UNIVERSITI PUTRA MALAYSIA

CONTROLLING ANTHRACNOSE AND POSTHARVEST QUALITY OF PAPAYA (*CARICA PAPAYA* L. CV. FRANGI) USING *BACILLUS SUBTILIS* STRAIN B34

MD. FAKHRUL HASAN

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By

MD. FAKHRUL HASAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2012

DEDICATION

To my beloved homeland BANGLADESH, a place of peace and love.

To my affectionate parents and beloved wife for encouraging me to the higher ideals of life.

To my daughters Fatima and Aisha, for giving me happiness and joy.

Finally to my Zannati son Mohammad, who have helped me to understand the reality of life.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in partial fulfillment of the requirement for the degree of Doctor of Philosophy

CONTROLLING ANTHRACNOSE AND POSTHARVEST QUALITY OF PAPAYA (*Carica papaya* cv. FRANGI) USING *Bacillus subtilis* STRAIN B34

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Chairman : Professor Mahmud Tengku Muda Mohamed, PhD

Faculty : Agriculture

Papaya (*Carica papaya* L.), a climacteric fruit, is highly vulnerable to several postharvest diseases. Among them, anthracnose caused by *Colletotrichum gloeosporioides* is the major postharvest disease which demands an effective control. This study was carried out aiming at developing an eco-friendly biocontrol agent to control the disease. Three antagonistic bacteria identified as B34, B60 and B71 were screened from 81 epiphytic bacteria isolated from leaf and fruit surfaces of papaya. These three isolates were identified as *Bacillus subtilis* based on Biolog Omniliog Gen III identification system and its 16S rDNA sequence which was compared with the related bacteria in the GenBank. All the three strains were proved to be efficacious for the control of anthracnose of papaya in *in vitro* biocontrol assays. Bacterial strain B34, B71 and B60 produced antibiotic substances which restricted the fungal growth up to 84.6, 80.6 and 79.3%, respectively compared with the control. Moreover, these strains not only able to completely inhibit the fungal growth by secreting diffusible antibiotics but also restricted 40.6, 33.4, and 34.6% of fungal growth through the antibiotic volatiles produced by strains B34, B60 and B71, respectively. In spore germination test, germination percentage decreased gradually with increased concentrations of strains'

suspension. No spores of the test fungus germinated at the concentration of 10^8 CFU mL^{-1} but the performance of strain B34 was quite better than B71 and B60 at lower concentration. Mycelial growth of *C. gloeosporioides* was completely absent in 10^8 CFU mL^{-1} cell suspensions of all strains. On the other hand, strain B34 showed statistically highest inhibition of growth of mycelial plug in sterilized culture filtrate treated plates compared to strain B60 and B71. However, *in vitro* performances of *B. subtilis* strain B34 against *C. gloeosporioides* showed its better efficacy compared to strains B60 and B71. The growth of strain B34 was faster in nutrient broth medium and the biomass production reached the highest level in temperatures between 30-36ºC three days after incubation. The strain B34 showed its different mechanisms in controlling *C. gloeosporioides* by reducing nutrients for test fungus, producing proteolytic enzymes and secreting antibiotics to quash the cell wall as well as dissolve the fungus. Moreover, strain B34 was able to attack and degrade the hyphae of pathogen when observed under scanning electron microscope. This bacterium was found to be highly compatible with 20% *Aloe vera* gel (aloe gel) and 2% sodium bicarbonate (SBC) or mixture of both. Both of these have suppressive activity against *C. gloeosporioides* of papaya and could be used as enhancer for biocontrol efficacy of strain B34 during fruits storage. The survival and proliferation of strain B34 in papaya wounds and on fruit surfaces was not affected by aloe gel and SBC throughout the storage period. In addition, as observed from scanning electron microscopy micrograms, strain B34 was able to colonize and multiply on the surface of papaya fruits. The combination of strain B34 with aloe gel and SBC was more effective in controlling the anthracnose disease than strain B34 alone or other treatments, even superior to that obtained with fungicide Benocide® (benomyl 50% WP) both in inoculated or naturally infected fruits stored at 12±1ºC and 90±5% RH for 18 and 14 days, respectively. However, this combination offered a greater control by reducing 98.2% of the disease over control in naturally infected fruits at the end of 14 days storage at 12±1ºC and 90±5% RH and six days post ripening at 28 ± 2ºC, which was superior to that found with Benocide® or other treatments tested. Furthermore, fruits treated with the combination of *B. subtilis*-aloe gel-SBC showed delayed climacteric respiration and ethylene evolution more than seven days compared to control with reduced rate of CO_2 and C_2H_4 productions. This combined treatment reduced weight loss by more than 25% compared to the control. It also markedly slowed down the ripening of fruits as shown by their retention of firmness after storage. Moreover, it also delayed changes in external colour, titratable acidity and pH without compromising fruit quality was observed in fruits that were subjected to the combined treatment. The storage life was thus extended by 15 days when compared with control. Inhibitory activity of strain B34 preserved in glycerol was also observed in this study. strain B34 preserved in 2% glycerol based liquid formulations showed economically better performance in terms of controlling *C. gloeosporioides* and number of viable propagules (CFUs) over 120 days of test period. Finally, it is clear that strain B34 not only able to store at 2% glycerol for longer time but also effective when incorporated with SBC and *Aloe vera* gel to control *C. gloeosporioides* as well as improve the postharvest quality during cold storage compared to Benocide®. This suggested that this composite coating is promising and eco-friendly to be used in commercial postharvest applications for prolonging the storage life of Frangi papaya.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGAWALAN ANTRAKNOS DAN KUALITI LEPAS TUAI BETIK (*Carica papaya* cv. FRANGI) MENGGUNAKAN *Bacillus subtilis* STRAIN B34

