FINAL REPORT

SUMMARY

Project title: Studies on *Lecanicillium muscarium* as a potential mycoparasite of the soybean rust fungus, *Phakopsora pachyrhizi* and its use as a biocontrol agent against soybean rust.

In this study, a Isolate N-08, a mycoparasitic fungus, was isolated from Assagay coffee farm, Cato Ridge, KwaZulu-Natal, South Africa, where it was observed parasitizing *Hemileia vastatrix*, the causal agent of coffee rust. Based on morphological and molecular studies the Isolate N-08 was identified as *Lecanicillium muscarium* and it was deposited into the National collection of fungi (Accession number PPRI 13715).

Co-inoculation studies of *L. muscarium* and *P. pachyrhizi* were done in UKZN Plant Pathology disease garden. The Environmental Scanning Electron Microscope (ESEM) observation of the interactions showed a mycophilic attraction of *L. muscarium* to *P. pachyrhizi* urediniospores. Long *L. muscarium* phialides were observed penetrating and wrapping tightly around *P. pachyrhizi* urediniospores.

In vitro studies to test the effect of the *L. muscarium* strain N-08 on *P. pachyrhizi*, the soybean rust fungus, were done. *L. muscarium* strain N-08 was observed colonizing *P. pachyrhizi* under light microscope and ESEM. Laboratory experiments were conducted to assess the effects of different growing conditions (temperatures, artificial growing media, natural substrates and UV radiation) on colony growth and conidia production. Optimization of growing conditions is one of the essential aspects which must be taken into consideration to produce an effective biocontrol agent. *L. muscarium* strain N-08 grows best at temperatures between 21 to 25ºC. The highest radial growth was observed at 24ºC (46.54 mm). V8 juice agar was the best media for colony growth with the mean value of 42.75 mm followed by SDA with 37.86 mm. When the isolates were exposed to UV light, the results did not show a significant difference between different media on mycelia growth. The highest conidia production occurred on millet cereal (4.2 x 10^9 conidia/ml) followed by wheat bran (3.2 x 10^9 conidia/ml) and pearled barley (2.9 x 10^9 conidia/ml).

The optimal dose level for disease control was assessed in the greenhouse and in field trials. It was found that 10^6 and 10^8 conidia/ml were more effective and 10^6 conidia/ml was chosen as the optimum dose for the field application.

Effect of *L. muscarium* against soybean rust was evaluated in the field. Two field experimental trials (2014/2015 and 2015/2016) were run at Ukulinga Research Farm. Compared to the pathogen inoculated control, all the three *L. muscarium* doses (10^4, 10^6, 10^8 conidia/ml) and the fungicide control (Score) decreased disease severity by 73.3%, 88.2%, 89.1%, and 90% respectively. The Area Under the Disease Progress Curve (AUDPC) for the treatments were as follows: 1st trial, Score (172.2 units), 10^8 conidia/ml (186.2 units), 10^6 conidia/ml (202.16 units), 10^4 (457.8 units) and pathogen inoculated control (1716.8 units). 2nd trial, score (259.7 units), 10^8(284.9 units), 10^6 (319.9 units), 10^4 (462.7 units) and the pathogen inoculated control (1053.5 units). Treated plots showed higher yield increase compared to the pathogen inoculated pathogen. However, dry seed weight did not significantly differ between the *L. muscarium* strain N-08 treated and score fungicide treated plots.

Introduction

Soybeans are grown in temperate to tropical regions of the world, with production being highest in Argentina, Brazil, China, India and the United State of America (Karr-Lilienthal *et al*., 2004). Emphasis on research has been on breeding of soybeans, suitable for tropical environments (Hartman *et al*., 1999, Hartman *et al*., 2005; Bonde *et al*., 2006). In the past 30 years, world production of soybeans increased to more than 100 million metric tons. Of this, 51% is produced in the USA, 20% in Brazil, 10% in Argentina and 10% in China. Due to an increase in soybean production throughout the world, diseases that affect this crop have, therefore, also increased in number and severity (Nunkumar, 2006).

Soybean is a victim of many diseases caused by fungi, bacteria, nematodes, viruses and aphids (Kevin *et al*., 2009). The United States Department of Agriculture reported over 15% losses in the USA and over 10% worldwide. Asian soybean rust (SBR) is one of the most important diseases of soybean. It is caused by *P. pachyrhizi*, an obligate parasitic fungus first reported in Japan, hence the name Asian Soybean Rust (Kendrick *et al*., 2011).

Since then, the disease has caused major crop losses in many Asiatic countries, such as Japan, China, Taiwan, Thailand and India (Sinclair & Hartman, 1999). In Africa, the pathogen was first reported in East African countries: Uganda, Kenya and Rwanda via monsoon winds from India in 1996 (Oloka *et al*., 2008). After a short time SBR appeared in Zimbabwe, Zambia and it reached Nigeria in 1999 and Mozambique in 2000, and was reported in South Africa (SA) in 2001 (Pretorius *et al*., 2001, du Preez *et al.*, 2005 and Nunkumar *et al*., 2008). Finally, in 2004, *P. pachyrhizi* was found in the USA, infecting soybean and kudzu in many southern states (Schneider *et al*., 2005).

