Use of *cis*-abienol or T-phylloplanin to prevent black shank disease in tobacco

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Abstract

Black shank is the biggest annual disease threat to Kentucky tobacco. It is caused by the fungus *Phytophthora parasitica* var. *nicotianae* which attacks plant's roots and stems and causes dark-brown to black lesions. So far, the major approaches to control the disease are a creation of resistant burley varieties, fungicide treatments, and crop rotation.

In our project we suggested a preventive treatment with natural products T-phylloplanin and cis-abienol synthesized by tobacco and secreted in tobacco gum. Phylloplanin and cis-abienol were shown to suppress *P. parasitica* spore germination and the disease development in dark tobacco (King, 2011) in laboratory conditions.

We hypothesized that these compounds, and also labdene-diol and sclareol, will suppress the race 0 and race 1-caused disease in burley tobaccos KY14 and KY14xL8 in green house conditions.

We prepared T-phylloplanin, cis-abienol, and labdene-diol stocks, developed methods for spore preparation, and established the working concentration for spores and for the all the natural compounds tested. Experiments were performed on the 5 to 8 week-old seedlings grown in 4-inch pots in the green house.

Phylloplanin reduced plant infection from both races by 50% in KY14, and race1 infection by 60% in KY14xL8. In older (8 weeks) plants suppression of race1 disease was 30%. Infection was delayed by 4 days in younger and older plants. In KY14xL8 the disease was suppressed by 13 days and infection was reduced by 70%. Phylloplanin was less suppressive comparatively to cis-abienol, sclareol and labdene-diol in both tobacco lines. Cis-abienol showed high inhibitory properties toward the disease. Race 0 infection was completely subdued, while race 1 infection was delayed by 10 day and was reduced by 80%. Sclareol was also very effective in inhibiting the disease in both lines and against both races. Race 0 disease was completely suppressed, and race 1 infection was delayed by 10 day and was reduced by 85%. Labdene-diol reduced the disease by half in 8 week-old KY14 plants, but was not effective in

preventing the disease in KY14xL8. We consider sclareol to be the best candidate for future studies and for practical application (majorly in a float bed) due to its inhibitory properties and availability.

Introduction

Black shank is the largest annual disease threat to Kentucky tobacco. It is caused by the fungus *Phytophthora parasitica* var. *nicotianae* that attacks plant roots and stems and causes dark-brown to black lesions, eventually killing the plant. Currently, black shank is found in majority of tobacco fields in the southern United States, causing damage in 2 to 5.5% of them, but in some field losses can be 100% (Gallup et al, 2006). The annual losses for dark tobaccos in Kentucky and Tennessee are estimated to be 1.25 million dollars (Bailey, 2009). Thus far, the major approaches to control this devastating disease are: creation of resistant hybrids; fungicide treatment, crop rotation and biological control (Pears et al, 2013-2014; Cartwright and Spurr, 1998; Ren et al. 2012). Breeders have developed resistant burley tobacco varieties (i.e. L209, MS KY14xL8 or MS Burley 21xL8) in some of which one of the parents is the resistant to race 0 Line 8 (L8). When L8 hybrids have been grown on soils infested with Race1, severe loses have been recorded (Bost and Hensley, 2005). Resistant varieties can tolerate the disease to some extent so long as they do not get water-stressed. In 2012 there was a lot of disease in Kentucky fields planted with resistant burley varieties because of the prolonged dry weather and heat (Seebold, 2012). Fungicide application (Subdue, Ridomil) is efficient in preventing the disease but have a long-term deleterious effects such as acquired resistance to them, collateral toxicity, and environmental pollution. Because of the adverse effect of the fungicides, there is a need of alternative fungicides that are sustainable and eco-friendly. Plant-made products, such as sugar esters and terpenoids are shown to have antibacterial, antifungal, and insecticidal properties (Chortyk et `al., 1997; Puterka et al., 2003, Wang et al., 2004). Essential oils (Lu et al, 2013) and tobacco root constituents (Snook et al. 1991) were found to be active against black shank disease.
Our laboratory is specialized in studying surface chemistry of tobacco leaves. Recently, it was discovered that tobacco trichome-produced phylloplane protein (T- phylloplanin) is active against blue mold spores (Shepherd et al, 2005, Kroumova et al, 2007, Shepherd and Wagner, 2012). Phylloplanin was tested also against *P. parasitica* in a tube assay, but it has not been tested in a pot assay (King, 2011). Another tobacco product - *cis*-abienol was found promising in preventing the black shank disease in dark tobacco (King, 2011). Results with cis-abienol and phylloplanin were preliminary, and quantification was not done for either compound. Additional work was needed to determine if either or both of the natural products could be used to control black shank in green house conditions. We hypothesized that

treatment with cis-abienol and T-phylloplanin, and also with two other natural products -sclareol and labdene-diol, will inhibit spore penetration into the roots and will prevent/reduce disease symptoms.

Specific objective:

Green house assessment of the inhibition of black shank spore infection of race 0- and race 1- sensitive burley tobacco line KY14 and race 1- sensitive KY14xL8 line by treatment with *cis*-abienol, T-phylloplanin, sclareol and labdene-diol in soil drench assay.

