AHDB Horticulture

Project title:	Biocontrol as a key component to manage brown rot disease on cherry
Project number:	CTP_FCR_2017_3
Project leader:	Xiangming Xu, NIAB EMR Michael Shaw, University of Reading
Report:	Annual report, October 2018
Previous report:	NA
Key staff:	Sophia Bellamy
Location of project:	NIAB EMR
Industry Representative:	NA
Date project commenced:	October 2017

DISCLAIMER

While the Agriculture and Horticulture Development Board seeks to ensure that the information contained within this document is accurate at the time of printing, no warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Sophia Bellamy

PhD Student

NIAB EMR

Signature ..... Date .....

Prof Xiangming Xu

Head of Department

NIAB EMR

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

- Two microbial biocontrol agents (BCAs) (*Aureobasidium pullulans* and *Bacillus subtilis*) have been identified and have shown promise as a potential control method for brown rot disease of stone fruits.

Background and expected deliverables

Brown rot, caused by *Monilinia* spp. is one of the most important diseases of stone fruits worldwide. *Monilinia laxa* infections reduce crop yield in the field by causing blossom blight, twig cankers and fruit rot, and they can also cause latent infection on fruits. *Monilinia laxa's* low optimal temperature for conidial growth (5-10 ^{o}C) can lead to the rapid spread of rot within cold storage and reduce fruit shelf life. With the restriction of post-harvest fungicide application, the spread of rot can lead to significant post-harvest crop loss (Martini & Mari, 2014).

The primary source of inoculum is from overwintering on mummified fruits left on the trees and the orchard floor. The main control method relies on fungicide spraying. NIAB EMR recently identified two microbes that significantly reduced sporulation of the brown rot fungus *M. laxa* under laboratory conditions. We are currently investigating how to optimise the use of biocontrol products in practice, regarding suppressing sporulation on overwintered fruit mummies and preventing infection of blossoms and fruit.

A better understanding of the effect of interactions between BCAs and the brown rot pathogen over time is needed. Combined with an understanding of the disease epidemiology, this knowledge will inform the best application time for improved efficacy of the BCAs. We expect that an increased interaction time of a BCA with a pathogen, will lead to increased biocontrol efficacy. The ability of the BCAs to colonise and survive on the fruit or blossom is tantamount to its effectiveness. Therefore, more research into the behaviour of the two new BCAs when applied to the target area of the crop, the trophic networks and overall ecology, are crucial in their effective use (Ruano-Rosa *et al.,* 2016).

The two microbial antagonists initially characterised by Rungjindamai *et al.* (2013) show promise to become commercialised as biocontrol products. Future research to better understand their ecology, such as survival on different plant organs under different conditions, will lead to improved deployment to increase biocontrol efficacy. This knowledge will help to develop new biocontrol based strategies that are needed for more sustainable food production.

Objectives of the project

This CTP studentship project aims to answer the following questions:

- Can biocontrol organisms reduce primary inoculum of *M. laxa*?
- Can biocontrol organisms reduce blossom wilt and subsequent infection of young fruitlets by *M. laxa*?
- What is the effect of biocontrol organisms on the cherry fruit microbiome and to what extent does the microbiome affect post-harvest rots?
- Can *B. subtilis* also reduce the inoculum source of bacterial canker on cherry leaves?

Summary of the project and main conclusions

In the first year of this PhD study, molecular techniques for assessing the living cells on the fruit surface were refined. A dose-response experiment was conducted in the laboratory to identify the optimum BCA concentration to successfully reduce brown rot on cherry. Two field experiments were conducted in the winter and the spring to assess the survival rate of the BCAs on mummified fruits (winter) and blossoms (spring). Once a survival rate is established, it can be cross-referenced with the dose-response experiment to assess if the BCAs can successfully colonise and survive at high enough levels to be effective. The results of these experiments will be completed in December 2018, and reported in the next annual report.

Financial benefits

This is only the first year of a four-year project so there are no results that will have direct effects on commercial fruit production at this stage.

Action points for growers

- There are no grower action points at this early stage of the project.

