# Forschungsprojekt – Nr.100830/1

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**ERA-NET CORE Organic II: Einbeziehung von Pflanzenresistenz, Anbaumethoden und Biopestizide zur Verbesserung der Bekämpfung von Rebkrankheiten, zur Steigerung der Ertragseffizienz und zur Erhöhung der Biodiversität im europäischen Bioweinbau**

**ERA-NET CORE Organic II: Integration of plant resistance, cropping practices, and biocontrol agents for enhancing disease management, yield efficiency, and biodiversity in organic European vineyards**

# 2. Zwischenbericht

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# 1. Inhaltsverzeichnis

## 2. Einleitung

Anorganische Fungizide wie Kupfer und Schwefel sind wichtige Säulen der Pilzbekämpfung im biologischen Landbau. Der Einsatz von kupferhältigen Fungiziden wird aufgrund ihrer toxischen Eigenschaften für Bodenleben zunehmend in Frage gestellt. Daher sind die Verwendung von Mikroorganismen in Form von biologischen Pflanzenschutzmitteln (biological control agents - BCAs) und eine spezifische Nutzung von Wirt-Pathogen-Beziehungen wie die induzierte Resistenz umweltfreundliche und vielversprechende Alternativen. Die Wirkung von biologischen Pflanzenschutzmitteln beruht am häufigsten auf einem antagonistischen oder hyperparasitären Wirkungsmechanismus.
Für die praktische Anwendung von BCAs sind vor allem eine ausreichende und stabile Wirksamkeit wichtig. Diese wird vor allem durch Umweltbedingungen beeinflusst und ist von einer präventiven Anwendung abhängig, da die Mikroorganismen die Pflanzenoberfläche in ausreichend besiedeln müssen um die Schaderreger zu verdrängen.
Um die Fitness und Wirksamkeit der BCAs zu verbessern wird in diesem Forschungsprojekt mit modernen molekulargenetischen Methoden wie quantitative realtime PCR (qPCR) ein sog. „Biopesticide Tracking" durchgeführt. Dabei wird die Etablierung und Vermehrung der antagonistischen Mikroorganismen sowohl unter kontrollierten als auch unter Freiland-Bedingungen studiert. Durch Kompatibilitätsuntersuchungen und sequentielle Applikationen von BCAs und Pflanzenhilfsstoffen sollen die Bekämpfungserfolge verbessert werden. Ziel ist es, mit den Ergebnissen dieser Untersuchungen neue Bekämpfungsstrategien zu entwickeln und das Einsatzgebiet der BCAs im Bioweinbau zu erweitert.

## 3. Zusammenfassung

Die Fitness und die Wirksamkeit von 4 ausgewählten, bereits in der EU zugelassenen biologischen Pflanzenschutzmitteln (biological control agents – BCAs) werden zur Bekämpfung von Schlüsselschaderregern im biologischen Weinbau getestet. Um die Fitness zu evaluieren wird ein sog. Biopesticide Tracking durchgeführt. Dazu müssen für die ausgewählten BCAs stammspezifische qPCRs entwickelt und getestet werden. Im vorliegenden Zwischenbericht wird die Entwicklung und die Validierung einer stammspezifischen qPCR für *Bacillus amyloliquefaciens* QST713 (Serenade$^{TM}$) beschrieben. Dazu wurde zuerst die Spezifität von publizierten Primer verglichen. Die Spezifität der getesteten Primer war für ein stammspezif. Tracking nicht ausreichend. Zur Entwicklung neuer Primer mit verbesserter Spezifität wurden variable Regionen aus einer Reihe nahe verwandter *Bacillus*-Stämme amplifiziert und sequenziert. Im Bereich des *yngG*-Gens wurden Polymorphismen gefunden, die die Entwicklung neuer Primer ermöglichten. Die entwickelten Primer waren für *Bacillus amyloliquefaciens* QST713 hoch spezifisch. Auf Basis dieser Ergebnisse wurde ein zweistufiges qPCR Protokoll erstellt und validiert. Als Vortest für das Biopesticide Tracking an Pflanzenmaterial wurden Potter Tower Experimente mit Rebblättern durchgeführt und erste quantitative Analysen gemacht. In diesen Versuchsreihen konnte gezeigt werden, dass QST713 bis zu neun Tage nach der Applikation auf Weinblättern nachweisbar ist. Während Kupfer einen tendenziell negativen Einfluss auf das Überleben von QST713 auf der Blattoberfläche hat, scheint sich Schwefel positiv auszuwirken. Bei einer

Vorbehandlung von Weinblättern mit Schwefel ist QST713 selbst nach zwei Wochen eindeutig auf der Blattoberfläche nachweisbar.
Flavescence dorée ist eine gefährliche Quaratänephytoplasmose im Weinbau. Die rebpathogenen Phytoplasmen werden durch die Amerikanische Rebzikade Scaphoideus titanus übertragen. Die Bekämpfung dieses Vektors ist obligatorisch und stellt daher gerade im Bioweinbau eine Herausforderung dar. Daher wird nach alternativen, biologisch verträglichen Kontrollmethoden gesucht. In Laborversuchen konnte der insektenpathogene Pilz *Lecanicillium lecanii* (MycotalTM) das 2. Larvenstadium der Amerikanischen Rebzikade infizieren.
Die kumulativen Mortalitätsraten korrigiert nach Abbot (1925) betrugen 73% (Experiment 1), 54% (Experiment 2) und 33,3% (Experiment 3). Die höchste Mortalität wurde in dem Tauchversuch erreicht. In allen drei Experimenten ist ab Tag 5 die Überlebenswahrscheinlichkeit der Larven in der mit *L. lecanii* behandelten Gruppe signifikant geringer als in der Kontrollgruppe ($p<0.001$; Log-Rank Test).

## 4. Summary

Fitness and efficacy of four biological control agents (BCAs) that are already registered according to the EU regulations, should be evaluated in relation to grape disease control under organic practices. Fitness will be evaluated through long term monitoring by strain specific quantitative PCR methods based on DNA markers (Biopesticide tracking). In a first stage, published primers were evaluated for their strain specifity. Due to the low strain specifity of the tested primers variable regions of closely related *Bacillus* strains were amplified and sequenced. Polymorphisms were detected in the region of the *yngG* gene. Based on these findings new primer for *Bacillus amyloliquefaciens* QST713 were designed and validated. The high strain specifity could be verified. With the specific gene markers at strain level, a real time PCR procedure was set-up for *Bacillus amyloliquefaciens* QST713 (Serenade$^{TM}$). To pretest the optimized qPCR protocol for strain specific detection in field, a potter spray tower experiment was conducted. More experiments to clarify the rate of quantitative recovery of the BCA from grape leaves are currently under way.

It could be shown that QST713 can be detected up to nine days after application on vine leaves. While copper has a tendency to negatively influence the persistence of QST713 on vine leaves, the effect of sulfur was positive. On vine leaves pretreated with sulfur, QST713 could be detected even two weeks after application.

Flavescence dorée is a severe grapevine yellows disease caused by Grapevine Flavescence dorée phytoplasma. It is transmitted by its principal vector, the Nearctic leafhopper *Scaphoidues titanus*. In organic viticulture the mandatory control of the vector is challenging. Therefore alternative and biocompatible control methods are investigated. Results from laboratory testing showed that *Lecanicillium lecanii* (Mycotal$^{TM}$) was virulent to the second larva instar of *Scaphoideus titanus*.

The percentage of the cumulative mortality of *S. titanus* nymphs corrected after Abbot's formula (1925) ranged from 73% (experiment 1), 54% (experiment 2) to 33.3% (experiment 3). The highest larval mortality was achieved by immersion of Chardonnay leaf disc. In all three experiments the probability of a larva to survive from the day five is significantly lower in the *L. lecanii* treated group than in the control group ($p<0.001$; log-rank test).

