Studentship Project: Annual Progress Report October/2019 to September 2020

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 aims and objectives

Our research question is "What role might auxin play in plants challenged with *Phytophthora* species and how can this knowledge be applied to combat Raspberry root rot (PRR) disease and develop novel strategies for disease control". The overall aims of the project are:

1. Investigate the effect of auxin, activators and inhibitors on *Phytophthora rubi* isolates *in vitro*
2. Investigate the role of auxin on raspberry root rot disease development
3. Functional analysis of ABP19a on auxin signalling and disease resistance

2. Key messages emerging from the project

We are exploring the role of ABP19 (Auxin binding protein 19) in resistance to PRR. ABP19 was highly upregulated in the *P. rubi* infected resistant cultivar, Latham. This gene is also closely located to the genetic marker for root rot resistance, Rub118b.

The raspberry ABP19a belongs to the germin-like superfamily proteins (GLPs), which are known for their involvement in host resistance. Sequence alignment and comparison showed that the conserved protein domains of GLP members were also conserved in the *Rubus idaeus* ABP19a (Figure 3B). Furthermore, the significant upregulation of ABP19a in the roots of infected Latham was confirmed by qPCR (Figure 4). Additionally, the silencing of *Nicotiana benthamiana*, NbABP19, resulted in reduced immunity response (Figure 5). These data support the role of ABP19 in host resistance. It is now important to understand how ABP19 confers resistance.

One hypothesis is that, if ABP19a is an auxin binding protein and binds to auxin specifically, the binding of auxin could initiate auxin signalling, resulting in root vigour thus providing resistance. One of the main phenotypic differences between the susceptible raspberry cultivar Moy and the resistant cultivar Latham is their root architecture. Latham has a thick, vigorous root system compared to Moy, which has unbranched weak root system making it suitable for pathogen invasion. A second hypothesis is that, like other GLPs, ABP19a may have oxalate oxidase (OXO) or superoxide dismutase (SOD) activity generating hydrogen peroxide (H_2O_2), which is an important signalling molecule for host resistance. The

upregulation of ABP19a in infected Latham may indicate higher production of H_2O_2. Our chemical screening data shows that H_2O_2 inhibits *P. rubi* mycelial growth (Figure 2A).

The second finding of the project is that the auxin transport inhibitor, 2,3,5-Triiodobenzoic Acid (TIBA) has been shown to inhibit *P. rubi* mycelial growth across all the nine isolates tested (Figure 2).Therefore, TIBA can be used as a means of controlling PRR. However, TIBA can affect plant growth and development because it is an auxin transport inhibitor. It is now important to understand the effect of TIBA on host plants. We also want to test the effect of other benzoic acid derivatives on *P. rubi* growth. This will help to clarify whether TIBA is causing inhibition due to disruption of auxin transport or due to the presence of benzene ring, which is a component of many synthetic fungicides developed for oomycete control.

3. Summary of results from the reporting year

Chemical Screening of nine different isolates of *P. rubi*:

To investigate the effect of auxin-related chemicals on mycelial growth of *P. rubi*, rye agar plates were prepared containing a dilution series of concentrations of 0, 0.1, 1, 10 parts per million range (ppm) using an established protocol (Randall et al., 2014); Bezanger et al., *in preparation).* The following chemicals have been tested: auxin IAA (Indole-3-Acetic acid), a potent synthetic auxin mimic NAA (1-Napthaleneacetic Acid), the auxin herbicide, 2,4-D (2,4-dichlorophenoxyacetic acid), the auxin transport inhibitors TIBA and NPA. The inhibition of mycelial growth of nine *P. rubi* isolates from a range of locations and years were screened (Figure 2.A)