SCIENCE SECTION

Introduction

Brown rot caused by *Monilinia* fungi is a major disease in rosaceous fruits and can be found in all temperate regions where stone fruits are grown. The disease is responsible for reducing yields and infecting blossoms as well as ripe fruits in the field and post-harvest. The fungi large host range includes economically important fruit crops, leading to extensive research into the causal agent. Thorough reviews of research into *Monilinia* species were conducted by Wormald (1954) and Byrede and Willetts (1975). Since then advancements in techniques have led to significant development of potential control agents of this pathogen with a focus on moving away from chemical fungicides. Despite these extensive studies, there is still no effective biological control of brown rot on cherry. New legislation is reducing the number of fungicides available to growers, therefore more needs to be done to fill the gap this creates. Many researchers have turned to biocontrol agents to help reduce the impact of pathogens. This review will look into the cherry industry and how it currently manages fungal diseases such as *Monilinia laxa* and the potential of biocontrols to fill the gap created by current fungicide legislation.

Project aims:

- The project aims to understand microbial ecology that underpins the use of microbial biocontrol agents (BCAs) to manage brown rot on stone fruit
- To develop new biocontrol based strategies for managing brown rot and evaluation of such strategies in small scale and commercial scale studies.
- The project will look at minimise overwintering inoculum and protect flowers from infection. It is expected that better control of the early epidemic stage will greatly reduce the disease as fruits mature.

Anticipated outcomes:

The research outcomes will assist in the development of management strategies for brown rot on stone fruit, integrating BCAs with other management practices based on our understanding of ecological characteristics of available BCAs.

Materials and methods

Experiment 1: The use of biocontrol to reduce primary inoculum source of *Monilinia laxa*

This experiment will determine whether targeting BCAs against disease on mummified fruit is an effective means to reduce primary inoculum and to work out key times of BCA application.

Overwintering experiment in the field

This experiment aims to evaluate the survival and efficacy of biocontrol agents *B. subtilis* (B91) and *A. pullulans* (Y126) on mummified fruit over winter. Preliminary analysis shows that they may not survive below 0°C (Y126) and 10°C (B91) and therefore we hypothesise a rapid decline in the viable population size, potentially affecting biocontrol efficacy. The experiment was conducted from December 2017 to March 2018 at NIAB EMR within a non-commercial cherry orchard. Four trees within the orchard were used to create a randomised block design with five treatments; each sampled six times over the winter period.

The five treatments included the two BCAs, *Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126), a fungicide treatment (Luna Sensation), a commercial biocontrol (Serenade) and sterile distilled water (SDW) as a control. For each treatment, samples were taken at six-time points throughout the winter. There were four repeats/blocks per treatment and time point. Michael Shaw, University of Reading, supplied 390 mummified plums from two unknown cultivars. Plums were used in this experiment because they have a greater surface area that leads to greater microbial recovery when compared to mummified cherries. Mummified plums of the same cultivar were separated into five groups, each randomly assigned to one of five treatments. The fruits were then sprayed with treatments using a handheld sprayer until run-off within separate plastic bags to avoid drifts. Treated fruits were placed in separate mesh bags and hung roughly 1.5 meters above the ground on orchard trees. Fruit from a single combination of treatment and sampling time point were placed in one bag.

Once collected, mummified fruits were washed in sterile distilled water and the wash subjected to propidium monoazide (PMA)-based DNA extraction and subsequent qPCR assay to determine the viable population of the two biocontrol microbes applied (Soto-Muñoz *et al.,* 2014). All samples have been taken, PMA treated and DNA has been extracted. The next step is to complete qPCR analysis to assess the number of viable cells within each sample collected. Then statistical analysis will be used to assess microbial survival and biocontrol efficacy. This will be completed by the end of 2018.

Experiment 2: The use of biocontrol to reduce blossom wilt and subsequent infection of young fruitlets

This experiment will look at whether BCAs are effective in reducing blossom wilt and subsequent infection of young fruitlets and what is the best time to apply BCAs for protecting blossom and fruit.

Blossom wilt field trial

This field trial was conducted in the spring of 2018 through to summer/fruit harvest 2018. Potted trees within the polytunnel were set up to run alongside the field experiment to replicate conditions of trees grown undercover. However, these trees were too young and did not produce fruit so the experiment will be redone for the spring-summer 2019. The experiment aimed to see to what extent can the BCAs colonise and survive on blossoms and through to fruit set.