Methods:

Preparation of T-phylloplanin and cis-abienol stocks

T-phylloplanin and cis-abienol are two natural chemicals, produced in substantial amounts by some representatives of *Solanaceae* family. *N. tabacum* T.I.1068 is unique in producing both of them. We grew tobacco T.I. 1068 in the greenhouse from existing seed stock for four months. More than 200 mature leaves were washed individually with water for 30 sec to recover T-phylloplanin (a protein synthesized by tobacco short glandular trichomes). The water solution was concentrated and T-phylloplanin was recovered in solid form by freeze-drying. The weight of the concentrated phylloplanin was measured and dry aliquots were stored at minus 20°C until use. Quality analysis of the protein was performed using gel electrophoresis, as done previously (Shepherd, 2004).

Cis-abienol is a minor natural product found in tobacco gum, but it is absent in many commertial tobacco lines. We have in our seed collection a line of T.I. 1068 that produces increased (at least 3 times) amount of the chemical. Plants from this line were grown to maturity in the greenhouse. Two hundred and fifty leaves were washed with acetonitrile for 30 sec per leaf. It is not soluble in water. The samples were concentrated via rotor evaporation to dryness. Total amount of exudate collected was 6.3 g, where cis-Abienol consisted 14%. Cis-abienol was further purified by partitioning between hexane and 80% methanol (Severson et al, 1988). At each step of purification samples were analyzed by GC-MC. We achieved preparations enriched in cis-abienol up to 85% (Fig. 1). The chemical was stored in 95% ethanol at 4°C.

Preparation of labdene-diol stock

We have generated in our lab a tobaco line having labdene-diol as a major compound in tobacco gum (50% of total gum or 90% of total diterpenes). The stock was prepared following the procedure for cis-abienol stock preparation, but without purification. Sclareol was purchased from Sigma-Aldrich Co., LLC.

Preparation of spores from P.parasitica fungus

There is a limited amount of literature regarding spore preparation and plant infection performed in laboratory conditions. Methods are very different, depending on the planned experiments (Gutierrez and Mila, 2007;   Csinos AS, 1999; Litton et al. 1970), but were not appropriate for our experiments. We had to develop our own method for spore preparation and plant infection. The protocol of King (2011), served as a reference for further customization. Isolate for race 1 was obtained from Ed Dixon (Plant Pathology, UK, Lexington, KY) and race 0 – from N. Martinez (KTRDC, UK, Lexington, KY). We prepared long-term storage mycelium plugs (1x1cm^2 of agar, containing fungus mycelium) in water, and stored them at room temperature until use (Litton et al. 1970, modified by Martinez N., personal communication). A single fungal plug was placed on a basal V8 medium, containing buffered V8 tomato juice, (Jeffers and Martin, 1986), and kept in the dark for 8 days. 5 plugs (1x1cm) of agar-mycelium were cut from the edge of the mycelium math, and incubated in sterile water, in the dark, at room temperature for 7 -14 days. From the 7th day on mycelium was monitored under scope for formation of sporangia, yellow balls at the end of the hyphae. This is an indication of successful zoospore formation. Zoospores were released after short 30 min cold shock, followed by incubation at 37°C for 30 min. Spores were counted by hemocytometer after immobilization with sodium azide.

For both race 1 and race 0, the harvest was from 50,000 to 100,000 spores per ml. They were further diluted to reach the desired concentrations.

Plant material and chemicals

KY14 seeds were obtained from KTRDC collection, and K14xL8LC and 209L seeds - from Dr. B. Pearce (Plant and Soil Science Department, UK).
Seeds were grown in 6-inch pots in the laboratory (14/10h, l/d, and 22°C and 23% humidity). After 20 days, individual seedlings were transferred to 4-inch pots and grown for another 15- 35 days. Several

days before the infection, the plantlets were transferred to the green house in a designated area restricted by black plastic in a wooden frame.
Sclareol and BSTF were purchased from Sigma-Aldich. Acetonitrile, hexane, methanol, dimetylformamide and ethanol were purchased from Fisher.

***Treatment with spores, T-phylloplanin, cis-abienol, sclareol, and labdene-diol*.**

Treatments included application of 1 ml of designated chemical around the base of the stems. T-phylloplanin concentrations were 100-150 mg/ml water; cis-abienol concentrations were in the range of 6 to 20 mg/ml alcohol; sclareol – 20-40 mg/ml methanol; and labdene-diol – 20-40 mg/ml methanol. Fungal spores were diluted with water to the desired concentration (from 15 to $48x10^3$ per ml) and one ml of suspension was applied around the base of each seedling, after treatment with natural products. Disease development was observed 6th-13th day post treatment. The infection stages included wilting and yellowing of the leaves, and development of a black stem at the base of the plant. We used methanol (solvent for all natural products, but phylloplanin) as negative control). The spore suspension in water was used as the positive control. Experiments were repeated at least three times. Treatments were done similarly for both race 0 and race 1 spores.

Results:

Selection of working concentrations for spores, phylloplanin, cis-abienol, clareol and labdene-diol

Spore concentration
We tested three concentrations – 15, 33, and 48 $x10^3$ spores per ml. There was not a significant difference in the infectivity rate. However, the concentration of $33x10^3$ /ml gave better infection (Fig. 2). It was selected to be working concentration for both race0 and race 1, in all tobacco lines.

