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FINAL REPORT 2020

Winetech Number : IWBT P 16-01

1. PROGRAMME & PROJECT LEADER INFORMATION

	Research Organisation leader	Project leader
Title, initials, surname	Prof M Du Toit (DVO), Prof M Vivier (SAGWRI)	Dr JP Moore
Present position	Professors	Senior Researcher
Address	South African Grape and Wine Research Institute (SAGWRI), Department of Viticulture and Oenology (DVO), Stellenbosch University, Matieland 7602, SA	South African Grape and Wine Research Institute (SAGWRI), Department of Viticulture and Oenology (DVO), Stellenbosch University, Matieland 7602, SA
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2. PROJECT INFORMATION

Project title	Towards protecting grapevine using natural bioprotectants
Short title	Priming for protection

Fruit kind(s)	wine grapes		
Start date (mm/yyyy)	2016-01	**End date** (mm/yyyy)	2020-12

Key words	priming, bioprotectants, grapevines, disease resistance

	CFPA	DFTS	HORTGRO	SATI	WINETECH	ARC	OTHER
TOTALS All years	R 0	R 0	R 0	R 0	R 858842	R 0	R 0

Total cost of entire project	R 858842

3. EXECUTIVE SUMMARY

Objectives and Rationale

The South African wine industry needs to develop innovative environmentally friendly technologies to control fungal diseases, such as *Botrytis cinerea*, in nurseries and vineyards. In this project, biostimulants such as strigolactone treatments were tested as promising natural compounds to enhance the plants immune system in laboratory and greenhouse conditions.

Methods

Strigolactone preparations (±)-GR24 and Nijmegen-1 were applied as root-soaks to *in vitro* plantlets of tobacco (*Nicotiana tabacum*) and grapevine (*Vitis vinfera*) cv Cabernet Sauvignon; and Cabernet Sauvignon and Sauvignon Blanc nursery vines. Treated plants challenged with *Botrytis cinerea* were then assessed for lesion size and disease progression.

Key Results

Strigolactone preparations (±)-GR24 and Nijmegen-1 treated tobacco and grapevine (cv Cabernet Sauvignon) *in vitro* plantlets and glasshouse plantlets showed decreased susceptibility to *Botrytis cinerea.* Treatment of grafted nursery vines (cv Cabernet Sauvignon and Sauvignon Blanc) showed no impact on disease susceptibility to *Botrytis cinerea* in glasshouse conditions.

Key Conclusion of Discussion

Strigolactone preparations (±)-GR24 and Nijmegen-1 have potential as bioprotectants by influencing plant-fungus interactions in grapevine plantlets. Strigolactone preparations could form part of a bioprotectant treatment programme in combination with other plant hormones used on grapes. Further research is necessary to assess the molecular mechanism-of-action *in planta*.

Take Home message for Industry

Strigolactone preparations (±)-GR24 and Nijmegen-1 are biostimulants and plant hormones that could help prevent vine losses in grapevine nurseries. These preparations should be tested in vine nursery trials to confirm this.

4. PROBLEM IDENTIFICATION AND MOTIVATION

Problem Identification

The South African Wine Industry is vulnerable to fungal pathogens such as Botrytis (*Botrytis cinerea*) and the mildews (Powdery - *Erysiphe necator* (or *Uncinula necator*) and Downey - *Plasmopara viticola*) which cause significant crop losses and reduce wine production with the negative economic consequences. Given the negative environmental consequences of chemical pesticide and herbicide usage, developing strategies to enhance natural plant defence systems is of considerable interest, and potentially cheaper than reliance on expensive chemical products.