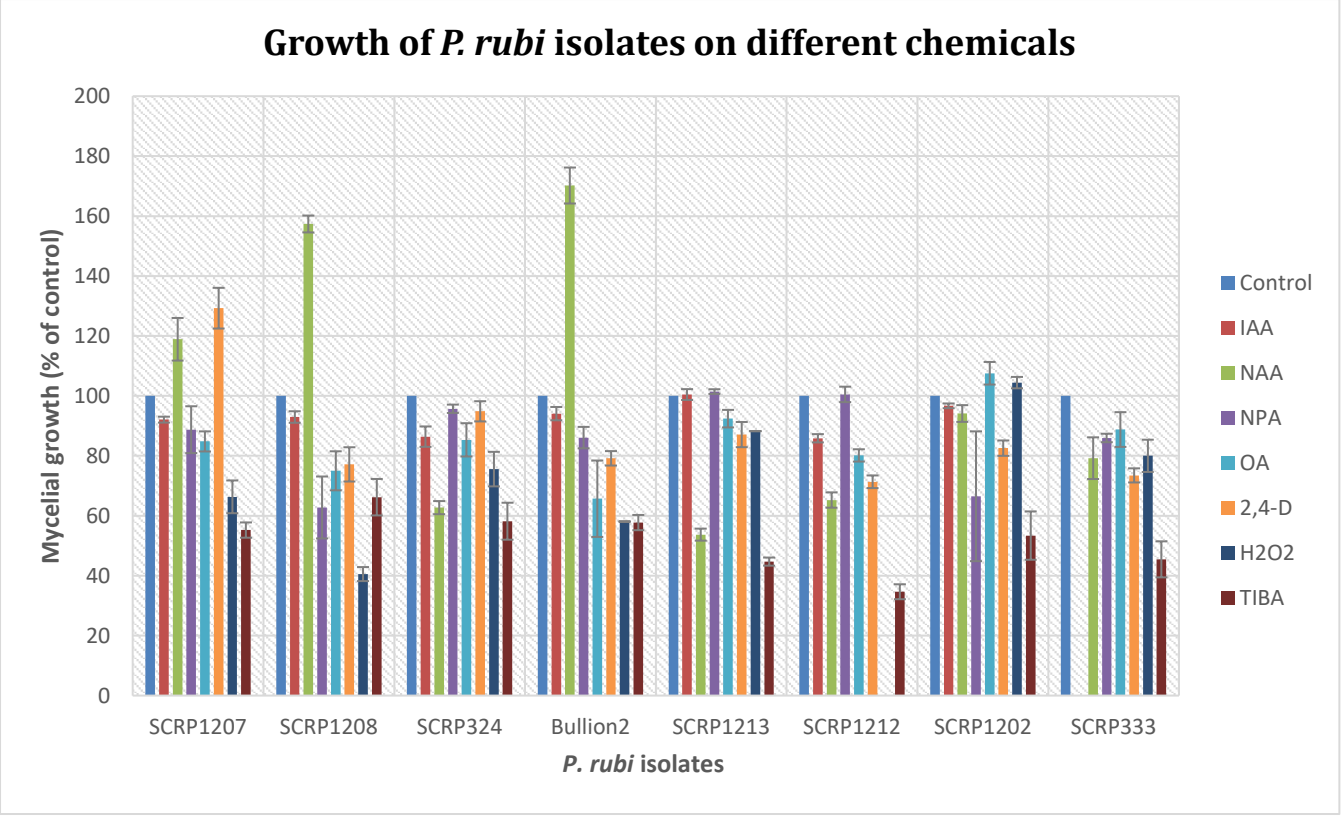


Figure 2.**A**: The effect of different chemicals at 10 ppm on the growth of *P. rubi* isolates. Growth was measured at 7dpi and calculated in % compared to the control. Here, 100% = the same as the control and less than 100% implies inhibition and over 100% promotes more growth compared to the control.