There were five treatments, as in experiment 1: two BCAs, *Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126), a fungicide treatment (Luna Sensation), a commercial biocontrol (Serenade) and a sterile distilled water treatment as a control. For treatments B91, Y126 and control, samples were taken at six-time points to assess biocontrol viability and microbiota. For Serenade and Luna Sensation only the first and last time points were taken to asses microbiota. There were three repeats. Each branch was a time interval and each tree a treatment. Four trees make up one block/ repeat with twelve trees in total; Serenade and Luna Sensation will only be on one tree as there are only two time points. The treatments and time intervals and experimental design are shown in Table 1.

Treatments were prepared following the same procedure as in the overwintering experiment, using handheld sprayers, and blossoms were treated until runoff. Branches were prepared by removing old and new (un-opened) blossoms treated blooms of similar age. Blossoms and fruit were collected from April to June in 6 separate intervals. These intervals coincided with key growth stages: three intervals during blossom and three during fruit stages. Once collected, the fruit/blossoms were counted and washed in maximum recovery, an isotonic medium that reduces multiplication of microorganisms, and SDW to collect surface microbes (including our applied biocontrol organisms). The wash collected was then divided in two. One half was used for PMA-based DNA extraction and subsequent qPCR assay to determine the viable population of applied two biocontrol microbes. The other half will be for amplicon-metabarcoding, to study the effect of treatments on fruit surface microbiota. DNA extraction has been carried out on all samples and qPCR is planned to be carried out in December 2018 (see Gantt chart).

Table 1: Summary of the treatments and sampling times for the blossom wilt field trial

Time interval		Treatments*				
		Y (Y126)	**B (B91)**	**C (control)**	**F (fungicide)**	**S (Serenade)**
April 11th	Day 1 after application	Y1	B1	C1	F1	S1
April 16th	Day 5 after application	Y2	B2	C2		
April 19th	Day 8 after application	Y3	B3	C3		
April 30th	Green Cherry	Y4	B4	C4		
May 28th	Ripening fruit	Y5	B5	C5		
June 18th	Ripe fruit	Y6	B6	C6	F6	S6

**Aureobasidium pullulans* (Y126); *Bacillus subtilis* (B91); sterile distilled water treatment (control); fungicide treatment (Luna Sensation); commercial biocontrol (Serenade)

Experiment 3: Dose-response

A dose-response experiment was conducted to assess the efficacy of each biocontrol at different concentrations. Combined with the BCA survival studies, it will help decide best application time at specific times.

South African cherries, cultivar Lapins, purchased from a supermarket, were surface sterilised and treated by pipetting 5 µl of treatment into a shoulder wound. Twelve treatments where used: Four concentrations of each Biocontrol (10^8, 10^7, 10^6, 10^5 CFU/ml), Serenade (10^9), Luna Sensation and a negative and positive control (both sterile distilled water). The negative control was not inoculated with *M. laxa* and the positive control was inoculated 24 hours after treatment. Once treated the cherries were placed in sterilised honey jars. There was one honey jar for each treatment to keep from cross-contamination and there were three cherries per Jar. The Jars were arranged at random in three repeat blocks (three Jars each with three fruit per treatment) within a 20˚C incubator. After 24 hours the cherries were inoculated (except the negative control) with *M. laxa* (10^5 Spores/ml) by pipetting 5 µl into the wound. The wound/lesion size was assessed throughout a week using a digital calliper. Measurements were recorded in mm five days after *M. laxa* inoculation.

Results

Experiment 3:

An ANOVA and post hoc Tukey test were performed using R to see if there was any statistical significance difference between the treatments.

Aureobasidium pullulans (Y126):

Though there is a trend in increasing lesion size as the concentration of Y126 decreases (Figure 1), there is no significant difference between the concentrations. This may be due to the high variance within the data. There is a significant difference (P = 0.022686) between the positive control and the first concentration (10^7 CFU/ml), however no significant difference with the other three concentrations. This would indicate that a concentration of 10^7 CFU/ml may be needed to be maintained within the field for Y126 to be competitive with *M. laxa*.

Although there was no statistically significant difference between the fungicide and Y126 (conc. 10^7 and 10^9) with P = 0.9 and 0.19, respectively, brown rot was present in 50% of the cherries treated with Y126 concentration 10^7 and 10^9 where there was no fruit with brown rot in cherries treated with fungicide, Luna Sensation.