Soybean rust is the most destructive foliar disease of soybean, especially in regions with moderate winters, such as Central-East Africa, Asia and southern America, where crop losses caused by *P. pachyrhizi* are estimated from 10 to 80% (Miles *et al.,* 2011). The disease can have a significant financial impact in affected areas. In east-central African countries where this pathogen first appeared on the continent, soybean rust continues to cause huge yield losses. Studies done at Makerere University, Uganda, estimated crop loss due to soybean rust at 15-80% (Oloka et al., 2005). In South Africa, yield losses caused by *P. pachyrhizi* are reported to be 10-80% (Nunkumar, 2006). Worldwide yield losses due to *P. pachyrhizi* have been estimated at 10-90%. The highest yield loss has been reported in Taiwan and Africa, where the disease can cause crop losses of up to 100 percent (Pivonia and Yang, 2004; Glen *et al.,* 2005).

Since its discovery, many studies have been done on *P. pachyrhizi* and disease management to reduce yield loss due to the pathogen. Many practices to decrease soybean crop losses have been suggested and adopted, such as cultural practice, fungicide applications and biological control (Hassan *et al.,* 2014). Fungicide applications have shown a positive effect in reducing crop damage caused by the Asian Soybean Rust; however, the pathogen has a tendency to develop resistance to fungicides such as the strobilurins (Ward, 2011). Few studies have been done on biological control as an option to control the soybean rust pathogen. Studies has been conducted on *Simplicillium lanosoniveum* to control soybean rust (Ward *et al.,* 2011). So far there is no registered commercial biocontrol product against SBR.

In February 2013, the fungus *Lecanicillium muscarium* was observed attacking and feeding on *Hemileia vastatrix*, the coffee rust at Assagay coffee farm at Cato Ridge, KwaZulu-Natal, South Africa. The mycoparasite fungus was isolated and tested on different plant rusts e.g. oxalis rust

(Nxumalo, 2015) and in this current study on soybean rust. This locally adapted strain of *Lecanicillium muscarium* could be a successful biological control agent against soybean rust. The success of this mycoparasite for controlling soybean rust could reduce the frequent and costly use of synthetic fungicides on soybeans. This will reduce input cost for farmers and increase yield by reducing soybean rust severity to low levels.

Aim and objectives of the study

The primary aim of the present study is to evaluate the potential of *Lecanicillium muscarium* to control *Phakopsora pachyrhizi* on soybeans.

The specific objectives include:

1. Review available literature on Soybean rust in order to properly understand the pathogen, *Phakopsora pachyrhizi*, and the use of fungi as potential biocontrol agents for rusts disease on soybean plants

2. Isolation and identification of local strain of *L. muscarium* from coffee leaves infected by *Hemileia vastatrix*, where the hyperparasite was seen colonizing the coffee rust pustules.

3. Obtain pure culture of *L. muscarium*, store under short- and long-term conditions. Determine optimum conditions for its growth and spore production in the laboratory.

4. Colonization and mycoparasitic studies using *L. muscarium* on *P. pachyrhizi*. This will involve *L. muscarium*-*P. pachyrhizi* interaction studies using environmental scanning electron microscopy (ESEM), as well as a leaf disc bioassay using *L. muscarium* and the soybean rust pathogen.

5. Evaluate the effects of *L. muscarium* on *P. pachyrhizi* under greenhouse conditions using potted soybean plants as subjects for the study. The greenhouse study will also identify the optimal dose level for disease control and assess disease development over time.

6. Evaluate the effect of *L. muscarium* on soybean rust at UKZN Ukulinga Research Farm facility. This will involve monitoring the impact of *L. muscarium* on soybean rust in the field (disease severity levels).

Field survey, sample collection and isolation of *Lecanicillium muscarium*

In January 2013, a local strain of *Lecanicillium muscarium*, was observed feeding on *Hemileia vastatrix*, also known as coffee rust at Assagay coffee farm, Cato Ridge, KwaZulu-Natal, South Africa (29°45′49.41′′S and 30°37′25.68′′E) (Figure 1). It was assumed that this adapted local strain of *Lecanicillium* spp. can be developed and used as a biocontrol agent for controlling many rust fungi. The selection of virulent isolates adapted to local environmental conditions is one of the most essential aspects for the development of efficient biological control agents.