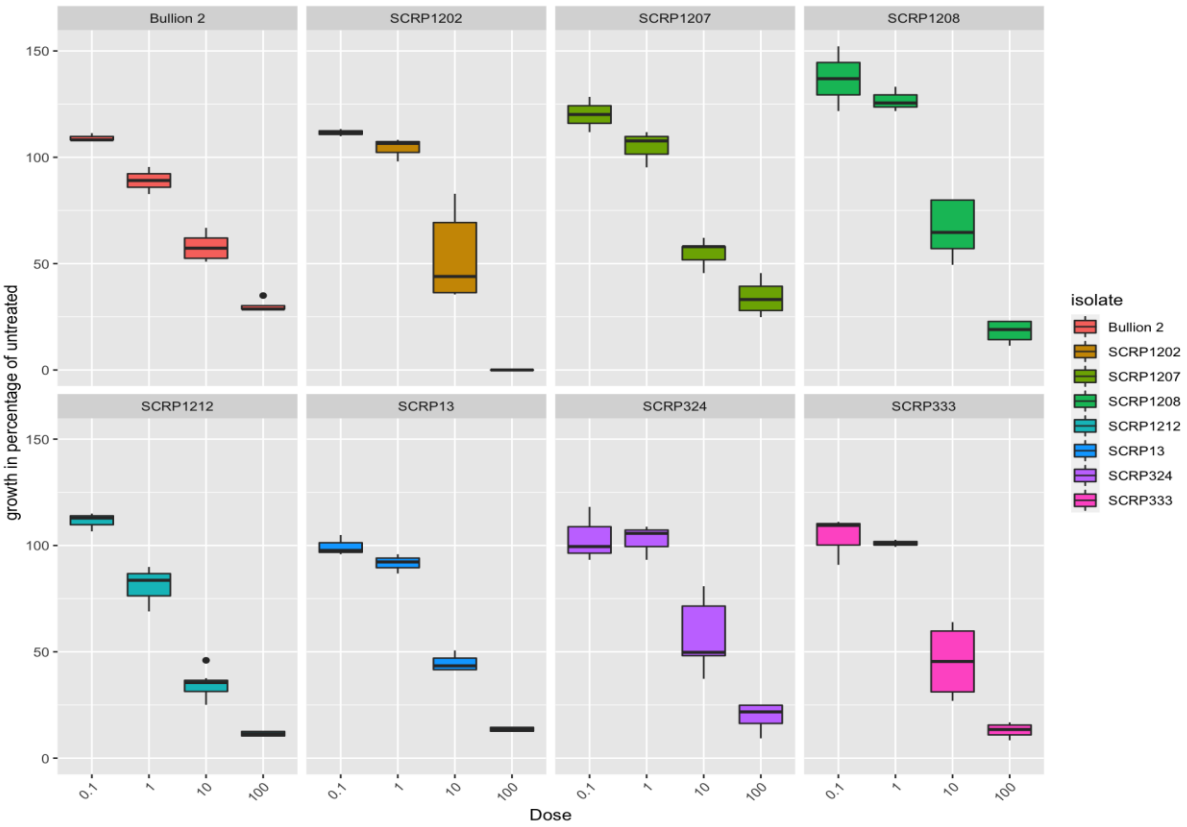


Figure 2.**B**: The inhibiting effect of TIBA on mycelial diametral growth of different *P. rubi* isolates. TIBA concentrations on growth media are 0.1, 1, 10 and 100 ppm. Growth measured at 7 dpi.