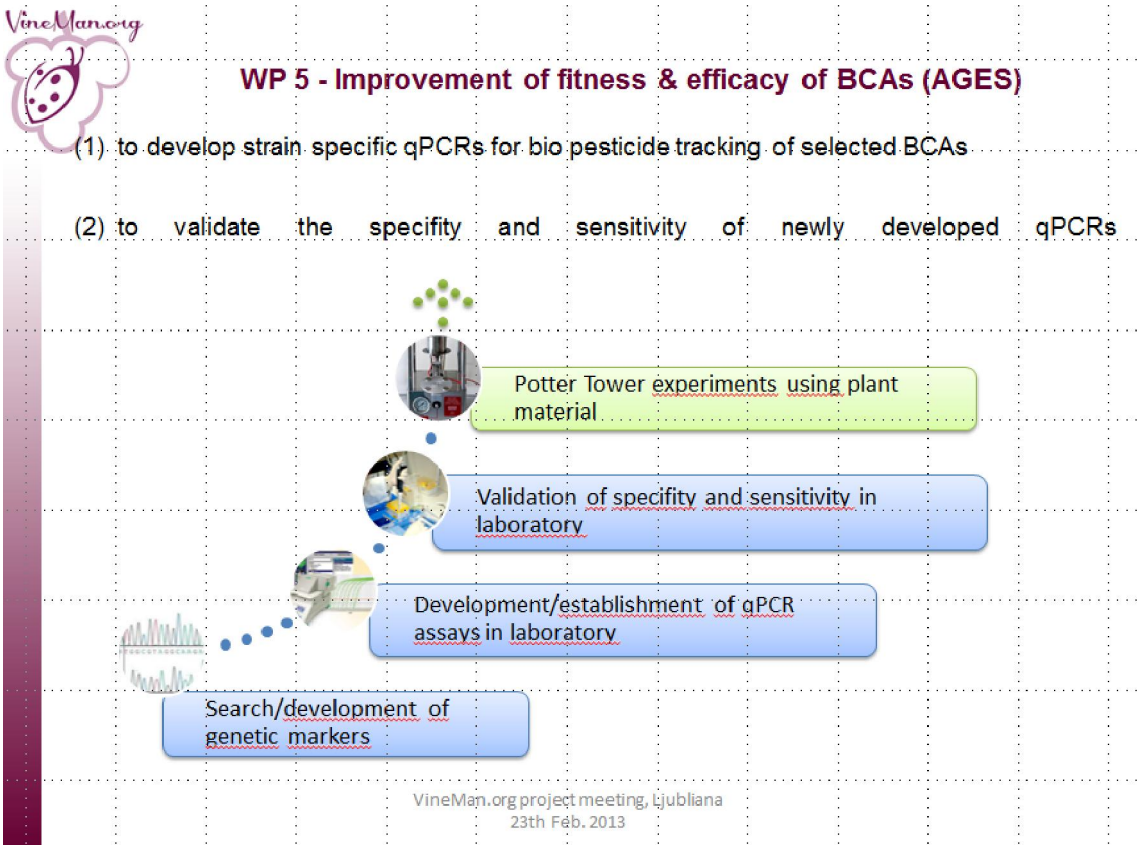
# 5. Improve fitness & efficacy of BCAs

## 5.1 Introduction and objectives of the work package

Inorganic fungicides like copper, sulphur or acid clays should be used to manage fungal problems as a last resort; the use of copper is problematic because of its poisonous effect on the soil's flora and fauna (Tivoli et al. 2009). The use of microorganisms and the exploitation of beneficial plant–microbe interactions offer promising and environmentally friendly strategies for organic agriculture (Berg, 2009). Biological control can result from many different types of interactions between organisms; the most effective BCAs appear to antagonize pathogens using multiple mechanisms (Montesinos et al. 2009). Practical implementation of bio control has been constrained by a number of factors, including efficacy and reliability of BCAs (Bonaterra et al., 2007; Peressitu et al, 2010). The greatest successes were achieved when environmental conditions are most controlled or predictable and when BCAs can pre-emptively colonize the host or the plant surfaces. Therefore, strategies must be developed to improve efficacy and reproducibility of the BCAs, and to increase the action spectrum of biological control of grapevine diseases under organic management practices.

**The objectives of the work package are:**

1. To develop strain specific qPCRs for bio pesticide tracking of selected BCAs
2. To validate the specifity and sensitivity of newly developed qPCRs
3. To evaluation the ability of *Lecanicillium lecanii* (Mycotal$^{TM}$) to infect *S. titanus* larvae
4. To monitor the population density of selected BCAs under controlled environment and field conditions using qPCRs
5. To improve control strategies by testing the compatibility of mixtures or sequential applications of BCAs or BCAs and natural products




Work flow for development and validation of strain specific qPCRs

## 5.2 Development of a strain-specific detection system for *Bacillus amyloliquefaciens* QST713

**2012:**

Development of the strain-specific detection system for *Bacillus amyloliquefaciens* QST713 (previously *B. subtilis* QST713), the active ingredient of Serenade Max™, was based on genetic markers previously shown to be specific for bio control strains (Joshi et al. 2006) Some of the target genes are known to be associated with bio control activities like production of antimicrobial substances. Published primers (see Table 1) were first tested on a set of Bacillus strains from the in-house strain collection. No primer pair gave amplification products with strains outside the *B. subtilis* complex, and all primers but one successfully amplified a product in the expected size from QST713 and some other strains from the *B. subtilis* complex. With one exception, the strains that gave positive results originated from bio control products. These products are however not considered to contain QST713. The one strain not originating from a bio control product was isolated from a metal working fluid. Nothing is currently known whether it has bio control activities.

PCR-products obtained from positive strains with primer pairs 135 and 147 were sequenced, and based on sequencing data new primers with improved specificity were developed. PCR-products obtained with primer pair 147, which amplifies part of the yndJ gene, did not show

enough variation to allow development of a QST713-specific detection system. In gene yngG, which was amplified by primer pair 135, two QST713-specific nucleotides could be found. Three forward and three reverse primers targeting these two nucleotides were developed and were tested in all possible combinations. Primer pair yngG01F/yngG04R (see Figure ) resulted in strong and specific amplification. The primer pair was tested against a representative selection of *B. amyloliquefaciens* strains at different annealing temperatures. With an annealing temperature of 67 °C highly specific and strong amplification of DNA from strain QST713 could be obtained (see Figure 3).

Table 1: Published primers for detection of *Bacillus amyloliquefaciens* QST713

| Name | Sequence | Gene | QST713 [a] | Specificity [b] |
|---|---|---|---|---|
| 44 | F: TATAGCGCAATGTATGTAT<br>R: GTACTCAAAATCCATCTGG | cloneQ044 | – | n.d. |
| 147 | F: CAGAGCGACAGCAATCACAT<br>R: TGAATTTCGGTCCGCTTATC | yndJ | + | some *B. subtilis* |
| 2 | F: TTCCACGGCCATTCCTATAC<br>R: TTTGTCCCCTTATCCTGCAC | bioA | + | some *B. subtilis* |
| SRFA | F1: GAAAGAGCGGCTGCTGAAAC<br>R1: CCCAATATTGCCGCAATGAC | srfAA | + | most *B. subtilis* |
| FND | F1: CCTGCAGAAGGAGAAGTGAAG<br>R1: TGCTCATCGTCTTCCGTTTC | fenD | + | some *B. subtilis* |
| 110 | F1: GTTCTCGCAGTCCAGCAGAAG<br>R1: GCCGAGCGTATCCGTACCGAG | srfAB | + | some *B. subtilis* |
| 135 | F: GAACTGTCCGAAACATGTCCG<br>R: CTGAGCTCTTGAACGGTCCGG | yngG | + | some *B. subtilis* |
| BMB | F2: TGAAACAAAGGCATATGCTC<br>R2: AAAAATGCATCTGCCGTTCC | bmyB | + | some *B. subtilis* |
| ITUC | F1: TTCACTTTTGATCTGGCGAT<br>R3: CGTCCGGTACATTTTCAC | ituC | + | some *B. subtilis* |

[a]: PCR-results with *B. amyloliquefaciens* QST713; +, PCR-product of the expected size; -, no PCR-product

[b]: positive results with most or only with some strains from the *Bacillus subtilis* complex

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QST713     TCATTCGTGCATCCGAGATGGATTCCGGCTCTTCGTGACAGCCTGGACGTCGCAAAAGGCATTACCAGATCTGAACATACGGTCTACGCGGCTCTCG
I_03_08    TCATTCGTGCATCCGAGATGGATTCCGGCTCTTCGTGACAGCCTGGACGTCGCAAAAGGCATTGCCAGATCTGAACATACGGTCTACGCGGCTCTCG
I_03_09    TCATTCGTGCATCCGAAATGGATTCCGGCTCTTCGTGACAGCCTGGACGTCGCAAAAGGCATTGCCAGATCTGAACATACGGTCTACGCGGCTCTCG
DQ011345   TCTTTCGTGCATCCGAGATGGATTCCGGCTCTTCGTGACAGCCTGGACGTCGCAAAAGGCATTGCCAGATCTGAACATACGGTCTACGCGGCTCTCG
ACC13      TCTTTCGTGCATCCGAGATGGATTCCGGCTCTTCGTGACAGCCTGGACGTCGCAAAAGGCATTGCCAGAACTGAACATACGGTCTACGCGGCTCTCG
EHC017     TCATTCGTGCATCCGAGATGGATTCCGGCTCTTCGTGACAGTCTGGATGTCGCAAAGGGGATTGCCAGATCAGAAGATACGGTCTACGCGGCTCTCG

QST713     TTCCGAACCTCATCGGTCTGGAGCATGCGGCAGAAGGCGGAATCGATCAGGCATGTGTATTTTTATCTGCAAGCGAAACCCATAACCAAAAAA
I_03_08    TTCCGAACCTCATCGGACTGGAGCATGCGGCAGAAGGCGGAATCGATCAGGCATGTGTATTTTTATCTGCAAGCGAAACCCATAACCAAAAAA
I_03_09    TTCCGAACCTCATCGGACTGGAGCATGCGGCAGAAGGCGGAATCGATCAGGCATGTGTATTTTTATCTGCAAGCGAAACCCATAACCAAAAAA
DQ011345   TTCCGAACCTCATCGGACTGGAGCATGCGGCAGAAGGCGGAATCGATCAGGCATGTGTATTTTTATCTGCAAGTGAAACCCATAACCAAAAAA
ACC13      TTCCGAACCTCATCGGACTGGAGCATGCGGCAGAAGGCGGAATCGATCAGGCATGTGTATTTTTATCTGCAAGCGAAACCCATAACCAAAAAA
EHC017     TTCCAAACCTCATCGGACTGGAACATGCAGCAGAAGGCAGAATTGATCAAGCCTGTGTGTTTTTATCAGCAAGCGAAACCCATAACCAAAAAA
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Figure 2: Multiple alignment of partial *yngG*-sequence from a subgroup of *Bacillus amyloliquefaciens* strains

Sequence DQ011345 was taken from published data, the remaining sequences were obtained within the frame of this project. Strains I_03_08, I_03_09 and EHC017 were obtained from biocontrol products, strain ACC13 originated from a metal working fluid. Sequences identical to the herein shown were removed from the alignment. Binding sites for QST713-specific primers are highlighted. Both the forward and the reverse primer (yngG01F and yngG04R) deviate from the consensus at the 3'-end and are highly specific for *B. amyloliquefaciens* QST713.