Phylloplanin concentration
We tested several concentrations of phylloplanin against race 1 spores-caused disease in younger 5-6 weeks-old KY14.. Concentrations of 100 to 150 mg/ml reduced the disease by 30 to 60% while concentrations of 75 mg/ml or less did not prevent the infection (Fig. 3a).

Cis-abienol concentration

Five week-old KY14, KY14 x L8 and 209 plants were treated with 2 and 6 ml cis-abienol per ml of ethanol. Cis- abienol is completely soluble in ethanol, and partially in other organic solvents. All treated plants died, including line 209. No infection was observed. Line 209 is resistant to both races of black shank, so the death could not be due to the disease. The result was unexpected, as according to King (2011) dark tobacco plants, were not affected by the alcohol. We attempted to use diluted ethanol and found out that twenty five percent ethanol or below was not toxic. However, at this concentration cis-abienol precipitated and could not be used for treatment.
In order to achieve cis-abienol solubilization**,** we experimented with several other organic solvents, such as methanol, ethyl acetate, ethyl lactate**,** and butyl butyrate. Methanol did not damage 5-8 week-old plants. Ethyl lactate treatment of small-size plants showed similar to black shank features such as wilting, yellowing of the leaves, and browning of the vascular system. Ethyl acetate and butyl butyrate were also toxic to the plants. Cis-abienol formed a fine suspension in methanol that was applied evenly to the plants. Treatment of KY14 plants with 20 mg/ml cis-abienol in methanol suppressed the disease more than 80% (fig 3b). This concentration was used in further experiments.

Sclareol and labdene-diol:
Sclareol and labdene-diol were also well solubilized in methanol. Sclareol formed a fine suspension, while labdene-diol dissolved completely. Sclareol showed disease inhibition in concentration of 30-40 mg/ml. Labdene-diol was effective with older KY 14 plants at a concentration of 30 mg/ml.

Comparative analysis of black shank suppression in 7 week-old KY14 and KY14xL8 plants by several natural products

Seven-week old plants were concomitantly inoculated with phylloplanin, sclareol, and cis-abienol. Water and methanol were the negative controls, and spore suspension - a positive control. We used optimized concentrations as follows: for phylloplanin – 100 mg/ml; cis-abienol – 20 mg/ml, and sclareol – 40 mg/ml. The disease development was monitored in progress at 6th, 10th and 13th day post-infection. We were looking for wilting and yellowing of the leaves and appearance of a black stem above the crown of the plant. Experiments were done with both race 0 and race 1 spores. All three chemicals suppressed or inhibited plant disease, caused by both races. Cis-abienol and sclareol inhibited race 0 disease completely and suppressed race 1 disease by 80% (Fig.4a; Fig.7). Phylloplanin suppressed disease development from both races by 50%. Moreover, where complete inhibition was not achieved, there was a delay of disease symptoms by 4 days. In race 0 experiment only 20% of phylloplanin-treated plants (vs 80% control) were infected on the 6th day, 25% - on the 10th day (vs 80% control), and 40% on

the 13th day (vs 80% control) (Fig. 4b). In race1 experiments, there was no infection on the 6th day when plants were treated with cis-abienol and sclareol, and less that 20% infection in phylloplanin-treated plants. Only 15% of plants treated with cis-abienol and sclareol were infected on the 10th day post-inoculation, and 40% of phylloplanin treated plants were infected (Fig. 4c).
Sclareol inhibited race 1infection of KY14xL8 seedlings completely (Fig. 9).. The line is resistant to race0 and was not affected by the treatment (Fig.5). Phylloplanin suppressed the infection for 10 days (vs 50-60% infection in control), and on the 13th day post-treatment it was 20% (vs 65% in control) (Fig.5b). Cis-abienol and sclareol were also reported to be active against bacterial wilt disease in tobacco (Seo et al, 2012).

Comparative analysis of black shank suppression by several natural products in 8 week-old KY14 plants

The plants' treatment and disease monitoring were done the same way as for 7-week old seedlings. We used labdene-diol (optimized concentration of 30 mg/ml) instead of cis-abienol (Fig. 6a). Phylloplanin, sclareol, and labdene-diol suppressed partially the disease caused by both races. Labdene-diol reduced infection to 30% (vs 80% in control) for both races. Sclareol was more efficient with race 1 (20% infection) than with race 0 (45% infection). Phylloplanin was also more efficient with race 1 (30% infection) than with race 0 (55% infection). The three compounds also helped the delay of the disease symptoms by 6- 10 days (Fig. 6b and c, and Fig. 8). There was no suppression of the disease in 8 week-old KY14xL8 plants (not shown).

Conclusion remarks:

Sclareol showed to be the best candidate for future study and practical application, majorly in a float bed. It strongly suppressed the disease from both races in younger plants from both lines. Sclareol is a natural product synthesized in some Nicotiana species. It is commercially available as clary sage (*Salvia sclarea*) extract at a price of $70-100/kg (good for 25,000 treatments).
Phylloplanin is not as efficient as the other natural products. It is easy to collect, but the procedure is laborious and lengthy. For these reasons it is not suitable for commercial application.
Cis-abienol was as effective as sclareol in preventing black shank disease. However, it is difficult to obtain a sufficient amount of it, because of the laborious and lengthy procedure. In addition, it is not stable under UV light and heat. These disadvantages make it unsuitable for commercial application.

