

The Importance of Monitoring Antagonists Survival for Efficient Biocontrol

E. Towsen¹, S. van Wyngaardt², J.A. Verschoor² and LKorsten¹

Department of Microbiology and Plant Pathology

Department of Biochemistry, University of Pretoria, Pretoria, 0002, RSA

ABSTRACT

*Biological control of avocado fruit diseases has been investigated at a pre-and post-harvest level at the University of Pretoria, South Africa, for the past eight years. Biological control can be applied more easily post-compared to pre-harvestly, and environmental conditions can be manipulated more effectively to enhance antagonist survival. Poor antagonist survival under pre-harvest field conditions can affect the efficacy of the biocontrol program due to fluctuating environmental conditions. Therefore, monitoring antagonist survival is of prime importance to ensure effective disease control. In this study different antagonist detection methods were evaluated and compared to monitor antagonist survival during field spray applications. These methods include electron microscopy, leaf imprint-and dilution plate techniques. However, none of these methods were found effective for accurate and rapid field monitoring of antagonist survival. Monoclonal antibodies were subsequently raised against the antagonist, *Bacillus subtilis*, which are currently used in the avocado biocontrol programme. The ELISA technique used to detect bacteria on plant material was selected and optimised to monitor different antagonist concentrations on the avocado phylloplane under greenhouse conditions. The ELISA technique is currently being evaluated for field monitoring of antagonist survival as part of the pre-harvest biocontrol spray programme.*

INTRODUCTION

Pre-and post-harvest diseases of avocados are currently controlled by chemicals (Darvas & Kotzé, 1987). For instance, copper-oxychloride and benomyl are registered for use as a pre-harvest spray to control avocado fruit diseases while prochloraz and thiabendazole have been registered as a post-harvest treatment to control post-harvest avocado diseases (Nel *et al*, 1993). However, copper-oxychloride leaves visible residues on fruit, benomyl can lead to build up of pathogen resistance and prochloraz is not registered for use on fruit destined for the export market to France. Furthermore, international awareness over the indiscriminate use of chemicals has resulted in increased interest in alternative control strategies such as biocontrol.

Biocontrol of avocado fruit diseases has been evaluated at the University of Pretoria on a semi-commercial basis (Korsten, 1993). The biocontrol agent can be applied preor post-harvestly for control of avocado fruit diseases. Biocontrol applied post-harvestly

can be more successful than pre-harvest applications, due to the manipulative environmental conditions in the packhouse, during shipping, and storage due to more effective targeting of the antagonist to fruit during packing; and the short period of protection required post-harvestly (Vorster *et al*, 1991) (Wisniewski & Wilson, 1992). This compared to pre-harvest applications where the biocontrol agents are more exposed to fluctuating microclimate on the plant surface and to seasonal changes which influences growth and survival of the antagonists (Blakeman, 1985).

Biocontrol agents have been applied according to commercial spray schedules used for fungicide applications. However, these schedules might not necessarily be optimal for biocontrol disease suppression (Sutton & Peng, 1993). Timing of antagonist application is of crucial importance in biocontrol programmes (Bhatt & Vaughan, 1962) and poor survival of the antagonist in the field will result in inadequate diseases control (Knudsen & Spurr, 1987). Population fluctuations of antagonists applied preharvestly should be monitored in the field at several intervals starting from the time of application (Spurr & Knudsen, 1985). This information is necessary to predict the survival of the antagonist in the field and can provide information necessary to improve biocontrol effectiveness through better formulation, adjustment of dosage and spray scheduling (Knudsen & Spurr, 1987). This information is also necessary to minimize wasteful application of inoculum to non-target organisms (Sutton & Peng, 1993).

The majority of research on detection and quantification of microorganisms has been studied in controlled or relatively simple environments (Donegan *et al*, 1991). Interaction between pathogenic bacteria and plant cells is better described than epiphytic interaction (Romantschuk, 1992). Another method used to detect epiphytic survival was done using a rifampicin-nalidixic acid mutant of *Pseudomonas viridiflava* (Mariano & McCarter, 1993). Persistence and efficacy of five bacterial preparations against *Cercospora* leaf spot on peanut were also monitored using a dilution plate technique (Knudsen & Spurr, 1987). However, in most pre-harvest biocontrol programmes antagonist survival are not monitored.

Methods used for monitoring antagonists need to be consistent and independent of the application time (Donegan *et al*. 1991). In the following report, different methods for monitoring *B. subtilis* survival and colonisation in avocado biocontrol field trials were evaluated. Direct (Scanning Electron Microscopy) and indirect (dilution plate and imprint techniques) were compared. Monoclonal antibodies were produced as an alternative method for monitoring antagonist survival.

