Management of Gray Mold of Iceberg Lettuce by Biological Control Agents and Chitosan Formulations

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ABSTRACT

Gray mold caused by *Botrytis* is the major problem in iceberg lettuce cultivation in poly tunnels in Sri Lanka. Currently management of this disease of lettuce depends mainly on foliar application of fungicides. Continues application of fungicides for control of gray mold could not be recommended as lettuce mainly consume as fresh vegetables. Therefore, studies were conducted to identify the causal agent, varietal resistance to pathogen and effective chitosan formulation and biological control agents on control of gray mold diseases of ice berg lettuce in poly tunnels. Causal agent of this disease was identified as *Botrytis cinerea*. Commercially grown varieties Eden and Maruli were equally susceptible to the disease. Different isolates of pathogen were shown different virulence levels on iceberg lettuce variety Eden. In vitro test showed that, almost complete inhibition of mycelia growth of all *Botrytis* isolates at 600ppm chitopower 2 and liquid formulation of *Trichoderma asperellum* ($4x10^5$ conidia/ml) but 600ppm chitopower 1 or liquid formulation of fluorescent *Pseudomonas* (10^6 bacteria/ml) or fungicide- Dicloran 75WP (3000ppm) were suppressed some isolates only. Studies in poly tunnel showed that lowest disease severity index (3.6% DSI) of gray mold in iceberg lettuce plants treated with *Trichoderma asperellum* and highest DSI (77.7 %) in control treatment. Dicloran 75WP, chitopower 2 and fluorescent *Pseudomonas* were recorded 16.6%, 18.5 and 46.2% DSI respectively. Results revealed that *Trichoderma asperellum* bio control agent and chitopower 2 could be used as alternatives to synthetic fungicides in controlling of gray mold disease of iceberg lettuce grown in poly tunnels.

Key Words: Biological control, Chitosan, Gray mold, Fungicide

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1. INTRODUCTION

Gray mold disease is become an epidemic level of iceberg lettuce cultivations especially in poly-tunnels in Sri Lanka. Currently management of botrytis disease of crops depends mainly on foliar application of fungicides. Several fungicides are currently used in Sri Lanka to control the disease by farmers. These are Azoxystrobin, Chlorothalonil, Mancozeb, Pyraclostrobin, Thiophenate Methyl, and Captan. Farmers, however, claimed that application of fungicides is not effective when symptoms have developed in cool and humid condition in poly tunnels (Personal communication with growers).

Rajapakse and Premarathna, 2015 reported that *Botrytis cinerea* is causal agent of gray mold of lettuce in poly tunnels. They also revealed that continuous foliar spraying of 4g/l concentrations of fungicide, Dicloran 75 WP were reduced the gray mold of lettuce caused by Botrytis. However, continues application of fungicides for control of Botrytis disease could not be recommended as lettuce mainly consume as fresh vegetables. Chitosan a natural biodegradable de-acetylated form of chitin has been proven to control numerous pre and post-harvest disease on various horticultural commodities (Aziz Aziz *et al.,* 2005, Kareem *et al.,* 2006, Wang *et al.,* 2007, Bautista-Banos*et al.,* 2005). It has reported that chitosan induces a series of defense reactions in plants correlated with enzymatic activities and also it has a direct effect on microbes by fungistatic or fungicidal potential (Meng *et al.,* 2010, Raafat and Sahl, 2009). Efficacy of the different chitosan compounds on growth inhibition of fungal pathogen such as *Colletotrichum* and *Fusarium* species was demonstrated by in vitro test (Rajapakse *et al*., 2014). Chenyan Hua *et al.,* 2009 reported that chitosan has a grater inhibitory effect on Kiwi fruit gray mold and Ben Shalon *et al.,* 2003 reported that almost complete inhibition of *Botrytis* conidia germination (in vitro) and controlled the gray mould of cucumber caused by *B. cinerea* by chitosan. There was several research reports indicated that selected strains of biological control agents *Pseudomonas fluorescens* and *Trichoderma* species had significantly reduced lesion areas on whole lettuce plants caused by *Botrytis cinerea* (Card, 2005, Freeman *et al.,* 2004, Walker *et al.,* 2001). It has been reported that *Trichoderma harzianum* control *Botrytis cinerea* and many other fungi by acquiring induce systemic resistance of plants and antagonism of nutrient activated conidia against *Botrytis cinerea* (Elad, 2000, Freeman *et al.,* 2004, Hjelyord *et al.,* 2001, Meyer *et al.,* 1998). Similarly Walker *et al.,* 1996 and 2001 demonstrated that antifungal activity of *Pseudomonas antimicrobica* by inhibiting conidia germination and outgrowth of *Botrytis cinerea*. Application of agro-chemicals are the major plant protection method over decades even though they are associated with many disadvantages including their expensive applications, environmental pollution and human health hazards due to excessive usage. This has emerged a worldwide huge trend to explore other environmental friendly alternative methods for plant protection. Therefore, the objectives of this study were to identify the causal agent of grey mold disease, resistance varieties and effective chitosan formulations and biological control agents on control of gray mold of iceberg lettuce in poly tunnels.