Labdene-diol worked well in inhibiting the disease in older KY18 plants. It is very stable and has complete solubility in methanol. The drawbacks are that it could be obtained in sufficient amount only from a specific line created in our lab, and the extraction procedure is laborious and lengthy.

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Fig. 1 GC of purified cis-abienol

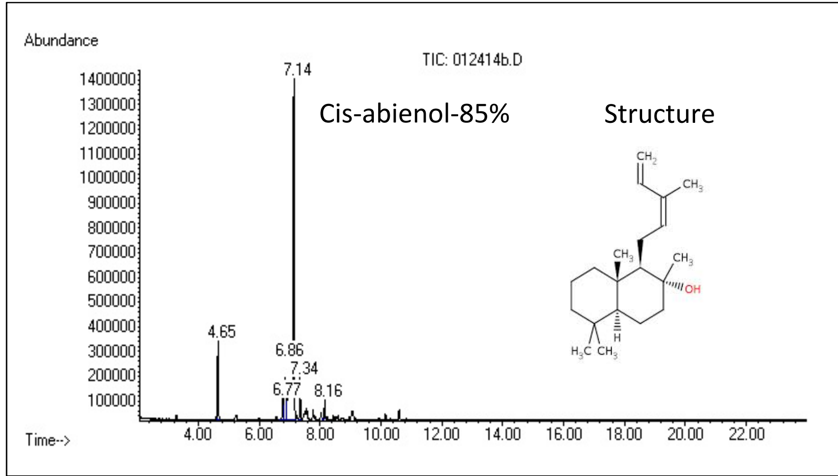
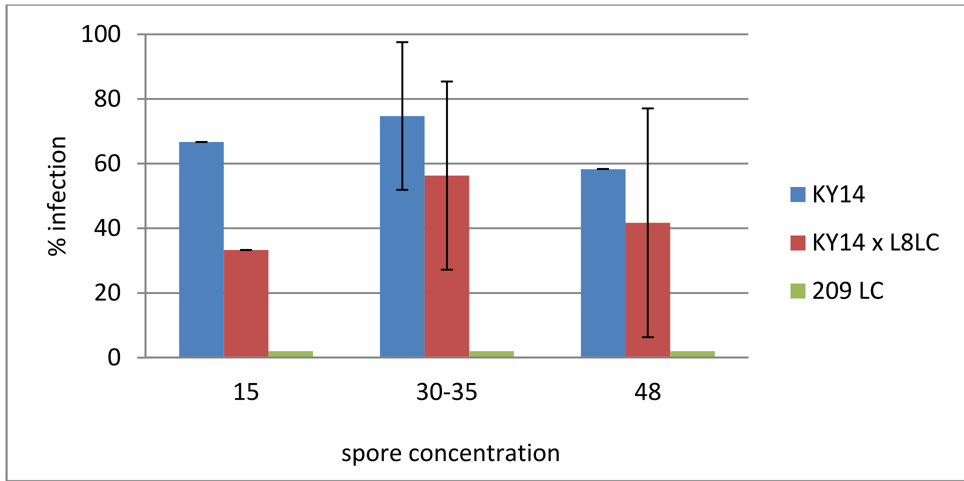
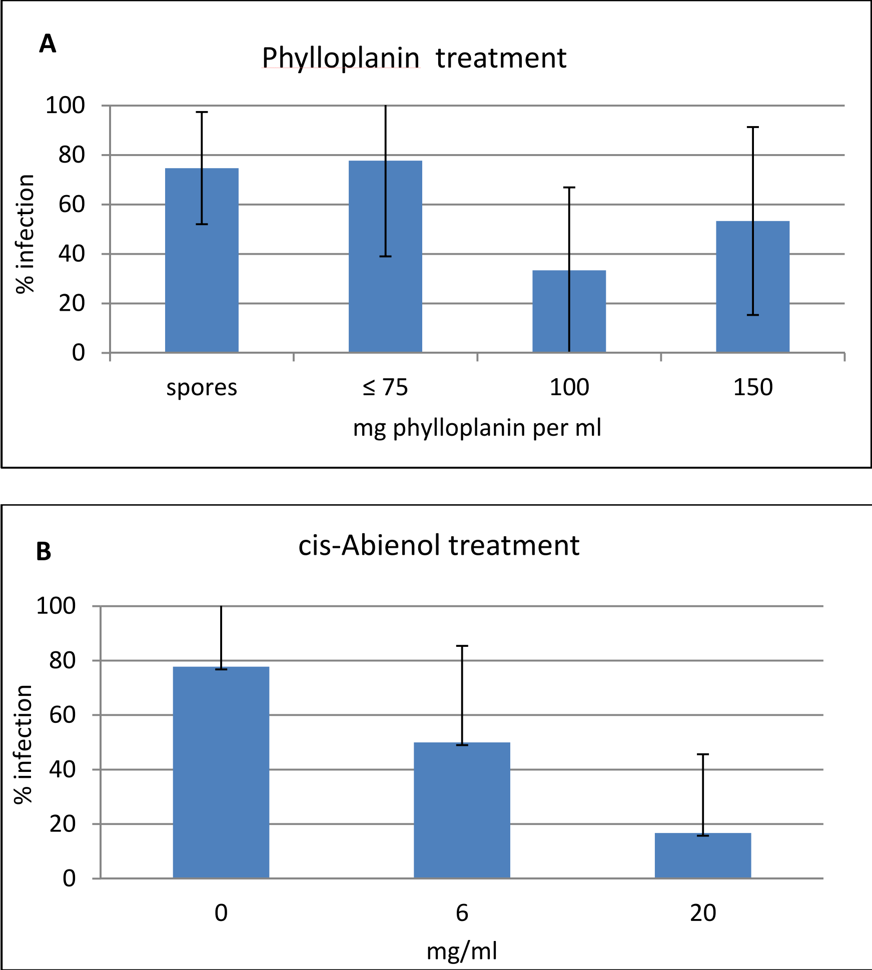