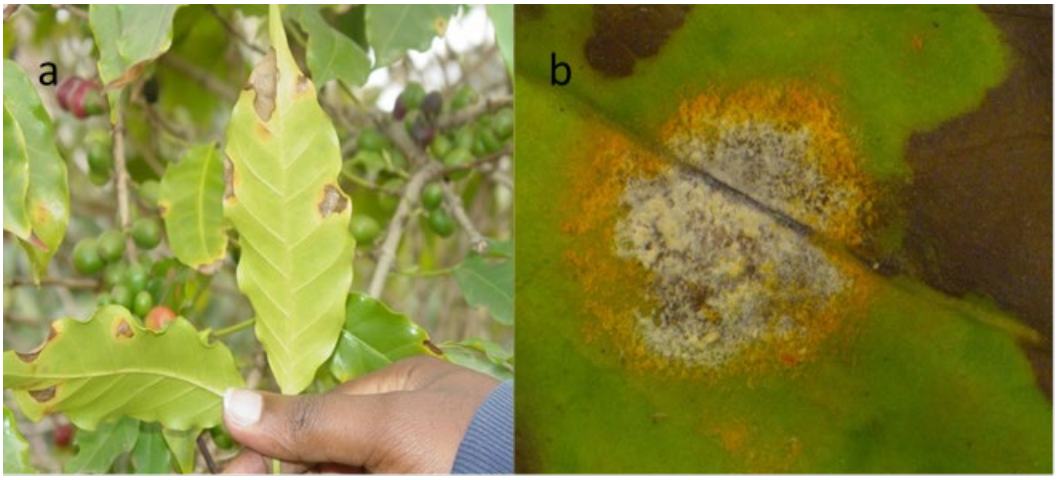

Figure 1a and 1b. *L. muscarium* colonising and hyperparasiting the rust fungus of coffee, *H. vastatrix*, at Assagay coffee farm, Cato Ridge, South Africa.

Infected coffee leaves were collected from Assagay Coffee Farm, transported to UKZN plant pathology laboratory and stored overnight at room temperature (between 25 and 26 ºC). The following morning, the whitish powdery fungus was washed out of coffee leaves and transferred to Sabouraud dextrose yeast extract (SDAY) medium. The culture was checked every 3 days for a period of 12 days. After 12 days, pure cultures were made on potato dextrose agar (PDA). The *L. muscarium* isolates were maintained through periodic re-inoculation of pure culture on PDA. For storage and short-time preservation, different methods were used to preserve the pure isolate, such as distilled water. This method can keep the culture alive from 3 to 6 months and on agar slants.

Laboratory screening test and identification

Bioassay studies using detached soybean leaves inoculated with *L. muscarium* and *P. pachyrhizi* under controlled laboratory conditions showed *L. muscarium* hyphae developing on SBR sori. See Figure 2 (1) and Figure 2 (2).

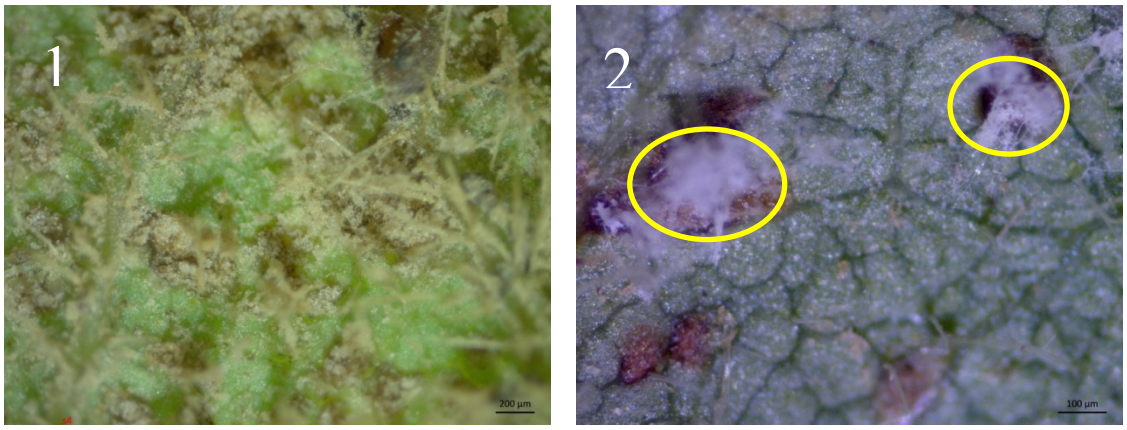

Figure 2, (1): *P. pachyrhizi* sori on detached soybean leaf: (2): Development of *L muscarium* on SBR sori (encircled).

Co-inoculation studies of *L. muscarium* and *P. pachyrhizi* were done in UKZN Plant Pathology disease garden. The Environmental Scanning Electron Microscope (ESEM) observation of the interactions showed a mycophilic attraction of *L. muscarium* to *P. pachyrhizi* urediniospores. Long *L. muscarium* phialides were observed penetrating and wrapping tightly around *P. pachyrhizi* urediniospores. (Figure 3 A and B).

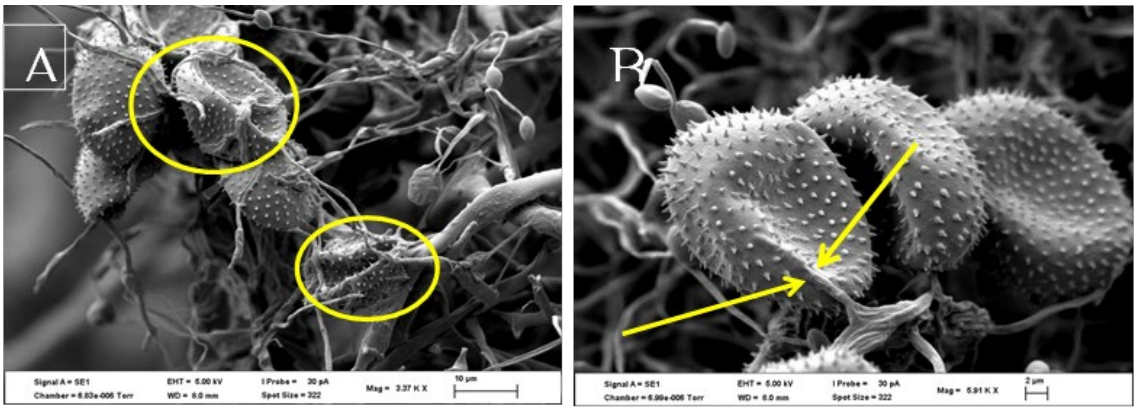

Figure 3(A): *L. muscarium* phialides surrounding and squeezing tightly SBR urediniospores (encircled) and (B): penetration of *L. muscarium* inside urediniospore (arrowed).