2. MATERIALS AND METHODS

2.1 Pathogen isolation and identification

Disease samples of Iceberg lettuce were collected from poly tunnels in hill country Nuwara Eliya area. Pathogen was isolated from lesions of disease affected leaves of iceberg lettuce on Potato Dextrose Agar (PDA). Single spore isolates of pathogen were prepared from mycelia of single conidia cultures grown on PDA. Pathogen was identified on the basis of microscopic observations of size and shape of conidia, mycelia arrangements, culture morphology on PDA and comparison with published data (Card 2000, Ellis and Waller, 1974). Five isolates of *Botrytis* was selected for further studies and mycelia growth rate on PDA and conidial diameter of each isolate was measured at 7 days after inoculation. The isolates of pathogen were cultured on PDA slant and stored for further studies.

2.2 Pathogenicity of botrytis isolates on iceberg lettuce

Pathogenicity of all isolates was tested by wound inoculation with conidia suspension and subsequent symptoms development on leaves. Five isolates of *Botrytis* obtained from diseased plants and iceberg lettuce plants of variety Eden was used for the pathogenicity test. One month old lettuce plants grown in poly tunnel under 18-26°C temperature and 85-90% relative humidity were inoculated with isolates of *Botrytis* by pin prink methods using conidia suspension (10^6 conidia/ml) of different isolates of *Botrytis*. Five iceberg lettuce plants were used to inoculate each *Botrytis* isolate. Un-inoculated plants were served as control. Disease symptoms were observed after 5 days of inoculation and re-isolation of pathogen was done on PDA medium to perform Koch postulation (Card 2000, Fernandez-Ortuno *et al*., 2011).

2.3 Response of iceberg lettuce varieties to infection of Botrytis isolates.

Experiment was conducted in poly tunnel (16-28°C, 80-90% relative humidity) at Dolosbage area (WU1) in Kandy District, Sri Lanka. Two commercially grown iceberg lettuce varieties Eden (V1) and Maruli (V2) and five *Botrytis* isolates (Br, Bs, Bl, Bp and Bt) were used for inoculation with lettuce varieties. Experimental design was completely Randomized design with two factor factorial. One month old ice berg lettuce plants of both varieties were inoculated with conidia suspension (10^6conidia/ml) of different *Botrytis* isolates using pin prink method to observe subsequent symptoms development on plants. Disease Severity of gray mold in Iceberg lettuce varieties was measured using rating scale at harvest (Elad *et al.,* 1992). Symptoms of leaf tissue were evaluated according to Disease Severity Index of 0-4, where 0=no disease, 1=1-12% rot, 2=13-25%, 3=26-50%, 4=51-100%. The data obtained were tabulated and analyzed subjected to the analysis of variance procedure of SAS 9.1 software. Duncan's Multiple Range Test was performed to compare the differences among treatment means at p=0.05.

2.4 Effect of biological control agents, Chitosan formulations and fungicide (Dicloran 75WP) on mycelia growth of *Botrytis* isolates (*in vitro*).