Grapevine has evolved natural (innate) defence systems for protection from attack by viral, bacterial, fungal and insect pathogens; although these mechanisms may have been limited by

domestication. A novel and 'untapped' area of study is understanding how plants are 'primed' by external biomolecules which confer the ability of *Vitis vinifera* to respond faster and/or stronger to a potential stress (e.g. an infection).
The ultimate aims of this study are: (1) to understand priming in tobacco and grapevine plants of which very little is known, (2) to identify natural bioprotectants, and (3) to demonstrate the 'proof-of-concept' that such biomolecules have potential value for the grape and wine industry; lead biomolecule preparations could be taken to further nursery/field trials.

Motivation
This study was performed to understand priming in tobacco and grapevine plants; to identify natural bioprotectants and to demonstrate the "proof-of-concept" that such biomolecules have potential value for the industry, i.e. marked resistance against *Botrytis cinerea* in grapevine plantlets. It was shown that strigolactones as defence molecules have potential value for industry.
It was determined that the strigolactones (±)-GR24 and Nijmegen-1 proved the most effective in growth and challenge experiments. Pathogenic challenge experiments resulted in clear data for (±)-GR24 and Nijmegen-1 strigolactones as being the most promising defence candidates. Strigolactone preparations (±)-GR24 and Nijmegen-1 when pre-applied as a root-soak induced some marked resistance against *Botrytis cinerea* in tobacco and grapevine plantlets. The mechanism-of-action remains unclear, currently it is believed to be due to some sort of priming phenomenon modifying the antioxidant response pathways of plants and fungus. However, treatment of 1 year old grafted vines in greenhouse conditions did not result in protection against Botrytis.
The use of these compounds in vine nurseries appear the most promising route for technology transfer in the area of plant protection and plant care. This study provides a solid base for a more thorough investigation of these promising natural bioprotectants in the context of industry applicability.

5. ACCUMULATED PROGRESS TABLE

Objectives	Milestones (Significant event or stage in a project)	Date Achieved
1. Preparation of potential bioprotectant biomolecules	Milestone 1: Completion of preparations of the bioprotectants to be tested (e.g. SLs)	2016-12-31
2. Preparation of tissue culture plant populations and high-throughput screen	Milestone 1: Preparation of tissue culture plants and methods to deliver and evaluate bioprotectants	2017-07-31
3. Measurement of Pre-Priming, Primed and Post-Priming (challenged) states	Milestone 1: Investigation and development of methods to assess stages of priming	2017-12-31
4. Evaluation of datasets showing if priming and post-priming occurred	Milestone 1: Implementation of univariate and multivariate statistical approaches to the datasets obtained.	2018-07-30

due to the application of the potential bioprotectants		
5. Challenge experiments (depends on confirmation of primed states and the time available)	Milestone 1: Evaluation and decision on potential challenge experiments viable in a tissue culture plant system	2017-12-31
6. Determination of the length of the protection of the bioprotectant – how long does it last? Is a top-up application necessary?	Milestone 1: testing for tissue culture priming protection and duration of protection in plantlets (tissue culture) Milestone 2: testing for the effects of top-up applications in tissue culture plantlets	2020-03-31
7. Assessment of strigolactones on vine cultivar-rootstock combinations in the context of plant growth and protection against fungi (e.g. Botrytis).	Milestone 1: evaluating the effect of strigolactones on growth of cultivar-rootstock vines in pots in glasshouse Milestone 2: comparing the influence of strigolactones on potted vines and evaluating if a protection response to pathogens is observed	2019-12-31
8. Evaluation of the effect of Strigolactones on other diseases – powdery and downy mildew as well as root-rot.	Milestone 1: developing a bioassay for powdery and downy mildew on tissue culture plantlets and/or potted vines Milestone 2: developing a bioassay for blackfoot disease on tissue culture and/or potted vines	2020-12-31
9. Cost of using bioprotectants. Assessment of viability (e.g. financial) for use by the industry. Especially if more applications during the season are needed.	Milestone 1: Determining the strigolactone enantiomers and synthetic analogues that provide optimal protection	2018-12-31
10. The application of the bioprotectants in nursery conditions on young vines.	Milestone 1: Determining the optimal method to apply strigolactones to nursery vines and assessing protection to pathogens, priming	2019-12-31