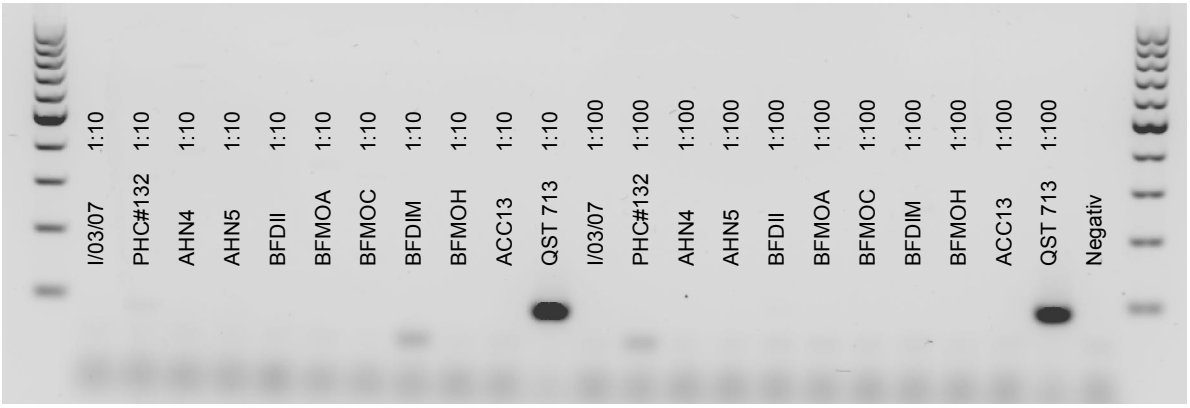



Figure 3: PCR amplification with primer pair yngG01F/yngG04R

Genomic DNA from *Bacillus amyloliquefaciens* strains was used in two dilutions (1:10 and 1:100). The amplification program was set to an annealing temperature of 67 °C and to 35 cycles. All genomic DNAs gave positive results in control reactions with universal primers.

Based on the obtained results with conventional PCR, a qPCR protocol for quantitative detection of QST713 was developed. Optimal results were obtained with SsoFast EvaGreen Supermix™ from BioRad at 67 °C annealing temperature. Sensitivity could be increased by approximately two orders of magnitude by introduction of a preamplification step with primer pair 135 for 15 cycles.

## Activities 2013

### 5.2.1 Potter spray tower experiment

To pretest the optimized qPCR protocol for strain specific detection of *B. amyloliquefaciens* QST713 in field, a potter spray tower experiment was conducted. Serenade Max™ was prepared according to the manufacturer's instructions and applied to vine leaves which were collected from field. Application doses were 5×, 1×, 0.5× and 0.1× the recommended amount. Immediately after application, leave samples were taken with a cork borer and stored in DNA-extraction solution (Qiagen DNeasy Plant Mini Kit) until further processing. To check deviations of application doses 2 discs at defined places on each leaves were taken. All experiments were carried out in fivefoldreplica. Sample DNA was used for Q713 strain specific nested qPCR. Current results indicate that in spite of extensive testing of specificity, even on non-inoculated leaves positive signals could be obtained. Variation was, however, extremely high.

Further optimization of the nested qPCR with inoculated leaf material was performed. Reduction of primer concentration in the preamplification step and a further increase of annealing temperature in the qPCR step from 67 °C to 69 °C finally resulted in loss of signal from non-inoculated plants and lower variation between replicates.

To allow high sample through-put, an automated DNA-isolation protocol was tested in collaboration with Michael Stierschneider from AIT. Although DNA-yield with automated protocols was similar to yield from the standard protocol (see 5.2.2), negative controls again gave positive results. The reason for this discrepancy is not known, but it is speculated that improved disruption of plant material by the automated protocol might release DNA from endophytic Bacilli that show high sequence similarity to the biocontrol strain QST713. All further experiments were therefore carried out with the standard protocol.

With the optimized DNA-isolation and qPCR-protocols, distribution of QST713 on vine leaves after application of Serenade was evaluated to set the parameters for future sampling. Serenade was applied to vine leaves with a potter tower and a hand sprayer respectively and five leaf discs were taken directly afterwards. Discs were treated as separate samples to analyze for equal distribution of QST713 on the leaf surface. Results are shown in Figure 1. While considerable variation was found between application by potter tower and by hand sprayer, and within one leaf after hand spray application (see sample 4 in Figure 1), little variation between and within leaves was found after application of Serenade with the potter tower.

For future experiments it was therefore decided to combine three discs from one leaf in one sample to represent the QST713 population on one vine leaf.

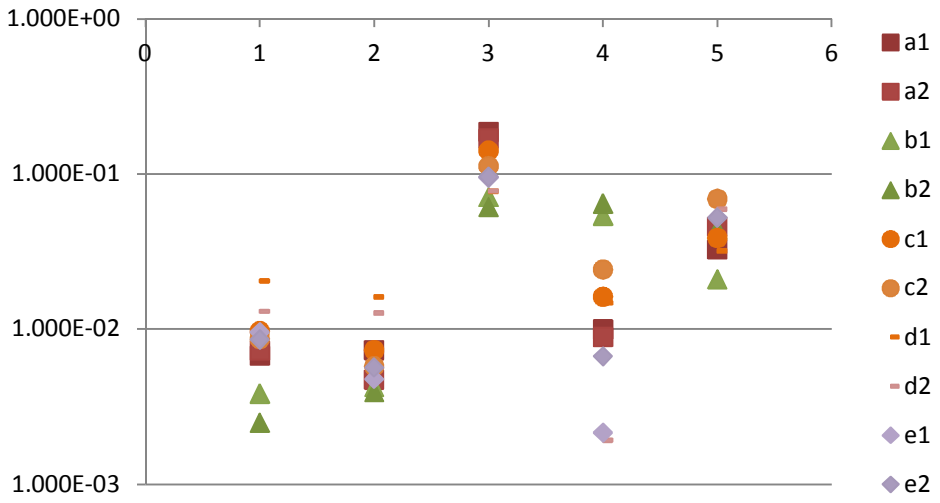



Figure 1: Distribution of *B. amyloliquefaciens* QST713 on vine leaves. Serenade was applied at the recommended dose with a potter tower (samples 1 and 2) or with a hand sprayer (samples 3, 4 and 5). Five leaf discs (a-e) were taken from each leaf and analyzed in duplicate by qPCR. Concentration of QST713 is given in relative to a standard DNA from a laboratory grown culture of QST713.

### 5.2.2 Protocol for sensitive and specific quantitative detection of *B. amyloliquefaciens* QST713

**DNA-Extraction (Modified from Qiagen DNeasy Plant DNA Isolation Kit):**

- 2-3 leaf discs in 2-ml screw cap tube with Lysing Matrix A (www.mpbio.com, 116910500)
- + 600 µl AP1
- + 6 µl RNase A
- Homogenization in FastPrep 2 × 30 sec, speed 6.0
- 10 min at 65 °C
- + 195 µl AP2
- 5 min on ice
- 5 min centrifugation (max. speed)
- Transfer supernatans to fresh tube
- 5 min centrifugation (max. speed)
- Transfer supernatans to lilac QIAshredder spin column
- 2 min centrifugation
- Add 1.5 Vol AP3 to flow-through and mix

- Add to white DNeasy Mini spin column in two steps (max. 700 µl at once)
- Spin for 1 min
- Discard flow-through
- Place spin column in new 2-ml collection tube
- Wash twice with 500 µl AW
- Discard flow-through between and after washing steps
- Spin dry for 5 min
- Elute DNA with 2 × 75 µl AE (prewarmed to 65 °C), incubate at RT for 5 min between elutions
- Transfer DNA to new tube with cap

| | | | |
|---|---|---|---|
| **Preamplification:** | 5 | µl | DNA |
| | 1 | µl | 135F (10 pmol/µl) |
| | 1 | µl | 135R (10 pmol/µl) |
| | 0,5 | µl | BSA (10 mg/ml) |
| | 2,5 | µl | $H_2O$ |
| | 10 | µl | 2 × PCR (GoTaq Green, Promega) |
| Initial Denaturation: | 1,5 | min | 95 °C |
| Amplification - 15 cycles: | 20 | sec | 94 °C |
| | 20 | sec | 62 °C |
| | 20 | sec | 72 °C |
| Final Elongation: | 5 | min | 72 °C |

| | | | |
|---|---|---|---|
| **qPCR:** | 10 | µl | DNA (1. PCR 1:10 diluted) |
| | 1 | µl | yng01F (10 pmol/µl) |
| | 1 | µl | yng04R (10 pmol/µl) |
| | 0,5 | µl | $H_2O$ |
| | 12,5 | µl | 2 × SsoFast EVAgreen Supermix (Biorad) |
| Initial Denaturation: | 2 | min | 98 °C |
| Amplification - 40 cycles: | 5 | sec | 98 °C |
| | 10 | sec | 69 °C |
| | 10 | sec | 72 °C fluorescence data collection |
| Melt Curve: | 65 - 95 °C | | |

**Preliminary tests - 2013**

Further studies were undertaken to clarify the applicability of qPCR protocols for biopesticide tracking.

