Mode of action of *Bacillus subtilis* ATCC 55466 as biocontrol agent of postharvest diseases of avocados

By

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I certify that the thesis submitted to the University of Pretoria for the degree of MSc (Agric) has not previously been submitted by me in respect of a degree at any other University

Signed on the 15 th of December 2004 at Pretoria.

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SUMMARY

Avocados are an economically important crop in South Africa and are mainly exported to Europe. As with any other tropical and subtropical crop, avocados are prone to pre- and postharvest diseases. Until recently, chemical control was the only effective measure to control fungal avocado pathogens. In 1987, a *Bacillus subtilis* isolate was found that showed promise as a biocontrol agent in both pre- and postharvest applications to control postharvest diseases. However, over time variable results has been obtained in semi commercial trials.

From the original *B. subtilis* isolate several subcultures have been made and used over a 15 year period in various experimental trials. The dual culture technique was used to compare the biocontrol activity of the subcultures against postharvest pathogens (*Colletotrichum gloeosporioides*, *Phomopsis perseae*, *Dothiorella aromatica* and *Lasiodiplodia theobromae*). The subcultures differed significantly in their effectiveness and genetic stability. No difference between the subcultures could be found when DNA fingerprinting using RISA PCR was used. The most effective subculture, MI-14, was used in further studies.

The mode of action employed by a biocontrol agent is of utmost importance and can be used to enhance its efficacy. In a previous study it was hypothesized that antibiosis as well as competition for nutrients and space is the modes of action involved in biocontrol of *B. subtilis* against postharvest pathogens of avocado. The direct interaction between *B. subtilis* and *C. gloeosporioides* on avocado fruit were observed using scanning electron microscopy. Cells of *B. subtilis* were observed to colonize the hyphae of *C. gloeosporioides*. In some instances, hyphal walls were lysed in the presence of *B. subtilis* and may be due to the presence of enzymes or antibiotic substances. Conidia of *C. gloeosporioides* did not germinate in the presence of *B. subtilis*. Diffusible inhibitory metabolites active against *C. gloeosporioides* were produced *in vitro* by *B. subtilis*. Inhibitory volatile substances were also produced by *B. subtilis* and were found to be active against *P. perseae*, *D. aromatica* and *L. theobromae* but not *C. gloeosporioides*. Siderophores production as well as chitinase, amylase, lipase and proteinase activity were also observed and may play a role in antagonism.

Antibiotic production by *B. subtilis* is a well-known phenomenon. Most antibiotics are polypeptides and lipopeptides. The involvement of phenolic metabolites in biocontrol by *B. subtilis* is less known. A seven-day-old culture of *B. subtilis* in a minimal medium was analyzed for the presence of free acid phenolic

compounds active against fungi. Free acid phenolic metabolites were found and separated using layer chromatography. TLC plates containing the separated spots were sprayed with *Cladosporium cladosporioides* and plates were observed for inhibition zones. The phenolic substances were present at 7.06 ± 0.95 mg gallic acid ml^{-1}. The phenolic substances fall in the hydroxycinamic acid group due to their fluorescent coloring under UV at 350 nm.

The mode of action involved is also influenced by environmental factors. The effect of temperature and carbon- and nitrogen sources on the *in vitro* inhibitory activity of *B. subtilis* against *C. gloeosporioides*, *P. perseae*, *D. aromatica* and *L. theobromae* were investigated using the dual culture technique. The most effective temperature range for *B. subtilis* was found to be between 20 and 37 °C. At temperatures lower than 15 °C, *B. subtilis* was found to be not very effective, suggesting why postharvest applications followed directly by cold storage do not always work effectively. D-arabinose and D-(+)-mannitol evaluated as carbon source as well as L-glutamic acid, L-glutamine and L-(+)-asparagine used as nitrogen sources support *in vitro* antagonism against the pathogens most effectively. They also do not support the growth of *C. gloeosporioides*, *P. perseae*, *D. aromatica* and *L. theobromae*. These nutrients can potentially be the most effective ones to incorporate in commercial *B. subtilis* formulations.

This study showed the potential role of antagonistic free acid phenolic substances, volatiles and siderophores on inhibition of fungal avocado pathogens. Further studies to confirm their *in situ* activity are required. In conclusion, various factors affect the efficacy of *B. subtilis* against postharvest pathogens of avocado. These factors should be kept in mind when applying the commercial product in order to achieve the best results.

OPSOMMING

Avokados is `n ekonomies belangrike gewas in Suid Afrika en word hoofsaaklik uitgevoer na Europa. Avokados is soos enige tropiese en subtropiese gewas vatbaar vir voor- en na-oes siektes. Tot onlangs was chemiese middels die enigste effektiewe beheermaatreel teen swam avokado patogene. In 1987 is `n *Bacillus subtilis* isolaat gevind wat belowende resultate getoon het as `n biobeheer middel teen avokado na-oes siektes tydens voor- en na-oes toedienings. Varieerende resultate is wel gekry in semi kommersieële proewe.