Figure 1 shows that as the concentration decreases the lesion size increases. This is to be expected as Y126 is known to be antagonistic towards *M. laxa* via competition for space and nutrients. As the concentration decreases so does Y126's ability to compete with *M. laxa.* As the biocontrol treatment was applied 24 hrs before the inoculation of *M. laxa*, this gave Y126 time to establish. A further experiment will be carried out to assess the effect of applying a Y126 treatment after *M. laxa* infection has occurred.

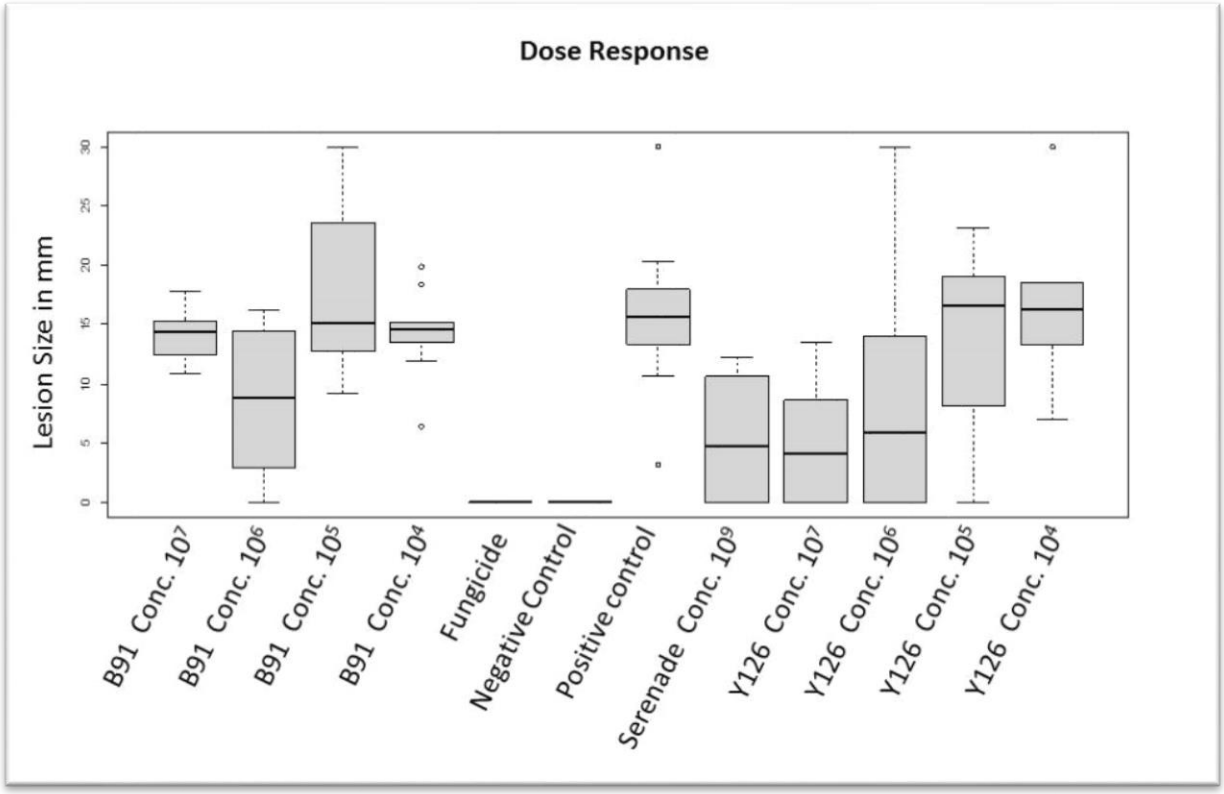

Figure 1. Box plot for dose-response in cherries (cultivar Lapins) showing lesion size in mm of six treatments including biocontrol agents *Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126) at four concentrations; fungicide Luna Sensation; commercial biocontrol Serenade; negative control and positive control with sterile tap water (negative was not inoculated and positive was inoculated with *M. laxa* 24 h after treatment). Concentrations in colony forming units (CFU) per ml

Bacillus subtilis B91:

There was no significant difference between the concentrations of B91 and the positive control. B91 antagonist property is the secretion of antibiotics; this may explain why there is little difference seen between the positive control and between the four concentrations. A follow-up experiment, applying B91 at higher concentrations and after *M. laxa* infection, will inform the best application time and dose to combat *M. laxa.*

A repeat of this experiment will be conducted in 2019 using the formulated product, higher concentrations and different treatment times (before, after and at the same time as *M. laxa* inoculation).