First test:

Potter spray tower experiments with Serenade, Botector, AQ 10, Mycotal, and the untreated control were carried out. Each treatment was replicated threefold with the authorized application dose. To analyse the colonization over a defined time frame we used water agar to keep the grapevine leaves fresh. The incubation conditions for the leaves were 24 °C and long-day light condition (16 hours). Samples were taken before application, directly after application and 2, 7, 9, 12, and 14 days afterwards. 3 discs of leave tissues per replicate were used for DNA extraction.

Quantification by qPCR showed that QST713 persists for up to 9 days on the leaves without significant changes in the concentration, but with a significant increase in variance (see Figure 2). After two weeks, all samples were close to or below the detection level.

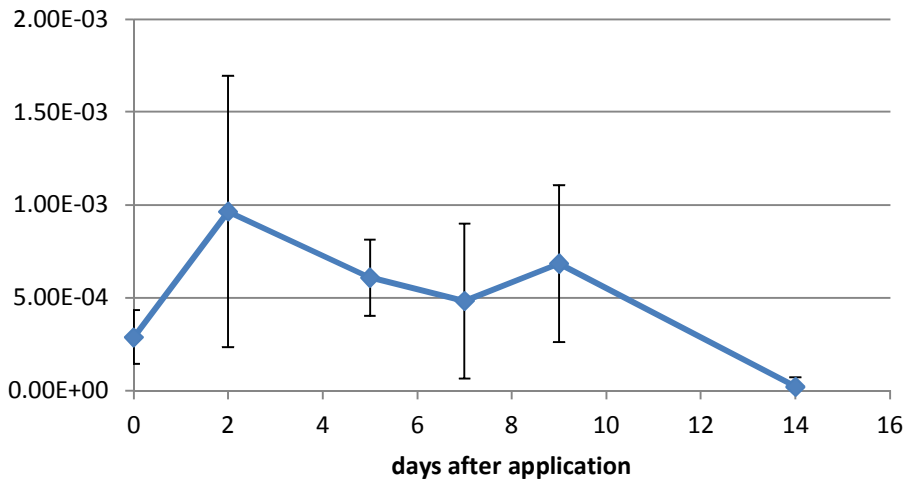



Figure 2: Persistance of *B. amyloliquefaciens* QST713 on vine leaves. Serenade was applied at the recommended dose with a potter tower to detached vine leaves kept on water agar. Three discs were taken from each leaf and analyzed in duplicate by qPCR. Concentration of QST713 is given relative to a standard DNA from a laboratory grown culture of QST713.

Second test:

To test the influence of copper and sulphur in the PCR reaction, a comparison series was conducted. All leaves were sprayed with copper or sulphur. After the leave surfaces were try,

the BCAs were applied. The incubation period, sampling and DNA extraction were similar to the first test.

While copper has a tendency to negatively influence QST713 on vine leaves, sulfur has apositive tendency. The only statistically significant difference ($p<0,01$) was found between "Serenade + Sulfur" and the other two treatments after 14 days of incubation. A clear positive effect of sulfur on the persistence of QST713 could be seen. All other differences were statistically not significant, due to high standard deviations.

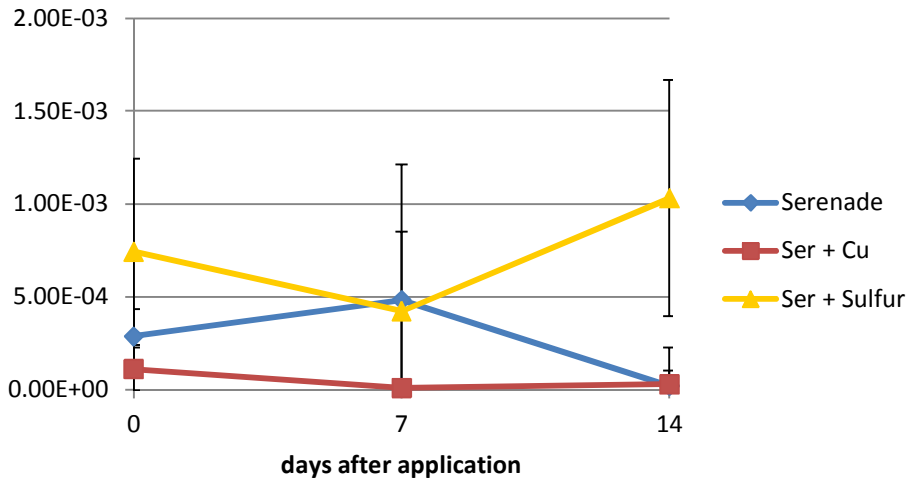



Figure 3: Compatibility of *B. amyloliquefaciens* QST713 with copper and sulfur on vine leaves. Serenade was applied at the recommended dose with a potter tower to detached vine leaves pretreated with copper and sulfur, respectively. Three discs were taken from each leaf and analyzed in duplicate by qPCR. Concentration of QST713 is given relative to a standard DNA from a laboratory grown culture of QST713.

The development of qPCR assays for Botector (*Aureobasidium pullulans* DSM 14941 und DSM 14940) have been started. The *in silico* testing of strain specific primers was successful. The optimization of the esthablised qPCR assay is under way.

5.2.3 Greenhouse experiments - Biopesticide tracking under controlled environment 2013

**Trial design:**

Creating an alternating cycles of high and low humidity in a greenhouse chamber

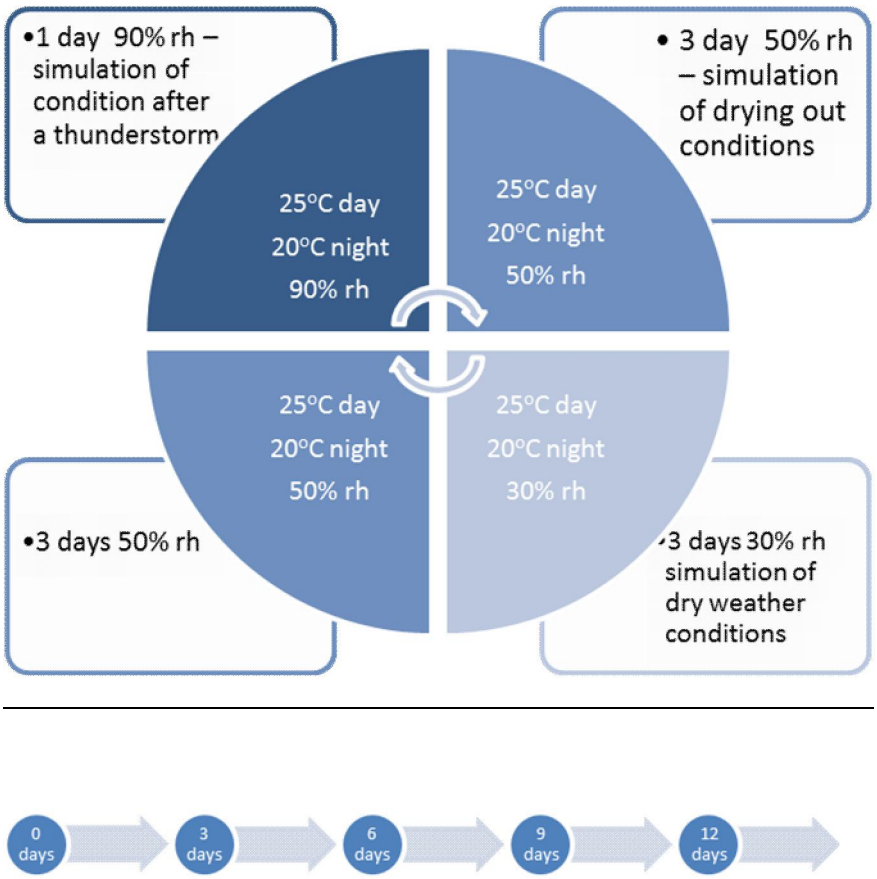



Figure 4: Sampling time, testing the quantity of fungal DNA on a defined leave surface by qPCR. In comparison a greenhouse chamber with constant temperature and humidity conditions was used.

Potted grapevines (cv Neuburger) were sprayed by hand with all four BCAs. Per treatment ten replicates of potted plants were used. Per sampling time three leaves were picked randomly from the potted grapes. 3 discs of leave tissues punching out with a cork borer were taken per replicate and used for DNA extraction. The trial lasted for one month. The DNA extractions are under way.

### 5.2.4 Field experiments - Biopesticide tracking under natural conditions - 2013

**Experimental set-up**

Trial locations:

Stammersdorf in Lower Austria

Cultivars: Neuburger,

Weather data were recorded.

Trial arrangement (plots):

Randomized complete block design.

Replicates: 4 (plot size: 160 $m^{2)}$

Equipment for spraying: Knapsack sprayer (Solo 473 P)

Date of applications:

First application: 2 July 2013

Second application: 19 July 2013

Third application: 5 August 2013

Fourth application: 23. August 2013

Sampling: To define the baseline 48 leaves were taken before the first spraying. At 20 sampling dates 3 leaves per replicate (12 leaves per BCA) were taken till the beginning of September. From each leave 3 discs of tissue were taken and used for DNA extraction. The DNA extractions are under way.