Verskeie subkulture is van die oorspronklike *B. subtilis* isolaat gemaak en is gebruik in verskeie proewe oor `n tydperk van 15 jaar. Die twee-kultuur tegniek was gebruik om die onderskeie subkulture se biobeheer aktiwiteit teenoor avokado na-oes patogene (*Colletotrichum gloeosporioides*, *Phomopsis perseae*, *Dothiorella aromatica* en *Lasiodiplodia theobromae*) te vergelyk. Die subkulture se effektiwiteit en hul genetiese stabiliteit het betekenisvol van mekaar verskil. DNS profiele van geselekteerde subkulture is deur RISA polimerase ketting reaksie gegenereer en het nie van mekaar verskil nie. Die mees effektiewe subkultuur, MI-14, is in verdere studies gebruik.

Die meganisme van werking wat deur `n biobeheeragent gebruik word, is van kardinale belang en kan gemanipuleer word om sy om sy effektiwiteit te verbeter. In `n vorige studie is dit gestel dat antibiose asook kompetisie vir nutriente en spasie die meganismes van werking is wat deur *B. subtilis* gebruik word in die biobeheer van na-oes avokado patogene. Die direkte interaksie tussen *B. subtilis* en *C. gloeosporioides* op avokado vrugte is deur skandeer elektron mikroskopie ondersoek. Dit is waargeneem dat *B subtilis* die hifes van *C. gloeosporioides* koloniseer. In sommige gevalle waar *B. subtilis* teenwoordig is, is die hifewande geliseer. Dit mag die gevolg wees van ensieme of antibiotiese stowwe. In die teenwoordigheid van *B. subtilis* het *C. gloeosporioides* konidia nie ontkiem nie. Daar is gevind dat *B. subtilis* diffundeerbare inhiberende metaboliete *in vitro* produseer wat aktief is teen *C. gloeosporioides*. Vlugtige inhiberende stowwe is ook deur *B. subtilis* geproduseer en is aktief teen *P. perseae*, *D. aromatica* en *L. theobromae* maar nie teen *C. gloeosporioides* nie. Siderofoor produksie deur *B. subtilis* asook die aktiwiteit van chitinase, amilase, lipase en proteïenase is waargeneem en kan `n rol speel in antagonisme.

Die produksie van antibiotiese metaboliete deur *B. subtilis* in `n bekende verskynsel. Die meeste antibiotiese metaboliete is polipeptiede en lipopeptiede. Die rol van fenoliese metaboliete wat deur *B. subtilis*

geproduseer is in biobeheer, is minder bekend. Sewe-dae-oue fermentasie kulture van *B. subtilis* in `n minimale medium is geanaliseer vir die teenwoordigheid van vrye-suur fenoliese verbindings wat aktief is teen swamme. Vrye-suur fenoliese verbindings is gevind en deur dunlaag chromatografie geskei. Plate wat die geskeide kolle bevat is bedek met *Cladosporium cladosporioides* en is daarna ondersoek vir die vorming van inhibisiesones. Fenoliese verbindings was teenwoordig teen teen `n konsentrasie gelykstaande aan 7.06 ± 0.95 mg gallig suur ml^{-1}. Die fenoliese verbindings wat gevind is word geklassifiseer in die hidroksiesinamiese suur groep na aanleiding van hul fluoresserende kleure onder UV lig by 350 nm.

Omgewingsfaktore beïnvloed ook die meganisme van werking wat gebruik word deur `n biobeheer agent. Die twee-kultuur tegniek is gebruik om die effek van tempratuur asook koolstof- en stikstofbronne op die *in vitro* inhiberende werking van *B. subtilis* teen *C. gloeosporioides*, *P. perseae*, *D. aromatica* en *L. theobromae* te ondersoek. Temprature tussen 20 en 37 °C het die werking van *B. subtilis* die beste ondersteun. By temprature laer as 15 °C is gevind dat *B. subtilis* nie baie effektief was nie, wat kan verduidelik hoekom na-oes toedienings dadelik gevolg deur koue stoor berging nie altyd effektief is nie. Daar was gevind dat *in vitro* antagonisme die beste deur D-arabinose of D-(+)-mannitol as koolstofbron, en L-glutamiensuur, L-glutamien of L-(+)-asparagien as stikstofbron ondersteun was. Hierdie bronne bevorder nie die groei van *C. gloeosporioides*, *P. perseae*, *D. aromatica* of *L. theobromae* nie. Dit kan die beste potensiaal hê om in `n kommersieële *B. subtilis* formulasie gebruik te word.

Hierdie studie toon die moontlike rol van antagonistiese vry-suur fenoliese verbindings, vlugtige stowwe en siderofore op die inhibisie van patogeniese swamme op avokado. Verdere studies om die *in situ* aktiwiteite hiervan te bepaal, is nodig. Verskeie faktore affekteer die werking van *B. subtilis* teen na-oes avokado patogene. Hierdie faktore moet in gedagte gehou word wanneer die produk toegedien word om die beste resultate te behaal.