Oleh

MD. FAKHRUL HASAN

Disember 2012

Pengerusi : Profesor Mahmud Tengku Muda Mohamed, PhD

Fakulti : Pertanian

Betik (*Carica papaya* L.), satu strain buah berklimakterik sangat mudah diserang beberapa penyakit lepas tuai. Di antaranya, antraknos yang disebabkan oleh *Colletotrichum gloeosporioides* merupakan penyakit lepas tuai utama yang memerlukan pengawalan yang berkesan. Oleh itu, kajian ini dilakukan bertujuan untuk membangunkan agen kawalan biologi mesra alam bagi mengawal *Colletotrichum gloeosporioides*. Tiga bakteria antagonis dikenali sebagai B34, B60 dan B71 telah disaring daripada 81 bakteria epifit yang diasing daripada permukaan daun dan buah betik. Ketiga-tiga bakteria tersebut telah dikenalpasti sebagai *Bacillus subtilis* berdasarkan sistem pengenalan Biolog Omniliog Gen III dan urutan rDNA 16S yang telah dibandingkan dengan GenBank bakteria berkaitan. Ketiga-tiga strain bakteria ini telah terbukti berkesan untuk mengawal antraknos betik secara biologi hasil ujian makmal. Bakteria strain B34, B71 dan B60 menghasilkan bahan antibiotik yang telah menghadkan pertumbuhan kulat masing-masing sehingga 84.6, 80.6 dan 79.3% berbanding kawalan. Selain itu, ia bukan sahaja dapat menghalang pertumbuhan kulat sepenuhnya dengan merembeskan antibiotik yang boleh tersebar,