Under the light microscopy, *L. muscarium* conidia were found to have ovoid to ellipsoidal shape. Under ESEM, conidia shape and length ranged between 1.9-3.9µm (Figure 4).

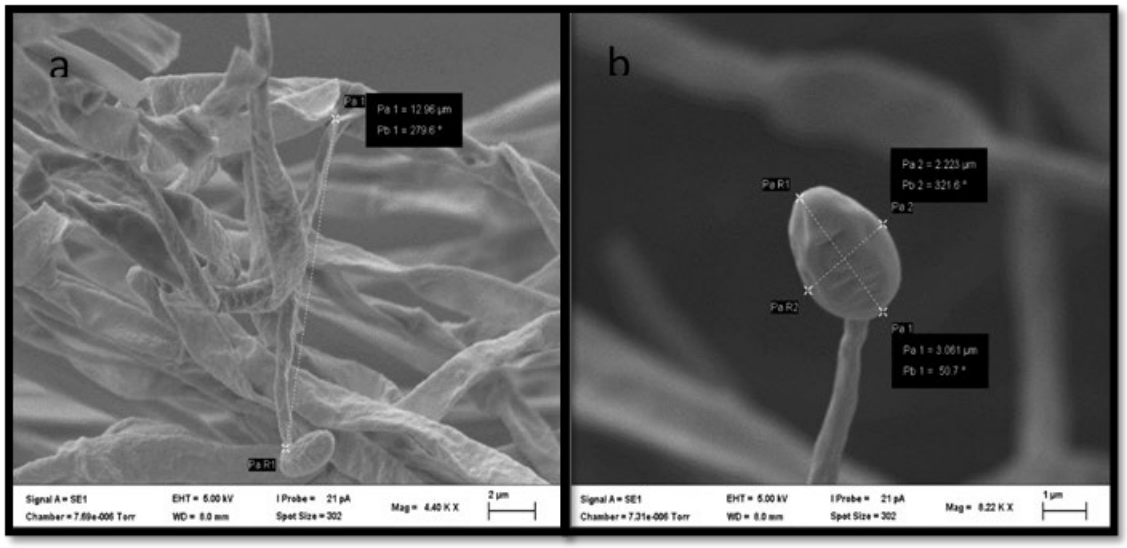

Figure 4. ESEM image and measurement of *L. muscarium* conidia

The DNeasy Plant Mini Kit (Qiagen, Germany) was used to extract total DNA from mycelium growing on agar plate. Polymerase chain reaction (PCR) was performed to amplify the internal transcribed spacer (ITS), and specifically the portion of the mitochondrial encoded NADH dehydrogenase subunits 1 (MT-ND1) and 3 (MT-ND3) genes. PCR was run according to the settings described by Kouvelis *et al.* (2008). The BLAST hits of the consensus sequences matched various species of *Lecanicillium*. This pattern was also noticeable in the phylogenetic tree

generated. The phylogenetic trees of MT-ND1 and MT-ND3 indicated a distant relationship between the South African Isolate (Nesta 08). Isolate Nesta_08 was considered to be a strain of *L. muscarium* because of its distant relationship with other *L. muscarium* isolates.

The study on growth and production of conidia by *L. muscarium* was done. This experiment targeted the production of inoculum for greenhouse and field experiments. To optimize growing and sporulation conditions, the isolate was cultured on different growing media, incubated at different temperatures and exposed to UV light.

Lecanicillium muscarium Isolate N-08 showed growth from 18°C to 28°C. The mean growth rate increased as temperature increase up to and including 24°C. Maximum growth occurred at 24°C (Figure 7).