The substrates and potential products of an auxin binding protein with putative OXO or SOD activity was investigated as it may convey resistance to *P. rubi* in raspberry resistant cultivar Latham. Both oxalic acid and H_2O_2 are regulators of plant defence and can be directly antimicrobial. Therefore, the inhibition of oxalic acid and H_2O_2 on *P. rubi* growth were also tested (Figure 2A). The auxin transport inhibitor TIBA showed a consistent ability to suppress the growth of *P. rubi* on plates (Figure 2B). To understand whether this growth suppression was due to the perturbance of polar auxin transport, NPA, the polar auxin transport inhibitor was tested. Interestingly, NPA showed no outstanding suppression of *P. rubi* growth which indicates that TIBA is inhibiting growth through other unknown mechanisms. However, TIBA has shown to inhibit polar auxin transport by interfering with vesicle trafficking of membrane proteins through the action of actin stabilization (Dhonukshe et al., 2008).The inhibition of *P. rubi* growth could be due to the same reason. The growth suppression of *P. rubi* could also be due to the fact that TIBA is a benzoic acid derivative. It has been published that benzoic acid and their derivatives such as salicylic acid are inhibitory to *Phytophthora* species (Christie, 1965). To test this hypothesis, we are going to conduct chemical screening of salicylic acid, benzoic acid and 2,4,6-Triiodobenzoic acid on the growth of *P. rubi* isolates in near future.

Growing raspberry cultivars in soil-free media

To understand the role of auxin, activators and inhibitors on PRR disease development, the chemicals screened on *P. rubi* will be tested on Glen Moy and Latham raspberry, growing on soil-free media. Growth and phenotypes of the raspberry plants will be recorded after foliar and root-based application of the chemicals. The effect of auxin, activators and inhibitors will also be tested on infected plants. To begin this experiment, raspberry plants were grown using a hydroponic technique called the nutrient film technique (NFT). Cuttings were taken from high health plants, dipped in rooting hormone, and then placed in rockwool plugs, which were previously soaked in a weak nutrient solution. The cuttings were then placed on a heat mat (25^{o}C) in a mist unit. Rooting were observed after 3-4 weeks. The rooted cuttings were transferred to the NFT tanks filled with weak nutrient solution (8g Solufeed dissolved in 16L of water). The nutrient solution was circulated from the reservoir with water pumps to the channel where the

plants were placed after rooting. Thus, the roots were not submerged in deep water rather the circulating solution provides oxygen, nutrient and water to the roots. A fibre mat was placed on the channel for even distribution of the nutrient solution, which was removed later when roots were older. It was observed that the PRR susceptible cultivar, Glen Moy, was difficult to initiate and develop roots.

ABP19 (Auxin Binding Protein 19) in Moy and Latham

In an in-house RNA-seq study, it was observed that when Latham and Moy were infected by *P. rubi*, several important genes were upregulated in the infected root. Among these, a gene with similarity to Auxin-binding protein 19 (ABP19) was significantly upregulated in the resistant cultivar, Latham. This gene was also closely located to the genetic marker for root rot resistance, Rub118b. Therefore, it is important to understand the role of ABP19 in resistance. Analysis of the genome sequences of Moy and Latham revealed the presence of multiple copies of ABP19 in both Moy and Latham sequences. Surprisingly, nine homologous copies of the gene were found closely located to each other (Figure 3A). Their sequences were compared to each other and to the published ABP19s i.e. peach (PpABP19/20) and cotton ABP19 (GhABP19) (Ohmiya, 2002; Pei et al., 2019). The raspberry ABP19 sequences were blasted against the genome sequence of the model plant *Arabidopsis thaliana*, to find the closest hit, AtGer3 (Staiger et al., 1999). Moreover, the databases of closely related species, strawberry (FvABP19-like) and black raspberry were also searched to find multiple copies of similar ABP19-like genes. The presence of multiple gene copies in different species and the multiple copies being present may indicate their functional importance. Sequence analysis and comparison showed that the ABP19 genes belong to the family of germin-like proteins with the highly conserved protein domains (Boxes A, B and C in Figure 3B).