## 5.3 Screening for novel antagonists against *Scaphoideus titanus* - 2012

Strains from the in-house strain collection with suspected activity against arthropods. This list mainly includes species in the fungal families *Cordycipitaceae* and *Clavicipitaceae*. Commonly used parasites of insects like *Metarhizium anisopliae*, *Cordyceps* (= *Beauveria*) *bassiana*, *Isaria farinos* and *Lecanicillium lecanii*. The strains tested for activity agains *Scaphoideus titanus* are listed in

Table 2. Additionally, the commercially available plant production product Mycotal™ containing *Lecanicillium lecanii* was tested.

Table 2: Strains tested for activity agains Scaphoideus titanus

| **Strain** | **Species** |
|---|---|
| NG_p28 | *Cordyceps bassiana* |
| NG_p37 | *Cordyceps bassiana* |
| KF1016_G | Cordycipitaceae |
| ISS1004 | *Isaria farinosa* |
| KF0909_H8 | *Isaria farinosa* |
| POC0701_A | *Isaria farinosa* |
| SAN1029 | *Isaria farinosa* |
| MB0701 | *Isaria farinosa* |
| KF0909_H3 | *Isaria* sp. |
| KF1016_M2 | *Lecanicillium lecanii/muscarium* |
| KF0909_H1 | *Paecilomyces lilacinus* |
| NG_p20a | *Paecilomyces* sp. (Clavicipitaceae) |
| NG_p35 | *Paecilomyces* sp. (Clavicipitaceae) |
| D_SC2 | *Paecilomyces variotii* |

All strains reisolated from infected *S. titanus* could be identified as *L. lecanii* by molecular methods.

### 5.3.1 Laboratory assessment to evaluation the ability of *Lecanicillium lecanii* (Mycotal™) to infect *S. titanus* larvae

### 5.3.2 Introduction

*Vertcillium lecanii* is one of the most important and common entomogenous Hyphomycetes occurring in all climatic regions, on coccids, aphids, thrips, Diptera, Homoptera, Hymenoptera, Lepidoptera and mites (Domsch et al 1980).
So far the potential of entomopathogenic fungi as control agents of leafhoppers in general has received little attention (Tounou et al. 2011). Nickel et al (2009) found strong evidence that the entomopathogenic fungus *Lecanicillium lecanii* (Mycotal™) can partly control cicades in medical herbs.
The present study was conducted to evaluate the effect of the entomopathogenic fungi *Lecanicillium lecanii* (*Lecanicillium lecanii* strain VI23 of the commercial product Mycotal™) on immature stages of *Scaphoideus titanus*, the vector of Grapevine Flavescene dorée phytoplasma under laboratory conditions as a possible alternative control measure and bio pesticide for *S. titanus* in organic vine production.

### 5.3.3 Methods and Materials

#### *Scaphoideus titanus* rearing

A *Scaphoideus titanus* colony was established in the laboratory at the Institute of Sustainable Plant Protection, at AGES. Vine wood material older than one year was collected in February (KW 8) 2012 after a cold period, from two grape arbors located in Misselsdorf, Styria, in Austria. In these grape arbors, many *S. titanus* were observed during the previous summers (2010 and 2011), and the collected woods were expected to contain eggs. The collect occurred after a cold winter period, when the eggs diapause was released.

#### Rearing cages

The wine wood material was immersed in a fungicide solution (25 liter water and 75mg benzimidazole) for 5 hours to prevent fungal growth during the storing. After that, the wine wood material was cut and stored on a grid in plastic boxes (44x33x17cm) filled with quarz sand (about 2cm high) in a climatic room with 6°C temperature and 95% RH for about 9 weeks (69 days) to keep eggs alive and prevent them from hatching, until the beginning of the experiments (Figure 5). The quarz sand was periodically humidified in order to avoid egg dehydration.

(19. March 2012): As on part of grapevine wood material fungi had developed, those cuttings were immersed a second time in a fungicide solution (25 liter Water and 75mg Benzimidazole) for 3.5 hours and dried at room temperature for 34 hours.

#### Egg hatching

By the end of April (KW 17) egg hatching was started, by placing the rearing cages in a climatic chamber at 24±1°C temperature, 16:8h light regime and 70-80% relative humidity.

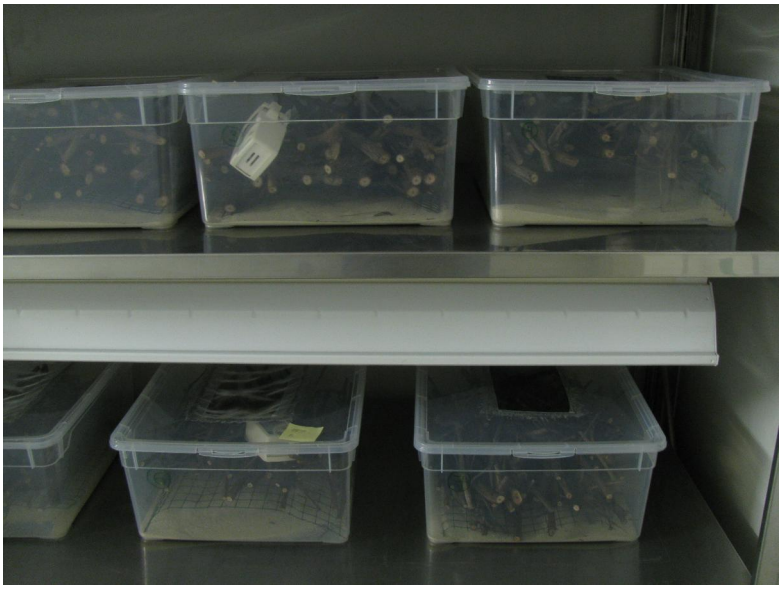


Figure 5: Rearing cages for *Scaphoideus titanus* colony establishment.

Bioassay

Insect development stage is important with regard to sensitivity to pathogens. The second larval stage of *S. titanus* was used in the three experiments, because this stage has a comparable long development of 9 days in average (Rak Cizej 2012).

In all experiments the *Lecanicillium lecanii* strain from the commercial product Mycotal™ (Koppert Ltd., The Netherlands) was tested as a pathogen of *S. titanus* larvae.
*S. titanus* individuals were incubated singly in petri dishes (77mm diameter, 26mm high), containing a Chardonnay leaf disk (67mm diameter) on water agar. Petri dish covers were ventilated with a screened whole (15 mm diameter) (see Fig. 6).

Experiment 1: Immersion

(11. June 2012)

A suspension of Mycotal™ (1g) and ADDIT (2.5g) in 1 liter water (22°C) was prepared.
*Vitis vinifera* (Chardonnay) leaf discs (diameter: 67 mm) were first washed with Aqua destillata and then immersed in the conidia suspension for 3 minutes.

Water-agar (12.5g/L) was prepared and poured in petri dished (diameter: 77 mm, height: 2.6 mm) which were previously cleaned with Ethanol. Leaf discs were put on the water agar at 38°-42°C temperature.

Eighteen (18) second instar nymphs of *S. titanus* were placed individually in a petri dish on the inoculated *V. vinifera* leaf discs and incubated. First, the larval development stage was checked individually with a binocular (della Giustina et al 1992).
For control, a further eighteen (18) second instar of *S. titanus* nymphs were maintained on Chardonnay leaf discs immersed for 3 minutes in water (24°C) alone.
The suspension or the water was let dry for 50 minutes.

Experiment 2: spraying with pump spray bottle

(18. June 2012)

The Mycotal™ - ADDIT–water suspension was sprayed on the *V. vinifera* leaf disc with a pump sprayer bottle. The leaf discs were put on the water-agar in the petri dishes at 38°C temperature. Then the suspension was sprayed with 5 puffs on each leaf disc. In the control the leaf discs were sprayed with water alone. On average 635.4 mg Mycotal™ - ADDIT–water suspension and 661.5 mg water was sprayed with five puffs of this pump spray bottle.

The suspension or the water was let dry for 40 minutes.

Thirty (30) second instar nymphs of *S. titanus* were placed individually in a petri dish on the inoculated *V. vinifera* leaf discs and further thirty nymphs were used for control. Again, larval development stage was checked individually with a binocular.

Experiment 3: spraying with Potter-tower

(21. June 2012)

The *V. vinifera* leaf discs were put on the water-agar in the petri dishes at 38-39°C temperature and were then sprayed with either 0.89 ml Mycotal™ - ADDIT–water suspension or water alone as a control with a Potter precision laboratory spray tower.

Afterwards, twenty (20) second instar larvae of *S. titanus* were placed individually in a petri dish on the inoculated *V. vinifera* leaf discs and further thirty larvae were used for control. Larva development stage was checked individually with a binocular before.

Incubation

The incubation conditions were the same for the three experiments except for the photoperiod light regime. After inoculation, the leafhopper larvae were incubated under controlled environmental conditions in an environmental chamber at 22±1°C, 80±5% R.H. In experiment 1, petri dishes were kept under a 16:8h light/dark regime. In experiment 2 and 3 petri dishes were kept in darkness for most of the time, to exclude possible negative effect of ultraviolet light on the fungus.

