Challenge plate testing to evaluate the inhibitory effects of *Bacillus amyloliquefaciens rm303* (Lolipepta) when combined with 2% w/v Sodium Bicarbonate against *Nigrosporum spp.* on Macadamia leaves.

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Summary

Lolipepta ™ - a *Bacillus amyloliquefaciens (rm303)* preparation was combined with 2% w/v Sodium Bicarbonate ($NaHCO_3$) applied *in vitro* in a challenge plating experiment against isolated from fresh plant tissue from a farm in the Bundaberg area. Six application rates were chosen *in vitro* – 2% Lolipepta, 5% Lolipepta, 10% Lolipepta, 2% Lolipepta/2% w/v $NaHCO_3$, 5% Lolipepta/2% w/v $NaHCO_3$ and 2% Lolipepta/2% w/v $NaHCO_3$. The combination of Lolipepta (of either concentration) and the 2% w/v $NaHCO_3$ was highly successful in holding the suspected pathogen after 4 and 7 days incubation.

Aims

To determine if the *in vitro* addition of Lolipepta™ in solution with 2% w/v $NaHCO_3$ suppressed *Nigrosporum* spp., a plant pathogen isolated from *Macadamia* sp. foliage.

Materials & Methods

Sample Preparation

Suspected plant pathogens were isolated from fresh *Macadamia* sp. leaf samples taken from an orchard in the Bundaberg region. Sabouraud Agar (SAB) plates were used to isolate fungi from sample material provided. Fungal colonies growing on plates were then re-isolated and grown separately on new SAB plates until pure cultures were obtained. Visual and laboratory preliminary inspections indicated pure samples similar in phenology to an Anthracnose pathogen, which subsequently was genetically identified as *Nigrospora* spp. – a plant pathogen that causes leaf spots.

Challenge Plate Preparation

Once sporulating (~ 9 days after plating), pure colonies were excised from the SAB plates using a scalpel to cut a plate into a regular grid pattern. Five mm square plugs were excised from the pure colonies, ready to be implanted into the centre of 37 SAB plates for challenge plating.

Efficacy of Lolipepta ™ and Sodium Bicarbonate

Lolipepta ™ was prepared at concentrations of 2%, 5% and 10% with sterile deionised water. Combination Lolipepta and Sodium Bicarbonate solutions were prepared by dissolving 2.00 g of $NaHCO_3$ in 100 ml of sterilised deionised water and swirling for 10

seconds, and then pipetting into the solution either 0.2, 0.5 ml or 1.0 ml Lolipepta to make combined 2% Lolipepta/2% w/v $NaHCO_3$; 5% Lolipepta/2% w/v $NaHCO_3$ or 10% Lolipepta/2% w/v $NaHCO_3$ solutions. In order to evaluate the possible inhibitory effects of the products against the pathogen, the product and the pathogen were applied to SAB plates using the *streak plate technique,* two parallel lines were drawn along the plate, approximately 20 mm from either 'side' of the plate. The products were applied along the lines using a sterile flame loop dipped in the corresponding concentration of the product. For each concentration of the product, there were three replicate SAB plates. In the centre of each plate, a 5 mm plug of the suspected pathogen was applied.

In order to determine efficacy of the products, streak plate sample diameters were measured at the start of the trial, and growth was compared to an untreated control. Successful inhibition of the pathogen was determined from challenge plates seven days after inoculation if the pathogen had not crossed the product barrier.

Measurements were recorded at four and seven days after incubations. The mean values of two measurements on each plate and standard deviations were presented. The percentage inhibition compared to the control was tabulated from the mean values.

Results & Discussion

Streak plate barriers became less effective with increasing rate of Lolipepta application in reducing growth beyond the treated margins after four and seven days, even when the combined Lolipepta and Sodium Bicarbonate solutions were used. The optimal rate at the end of the seven-day trial was 2% or 5% Lolipepta in combination with 2% Sodium Bicarbonate, with 116% efficacy, followed by the 2% Lolipepta only treatment with ~82% efficacy (Table One).

Table One. Inhibition of *Nigrospora* spp. on plates streaked with Lolipepta ™ at 2%, 5% and 10% application rates, with or without addition of 2% w/v Sodium Bicarbonate ($NaHCO_3$), as compared to a control (no treatment).

	4 days incubation mean ± S.E. (mm)	**Percent inhibition**	**7 days incubation mean ± S.E. (mm)**	**Percent inhibition**
Control	35.0 ± 0.0	0.0	35.0 ± 0.0	0.0
2% Lolipepta ™	-2.67 ± 5.22	88.15	-4.00 ± 6.00	82.22
5% Lolipepta ™	-13.33 ± 7.81	38.98	-15.33 ± 9.67	29.82
10% Lolipepta ™	-18.25 ± 1.77	25.76	-25.17 ± 0.60	-2.37
2% Lolipepta ™ + 2% $NaHCO_3$	6.25 ± 1.25	131.51	4.17 ± 0.44	116.87
5% Lolipepta ™ + 2% $NaHCO_3$	5.29 ± 1.37	131.51	2.83 ± 0.17	116.87
10% Lolipepta ™ + 2% $NaHCO_3$	-6.58 ± 11.13	65.87	-17.17 ± 8.58	11.02

