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Bioefficacy of *Bacillus amyloliquefaciens* Strain DGA14 as Potential Microbial Control Agent Against *Colletotrichum gloeosporioides* Causing Papaya Anthracnose

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A B S T R A C T

Anthracnose is an important fungal disease of papaya and caused major losses to its production worldwide. Common management of the disease involves the use of pesticides however, due to the increasing concern on the use of pesticides, alternative control have been encouraged. Use of microbial control agents are one of the potential alternatives to the management of the disease. Hence, this study was conducted to evaluate the bioefficacy of *B. amyloliquefaciens* strain DGA14as potential microbial control agent against *C. gloeosporioides* Penz causing papaya anthracnose under *in vitro* and *in vivo* test conditions and to determine the most effective level of *B. amyloliquefaciens* against *C. gloeosporioides*. Increasing levels of the bacterium such as 15 ml, 20 ml, 25 ml, and 30 ml per liter water were tested against the pathogen and were compared to control (sterilized distilled water). The experiments were laid out in Completely Randomized Design (CRD) with three replications. The data were statistically analyzed using the Analysis of Variance (ANOVA) and the treatment means were compared using Tukey's Honest Significant Difference (HSD). Results in *in vitro* test showed that *Bacillus amyloliquefaciens* at 30ml per liter of water significantly inhibited *C. gloeosporioides* with zone of growth of 13.30mm and subsequent growth inhibition of 61.75%. Results of the *in vivo* test likewise showed that *B. amyloliquefaciens* at 30ml per liter of water applied as fruit-dip delayed the appearance of anthracnose symptom by two days and significantly reduced the severity of papaya anthracnose (DSI=1.42).

Introduction

Papaya (*Carica papaya* L.) is an economically important fruit grown in and exported from the Philippines. International demand for papaya had an increasing trend and ranks as top five most traded tropical fruits in 2014 (Valencia fruits, 2012). However, despite of the increasing trend of its exports, papaya has a low export ratio globally with less than 3 percent of global papaya production (Evans *et al.*, 2015). In 1998 to 2008, there is an average of 2% increased share of papaya in the total value of agricultural crop and agricultural output in the Philippines (Lustria, 2009). Ripe papaya suffers from high levels of post-harvest diseases causing high levels of reductions of the fruit's availability worldwide (Mondal *et al.*, 1995; Hamim *et al.*,

2014). Fungal diseases are one of the major reasons of post-harvest losses of papaya production. Anthracnose is an important fungal disease caused by the fungus, *Colletotrichum gloeosporioides,* which was recognized globally as a major post-harvest disease. According to Maeda and Nelson (2014), this disease caused major losses to postharvest and marketable fruit of residential growers and local consumers of Hawai'i, thus, decreasing its productivity and yield performance.

The disease can usually controlled by the application of chemical fungicides (Mahoney and Tattar, 1980). However, public concerns continues to increase due to its hazardous residues (Jang *et al.,* 2010), resistance (Jung and Oakley, 1990), long-term and unknown effects on health (Miles and Frewer, 2001), and its adverse effects on biotic diversity in ecosystems (Kegley *et al.,* 1999). For these reasons, biological control using antagonistic microorganisms has emerged as an environmentally friendly alternative for plant pathogen control (Pal and Gardener, 2006).

Antagonistic microorganisms produce a wide variety of antimicrobial metabolites. *Bacillus* species have been reportedly effective in controlling various plant pathogen. The bacterium is epiphytic and colonized plants endophytically (McSpadden, 2004).

Suppression of plant pathogens from these bacteria might be due to its microbial antagonisms due to its production of different varieties of secondary metabolites (Chen *et al.,* 2009) and enzymes-like chitinase (Niazi *et al.,* 2014).

Hence, the study was conducted to explore the efficacy of this microbial control agent against *C. gloeosporiodes* causing papaya anthracnose.

Materials and Methods

In vitro and *in vivo* studies were conducted at the laboratory of the University of Southeastern Philippines, Tagum-Mabini Campus, Mabini Unit, Pindasan, Davao de Oro province. These were laid-out in a Completely Randomized Design (CRD).

Pure culture suspension of *B. amyloliquefaciens* was provided by Dr. Alvindia of Central Luzon State University. The bacterium was purified by streaking a loop-full bacterial suspension on a PDA medium using sterile wireloop. After 24 hours, a colony of this bacterium was then transferred in another plated PDA medium for purification. While, the pathogen, *C.gloeosporioides* was obtained from anthracnose-infected papaya that was collected from the nearest papaya orchard. Microscopic examination was likewise conducted to ensure the true identity of the fungus. The fungus was isolated using tissue planting technique described by Vicente *et al.,* (2014). The pure culture of the pathogen was then mass produced in PDA medium and incubated for seven days.