In experiment 1, some of the leaf discs showed brown areas after two days, which was due to the high temperature of the water agar that was too hot for the plant tissue apparently.
In experiment 3, the relative humidity in some of the petri dishes in the control group was too high so that fungus had developed on some leaf discs after day ten. The availability of food for the larva was probably negatively influenced and accounts for the comparably higher mortality rate in the control group. Apart from that, the leaves remain viable until the end of the experiment (13 days at maximum).

The Incubation period was twelve days in experiment 1 and thirteen days in experiment 2 and 3.

Mortality assessment was carried out for up to thirteen days, and was checked after:

- experiment 1: four, seven and twelve days
- experiment 2: five, seven, ten and thirteen days
- experiment 3. five, seven, ten and twelve days

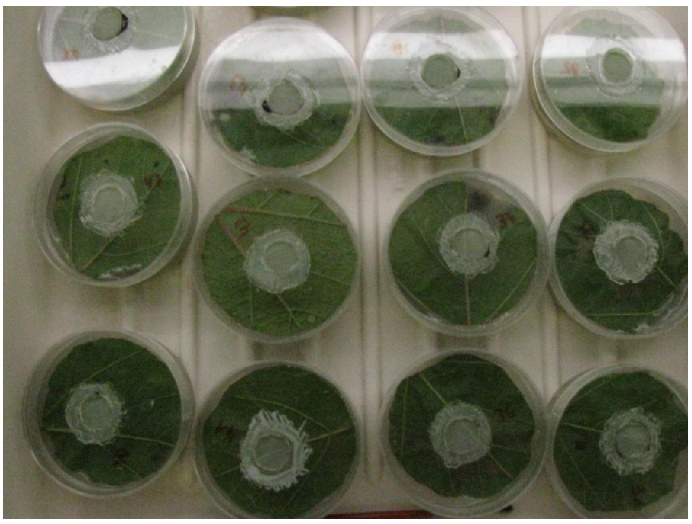


Figure 6: Example of incubated *Scaphoideus titanus* nymphs in petri dishes.

Mycosis tests:

To determine if a larva died of mycosis, colonization of the cadaver by *L. lecanii* was evaluated. Dead individuals of *S. titanus* were removed, their surface sterilized in 70% ethanol, dried and incubated on moist filter paper inside sterile petri dishes, which were placed on wet paper inside a plastic box (25x30 cm) in an environmental chamber at 22±1°C, 80±5% R.H. in darkness for 5 days to confirm death by entomopathogenic fungi.
Afterwards, the mycelium from the cadaver was transferred to an adapted PDA agar for *Paecilomyces lilacinus* (Mitchel et al 1987) and incubated at the same conditions. The fungus isolates were sequenced at the Fungal Genetics and Genomics Unit, University of Natural Resources and Life Science (BOKU), Austria by using molecular methods.

Figure7: *Scaphoideus titanus* larvae on a *Vitis vinifera* leaf disc treated with Mycotal™ by immersion.

The infection of the entomopathogenic fungus *Lecanicillium lecanii* (strain VI23, active ingredient of Mycotal™) initially starts at the legs of the larvae.

### 5.3.4 Results

The entomopathogenic fungi *Lecanicillium lecanii* (Mycotal™) proved to be virulent to the second instar nymphs of *Scaphoideus titanus*. The percentage of the corrected cumulative mortality after Abbott's formula (1925) ranged from 73 (experiment 1) to 54% (experiment 2) and 33% (experiment 3). Except for experiment 1, the mortality in the control group in experiment 2 and 3 were high, with about 20%. This was due to the relatively high humidity in the petri dishes in experiment 2 and 3, followed by leaf discs getting moldy. The leafhopper had less access to healthy plant tissue (see Fig. 7 to 15).

On all cadavers the initial growth of the fungi began on the legs (see Fig.7)

Fast fungal growth was observed: the cadavers were covered by a dense mycelium within few days (ca. 5 days) (see Fig. 8).

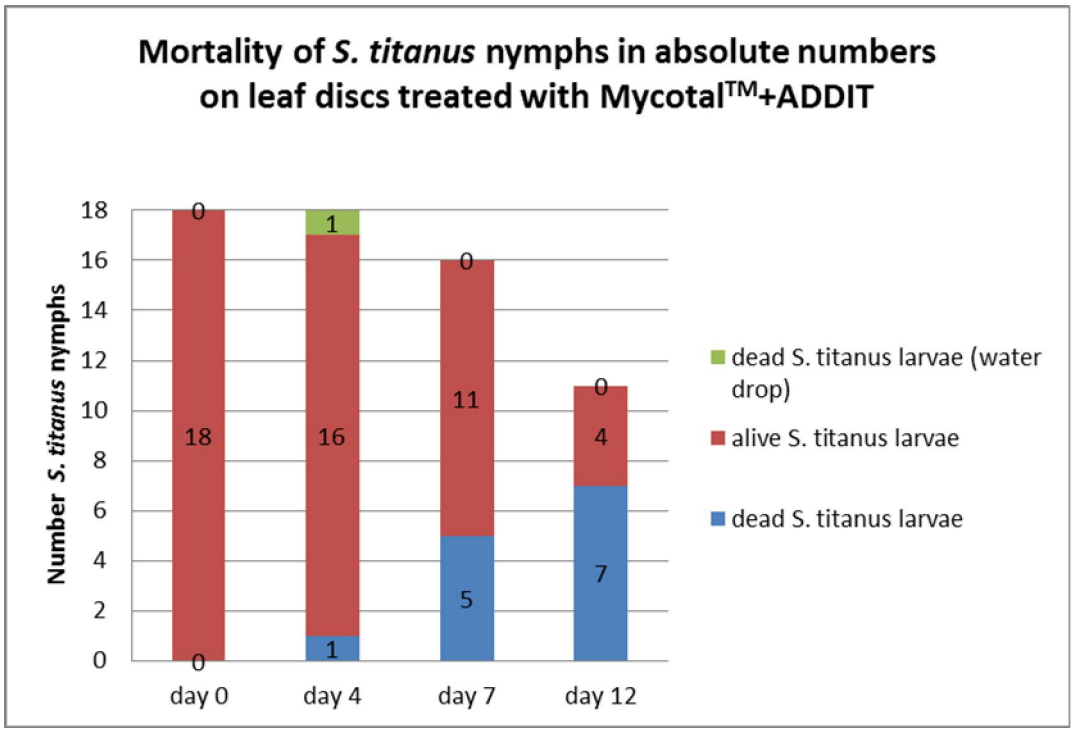
Figure 8 : *Scaphoideus titanus* nymph after inoculation on a *Vitis vinifera* leaf disc treated with Mycotal™.

*Lecanicillium lecanii* (strain VI23) covered its diseased host after 5 days incubation.

Results of mycosis tests

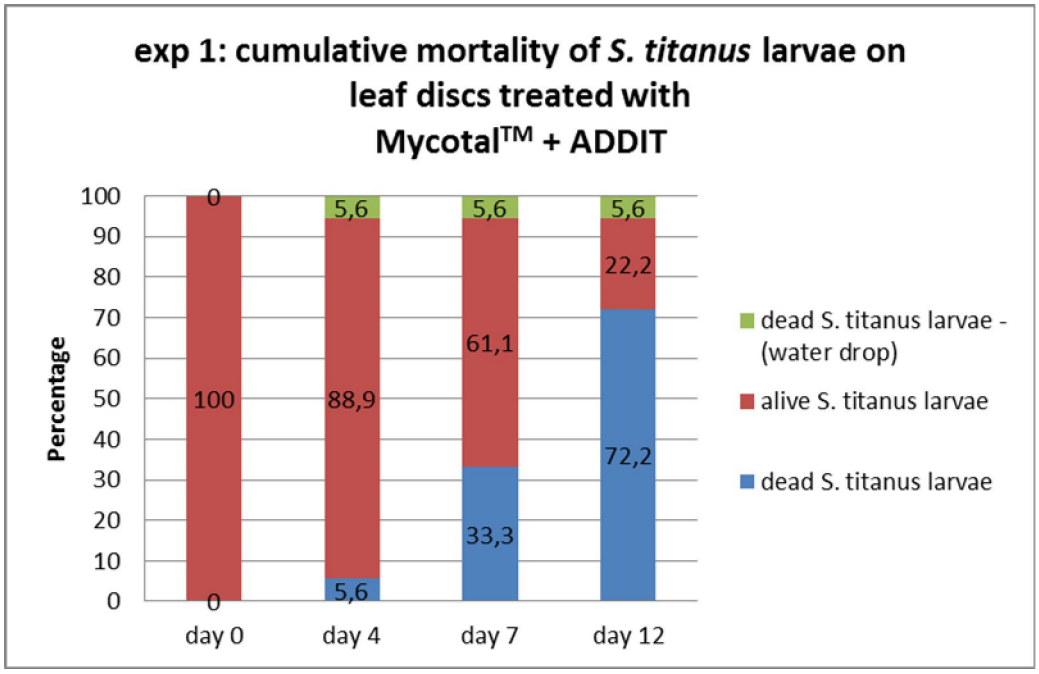
So far, seven reisolated fungus isolates from the three experiments were sequenced. All of them were identified as *Lecanicillium lecanii*.

The progressions of the mortality of the inoculated *S. titanus* larvae of the test and the control group in experiment 1 are shown in figures 9 to 11.