Fig. 2 Plant infection as a function of spore concentration



Spores were from race 1 and plants were 35 d-old. Line 209 is resistant to race0 and race1 spores and was included as a negative control.

Fig. 3 Establishing working concentrations of phylloplanin and cis-abienol for the suppression of black shank in KY14 plants



Race 1 spores are applied at 30 x 10^3 spores/ml. Plants are 5 week-old.

Fig. 4 Effect of phylloplanin, cis-abienol and sclareol on the suppression of black shank disease in 7 week-old KY14 plants

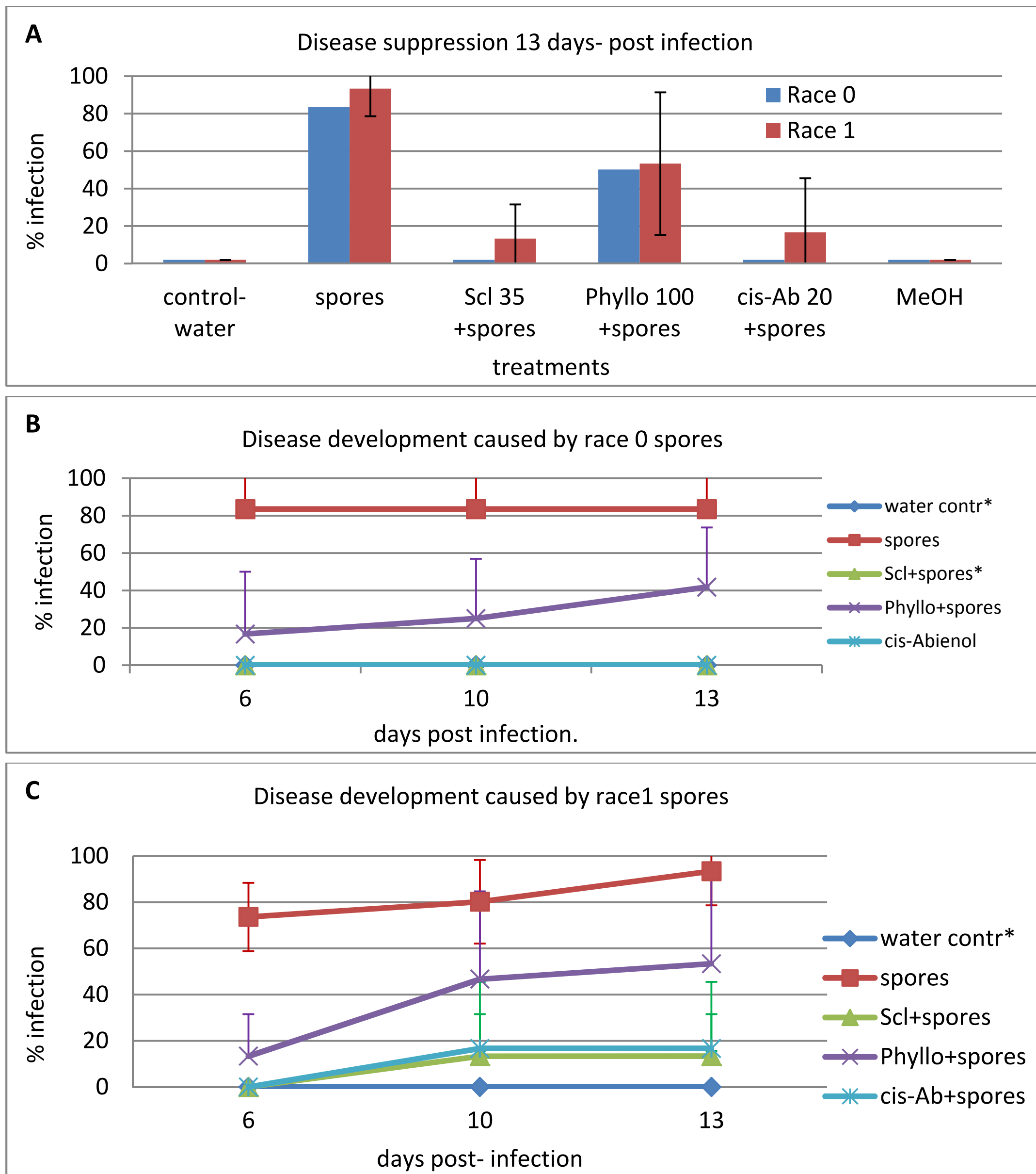


Fig. 5 Effect of sclareol and phylloplanin on suppression of black shank disease in 7 week-old KY14xL8