6. WORKPLAN (MATERIALS AND METHODS)

Objective 1: Preparations of the bioprotectants to be tested (e.g. Strigolactones)

Smoke-water and lumichrome solutions. Smoke water is prepared by drawing the smoke from a slow burning fire through distilled water. Alternatively, pre-prepared smoke water, derived from burnt fynbos material, was purchased from the seed room at Kirstenbosch Botanic Gardens and lumichrome was supplied by Dr PN Hills (Plant Biotechnology). Strigolactones (SLs) (±)-GR24 and Nijmegen-1 were purchased from Strigolab (https://strigolab.eu/) an academic lab supplier of strigolactone preparations.

Objective 2: Preparation of tissue culture plants and methods to deliver and evaluate potential bioprotectants.

SU Grapevine tissue-culture facilities (Prof MA Vivier and Mrs M Korkie) were used to generate SR1 tobacco (*Nicotiana tabacum*) and grapevine (*Vitis vinifera*) plantlets. Plantlets were also hardened off for planting out in a greenhouse. Evaluating foliar spray and root soak methodologies for SLs was performed. Preparing populations of *in vitro* tissue culture grapevine plants cv Cabernet Sauvignon.

Objective 3: Investigation and development of methods to assess stages of priming

The effect of plant associated compounds on plant growth were evaluated. Measurement of hydrogen peroxide activity was conducted. Measurement of superoxide dismutase (SOD) activity was performed.

Objective 4: Evaluation of datasets showing if priming and post-priming occurred due to the application of the potential bioprotectants

Univariate statistics (ANOVA etc.) were conducted on all relevant datasets with the help of Prof. Martin Kidd (SU).

Objective 5: Evaluation and decision on potential challenge experiments viable in a plant tissue culture system

Challenge experiments were conducted in a plant-infection chamber with *B. cinerea* BO5. 10 and *B. cinerea* grape strain (cultivated on apricot halves) on both tobacco and grapevine. Lesion index scale and lesion descriptions were used to define tolerance and susceptibility after infection of tobacco with *B. cinerea.* Disease susceptibility of treated tobacco plants to *B. cinerea* was assessed. *B. cinerea* branching bioassay and growth response to SLs was developed. Effect of GR24 and Nijmegen-1 on the hyphal branching structures of *B. cinerea* was assessed.

Objective 6: Determination of the length of the protection of the bioprotectant – how long does it last? Is a top-up application necessary?

Double applications of SLs (±)-GR24 and Nijmegen-1 were tested in potted grafted vines (2 weeks apart).

Objective 7: Assessment of strigolactones on vine cultivar-rootstock combinations in the context of plant growth and protection against fungi (e.g. Botrytis).

40 Cabernet Sauvignon (CS 37 C clone) and 40 Sauvignon Blanc (SB 11R clone) scions grafted onto Richter 110 rootstocks were purchased and potted out. Root-soak applications of SLs were trialled on these plants with 20 vines per cultivar designated as controls and 20 vines per cultivar designated as experimental.

Objective 8: Evaluation of the effect of Strigolactones on other diseases – powdery and downy mildew as well as root-rot.

Powdery mildew was obtained from the Department of Plant Pathology (SU) and maintained on host grapevines growing on their own roots in the plant infection chamber.

Objective 9: Cost of using bioprotectants. Assessment of viability (e.g. financial) for use by the industry. Especially if more applications during the season are needed.

Strigolactones (SLs) (±)-GR24 and Nijmegen-1 were purchased from Strigolab (https://strigolab.eu/) the costing was used to calculate a per plant application cost.

Objective 10: The application of the bioprotectants in nursery conditions on young vines.