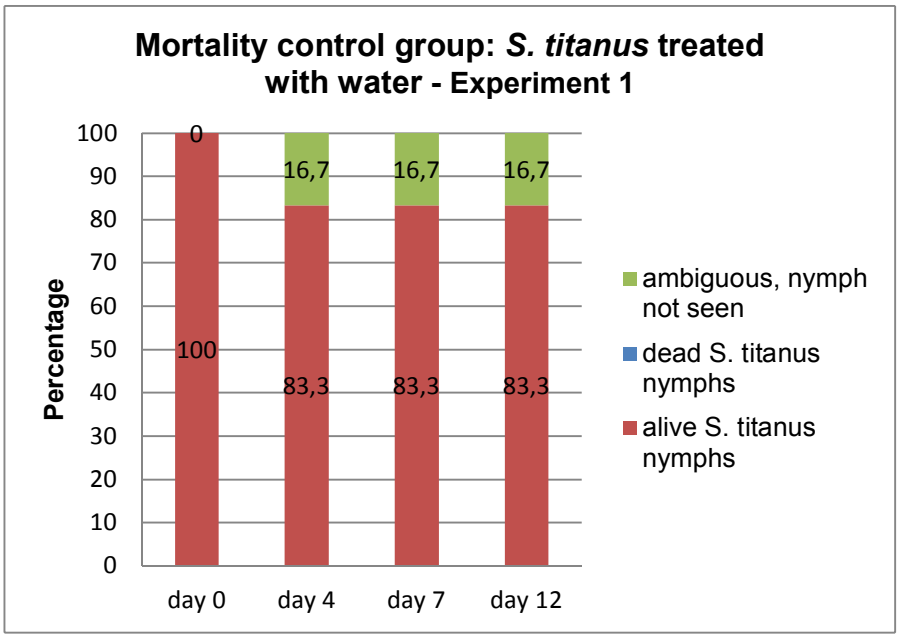

Figures 9: Mortality of *Scaphoideus titanus* larvae treated with *L. lecanii* in experiment 1.

The figure shows the absolute numbers of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with Mycotal™.




Figures 10: Mortality of *Scaphoideus titanus* larvae treated with *L. lecanii* in experiment 1.

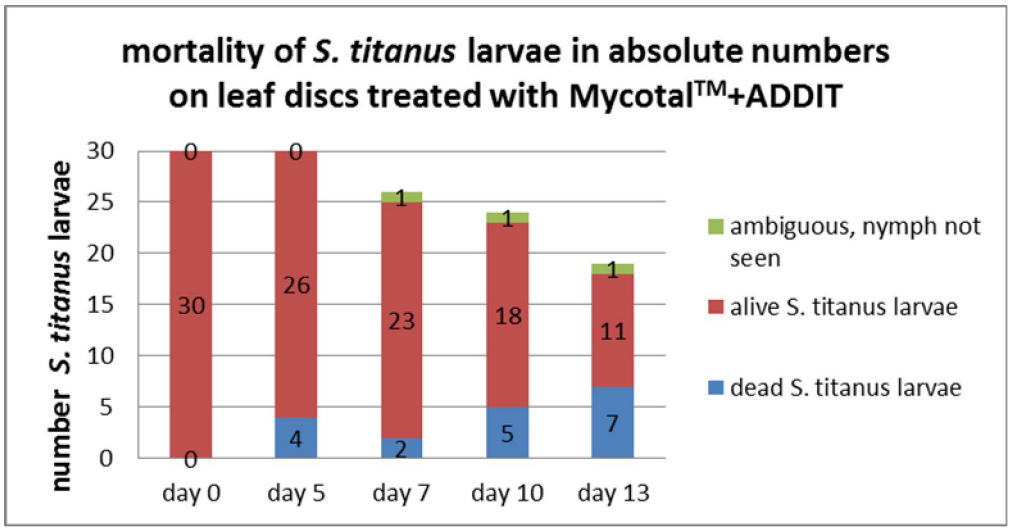
The figure shows the cumulative percentage of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with Mycotal™.



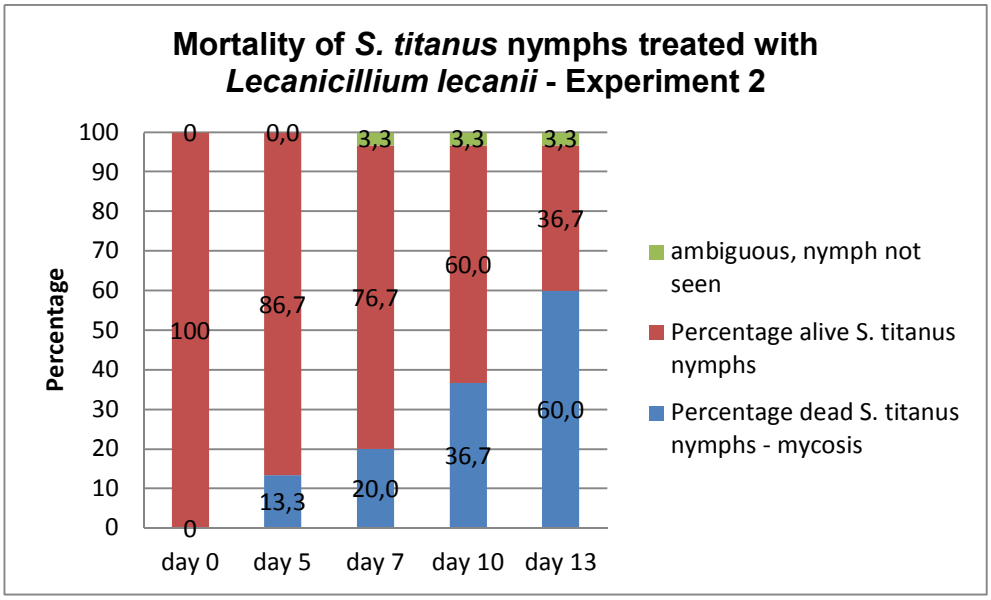

Figures 11: Cumulative percentage mortality of *Scaphoideus titanus* larvae of the control group in experiment 1.

The figure shows the mortality control group of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with water alone.

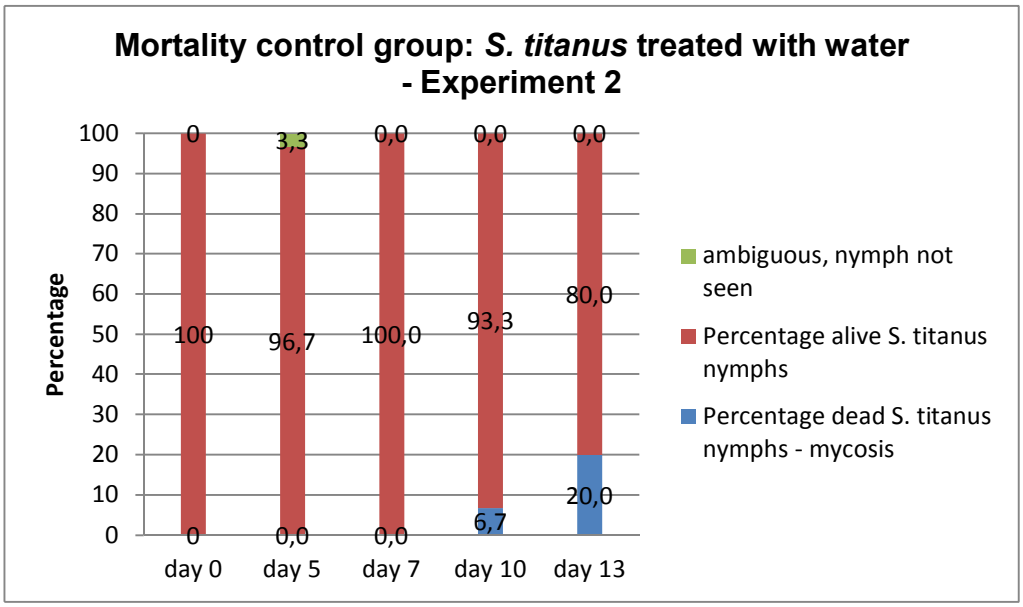
The progressions of mortality of the inoculated *S. titanus* nymphs of the test and the control group in experiment 2 are shown in figures 12 to 14.




Figures 12: Mortality of *Scaphoideus titanus* larvae treated with *L. lecanii* in experiment 2.
The figure shows the absolute numbers of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with Mycotal™.



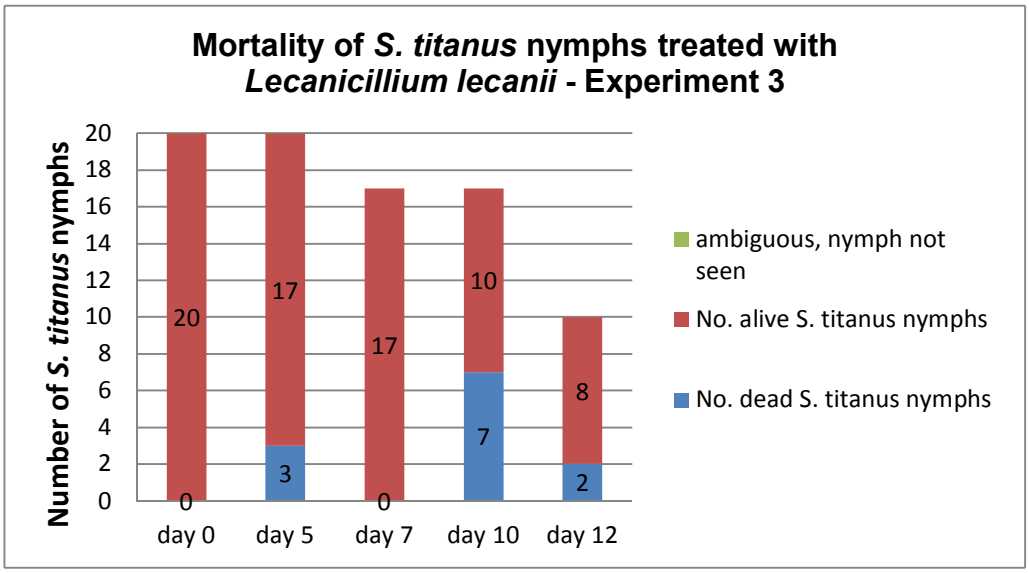

Figures 13: Mortality of *Scaphoideus titanus* larvae treated with *L. lecanii* in experiment 2.
The figure shows the cumulative percentage of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with Mycotal™.