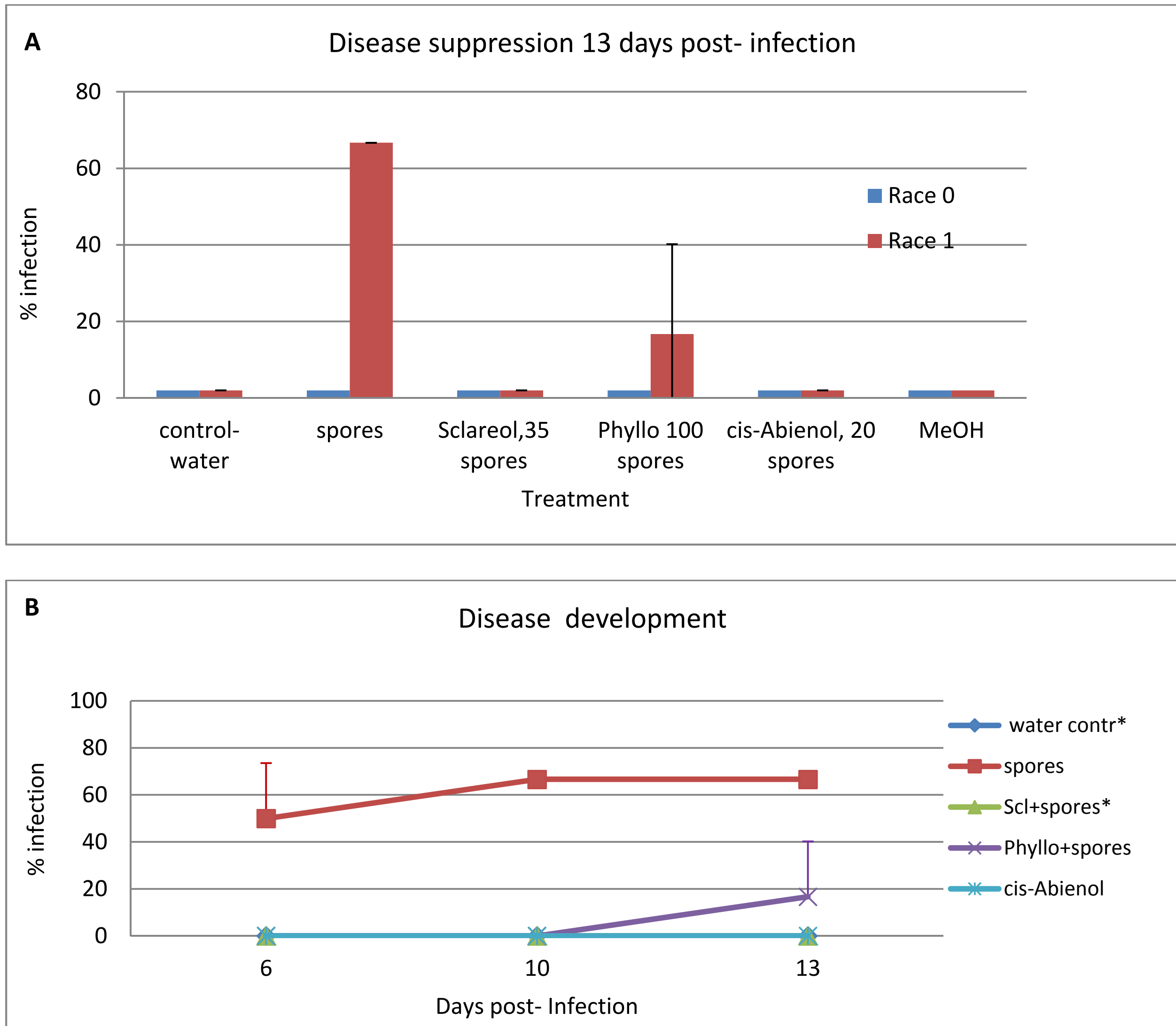


Fig. 6 Effect of labdene-diol, sclareol, and phylloplanin on suppression of black shank disease in 8 week-old KY14