In Vitro Test

To evaluate the efficacy of *B. amyloliquefaciens* under *in vitro* test, the following concentrations of *B. amyloliquefaciens* were tested against *C. gloeosporioides* (*C.g.*): T_1-control (SDW only); T_2- 15 ml *B. amyloliquefaciens* per liter water; T_3- 20 ml *B. amyloliquefaciens* per liter water; T_4- 25 ml *B. amyloliquefaciens* per liter water and T_5- 30 ml *B. amyloliquefaciens* per liter water.

This was done using a modified dual incubation method conducted by Han *et al.,* (2015). Bacterial suspension was imposed on a PDA plate with holes (4 mm in diameter) that were punched using a sterilized cork borer. Twenty microliter of two-day old bacterial suspension (based on 15 ml, 20 ml, 25 ml and 30 ml/ L of water) were dropped into the holes one day before inoculation of the seven-day old fungal pathogens. Cultures were then incubated at room temperature in the dark. This set-up was replicated thrice with five plates per replicate. The study was repeated twice to have a constant results.

In Vivo Test

Treatments used in this experiment were as follows: T_1-negative control (SDW only, without *C.*

gloeosporioides); T_2- positive control (SDW only, with *C. gloeosporioides*); T_3-15 ml *B. amyloliquefaciens* per liter water; T_4- 20 ml *B. amyloliquefaciens* per liter water; T_5- 25 ml *B. amyloliquefaciens* per liter water and T_6- 30 ml *B. amyloliquefaciens* per liter water.

For the pathogenicity assay, papaya fruits of the same age of susceptible papaya variety, Solo, at color index 4 (more yellow than green) were surface sterilized with 10% HCl. Papaya fruits were dipped in each bacterial culture suspension based on the rates reflected in the treatments for 15 minutes.

After 24 hours, a *C. gloeosporioides* spore suspension at 3.5×100 conidia/mlwere inoculated to papaya fruits through spraying and incubated at room temperature in a humidity box. After ten days, the diseased areas of papaya fruits were then rated visually (Alvindia, 2013). This study was replicated thrice also with five fruits per replicate. A total of 90 papaya fruits were used in this study. Same set-up was repeated to test the consistency of the results.

Data Gathered

In Vitro Test

Zone of Growth (mm)

The zone of growth was recorded by radial growth measurement in millimetre of *C. gloeosporioides* from center to last growing point of the mycelial growth after five days of incubation (mm) using a ruler.

Zone of Inhibition (%)

Zone of inhibition (ZI) of the different rates of *B. amyloliquefaciens* was calculated using the formula below (Alwathnani and Perveen, 2012)

Inhibition (%) = (C-T/C) 100

Where, C is the average radial growth in control plate and T is the average radial growth in treated plates.

In Vivo Test

Days to Appearance of Symptoms

This was recorded as soon as the earliest symptoms of anthracnose were noted.

Severity of Infection

The infection was rated using the scale below. This was taken ten days after inoculation. Disease severity rating was based on the scale provided by Suharban *et al.*, (1985).

Based on the standard rating scale, degree of infection was computed as follows:

$$DSI = \frac{\sum(\text{Number on scale x No. of fruits in that scale})}{\text{Number of treated fruits}}$$

Degree of efficacy of the treatments was based on the computed disease severity index using the following arbitrary scale:

Statistical Analysis

Data were analyzed using the ANOVA in CRD and the treatment means were compared using Tukey's HSD.

Results and Discussion

In Vitro Test

Inhibitory Effect of Different Levels of *B. amyloliquefaciens* on *C. gloeosporioides* causing Papaya Anthracnose

The inhibitory effect of different levels of *B. amyloliquefaciens* on *C. gloeosporioides* causing papaya anthracnose under *in vitro* test is shown in Table 1. Inhibitory effect of the bacterium on the growth of *C. gloeosporioides* is shown in Figure 1. ANOVA revealed significant differences among treatment means. Results showed that 30ml of *B.*

amyloliquefaciens suspension per liter of water had significantly inhibited (61.75% inhibition) the growth of the fungus with only13.30mm which is comparably lower as compared to 25ml of *B. amyloliquefaciens* per liter of water with 14.78mm zone of growth. Consequently, 15 ml and 20 ml of the bacterial suspension per liter of water had inhibited (51.97% and 55.60% inhibition, respectively) *C. gloeosporioides* with corresponding zone of growth of 16.70mm and 15.44mm which are significantly lower as compared to positive control (SDW only) with 34.78mm zone of growth.