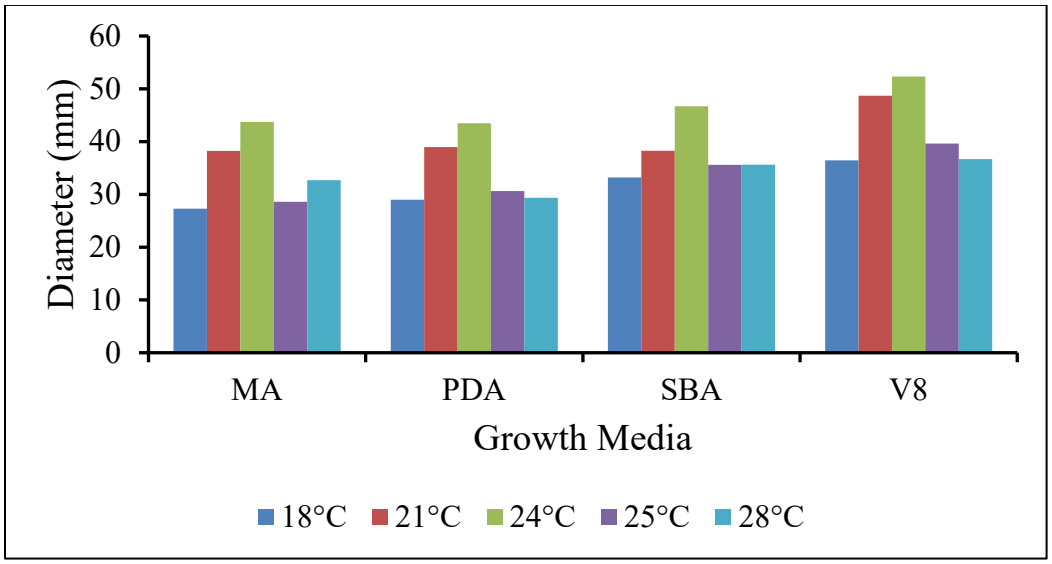

Figure 7. Radial growth (diameter in mm) of *L. muscarium* on different media and at different temperatures.

The mycelia growth of *L. muscarium* evaluated on different media indicated that *L. muscarium* strain N-08 was able to grow on all four media tested (PDA, Malt extract agar, V-8 tomato juice agar and Sabouraud dextrose agar). V-8 tomato juice agar was the best media for colony growth with a mean diameter value of 42.75mm followed by SDA with 37.86mm after 30 days of incubation.

Six different agro-industrial solid media were examined for *L. muscarium* N-08 sporulation: wheat bran, rice, rolled oats, millet, peeled barley and sorghum. *L. muscarium* strain N-08 sporulated on all grains used. The highest conidia production occurred on millet cereal (4.2 x 10^9 conidia/ml) followed by wheat bran (3.2 x 10^9 conidia/ml) and peeled barley (2.9 x 10^9 conidia/ml). The lowest spore production was observed on rolled oats (0.6 x10^9 conidia/ml) (Figure 8).

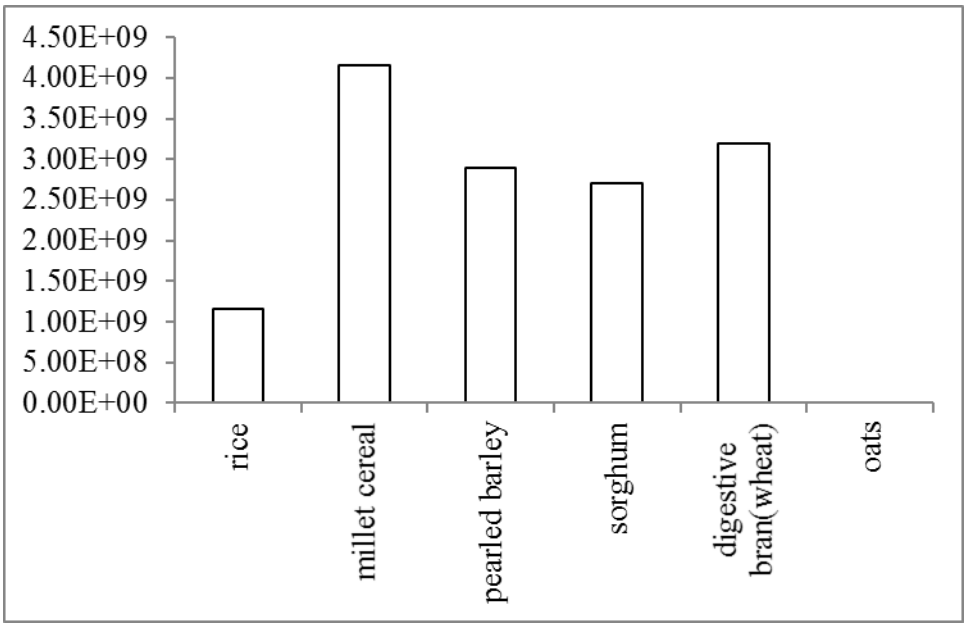

Figure 8. Sporulation of *L. muscarium* on different source of substrates incubated at 25ºC for 30 days.

Greenhouse trial

Greenhouse trials were conducted to determine the most effective dose for field application. Three *L. muscarium* dosages were used (10^4, 10^6 and 10^8 conidia/ml). It was found that 10^6 conidia/ml and 10^8 conidia/ml were more effective and 10^6 was chosen as the optimum dose for field application. Compared to the pathogen inoculated control, all three different *L. muscarium* inoculum levels (10^4, 10^6 and 10^8 conidia/ml) significantly reduced rust pustules on soybean leaves (Figure 9). The lowest disease severities were recorded for 10^6 and 10^8 conidia/ml with mean values of 15.6 and 12.6 respectively. Compared to the pathogen inoculated control, all three *L.*

muscarium treatments showed growth and development of *L. muscarium* strain N-08.