Discussion and Conclusions

The results from the dose response experiment have a high variance so a repeat of this experiment will be conducted in 2019 using the formulated products, higher concentrations and different treatment times (before, after and at the same time as *M. laxa* inoculation).

The initial results show that *Aureobasidium pullulans* Y126 will need to sustain a high concentration of 10^7 CFU per ml or more in order to compete with *M. laxa*. *Bacillus subtilis* B91 may need longer to establish and produce antagonist compounds in order to be effective, and this will be confirmed in the repeat experiment. If B91 needs a longer establishment time, it will have an effect on the application times of this BCA.

Data is still being collected and analysed for experiments 1 and 2.

Knowledge and Technology Transfer

The student attended and presented a poster at:

XV Meeting of the IOBC-WPRS - Poster presentation at the International organisation for biological and integrated control conference in Lleida, Spain. Secured 500 euro travel grant.

NIAB Poster day 2018 - Poster presentation at NIAB poster day 2018 in Cambridge, with the CTP.

Successful grant applications

Secured 500 euro travel grant for XV Meeting of the IOBC-WPRS**.**

£750 funding to attend the Biotechnology YES competition in October 2018 from the BBSRC.

Awards

- Primer Design Postgraduate Student Sponsorship scheme - Won Silver level sponsorship
- BSPP Grand challenges 2018 – Team winner

References

Allen, M., & Silver, B. (2010). Cherries. *Journal of Agricultural & Food Information*, *11*(4), 275–281. https://doi.org/10.1080/10496505.2010.516715

DEFRA (2016). (online) https://www.gov.uk/government/statistics/horticulture-statistics-2015 (Accessed Oct 2017).

Ippolito, A., & Nigro, F. (2000). Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. *Crop Protection*, *19*(8–10), 715–723. https://doi.org/10.1016/S0261-2194(00)00095-8

Janisiewicz, W. J., Kurtzman, C. P., & Buyer, J. S. (2010). Yeasts associated with nectarines and their potential for biological control of brown rot. *Yeast*, *27*(7), 389–398. https://doi.org/10.1002/yea.1763

Kappel F., Granger A., Hrotkó K., and SchusterM., (2011). Fruit Breeding. Handbook of Plant Breeding 8: 459-504.

Lima, G., Ippolito, A., Nigro, F., & Salerno, M. (1997). Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rots. *Postharvest Biology and Technology*, *10*(2), 169–178. https://doi.org/10.1016/S0925-5214(96)01302-6

Martini, C., & Mari, M. (2014). *Monilinia fructicola*, *Monilinia laxa* (Monilinia Rot, Brown Rot). In *Postharvest Decay* (pp. 233–265). Elsevier. https://doi.org/10.1016/B978-0-12-411552-1.00007-7

Ruano-Rosa, D., Prieto, P., Rincón, A. M., Gómez-Rodríguez, M. V., Valderrama, R., Barroso, J. B., & Mercado-Blanco, J. (2016). Fate of *Trichoderma harzianum* in the olive rhizosphere: time course of the root colonization process and interaction with the fungal pathogen *Verticillium dahliae*. *BioControl*, *61*(3), 269–282. https://doi.org/10.1007/s10526-015-9706-z

Rungjindamai, N., Xu, X.-M., & Jeffries, P. (2013). Identification and Characterisation of New Microbial Antagonists for Biocontrol of *Monilinia laxa*, the Causal Agent of Brown Rot on Stone Fruit. *Agronomy*, *3*(4), 685–703. https://doi.org/10.3390/agronomy3040685

Smilanick, J. L., Denis-Arrue, R., Bosch, J. R., Gonzalez, A. R., Henson, D., & Janisiewicz, W. J. (1993). Control of postharvest brown rot of nectarines and peaches by Pseudomonas species. *Crop Protection*, *12*(7), 513–520. https://doi.org/10.1016/0261-2194(93)90092-W