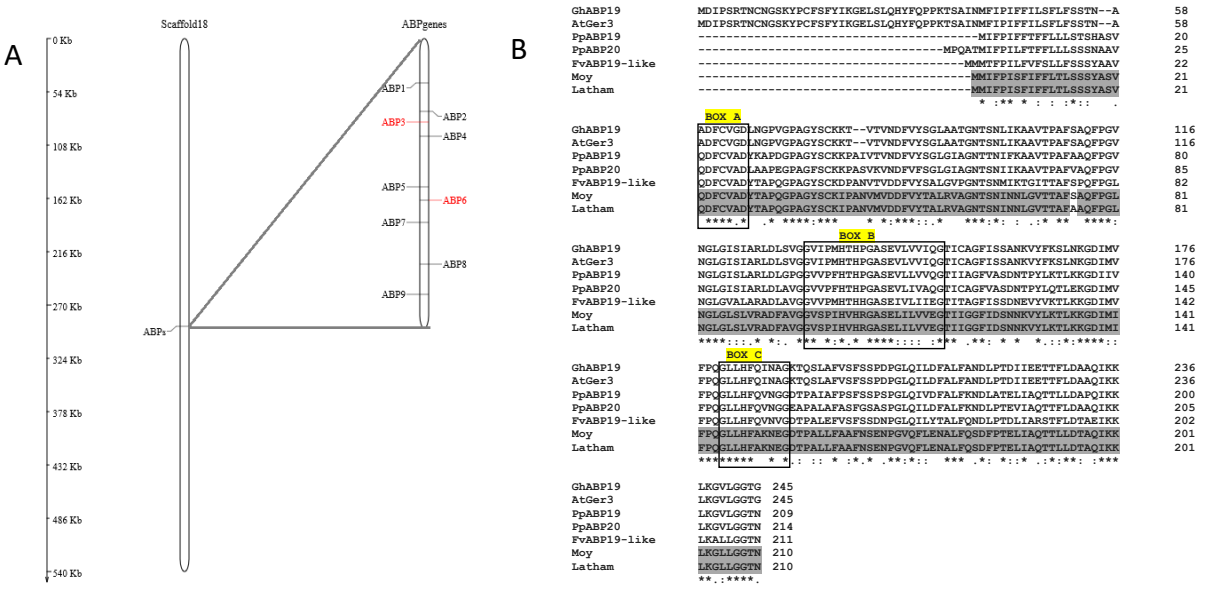


Figure 3.**A**. The location of nine ABP19 genes on Moy scaffold. **B**. Multiple sequence alignment and comparison of different ABP19 proteins.

To confirm the presence of ABP19 gene and to understand which genes among the nine copies are expressed, RT-qPCR was conducted using the Universal Probe Library (UPL, Roche Molecular Systems Inc.). Primer pairs were designed from Moy sequences, that was specific to the different copies of the ABP19 genes. Primers were also designed so that they were common to all copies of the genes. This was done to identify the expression of the different copies. The cDNA was synthesised using Takara RNA to cDNA EcoDry Premix. Primer validation was conducted in seven dilution series using pooled samples of infected/non-infected Moy and Latham root cDNAs. As a reference the housekeeping gene, Actin2, was used. Samples were run in the StepOne PCR real-time PCR system to enable precise quantitative

real-time PCR results. The use and testing of the different primer combinations are on-going. A primer pair designed to multiple ABP19 gene copies, ABP19.a, showed an expression that was less than the reference gene. ABP19.a primer pair was then used to check the expression of ABP19 in three biological reps of infected and non-infected Latham and Moy root samples. Relative quantification in relation to Actin2 expression was analysed using the Pfaffl method. It was observed that the fold change in gene expression was significantly higher in infected versus non-infected Latham when compared to Moy (Figure 4A). We also observed that the expression of ABP19 was 8 times higher in infected Latham when compared to Infected Moy (Figure 4B).