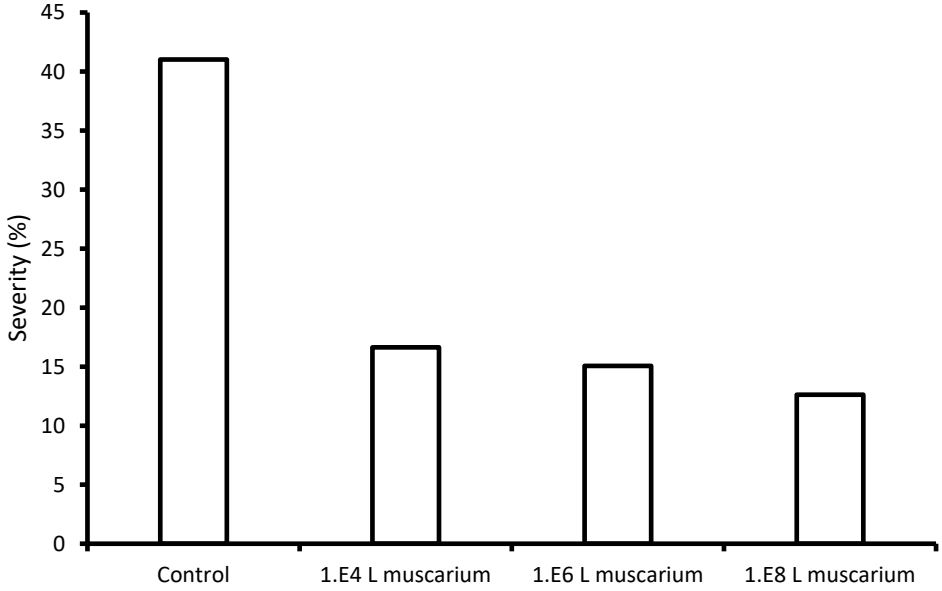

Figure 9**.** Percentage disease severity under greenhouse conditions.

Field trials (2014-2015)

Field experiment were conducted at Ukulinga research farm, Pietermaritzburg, South Africa (29°24′E; 30°24′S).

Plots treated with *L. muscarium* or Score fungicide caused a significant decrease in rust severity compared to the pathogen inoculated control. From Figure11, the AUDPC values for the treatments were: 172.2 units for Score, 186.2 units for 10^8 conidia/ml, 202.16 units for 10^6 conidia/ml, 457.8 units for 10^4 and 1716.8 units for the pathogen inoculated control. Three of the treatments (Score, 10^8 and 10^6 conidia of *L. muscarium*/ml) effectively reduced the disease (Figure12).

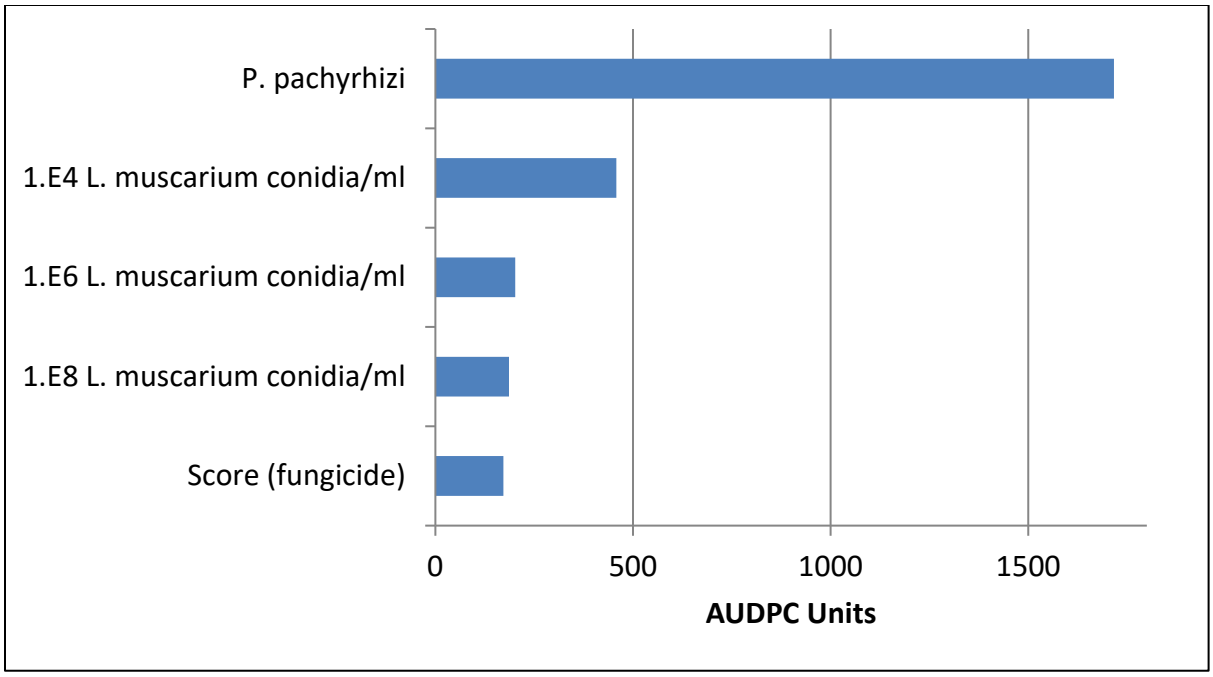

Figure 11: AUDPC units for the five treatments that were evaluated during 2014/2015 field studies.

