevink, P. C., Armstrong, M. R., Bukharova, T., Sukarta, O., Win, J., Kamoun, S., Birch, P. R. J. & Banfield, M. J. (2014). Phytophthora infestans RXLR effector PexRD2 interacts with host MAPKKKε to suppress plant immune signaling. *The Plant Cell*, *26*(3), 1345-1359.
3. Stulemeijer, I. J., Stratmann, J. W., & Joosten, M. H. (2007). Tomato mitogen-activated protein kinases LeMPK1, LeMPK2, and LeMPK3 are activated during the Cf-4/Avr4-induced hypersensitive response and have distinct phosphorylation specificities. *Plant Physiology*, *144*(3), 1481-1494.

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