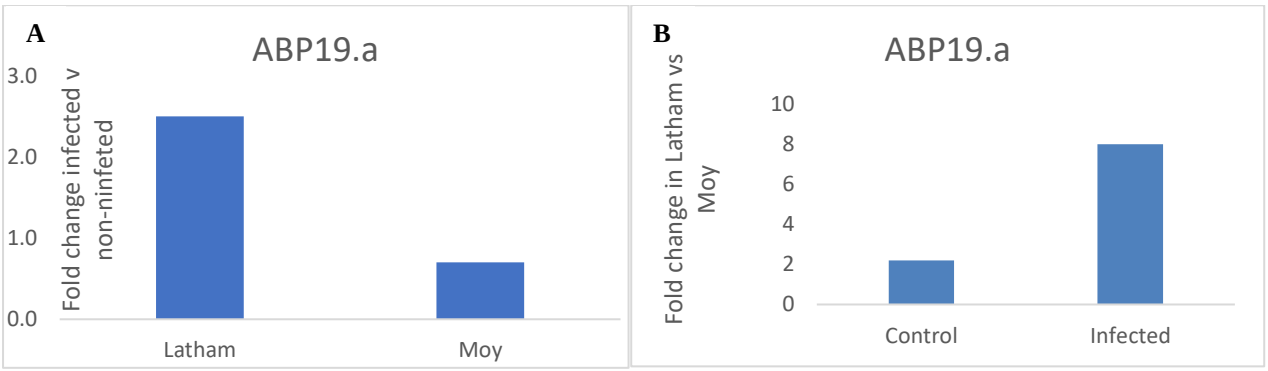
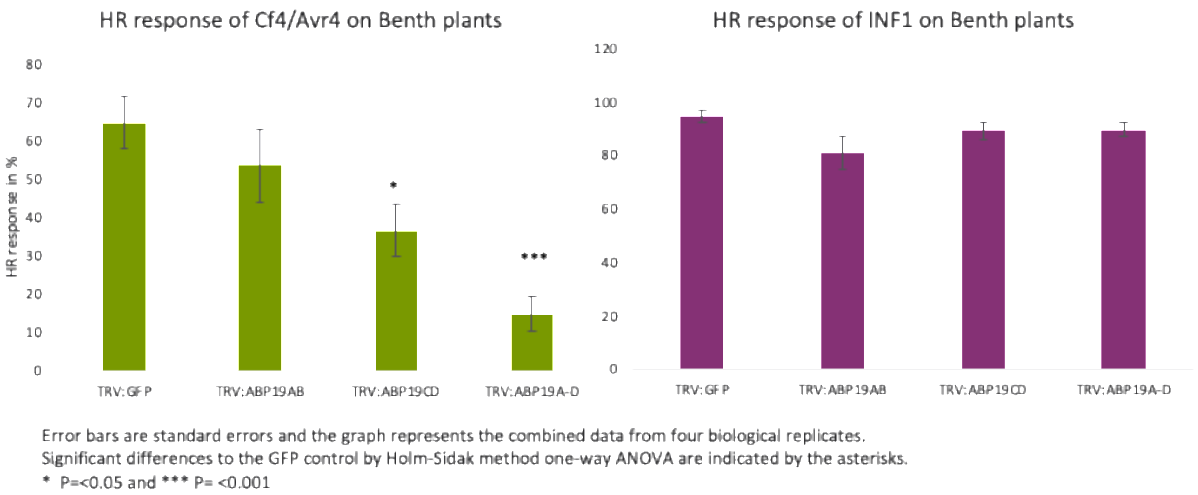


Figure 4 A. Fold change in ABP19 expression in infected vs non-infected Latham and Moy roots.
B. Fold change in ABP19 expression in Latham vs Moy control and infected plants.

The next step will be to find out which particular gene/genes are expressed among the nine copies. To do this we will perform qPCR with the primers that were designed specifically to each particular gene. After that, the ABP19 gene/genes of interest will be cloned into expression vectors for further analysis.