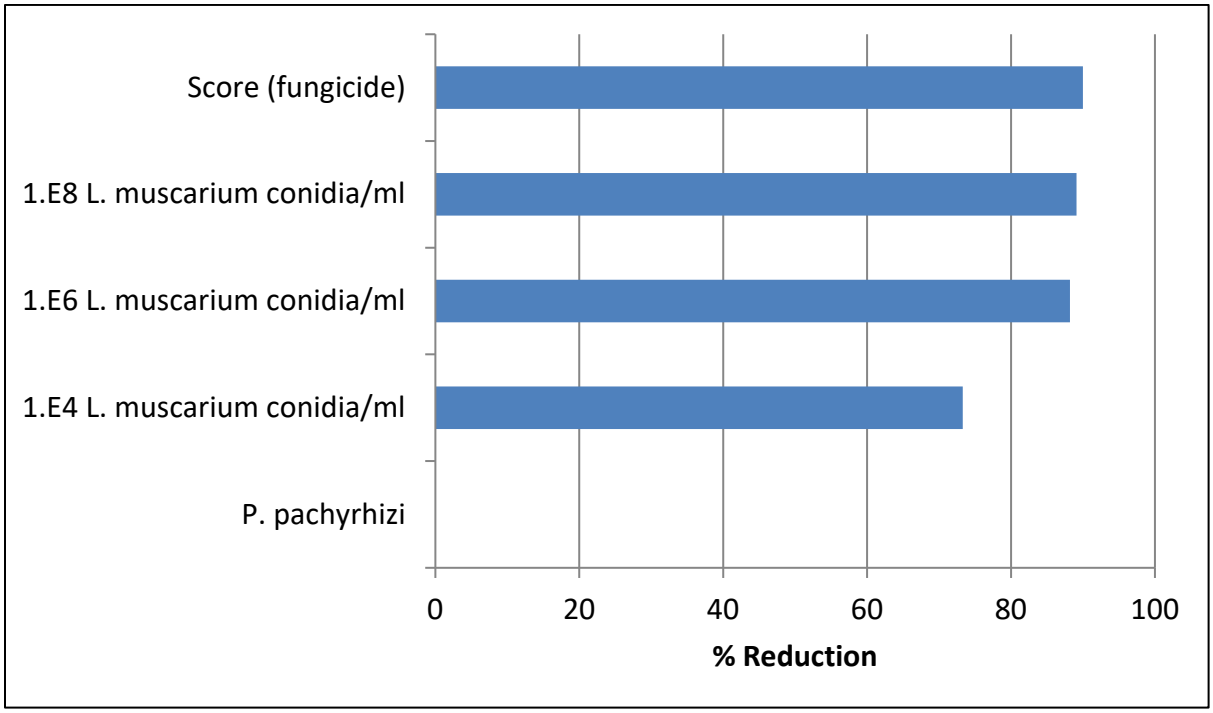

Figure 12: Percentage disease reduction as a result of using 10^4, 10^6, 10^8 *L. muscarium* conidia/ml and score fungicide on soybean plants inoculated with the *P. pachyrhizi*.

Field trials (2015/2016)

To study the effect of *L. muscarium* in the field, the 2014/2015 field trial experiment was repeated at Ukulinga Research farm, Pietermaritzburg, South Africa (29°24′E; 30°24′S). Disease severity was assessed 7 times from the 20 January to the 2nd March 2016. Compared to the pathogen inoculated control with the highest severity of 70%, all other three different *L. muscarium* inoculum levels (10^4, 10^6 and 10^8 conidia/ml) significantly reduced rust severity. The final disease severity recorded were 30%, 17.2%, 12% respectively for the three *L. muscarium* inoculum levels. The fungicide control has the lowest disease severity value of 7.2% (Figure 14). Area Under the Disease Progress curve (AUDPC) was calculated to give the summary of the disease severity over time. AUDPC units for the treaments were: Score (259.7 units), 10^8 *L. muscarium* conidia/ml (284.9 units), 10^6 *L. muscarium* (319.9 units), 10^4 *L. muscarium* (462.7 units) and the pathogen inoculated control (1053.5 units) (Figure 13). Compared to the pathogen inoculated control, *Lecanicillium* and fungicide treated plots showed yield increase. Dry seed weight showed no significant difference between *L. lecanicillium* and fungicide treated plots (Figure 14).

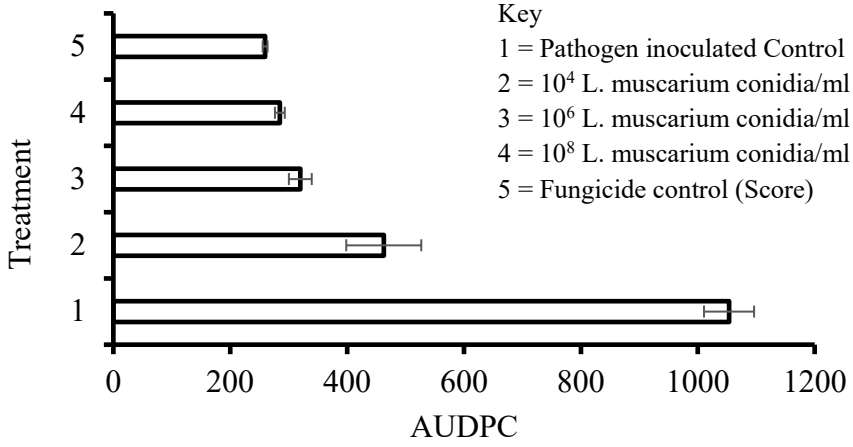

Figure 13: AUDPC units for the five treatments that were evaluated during 2015/2016 field studies.