Soto-Muñoz, L., Teixidó, N., Usall, J., Viñas, I., Crespo-Sempere, A., & Torres, R. (2014). Development of PMA real-time PCR method to quantify viable cells of *Pantoea agglomerans* CPA-2, an antagonist to control the major postharvest diseases on oranges. *International Journal of Food Microbiology*, *180*, 49–55. https://doi.org/10.1016/j.ijfoodmicro.2014.04.011

Wittig, H. P. P., Johnson, K. B., & Pscheidt, J. W. (n.d.). Effect of Epiphytic Fungi on Brown Rot Blossom Blight and Latent Infections in Sweet Cherry. Retrieved from https://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS.1997.81.4.383

Appendices

Raw data from dose response experiment 3:

No.	Treatment	Conc.	Block	Cherry	Lesion Size
1	P. control	0	1	a	17.35
1			1	b	13.37
1			1	c	16.03
2	P. control	0	1	a	15.63
2			1	b	25
2			1	c	NA
3	Y126	1	1	a	13.43
3			1	b	0
3			1	c	8.39
4	Y126	2	1	a	0
4			1	b	5.9
4			1	c	25
5	Y126	3	1	a	20.28
5			1	b	16.26
5			1	c	23.17
6	Y126	4	1	a	18.57
6			1	b	25
6			1	c	13.34
7	B91	1	1	a	17.83
7			1	b	15.33
7			1	c	10.88
8	B91	2	1	a	11.6
8			1	b	16.23
8			1	c	6.11
9	B91	3	1	a	17.13
9			1	b	25
9			1	c	11.99
10	B91	4	1	a	6.44
10			1	b	19.86
10			1	c	15.24
11	Serenade	1	1	a	10.68
11			1	b	11.89
11			1	c	12.32
12	Fungicide	1	1	a	0
12			1	b	0
12			1	c	0
13	N. control	0	2	a	0
13			2	b	0
13			2	c	NA
14	P. control	0	2	a	10.68

14			2	b	18.61
14			2	c	20.32
15	Y126	1	2	a	0
15			2	b	8.65
15			2	c	0
16	Y126	2	2	a	0
16			2	b	15.4
16			2	c	0
17	Y126	3	2	a	17.78
17			2	b	16.6
17			2	c	NA
18	Y126	4	2	a	17.23
18			2	b	15.4
18			2	c	10.41
19	B91	1	2	a	15.32
19			2	b	12.04
19			2	c	14.42
20	B91	2	2	a	NA
20			2	b	15.72
20			2	c	13.17
21	B91	3	2	a	15.59
21			2	b	25
21			2	c	NA
22	B91	4	2	a	14.16
22			2	b	14.65
22			2	c	18.37
23	Serenade	1	2	a	0
23			2	b	0
23			2	c	0
24	Fungicide	1	2	a	0
24			2	b	0
24			2	c	0
25	N. control	0	3	a	0
25			3	b	0
25			3	c	0
26	P. control	0	3	a	3.22
26			3	b	13.22
26			3	c	14.48
27	Y126	1	3	a	8.75
27			3	b	0
27			3	c	C
28	Y126	2	3	a	12.61
28			3	b	C
28			3	c	NA
29	Y126	3	3	a	0

29			3	b	0
29			3	c	NA
30	Y126	4	3	a	25
30			3	b	7
30			3	c	16.25
31	B91	1	3	a	12.81
31			3	b	14.32
31			3	c	C
32	B91	2	3	a	2.3
32			3	b	3.62
32			3	c	0
33	B91	3	3	a	13.62
33			3	b	14.77
33			3	c	9.2
34	B91	4	3	a	15.03
34			3	b	11.95
34			3	c	13.45
35	Serenade	1	3	a	0
35			3	b	6.48
35			3	c	4.81
36	Fungicide	1	3	a	0
36			3	b	0
36			3	c	0

Biocontrol Y126 and B91 (concentrations 10^8, 10^7, 10^6, 10^5 CFU/ml); Serenade (10^9 CFU/ml); fungicide Luna Sensation; a negative and positive control (both sterile distilled water). The negative control was not inoculated with *M. laxa* and the positive control was inoculated 24 hours after treatment.