tetapi juga menyekat 40.61, 33.36 dan 34.6% pertumbuhan kulat melalui penghasilan antibiotik meruap oleh bakteria masing-masing strain B34, B60 dan B71. Dalam ujian percambahan spora, peratusan percambahan menurun secara beransur-ansur dengan peningkatan kepekatan larutan bakteria. Tiada sebarang spora kulat yang diuji telah bercambah pada kepekatan sebanyak 10^8 CFU mL^{-1} tetapi prestasi strain B34 adalah lebih baik berbanding B71 dan B60 pada kepekatan yang lebih rendah. Pertumbuhan miselium *C. gloeosporioides* adalah tiada sepenuhnya dalam piring petri yang dirawat dengan larutan sel 10^8 CFU mL^{-1} bagi semua strain. Sebaliknya, strain B34 secara statistik menunjukkan perencatan pertumbuhan tertinggi bagi palam miselium dirawat dengan turasan kultur steril dalam piring petri berbanding strain B60 dan B71. Walau bagaimanapun, keputusan ujian dalam makmal bagi *B. subtilis* strain B34 terhadap *C. gloeosporioides* menunjukkan keberkesanan yang lebih baik berbanding strain B60 dan B71. Pertumbuhan strain B34 adalah lebih cepat dalam larutan media nutrien dan pengeluaran biojisim mencapai tahap tertinggi di antara suhu 30-36ºC tiga hari selepas pengeraman. Strain B34 menunjukkan mekanisme yang berbeza dalam mengawal *C. gloeosporioides* dengan mengurangkan nutrien bagi kulat yang diuji, menghasilkan enzim proteolitik dan merembeskan antibiotik untuk menghancurkan dinding sel serta melarutkan kulat. Selain itu, strain B34 mampu menyerang dan merosakkan hifa patogen apabila diperhatikan di bawah mikroskop imbasan elektron. Bakteria inididapati sangat serasi dengan 20% *Aloe vera* gel (gel lidah buaya) dan 2% natrium bikarbonat (SBC) atau campuran kedua-duanya. Kedua-duanya mempunyai aktiviti bersifat menindas terhadap *C. gloeosporioides* betik dan boleh digunakan sebagai penambah keberkesanan kawalan biologi strain B34 semasa penyimpanan buah. Kelangsungan hidup dan perkembangbiakan strain B34 dalam luka betik dan pada permukaan buah-buahan tidak terjejas oleh gel lidah buaya dan SBC sepanjang tempoh penyimpanan. Di samping itu, seperti yang diperhatikan melalui mikrogram mikroskop imbasan elektron, strain B34 dapat menjajah dan mengganda pada permukaan buah betik. Gabungan strain B34 dengan gel lidah buaya dan SBC adalah lebih berkesan dalam mengawal penyakit antraknos daripada strain B34 sahaja atau rawatan lain, malahan dilihat lebih baik daripada racun kulat Benocide® (benomyl WP 50%) samada bagi buah yang disuntik atau dijangkiti semulajadi yang masing-masing disimpan pada 12±1ºC dan 90±5% RH selama 18 dan 14 hari. Walau bagaimanapun, gabungan ini menawarkan satu kawalan yang lebih baik dengan mengurangkan 98.2% pengurangan penyakit berbanding kawalan bagi buah yang dijangkiti semulajadi pada akhir 14 hari penyimpanan pada 12±1ºC dan 90±5% RH dan enam hari selepas kemasakan pada 28±2ºC, yang mana adalah lebih baik berbanding Benocide® atau rawatan lain yang diuji. Tambahan pula, buah yang dirawat dengan gabungan *B. subtilis*-gel lidah buaya-SBC menunjukkan penangguhan respirasi klimakterik dan evolusi etilena lebih daripada tujuh hari berbanding kawalan dengan pengurangan kadar penghasilan CO_2 dan C_2H_4. Rawatan gabungan ini mengurangkan kehilangan berat lebih daripada 25% berbanding dengan kawalan. Ia juga melambatkan kemasakan buah secara ketara seperti yang ditunjukkan oleh pengekalan kepejalan selepas penyimpanan. Selain itu, ia melambatkan perubahan warna luaran, asid tertitrat dan pH tanpa mengkompromi kualiti buah pada buah yang dirawat dengan rawatan kombinasi. Jangkahayat simpanan dapat dipanjangkan selama 15 hari apabila dibandingkan dengan kawalan. Pengekalan aktiviti perencatan strain B34 yang disimpan dalam gliserol juga diperhatikan dalam kajian ini. strain B34 yang disimpan dalam rumusan 2% gliserol berasaskan cecair dari segi ekonomi menunjukkan prestasi tertinggi mengawal *C. gloeosporioides* dan bilangan bahan biak berdaya saing (CFUs) selama 120 hari tempoh penyimpanan. Akhirnya, adalah jelas bahawa strain B34 bukan sahaja dapat disimpan pada gliserol 2% untuk masa yang lama tetapi juga berkesan dalam penggabungan dengan SBC dan gel lidah buaya untuk mengawal *C. gloeosporioides* serta mengekalkan kualiti lepas tuai semasa penyimpanan sejuk berbanding untuk Benocide®. Ini mencadangkan bahawa salutan komposit ini menjanjikan sebagai sesuatu yang mesra alam untuk digunakan dalam aplikasi lepas tuai komersial bagi memanjangkan hayat penyimpanan betik Frangi.

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I certify that a Thesis Examination Committee has met on -/-/- to conduct the final examination of Md. Fakhrul Hasan on his PhD thesis entitled **"Controlling anthracnose and postharvest quality of papaya (*carica papaya* cv. Frangi) using *Bacillus subtilis* strain B34"** in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or any other institution.

MD. FAKHRUL HASAN

Date: 13 December 2012

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