40 Cabernet Sauvignon (CS 37 C clone) and 40 Sauvignon Blanc (SB 11R clone) scions grafted onto Richter 110 rootstocks were challenged with *B. cinerea* BO5. 10 and *B. cinerea* grape strain. Detached leaf assays were used to assess efficacy of protection against *B. cinerea.*

7. RESULTS AND DISCUSSIONS

Background: Strigolactones were initially described as plant root exudates which act as germination cues of parasitic seeds belonging to the Orobanchaceae family. It was later discovered that strigolactones function as stimulants to induce hyphal branching of arbuscular mycorrhizal (AM) fungi to aid in the establishment of beneficial symbiosis.
All experiments were performed independently twice, each with four biological representatives per data set. Data was presented as mean ± standard error of four independent determinations. For statistical analysis, one-way analysis of variance (ANOVA) test was used for all data.

Germinated tobacco seedlings were grown on commercial MS media under tissue culture conditions in the presence of varying concentrations of smoke water, lumichrome, (±)-GR24 and Nijmegen-1 respectively. Smoke water increased the average lateral root formation increased by ca. 37% while lumichrome increased lateral root formation by an average of ca. 45% compared to the control. Similar results were obtained for (±)-GR24 and Nijmegen-1 which were both able to increase lateral root formation by a cumulative average of ca. 40% and ca. 30% respectively. No significant differences were observed in the number of leaves for lumichrome, (±)-GR24 and Nijmegen-1. Smoke water, however, increased the number of leaves formed by an average of ca. 47%.
Fungal challenge (*B. cinerea*) studies on detached tobacco leaves (see Fig. 1.) was conducted. In order to define the range of lesion phenotypes, a 10-point lesion index scale was adapted from literature. The smoke water and lumichrome treated plants showed comparable disease progression patterns to the control throughout the experiment. For the compounds Nijmegen-1 and (±)-GR24, both resulted in a 24 h delay in disease progression, with initial infection onset only occurring after 72 h. After 144 h, disease symptoms had progressed for both Nijmegen-1 and (±)-GR24 but with limited or minor expansion. Nijmegen-1 and (±)-GR24, however, were able to reduce the infection lesions by ca. 40-50% with final lesion sizes averaging 7 mm for Nijmegen-1 and 5 mm for (±)-GR24. Only Nijmegen-1 and (±)-GR24 were therefore selected for downstream analysis. Nijmegen-1 and (±)-GR24 showed the highest percentage decrease in susceptibility with an ca. 80-100% decrease 48 h post infection and ca. 30-55% decrease 144 h post infection. Application of these strigolactones also restricted lesion development by ca. 40-50% in the inoculated leaves within the initial hours of infection.
The oxidative burst is among the early defence responses that is involved in inducing a primed response. The addition of the strigolactones (±)-GR24 and Nijmegen-1 as a root drench 48h prior to infection triggered a single and transient burst of ROS (reactive oxygen species) in the detached leaves within a few hours of treatment. The amount of H2O2 increased with the same kinetics for all concentrations for both (±)-GR24 and Nijmegen-1. Similar results were obtained for SOD (superoxide dismutase) determination, with both strigolactones exhibiting ca. 150% more SOD activity compared to the control 12 h post inoculation.