MATERIALS AND METHODS

Bacterial cultures

Bacillus subtilis (Ehrenberg) Cohn (B246) isolated from the avocado phylloplane, successfully evaluated *in vitro*, *in vivo*, in post-harvest packinghouse experiments and, in pre-harvest field trials for antagonism to control avocado post-harvest pathogens (Korsten, 1993), were selected for further study of antagonist attachment, survival and colonisation. B246 was maintained on standard 1 nutrient agar (STD) (Biolab) slants at 5 °C and in 30 % (v/v) glycerol-Ringers (Merck) solution at -78 °C.

The antagonist was grown in 100 ml STD broth for 32 h for mass cell production. Cell growth was harvested by means of centrifugation in a Sorval RC5b refrigerated superspeed centrifuge using a GSA rotor at 11 080.64 g for 20 min. Bacteria were counted with a Petroff-Hausser counting chamber and the concentration adjusted to 1×10^7 cells/ml.

Effectivity of leaf and fruit imprinting technique

Three Fuerte cv. trees were randomly selected at the experimental farm (University of Pretoria) and a northern and southern branch labelled on each tree. Thirty leaves and fruit were selected from each branch, labelled and wiped with 70 % ethanol for more effective counting of antagonists. The selected branches were sprayed with 100 ml freshly harvested *B. subtilis* at a concentration of 1×10^7 cells/ml water, using a 500 ml hand held spray bottle. Five marked leaves and fruit were picked from each branch, placed into paper bags, and transported to the laboratory for processing. Ad- or abaxial leaf imprints were made on Mundt and Hinkle (MH) selective medium, for 15 s, using as a weight a 250 ml Erlenmeyer flask with 200 ml water in. Fruit were rolled in MH selective medium. Leaves and fruit were imprinted after 1 h and 2, 3, 5 (in the case of abaxial samples) or 6 (in the case of adaxial samples) and 7 days. Plates were incubated at 28 °C for 48 h before c.f.u. were counted and statistically compared.

Scanning Electron microscopy (SEM)

Micro-droplet technique (MDT) — leaves and fruit

Newly selected leaves on the northern and southern side of the above mentioned three trees were wiped with 70 % ethanol, before 11 (25 mm²) blocks were drawn ad- and abaxially with a felt pen on each leaf interveinal area and fruit. 0.01 ml *B. subtilis* (1×10^7 cells/ml) were placed in each block, allowed to air dry, before samples were collected from each tree after 1 h and 2, 3, 5 or 6 (in the case of the adaxial samples 5 and abaxial samples 6) and 7 days. Blocks (25 mm²) were cut from each sample, placed in 2 % Gluteraldehyde (Biorad) in 0.1 M Cacodylate buffer and transported to the laboratory for SEM processing according to the method of Weakley (1987). Samples were dried in a Hitachi CHP-2 critical point dryer before specimen stabs were coated with gold palladium in an Eiko IB-3 ion counter and viewed in a Hitachi S-450 SEM (Hitachi Ltd, Tokyo, Japan) operating at 5 KV. Three spot counts were made at 25 000 x magnification on each sample evaluated and total counts were statistically compared.

Dilution plate technique

Leaf samples were picked from the northern, eastern, southern and western aspect from each tree one hour after spraying for the fourth time with *B. subtilis* at Westfalia Estate. Leaves were taken to the laboratory. Leaf washing and dilutions were made by placing 1 g of plant material (20 leaf discs obtained with a no 10 corkborer) in 9 ml quarter strength Ringers. After 15 sec sonification in an ultrasonic bath UMC 5, Ultrasonic manufacturing company (Krugersdorp, S.A.), aliquots were diluted and plated

out on STD + Chloramphenicol (Sigma). Plates were incubated for 48 h at 28 °C before antagonists were counted.

Monoclonal antibodies

B. subtilis used in above mentioned trials were inoculated separately into five 250 ml Erlenmeyer flasks each containing 100 ml STD 1 broth (Biolab, Merck). Flasks were shake incubated at 73 rpm at 26 °C. After 24 h one ml 39 % formaldehyde was added to each flask before centrifugation (Sorvall-SA Scientific) at 1288 g for 10 min at 4 °C. Pellets obtained from harvesting the antagonist were dissolved in 20 ml phosphate buffered saline (PBS) (pH 7.2). Total cell counts of the five antagonist preparation were determined with a Petroff-Hausser counting chamber before adjusting the final concentration of each antagonist 1×10^7 cells/ml PBS. Aliquots were frozen away for immunisation and subsequent screening.

Monoclonal antibodies were prepared against *B. subtilis* according to the method used by Köhler & Milstein (1975).

In vitro and in vivo screening of monoclonal antibodies

In vitro

B246 used as antigen source were aligned in a 96-well Microtiter plate (Cooke Microtiter system M299, Sterilin Products, Middlesex, England) where each vertical row was coated with concentrations of either 10^8 , 10^7 , 10^6 , or 10^5 cells/ml. The ELISA was carried out according to an optimised ELISA using supernate of subclone 6B7E5.