Plates spread with Lolipepta and/or Lolipepta and Sodium Bicarbonate yielded variable results. Again, the 2% Loli/Bicarb combination yielded the highest percent efficacy at 68%, whilst the 2% Lolipepta treatment was 61% effective against the pathogen (Table Two) (Figure Two). The other treatments, albeit higher in Lolipepta concentration, were less effective in holding the pathogen. The reasons for this are

postulated to be due to a mutualistic effect of the active ingredient in LoliPepta, *B. amyloliquefasiens,* which favoured the pathogens' growth *in vivo* at increasing concentrations. An improperly prepared stock solution is an unlikely cause of these results, as each stock was prepared on the day of incubation, and independently for both streak and spread plates (yet the trend was consistent across both plate types).

Table Two. Inhibition of *suspected* Anthracnose on plates spread with Lolipepta ™ at 2%, 5% and 10% application rates, with or without addition of 2% w/v Sodium Bicarbonate ($NaHCO_3$), as compared to a control (no treatment).

	4 days incubation mean ± S.E. (mm)	**Percent inhibition**	**7 days incubation mean ± S.E. (mm)**	**Percent inhibition**
Control	35.0 ± 0.0	0.0	35.0 ± 0.0	0.0
2% Lolipepta ™	12.6 ± 3.8	28.2	21.3 ± 0.5	61.0
5% Lolipepta ™	12.4 ± 2.7	53.0	8.8 ± 7.5	25.2
10% Lolipepta ™	12.4 ± 2.3	26.0	18.1 ± 5.5	51.7
2% Lolipepta ™ + 2% $NaHCO_3$	25.8 ± 3.6	25.5	24.1 ± 0.3	68.8
5% Lolipepta ™ + 2% $NaHCO_3$	23.6 ± 2.3	30.8	18.6 ± 5.8	53.1
10% Lolipepta ™ + 2% $NaHCO_3$	14.5 ± 5.7	78.0	0.0 ± 0.0	0.0

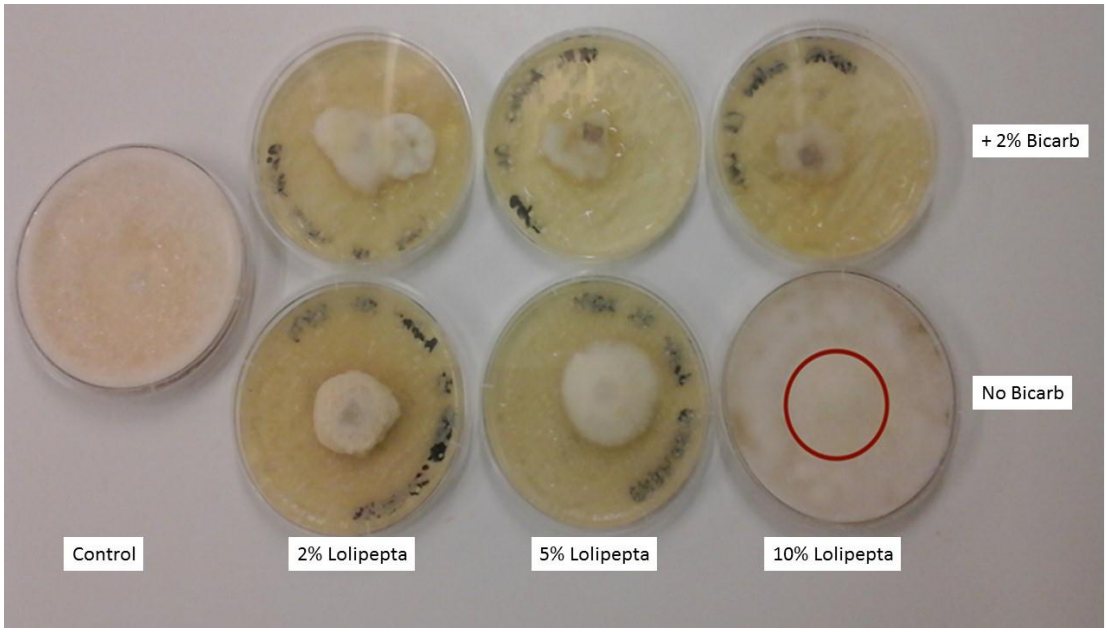

Figure Two. Suspected *Anthracnose* spread challenge plates, where the top row shows plates spread with (L-R): 2%, 5% and 10% Lolipepta plus 2% Sodium Bicarbonate, and the bottom row shows plates spread with (L-R): 2%, 5% and 10% Lolipepta only. These are compared with a control, to the far left of the image.

Conclusion and recommendations

Based on the results of this trial, it appears that higher concentrations of the product are facilitative to the growth of the Nigrospora pathogen. Supplementary Sodium Bicarbonate appears to mitigate some of this affect, with slightly higher rates of

efficacy afforded by the additive. It is recommended that the impact of supplementary $NaHCO_3$ addition to the Lolipepta product on the survival and activity of the active ingredient, *Bacillus amyloliquefaciens,* be evaluated prior to using a combined product in a commercial setting.