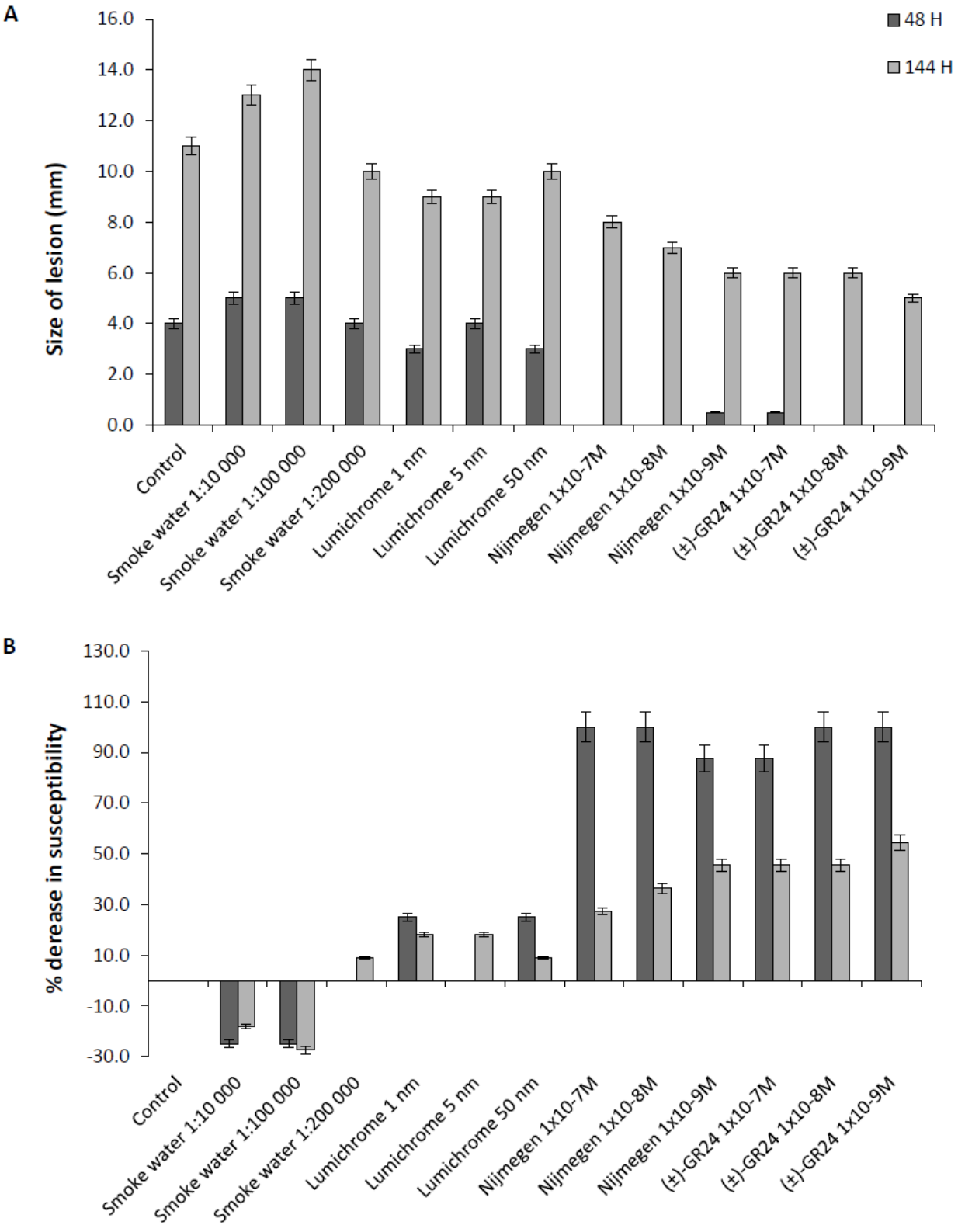

Fig.1. Disease susceptibility of treated tobacco plants to *B. cinerea*. A decrease in susceptibility to *B. cinerea* was determined by measuring the **(A)** size of lesions (mm) produced on detached leaves of the control and treated tobacco plants at 48 and 144 hours post inoculation. **(B)** The susceptibility to *B. cinerea* was expressed as a percentage of the decrease in susceptibility normalised against the tolerance of the control set, which was referenced as 0%. Error bars represent the means (±SE; n = 4) of two independent experiments.