The above results suggest that *B. amyloliquefaciens* at higher rate can significantly inhibit the growth of *C. gloeosporioides* causing papaya anthracnose.

In Vivo Test

Average Number of Days to Symptom Appearance and Disease Severity of Papaya Anthracnose after Treatment with different levels of *B. amyloliquefaciens*

Average number of days to symptom appearance and disease severity of anthracnose in papaya treated with different levels of *B. amyloliquefaciens* is presented in Table 2. Effects of different levels of the bacterium to the development of the disease are shown in Figure 3.

Analysis of Variance revealed no significant differences on the number of days to symptom appearance. However, disease severity of papaya anthracnose as affected by the bacteria, *B. amyloliquefaciens* was significantly different.

Results showed that papaya treated with 30ml *B. amyloliquefaciens* had delayed symptom expressions of papaya anthracnose with an average mean of 5 days to symptom appearance followed by papaya treated with 20ml of the bacterial suspension that ranged from 4-5 days after fungal inoculation. Papayas treated with 10ml, 15ml and 25ml of the bacterial suspension also had a delayed appearance of anthracnose symptoms with both average mean of 4 days after inoculation. And the earliest appearance of anthracnose symptom was observed in papayas without treatments with an average mean of 3-4 days after inoculation.

Significantly, lowest disease severity of anthracnose was observed in papaya treated with 30ml of *B. amyloliquefaciens* suspension with disease severity index (DSI) of 1.42 which is rated as very effective (VE). On the other hand, papayas treated with 15ml, 20ml and 25ml of bacterial suspension had lower disease severity (DSIs=2.25, 2.42 and 2.25, respectively) and were rated as effective (E). Highest disease severity was observed in untreated papaya with 3.39 disease severity index. While no disease was recorded in negative control.

The above results imply that *B. amyloliquefaciens* at the rate of 30ml per liter of water to papaya fruits can delay the appearance of anthracnose symptom up to two days and can significantly reduce papaya anthracnose with DSI=1.42.

Antifungal activities of *B. amyloliquefaciens* towards the pathogen. *B. amyloliquefaciens* were reportedly effective in managing multiple plant diseases caused by soil-borne or post-harvest pathogens. Previous studies reported that *Bacillus* species effectively suppress or control phytopathogenic fungi due to the production of various secondary metabolites that (Cho *et al.,* 2003; Souto *et al.,* 2004; Korenblum *et al.,* 2005).

Moreover, production of antibiotics, lytic enzymes, volatile compounds and siderophores were said to be the mechanisms of the pathogen inhibition by the antagonistic microbe (Mao *et al.,* 2006). Abnormal mycelia such as changed cell wall structures like as swelling, lysis and the formation of abnormal mycelia as balloon like structures in *Botrytiscinerea* were observed in the study.

Table.1

Rate	Disease Severity Index (DSI)	Degree of Infection
0	0	Healthy
1	1-20%	Less Severe
2	21-40%	Slightly Severe
3	41-60%	Moderately Severe
4	61-80%	Severe
5	80% and above	Highly Severe

Table.2

Disease Severity Index (%)	Degree of Effectiveness
1.0 to 1.5	Very Effective (VE)
Between 1.6-2.5	Effective (E)
Between 2.6-3.0	Moderately Effective (ME)
Between 3.1-4.5	Less Effective (LE)
Between 4.6-5.0	Not Effective (NE)
1.0 to 1.5	Very Effective (VE)

Table.3 Average radial growth (mm) and percent inhibition of *C. gloeosporioides* as affected by different levels of *B. amyloliquefaciens* after five days of incubation.

TREATMENTS	Replication			MEAN**	% Inhibition[a,] **
	I	II	III		
Control (SDW only)	35.00	35.00	34.33	**34.78^{d}**	**0.00^{d}**
15 ml *B. amyloliquefaciens* per L water	16.67	17.00	16.44	**16.70^{c}**	**51.97^{c}**
20 ml *B. amyloliquefaciens* per L water	16.11	15.44	14.78	**15.44^{bc}**	**55.60^{bc}**
25 ml *B. amyloliquefaciens* per L water	14.00	15.00	15.33	**14.78^{ab}**	**57.50^{ab}**
30 ml *B. amyloliquefaciens* per L water	12.11	13.78	14.00	**13.30^{a}**	**61.75^{a}**

CV (Growth Diameter) = 3.51% ; **CV (% inhibition) = 3.66%**

** significant at 1% level; Means with common letter superscripts are not significantly different at 1% level, Tukey's HSD.