Figures 14: Cumulative percentage mortality of *Scaphoideus titanus* larvae of the control group in experiment 2.

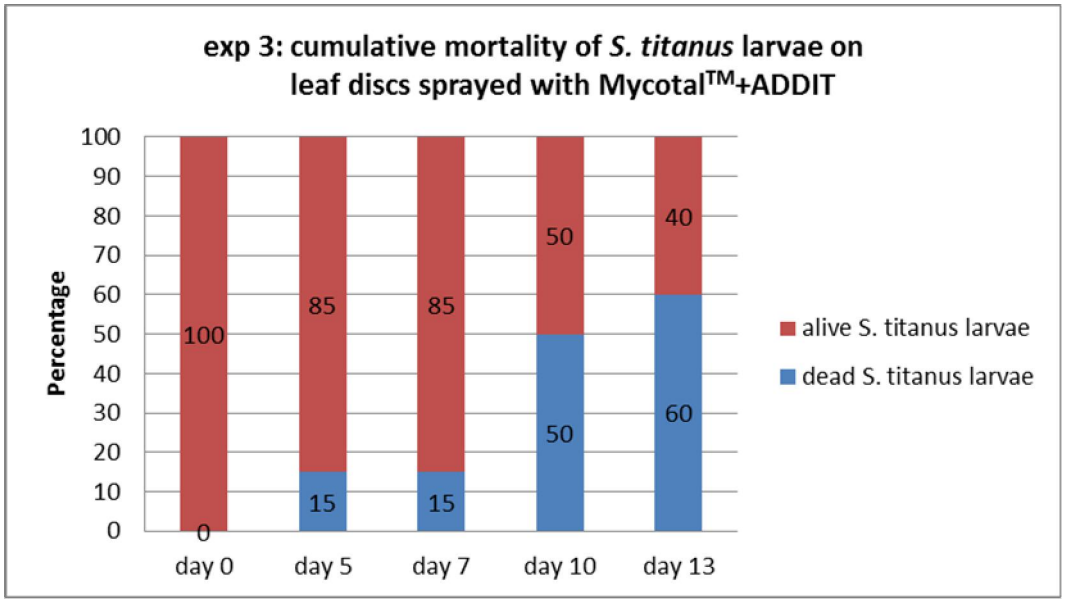
The figure shows the mortality control group of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with water alone.

The progressions of mortality of the inoculated *S. titanus* nymphs of the test and the control group in experiment 3 are shown in figures 15 to 17.



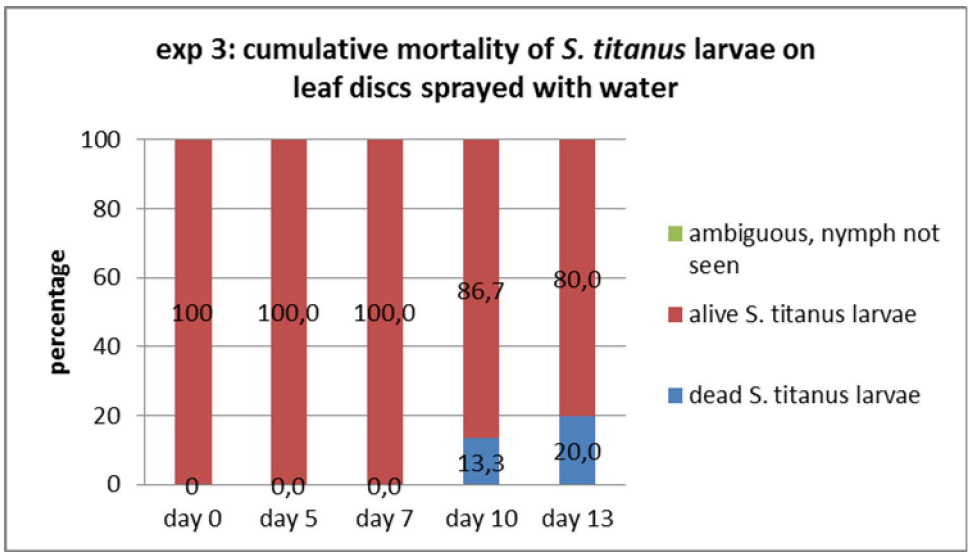

Figures 15: Mortality of *Scaphoideus titanus* larvae treated with *L. lecanii* in experiment 3.

The figure shows the absolute numbers of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with Mycotal™.




Figures 16: Mortality of *Scaphoideus titanus* larvae treated with *L. lecanii* in experiment 3.

The figure shows the cumulative percentage of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with Mycotal™.




Figures 17: Cumulative percentage mortality of *Scaphoideus titanus* larvae of the control group in experiment 3.

The figure shows the mortality control group of *S. titanus* larvae on inoculated *Vitis vinifera* leaf discs treated with water alone.

**Statistical analysis**

Kaplan-Meier estimates of the survival function S(t) was used to determine the overall mortality rates of *S. titanus* larvae. The probability of a larva surviving up to a time *t*, was calculated.

The probability to live for four days is the same for larvae in both groups. However as of day five the survival functions of *S. titanus* larvae in the *L. lecanii* treated group and in the control group are highly significant different ($p<0.001$; Log-Rank Test). *S. titanus* larvae in the *L. lecanii* treated group died earlier (Figure 18).

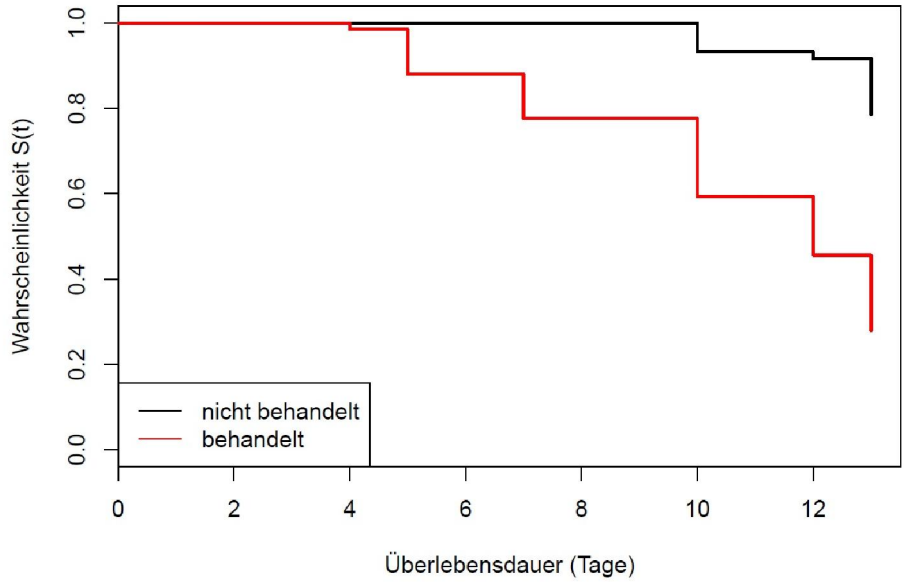



Figure 18: Kaplan-Meier cumulative risk curve for *S. titanus* larvae mortality in the *L. lecanii*-treated group (red line) and the control group (black line).

**Activities 2013**:

Based on the laboratory results of the *L. lecanii* pathogenicity to *S. titanus* larvae, a field test was planned for 2013 to test the entomopathogenic fungus under natural conditions. An organically farmed vineyard was chosen as experimental plot in south-east Styria because *S. titanus* is present in this area since 2004. On two dates in June (6.6. and 13.6. 2013) a systematic, visual monitoring for *S. titanus* larvae was performed in the vineyard. Contrary to the assumption that larvae should be present only one larva was found. The field test was not conducted therefore.

## 6. Presentations

Helga Reisenzein: Ersatzstrategien von Kupfer im Weinbau. Fachtagung "Kupfer im Pflanzenschutz", Austria, Vienna, 26.09.2012

Strauss G.: current research activities on *Scaphoideus titanus* and Grapevine flavescence doree in Austria; IOBC/WPRS Working group meeting 2013, 16.10.2013, Ascona, CH

Helga Reisenzein, Gudrun Strauß: Integration of plant resistance, cropping practices, and biocontrol agents for enhancing disease management, yield efficiency, and biodiversity in organic European vineyards (VINEMAN.org). Forschungsgespräche LFZ Klosterneuburg, 29.11.2013

Helga Reisenzein, Gudrun Strauß und Markus Gorfer: VINEMAN.org. Rebschutzgebietsleitertagung, AGES Wien, 23.01.2014

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