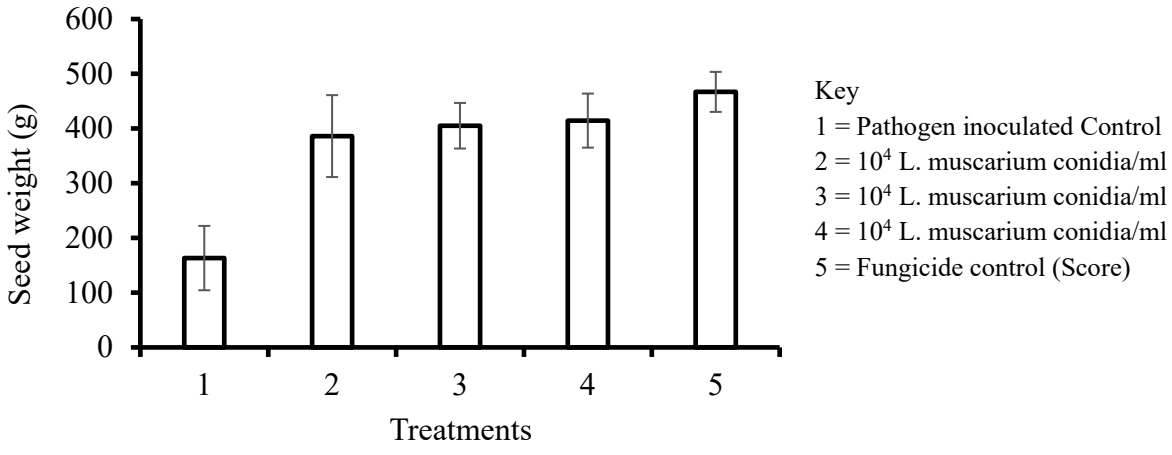

Figure 14. Average of seed weight (g) per treatment at harvest

Discussion

Many fungal biocontrol agents against plant diseases have been successfully registered and commercialised all over the world as alternative to fungicides use. There is no biocontrol product on the market for soybean rust control. In this study, the fungus was isolated, identified and tested against soybean rust.

Based on morphological and molecular studies, the potential biocontrol fungus was identified as *L. muscarium*. The isolate N-08 was deposited into the National collection of fungi with accession number PPRI 13715. In this study, temperature, artificial media, UV radiation and natural substrates was shown to have significant effect on the growth and spore production of *L. muscarium*. The optimal colony growth for *L. muscarium* strain N-08 was observed from 21 to 24˚C. Previous studies reported the genus *Lecanicillium* to be mesophilic with optimum growth temperature around 25˚C, and no growth has been recorded over 34˚C (Li et al., 1991; Lopez-Lorca and Carbonell, 1998; Vu et al., 2007; Rivas et al., 2014). The best substrate for spore production was millet cereal with an average of 4.15×10^9spores/ml. *L. muscarium* strain N-08 produced spores on all media used: wheat ($3.2\text{x}10^9$spores/ml); barley ($2.9\text{x}10^9$spores/ml); sorghum ($2.7\text{x}10^9$spores/ml); rice ($1.15\text{x}10^9$spores/ml) and oats ($0.6\text{x}10^9$spores/ml). Studies done

previously by Shinde *et al*. (2010) found pearl millet to be a suitable media for *Lecanicillium* spp. spore production ($10.17x10^{10}$spores/100g). The isolate grew on all artificial media tested, the best growth was observed on V8 juice agar (42.75mm). When exposed to UV radiation, results showed no significant difference on radial growth for all media tested. Biocontrol agents respond to UV radiation depending of the geographic origin, the one from regions with higher incidences of UV radiation are less affected (Piazena, 1996).

In vitro studies on the effect of *L. muscarium* isolate N-08 against *P. pachyrhizi* was done. Co-inoculation studies of *L. muscarium* and *P. pachyrhizi* using bioassay (detached soybean leaves *P. pachyrhizi*) show *L. muscarium* N-08 growing and colonizing *P. pachyrhizi.* Microscope studies show the interaction between both fungi. Long *L. muscarium* phialides were observed penetrating and wrapping tightly around *P. pachyrhizi* urediniospores. Samples from the zones of the leaves colonised by both fungi were observed under the light microscopy and SEM. It has been suggested that *L. muscarium* releases diffusible inhibiting compounds containing lytic and/or antibiotics into the media. The mycoparasitism of *L. muscarium* against *P. pachyrhizi* was test in the greenhouse, where the optimum dose for *L. muscarium* was estimated to 10^6 spores/ml. The same dose level of inoculum was tested in the field experiments. Plots treated with *L. muscarium* N-08 showed a disease severity reduction of 88.2% (10^6 spores/ml). Compared to the pathogen inoculated control, all *L. muscarium* doses tested were able to reduce disease severity. The fungicide control (Score) fungicide reduced the disease severity by 90%.

Conclusion

This report summarises the work done to evaluate the potential of *Lecanicillium muscarium* to control *Phakopsora pachyrhizi* from isolation of the mycoparasitic fungus to field experiments. In particular, we described its ability to colonize and overwhelm the soybean rust fungus. As a mycoparasite, *L. muscarium* uses physical force and enzymatic production to destroy the pathogen. Due to its strong mycoparasitic activity, it deserves to be exploited more and developed into a commercial biocontrol agent against soybean rust and other plant pathogens. However, more field studies need to be done.