Studies (*in vitro*) were conducted with the treatments of two formulations of chitosan i.e. Chitopower 1 (600ppm), chitopower 2 (600ppm) developed by Sri Lanka Atomic Energy Board and two biological control agents developed by Horticultural Crops Research and Development Institute, Sri Lanka i.e. liquid formulation of *Trichoderma asperellum* ($4x10^5$ conidia/ml) and fluorescent *Pseudomonas* ($1x10^6$bacteria/ml) and fungicide- Dicloran 75WP (3000ppm). Untreated plants were served as control. Five isolates of *Botrytis* were used for each study. Experimental procedure was Completely Randomized Design and each treatment replicated five times. Effectiveness of Chitopower 1, Chitopower 2 and fungicide Dicloran 75WP in controlling of mycelia growth of *Botrytis* isolates was measured by culturing them on amended PDA medium. Effectiveness of biological control agents in controlling mycelia growth of *Botrytis* isolates was measured by duel culture techniques on PDA medium. All PDA plats were incubated at 28 ^{0}C for 7 days. Percent inhibition of fungi (growth reduction over control) was calculated by the following equation.

$$I = \frac{100(C-T)}{C}$$

Where I is the % inhibition of mycelia growth, C the growth of fungus in control plate (mm), and T is the growth of fungus on the treated plate (mm).

2.5 Effect of biological control agents, chitopower 2 and fungicide Dicloran 75WP on gray mold of Iceberg lettuce grown in Poly tunnel.

Experiment was conducted in poly tunnels at Dolosbage area (WU1) in Kandy District, Sri Lanka. Mean day and night temperature in the location was 23 ^{0}C and 16 ^{0}C respectively. RH was over 90% throughout the day. Ice berg lettuce variety Eden was used for experiment.

Plants were established in pots and standard crop management practices were done throughout the study. Top of the areal part of each ice berg plants were artificially inoculated with conidial suspension (10^6conidia/ml) of most virulent Botrytis isolate Bl by pin prink method when plants were one month old. The treatments were selected based on the results of *in vitro* test (Table 3) and applied to the plants to cover whole aerial parts of the plant as an aqueous spray by using a knapsack sprayer one day after artificial inoculation of pathogen and continued 5 times with 7 days intervals. Fertilizer mixture of urea, super phosphate and MOP 6: 30: 30 was used as basal and top dressing fertilizer. The experiment was laid out in a Completely Randomized Design and five treatments were randomized in ten replicates. Data were collected at harvesting stage. Measurements were taken on Disease Severity Index (%) (Elad *et al.,* 1992) and yield loss compared to most effective treatment. The data obtained were tabulated and

analyzed subjected to the Analysis of Variance procedure of SAS 9.1 software. Duncan's Multiple Range Test was performed to compare the differences among treatment means at p=0.05.

Treatments were used as follows.

T1- Foliar spraying of liquid formulation of *Trichoderma asperellum* ($4.5x10^5$ conidia/ml)

T2- Foliar spraying of liquid formulation of fluorescent *Psuedomonas* ($1x10^6$ conidia/ml)

T3- Foliar spraying of Chitopower II (600ppm)

T4- Foliar spraying of Fungicide- Dicloran 75WP (3000ppm)

T5- control

RESULTS AND DISCUSSION

Gray mold symptoms were observed in iceberg lettuce plants grown in poly tunnels at Nuwara Eliya area in Sri Lanka. The symptoms observed were water-soaked, brown or gray blight, and abundant mycelia with conidia were appeared on the infected leaves. Isolates of Botrytis were identified by comparison of their colony morphology on PDA, microscopically observations of size, shape of conidia and mycelia arrangements with published data (Ellis and Waller, 1974).

The fungus produced light gray to gray colonies with dark mycelium and abundant conidia on PDA at 26ºC for 7 days. It was observed that significant variation of mycelia growth rate of different isolates on PDA. The conidia of all isolates were one-celled, ovoid in shape, dark brown, and average size of conidia of different isolates was varied from 12-14x9-11 µm. Identification of *Botrytis cinerea* was from its typical spore and conidiophores structure. Willets, 1997 reported that conidiophores are specialized structure that incorporates a terminal cluster of synchronously produced conidia, borne on a well-developed conidiogenous hypha, which resembles a bunch of grapes. Primary cultures of isolates on PDA have a light grey at first, later becoming grey or grayish-brown (Table 1) with dark walled erect septate hyphae. The dark coloured mature conidiophores, which branch alternately. This conformed to the description of *Botrytis cinerea* (Ellis and Waller 1974).