[a]=Percent growth of inhibition was computed using the formula: (C-T/C) x 100 where, C= radial growth of fungus in control plates and T=radial growth of fungus in treated plates. Data were subjected to arc-sine transformation prior to analysis.

Fig.1 Inhibitory effect of different levels of *B. amyloliquefaciens* on *C. gloeosporioides* after five daysincubation. a) Control (SDW only); b) 15ml of *B. amyloliquefaciens;* c) 20ml of *B. amyloliquefaciens;* d)25ml of *B. amyloliquefaciens;* e) 30ml of *B. amyloliquefaciens* per liter of water.

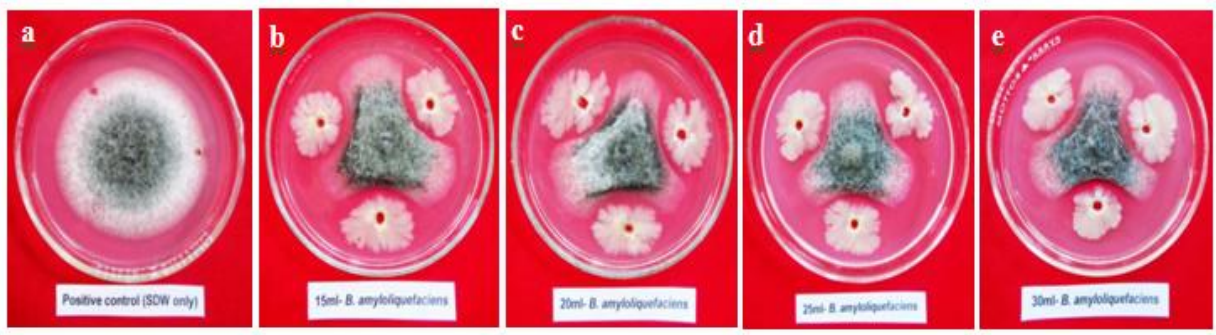


Table.4 Average number of days to symptom appearance and disease severity index[a] of *C. gloeosporioides*-inoculated papayas treated with different levels of *B. amyloliquefaciens.*

Treatments	Number of Days to Symptom Appearance[ns]	Disease Severity Index[a] **	Degree of Effectiveness
Negative Control (SDW only,without *C.g.*)	-	-	-
Positive Control (SDW only, with *C.g.*)	3-4	3.39[c]	-
15ml/L- *B. amyloliquefaciens* + *C.g.*	4	2.25[b]	E
20ml/L- *B. amyloliquefaciens* + *C.g.*	4-5	2.42[b]	E
25ml/L- *B. amyloliquefaciens* + *C.g.*	4	2.25[b]	E
30ml/L- *B. amyloliquefaciens* + *C.g.*	5	1.42[a]	VE
CV (%)=	**14.03**	**12.39**	-

[ns]=not significant; **significant at 1% level. Means with common letter superscripts are not significantly different at 1% level, Tukey's HSD

[a]= Disease severity index (DSI) was computed using the formula: DSI = ∑ (Number on scale x Number of fruits in that scale)/ Number of treated fruits.

Fig.2 Mycelial growth of *C. gloeosporioides* in (a) Control plate (SDW only); (b)Treated plate (with *B. amyloliquefaciens*) showing (c) abnormal mycelial formation (balloon-like structure) in treated plate.