In vivo

Four different cell concentrations of *B. subtilis* (10^8 , 10^7 , 10^6 , or 10^5) were sprayed separately onto ten mature Ryan cultivar leaves, one of the commercially available cultivars. Ten ml were sprayed on each leaf both abaxially and adaxially. Leaves were left to air dry before further processing. From each concentration sprayed 0.1 g of leaf tissue was separately grounded with a mortar and pestle using 2 ml PBS. Wells of a microtiter plate were coated with the crude extract and screening was done with the subclone 6B7E5.

RESULTS AND DISCUSSION

Antagonist survival on avocado leaves

Survival of *B. subtilis* on the avocado phylloplane varied between different monitoring techniques. An increase in total cell counts was observed on the abaxial side of the leaves when the antagonist was counted under the Scanning Electron Microscope (SEM) after three, five and seven days. An increase in total cell counts was observed abaxially on the southern compared to northern side of the tree. This can be due to a higher intensity of UV irradiation on the northern side of the tree. Leben & Whitmoyer

(1979) also found that bacteria die as soon as leaves are exposed to ultraviolet irradiation, which is much higher adaxially. Haas & Rotem (1976) found that whether upper or lower leaf surface was inoculated, survival was not affected. This was done in a growth chamber which excluded the effect of UV radiation. In contrast with the SEM studies a drop in colony forming units (c.f.u.) was observed with the leaf imprint technique as well as dilution plate technique. Higher numbers in c.f.u. were counted on the adaxial leaf compared to abaxial surface, with the leaf imprint technique, directly after application. This can be due to difficulty of applying antagonist on the abaxial surface.

Leben (1969) observed that naturally occurring organisms locate more appropriate sites after a certain time on the leaves. The same tendency was observed with the SEM where cells were in close proximity to stomata or trichomes after one day and one week. Cells were mainly between depressions of epidermal cells after one week. Mariano & McCarter (1993) also found that cells colonize in depressions between epidermal cells, around trichomes, along veins and around stomata 2-3 h after inoculation.

Antagonist survival on avocado fruit

A decrease in c.f.u. was observed after six days using the imprint technique. However, total counts with the SEM showed cell increase to the extent that cell counting was difficult after six days. Microcolonies appeared according to a packing manoeuvre described by Lawrence *et al.* (1987). Throughout the study antagonist cell multiplication was more prominent on the fruit than on the leaf surface. According to the investigation the inadequacy of these methods was evident, although SEM gave higher counts, detection of populations by means of this method is more time consuming and expensive.

Monoclonal antibodies

Viability of the MAB produced against *B. subtilis* was evaluated *in vitro* and *in vivo*. Four different cell concentrations of *B. subtilis* were sprayed on leaves, extracted and evaluated using the ELISA technique. A significant increase in absorbency values was observed for the different cell concentrations (Figure 1). The MAB are currently being evaluated in field trials using the ELISA technique.

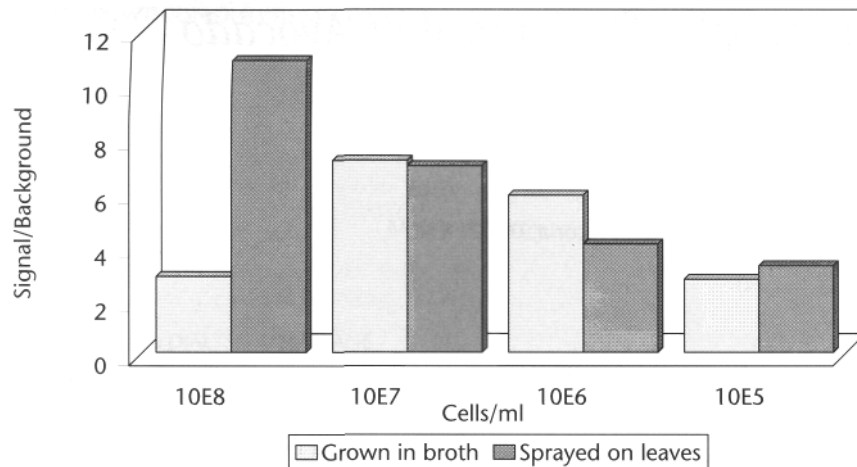


Figure 1

Results obtained with the ELISA technique using subclone 6B7E5, showing the specificity of differently prepared *Bacillus subtilis*.

CONCLUSION

Different detection techniques for monitoring *Bacillus subtilis* populations in avocado biocontrol programmes have been discussed. Monoclonal antibodies produced against *B. subtilis* have shown the most potential. The importance of monitoring antagonist populations must not be underestimated, because biocontrol agents operate in natural environments and are vulnerable to disruption. Antagonist survival information can be used to determine the adaptability of the agent and to test the performance of different formulations. Pre-harvest applications of the antagonist need more applications and higher volumes; therefore this information is of prime importance in enhancing cost efficiency of any biocontrol programme. This will amplify the positive attitude towards biocontrol as an alternative control method. As long as information of antagonist survival is not available, only slow progress can be made.

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