The growth and stress response profiles of *Botrytis cinerea* directly to the application of synthetic strigolactones (±)-GR24 and Nijmegen-1 were also investigated. Exposure of *B. cinerea* to the strigolactone analogues resulted in increased hyphal branching, with (±)-GR24 stimulating a stronger effect than Nijmegen-1 by affecting colony diameter and radial growth. An oxidative stress response was observed with *B. cinerea* exhibiting increased ROS (reactive oxygen species) and SOD levels when grown in the presence of (±)-GR24 and Nijmegen-1 respectively. These results suggest that strigolactones may potentially function by altering the ROS homeostasis of *B. cinerea* which resulted in both morphological and physiological changes which may contribute to a reduction in virulence.

Disease symptom development of (±)-GR24 and Nijmegen-1 treated tissue culture grapevine plants upon challenge with *B. cinerea*. A root soak was found to be more effective rather than foliar spray. Grapevine explants containing a single node with axillary buds, derived from a mother plant (cv. Cabernet Sauvignon) was used for own rooted studies (see Fig. 2.). For infection studies, completely hardened, representative grapevine plants were transferred to the fresh growth medium, consisting of equal ratios of sterile vermiculite and perlite. To allow for ex-vitro acclimatization magenta lids were used to gradually reduce the relative humidity to ambient levels by tilting the lids every second day for 1 week. Plants were treated with the respective compound 48 h prior to infection as 20 ml root drench applications at different concentrations and control plants treated with distilled water. The results obtained in the *in vitro* assay mirrored the pattern seen in the detached leaves of greenhouse grown plants. Similarly to the greenhouse grown tobacco plants, the infection process was not completely stopped by (±)-GR24 or Nijmegen-1 but the disease incidence was clearly reduced. The lowest concentration of $1x10^{-9}$M for both strigolactone analogues proved to be the most effective in reducing symptom development, which were evaluated, based on the degree of wilting, browning and fungal hyphal occurrence.

Cabernet Sauvignon (CS 37 C clone) and Sauvignon Blanc (SB 11R clone) scions grafted onto Richter 110 rootstocks were purchased from Vititec and potted out (soil-vermiculite) in the glasshouse. Costs of application of (±)-GR24 and Nijmegen-1 were in the upper range of 0.3 ZAR (30 cents) per plant to the lower range of 0.03 ZAR (3 cents) per plant, which are industry competitive. BC204 a growth promoting substance was also tested but this had no visible effect on disease susceptibility. 1x10-8 M and 1x10-9 M (±)-GR24 concentrations were applied as root soaks 48 h prior to conducting infection challenge experiments on detached leaves with *B. cinerea*. No differences in disease susceptibility were observed between control and treated plants in glasshouse conditions. 1x10-8 M and 1x10-9 M (±)-GR24 concentrations were also repeatedly applied to the same plants over two week intervals and no alteration in growth was observed as well as no differences in disease susceptibility to *B. cinerea* were found (detached leaf assays). It should be noted repeated treatments with fungicides (to control mildew) and insecticide were needed to control powdery mildew and red spider mite infestations. The Covid-19 pandemic has delayed any continuation of this research (powdery mildew or root rot challenge) with the plants in the glasshouse still subject to periodic spider mite damage. We are currently testing molecular genetic responses in tobacco and grapevine following strigolactone application to indicate defence gene signaling but the Covid-19 pandemic has seriously delayed progress here.