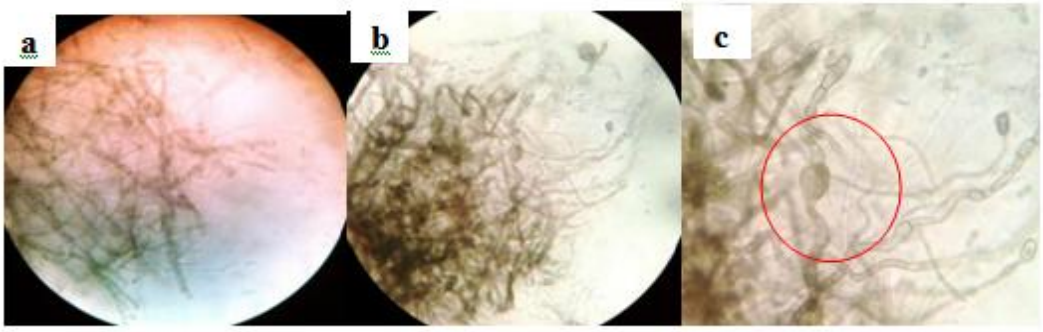
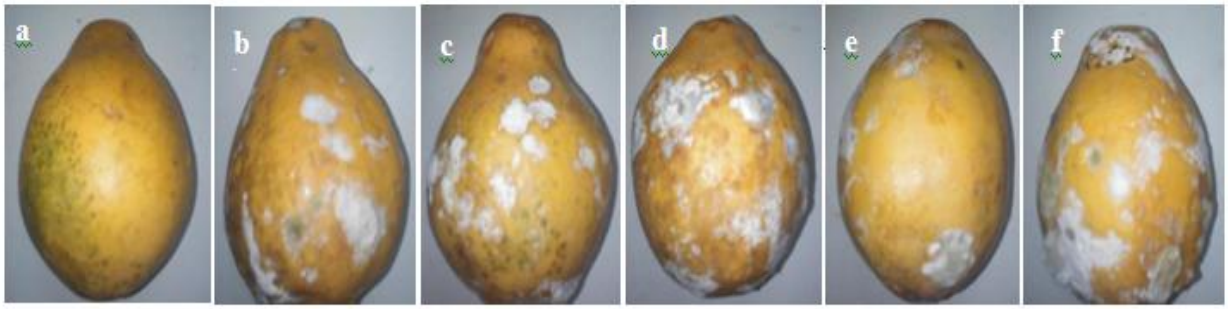


Fig.3 Effects of different levels of *B. amyloliquefaciens* to papaya anthracnose. a) negative control; b) positive control; c-f) 15 ml, 20 ml, 25 ml, 30 ml of *B. amyloliquefaciences* per liter of water.



Moreover, Kim and Chung (2004) reported that another strain of *B. amyloliquefaciens* (MET0908) can decomposed fungal hyphal walls of *C. lagenarium* due to its secreted extracellular enzyme, ß-1,3-glucanase. Meanwhile, similar results on the study were observed on the morphological structure of *C. gloeosporioides* showing physiological disorders such as abnormal swelling and degradation and in worst cases is bursting of its hyphae by the excretion of lytic enzymes from the antagonist as shown in Figure 2. which was likely observed also to the study of Jamal *et al.,* (2015) in *P. capsici* mycelia after treatment with 500 ppm of crude extract while 1000 ppm. Moreover, antifungal activity of *B. amyloliquefaciens* increased proportionally with the number of bacterial cells (Yoshida *et al.,* 2000). Mycelial growth of several plant pathogens such as *A. panax*, *B. cinerea*, *P. grisea* and *S. sclerotiorum* were strongly reduced by the crude extract of *B. amyloliquefaciens* in higher concentrations (50 ppm) (Ji *et al.,* 2013).

Mechanisms of control exhibited by the bacterium are also categorized as competition, parasitism or predation, and antibiosis (Yoshida *et al.,* 2000). On the study conducted by Mari *et al.,* (1996), severity of gray mold caused by *Botrytis cinerea* in pears had been reduced after the application of *B. amyloliquefaciens* 2TOE. They suggested that this was due to competition for nutrients.

Yoshida and his comrades (2000) reported that *B. amyloliquefaciens* strain RC-2 had effectively inhibited anthracnose disease in mulberry and antibiosis plays a major role in the inhibition of the pathogen, *R. solani*, causing the disease. This inhibition was also likely to be due to competition for nutrients rather than to production of antibiotics. They further discussed that application of the bacteria culture filtrate was the key in the strong inhibitory effect on conidial germination of *C. dematium*. Complete inhibition of conidial germination of the fungus on mulberry leaves pre-treated with undiluted culture filtrate of RC-2 was observed after scanning electron microscopy. These suggest that the preventive effect is due to inhibition of conidial germination on the leaves.

Therefore, *Bacillus amyloliquefaciens* can be a potential microbial control agent against *Colletotrichum gloeosporioides* causing papaya anthracnose. Moreover, increasing levels of this bacterium provides promising results in managing the pathogen and the disease it caused. However, efficacy of this bacterium as foliar spray during flowering stage of the crop must be also explored under field condition for further validation.

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