To determine the pathogenicity test, conidial suspension ($1x10^6$conidia/ml) was inoculated with iceberg lettuce plants variety Eden by pin prink methods and allow to grow in poly tunnel (16-28°C, 80-90% relative humidity). After 5 days, grey mold symptoms similar to the original symptoms were developed on inoculated plant leaves. The fungal pathogen was re-isolated from the disease lesions of the inoculated plants and the re-isolated pathogen exhibited the same morphological characteristics as those of the original isolates. Thus, the isolates of fungal pathogen fulfilled the criteria stipulated by the Koch's postulates and were identified as the causal agent of the gray mold on iceberg lettuce plants.

Table 1.Characteristic of fungal isolates of *Botrytis* collected from leaves of infected plants

Botrytis isolates	Colony colour on PDA at 7 DAI	Colony diameter on PDA at 7 DAI	Average size of conidia (µm)
Bs	Light gray at first, later Grey, reverse black,	5.6[c]	12x9
Bp	Grey at first, later grayish-brown, reverse black	9.2 [a]	13x9
Bt	Grey at first, later grayish-brown, reverse black	9.2 [a]	14x11
Br	Light gray at first, later gray, reverse black	6.4 [b]	13x10
Bl	Grey at first, later grayish-brown, reverse black	9.1 [a]	13x9

Note: Means with the same letter(s) on the column are not significantly different at P= 0.05

Varietal response of iceberg lettuce to different isolates of *Botrytis* showed that there were significant variations of percent disease severity index (DSI%) among *Botrytis* isolates (Table 2). Both iceberg lettuce varieties Eden and Maruli were equally susceptible to all *Botrytis* isolates. However, Botrytis isolate-Bl could be considered as most virulent isolates as it had the highest DSI on both iceberg varieties compared other isolates.

Table 2: Mean Disease Severity Index (DSI %) of grey mold of lettuce varieties inoculated with different isolates of Botrytis.

Varieties	Disease Severity Index (%)					
	Br	**Bs**	**B1**	**Bp**	**Bt**	**Mean**
V1	17.0	20.7	98.5	8.9	11.1	31.0
V2	12.6	11.8	92.6	8.9	2.9	25.8
Mean	14.8[b]	16.3[b]	95.6[a]	8.9[c]	7.0[c]	

Note: Means with the same letter(s) are not significantly different at P= 0.05

V1 & V2: lettuce variety Eden and Maruli Br, Bs, Bl, Bp&Bt: different Botrytis isolates

Study (*in vitro*) was conducted to identify the effect of two chitosan formulations (Chitopower 1 and Chitopower 2), two biological control agents (formulation of *Trichoderma asperellum* and fluorescent *Pseudomonas*) and fungicide- Dicloran 75WP (3000ppml) on growth of different isolates of *Botrytis* on PDA (Table 2).

Table 3:Effect of Chitopower 1, chitopower 2, *Trichoderma asperellum*, fluorescent *Pseudomonas* and fungicide- Dicloran 75WP on mycelia growth of *Botrytis* isolates (*in vitro* test).

Botrytis isolate	Growth reduction % of Botrytis isolates on PDA at 7 DAI				
	600ppm Chitopower 1	600ppm Chitopower 2	Dichloran 75WP (3000ppm)	*Trichoderma asperellum*	fluorescent *Pseudomonas*
Br	100	100	79.3	100	100
Bt	83.2	100	71.4	100	46.3
Bl	54.6	100	100	100	100
Bs	94.4	100	56.9	100	23.3
Bp	100	100	47.3	100	100

Results revealed that chitopower 2 (600ppm) and *Trichoderma asperellum* bio control agent completely suppressed mycelia growth of all tested isolates of *Botrytis* (in vitro) (Table 3). Biological control agent fluorescent *Pseudomonas* also completely suppressed most virulent *Botrytis* isolate i.e. Bl and other two isolates Br and Bp. Chitopower 1 (600ppm) completely suppressed only Br and Bp i.e. less virulent isolates on both iceberg lettuce varieties.