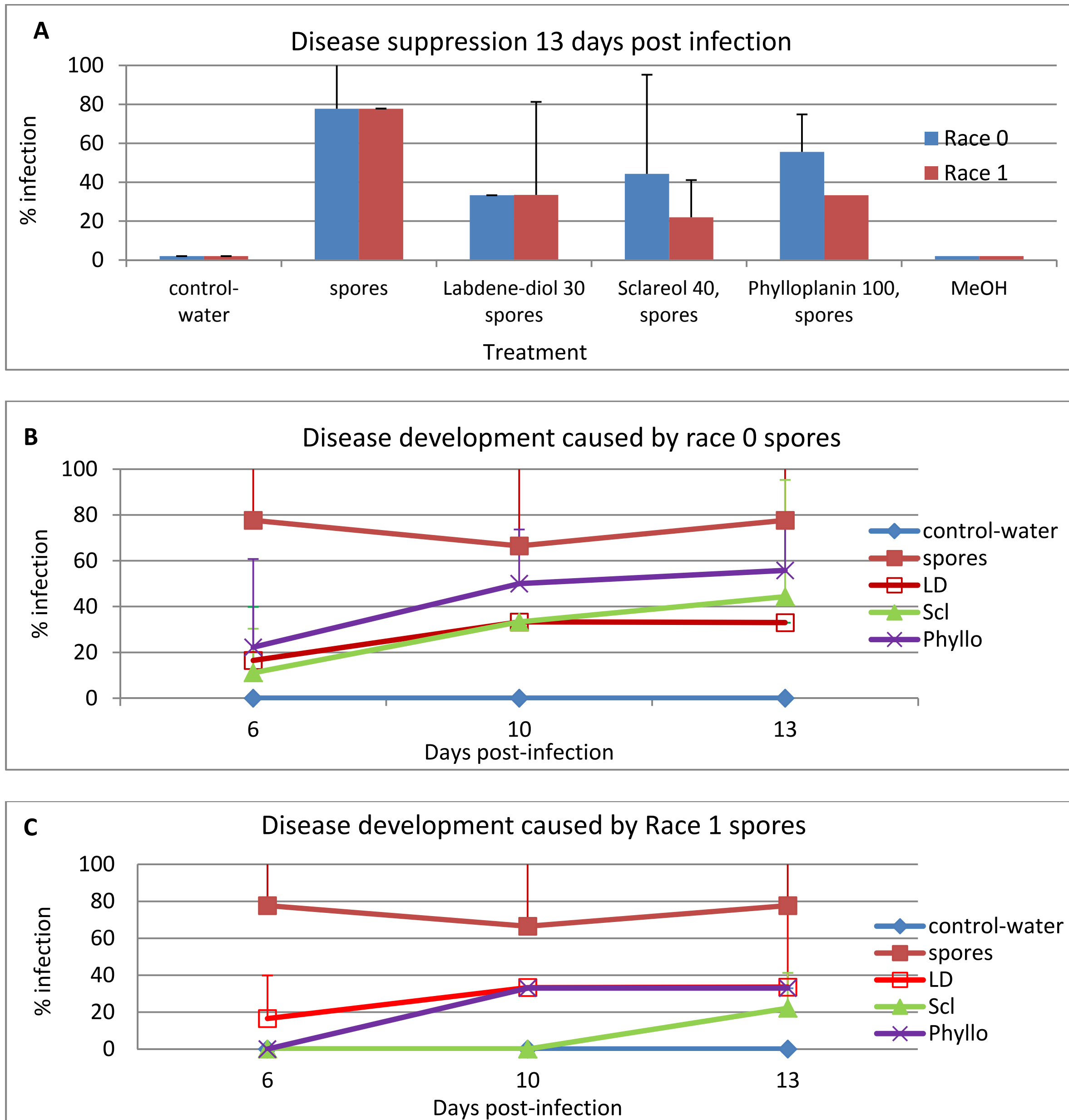
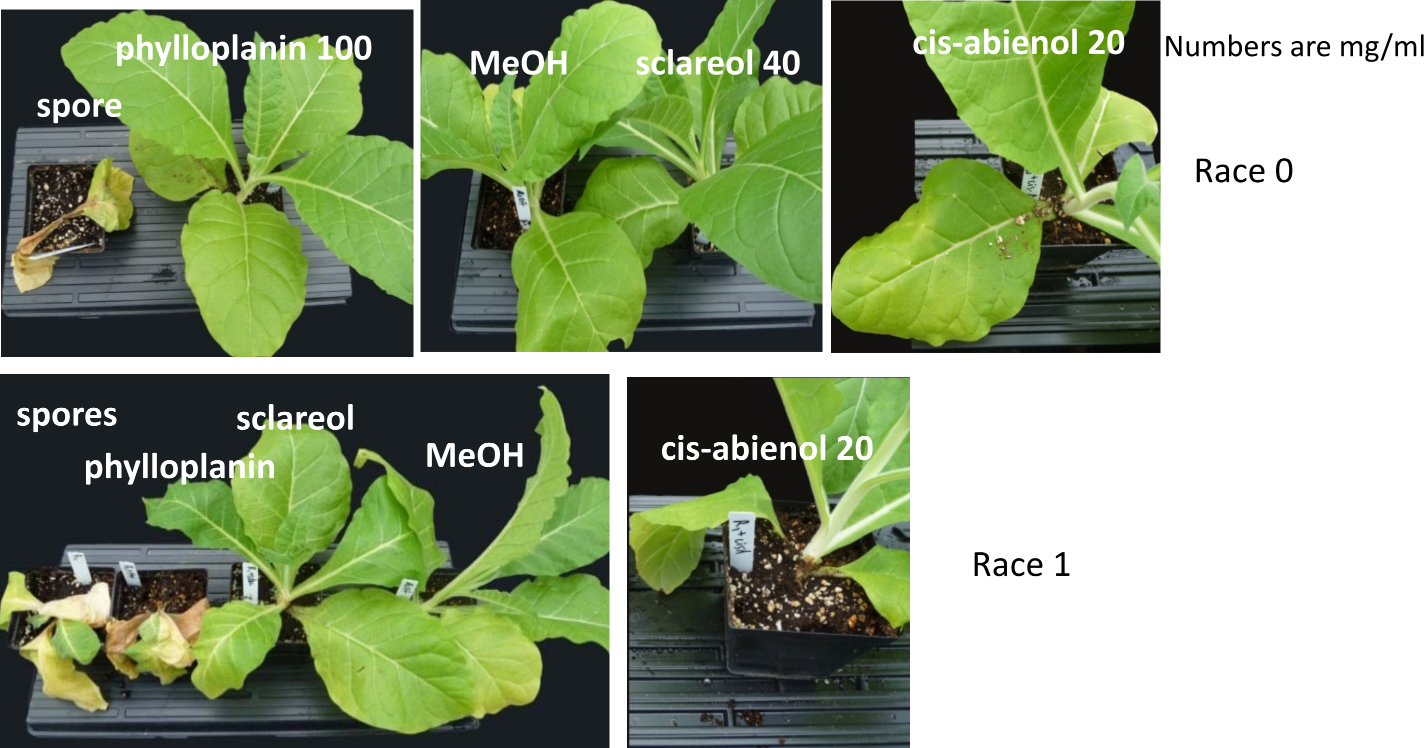
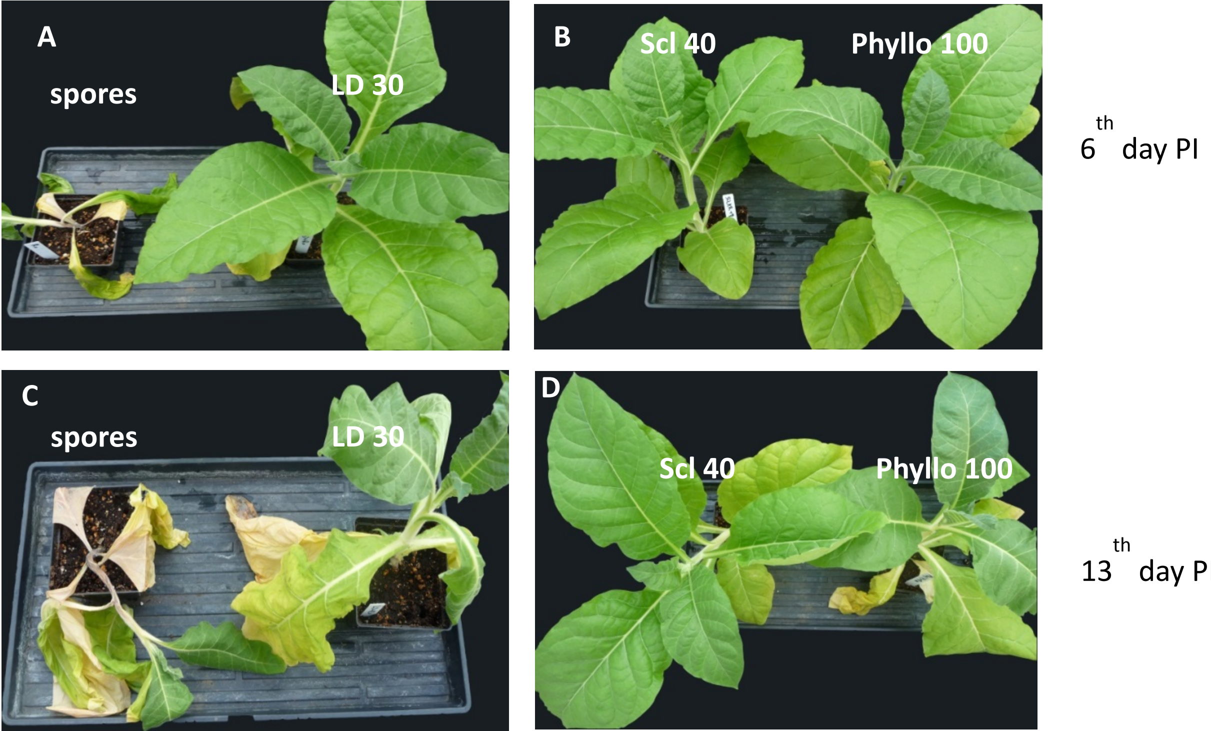


Fig. 7 Treatment of 7 week-old KY14 tobacco with natural products phylloplanin, sclareol and cis-abienol to suppress black shank disease



Non-treated controls were infected by spores of race 0 and race0. Phylloplanin-treated plant was infected by race 1 spores.

Fig. 8 Delay of race 0-caused black disease by phylloplanin and labdene-diol, and disease prevention by sclareol



Infection was delayed by 7 days after treatment with labdene-diol (Fig.8C) and phylloplanin (Fig.8D)

Fig. 9 Treatment of 7 week-old KY14xL8 with natural products to prevent race 1-caused black shank disease

spores	sclareol	phylloplanin	cis-abienol	water	methanol
	40 mg/ml	100 mg/ml	20 mg/ml		