Cell Death Assays conducted on NbABP19 silenced *Nicotiana benthamiana* plants

To further understand the importance of ABP19 genes in disease resistance, cell death (CD) assays were conducted on NbABP19 silenced *N. benthamiana* plants. Virus-induced gene silencing (VIGS) was used to knock down the expression of four *NbABP19*-like genes in *N. benthamiana* plants. Based on sequence similarity, the *NbABP19* genes were paired into two groups *NbABP19A/B* and *NbABP19C/D*. VIGS were constructed in such a way that in the first construct, TRV:ABP19AB, NbABP19A and B were silenced and in the second construct, TRV:ABP19CD, NbABP19C and D genes were silenced. In a third construct, TRV: ABP19A-D, all four *N. benthamiana* ABP19 genes were silenced. After two weeks of VIGS infection, the oomycete PAMP (Pathogen Associated Molecular Pattern) elicitin, INF1 and the fungal effector, Avr4, co-expressed with its receptor, Cf4, were inoculated in the VIGS plant to measure the effect of the silenced NbABP19 genes on the hypersensitive response (HR). INF1 is the extracellular elicitor protein secreted by *Phytophthora infestans*, that induce a HR in *N. benthamiana* plants (Kamoun et al., 1997). The tomato Cf4/Avr4 induced HR is known to function through a different immune signalling cascade compared to INF1 and is a well-established system to study immunity in *N. benthamiana* plants (Thomas et al., 1998; Thomas et al., 2000). Four biological reps of these analyses showed that ABP19 silencing, particularly targeting all four genes, significantly perturbs the Cf4/Avr4 CD but not INF1 CD (Figure 5). The conclusion so far is that silencing of NbABP19 resulted in reduced immunity of the host plant thus implying the involvement of NbABP19 in resistance. More reps of these CD assays will be continued to confirm this observation. We will also investigate other cell deaths in *NbABP19* VIGS plants.



Error bars are standard errors and the graph represents the combined data from four 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

Figure 5. Cf4/Avr4 and INF1 CD in *NbABP19* silenced plants. The graph depicts the average percentage of infiltrated sites that develop CD at 7 dpi. One-way ANOVA gave significance of P<0.001 for the Cf4/Avr4 cell death assays in TRV: ABP19A-D.

4. Key issues to be addressed in the next year

The milestones for next year have been arranged according to the project objectives:

1. **To investigate the effect of auxin activators and inhibitors on *Phytophthora rubi* isolates in vitro**
    - Investigate the effect of benzoic acid and derivatives on *P. rubi* mycelial growth on plates.
    - Investigate whether TIBA is disrupting vesicle trafficking in *P. rubi*.
2. **To investigate the role of auxin on raspberry root rot disease development**
    - Challenge raspberry cultivars in soil-free media with *P. rubi* isolates to re-evaluate the durability of the resistance.
    - Measure the growth and phenotype of raspberry cultivars after foliar and root-based applications of auxins, activator and inhibitors to soil-free growth media.
    - Perform transcriptomics on raspberry cultivars after infecting with *P. rubi* isolates.
    - Perform transcriptomics on raspberry cultivars challenged with *P. rubi* after auxin, activator and inhibitor treatment.
    - Use confocal imaging to monitor PRR disease development in raspberry roots after foliar and root-based application of a range of auxins, activator and inhibitor treatment.
3. **Functional analysis of RiABP19 on auxin signalling and disease resistance**
    - Cloning of raspberry ABP19 sequences into tagged-plant expression vectors.
    - Establish the importance of ABP19 in disease resistance using hydrogen peroxide staining of raspberry roots.
    - Investigate other cell deaths in *NbABP19* VIGS plants.
    - Investigate whether Cf4 and INF1 CDs are affected by TIBA.
    - Identify root exudate components in raspberry cultivars which may be attractants for zoospores.

5. Outputs relating to the project

(events, press articles, conference posters or presentations, scientific papers):

Output	Detail
Poster	Presented a poster at the Postgraduate event organised by the James Hutton Institute
Presentation	I presented my results so far at a group meeting (PRR meeting) organised at the Institute.
Report	I have submitted 9-month progress report to Warwick University and successfully passed the evaluation.

6. Partners (if applicable)

Scientific partners	
Industry partners	
Government sponsor	

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