Ben Shalon *et al.,* (2003) reported that almost complete inhibition of *Botrytis* conidia germination was found, in vitro by chitosan molecule which is similar to chitopower 2 but not with chitosan oligomers which is similar to chitopower 1. They also reported that chitosan controlled the gray mould caused by *Botrytis cinerea* compared with control but chitin oligomers did not show any effect on disease control. They further concluded that although a dual mode of action was involved in the control of gray mould by chitosan, the antifungal activity of the compound was an essential factor. It was evident that the induction of the defense response without the antifungal activity was not enough to suppress the diseases. Therefore, chitopower 2 (600ppm), *Trichoderma asperellum* and fluorescent *Pseudomonas* was selected to identify the effective and environmental friendly disease control practice for gray mold of iceberg lettuce grown in poly tunnels.

Table 4: Mean Disease Severity Index % of treatments and percentage yield loss compared to most efficient treatment when iceberg lettuce plants (var. Eden) treated with chitopower 2, biological control agents *Trichoderma asperellum* and fluorescent *Pseudomonas* and fungicide Dichloran 75WP.

Treatments	Mean DSI (%) of gray mold	Percentage yield loss compared to most effective treatment (T1)
T1-*Trichoderma asperellum*	3.6 [c]	
T2- fluorescent *Pseudomonas*	46.2 [ab]	33
T3- Chitopower 2	18.5 [bc]	11
T4- Dichloran 75WP	16.6 [bc]	9
T5 – Control	77.7 [a]	85

Note: Means with the same letter(s) are not significantly different at P= 0.05

The lowest yield loss was observed in *Trichoderma asperellum* treated plots while highest yield loss was observed in control plots (Table 4). Benitez *et al.,* (2004) reported that *Trichoderma* strains exert bio control against fungal pathogens either indirectly by competing for nutrients and space, modifying the environmental conditions or promoting plant growth and plant defensive mechanisms and antibiosis or directly by mechanisms such as mycoparasitism. Chitopower 2 and fungicide Dichloran 75 WP treated plots also recorded comparatively lower DSI (%) and yield losses (Table 4). Chenyan Hua *et al.,* 2009 reported that low molecular weight chitosan has a grater inhibitory effect on Kiwi fruit gray mold due to induction of accumulation of higher levels of hydrogen peroxide, greater level of activity of several defense-related enzymes, greater ability to penetrate the cell walls in kiwifruit epidermal peel tissue and activate a greater defense response. Similarly the low molecular weight chitosan which was used to produce Chitopower 2 formulation showed remarkable disease control ability of gray mold of iceberg lettuce. Ben Shalon*et al.,* 2003 reported that almost complete inhibition of *Botrytis* conidia germination (in vitro) and controlled the gray mould of cucumber caused by *B. cinerea* by chitosan. Results in DSI and yield loss data indicated that gray mold of iceberg lettuce was effectively controlled by spraying of liquid formulation of *Trichoderma asperellum* bio control agent as well as chitopower 2 starting from early crop growth stage and continuing up to harvesting stage with 7 days intervals.

CONCLUSION

Causal agent of gray mold of iceberg lettuce in poly tunnels was re-conformed as *Botrytis cinerea*. Commercially grown varieties Eden and Maruli were equally susceptible to disease. Almost complete inhibition of mycelia growth of all *Botrytis* isolates was found, in vitro, at 600 ppm chitopower 2 and liquid formulation of *Trichoderma asperellum*. Gray mold of iceberg lettuce caused by *Botrytis cinerea* in poly tunnels was effectively controlled by spraying of liquid formulation of *Trichoderma asperellum* bio control agent as well as Chitopower 2, starting from early crop growth stage and continuing up to harvesting stage with 7 days intervals.

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