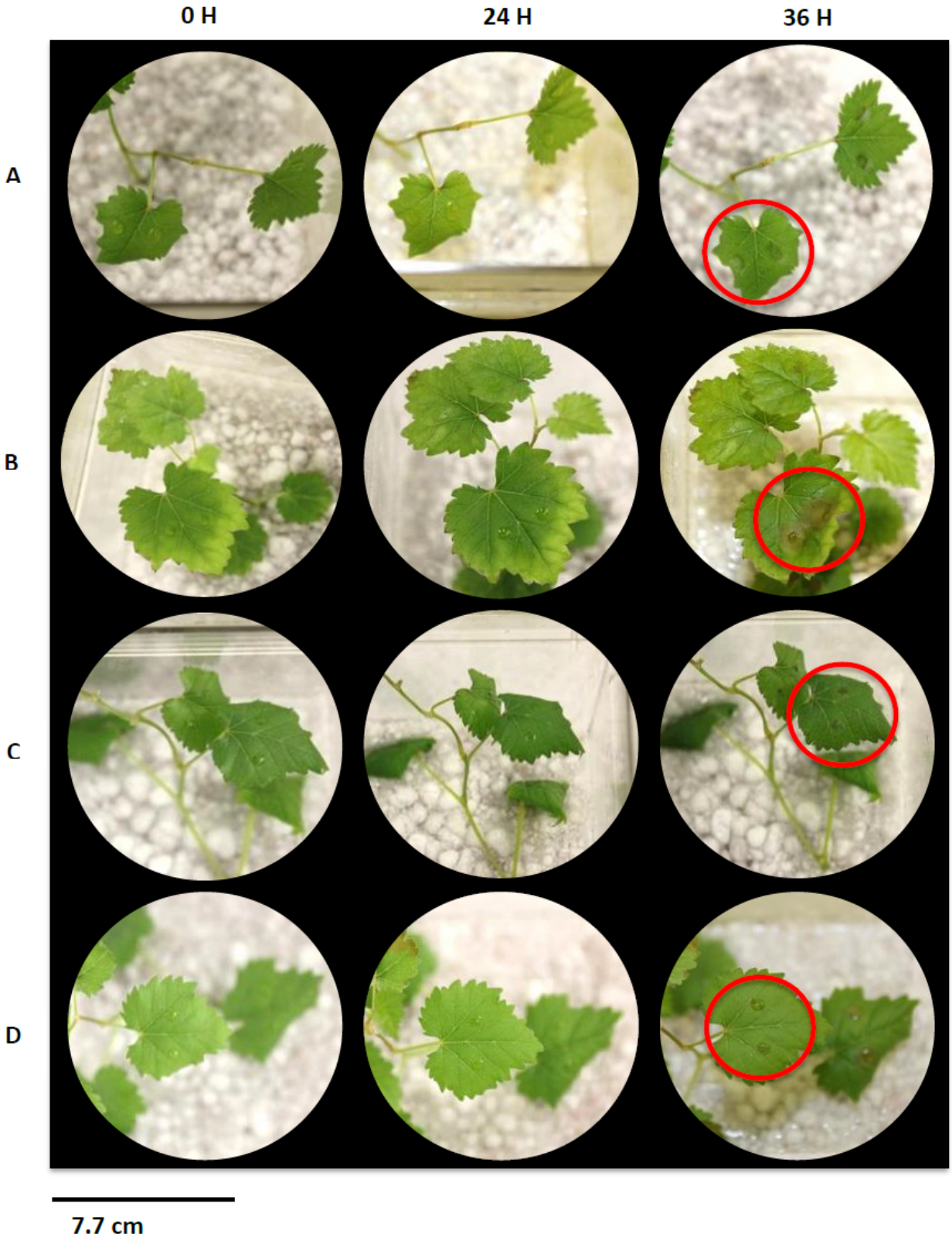

Fig.2. Disease symptom development of (±)-GR24 treated tissue culture grapevine plants upon challenge with *B. cinerea*. Intact leaves were inoculated a conidial suspension of *B. cinerea* and maintained at 22°C at 100% humidity. Treatments consisted of root drench applications of water for **(A)** mock **(B)** control or (±)-GR24 concentrations **(C)** 1×10^{-8} M and **(D)** 1×10^{-9} M. Images are representative of four biological repeats of two independent experiments. Disease development is indicated by the red circle.

8. CONCLUSIONS AND RECOMMENDATIONS

Two of the four tested compounds, namely (±)-GR24 and Nijmegen-1, were rapidly perceived by both tobacco and grapevine cv. Cabernet Sauvignon plants and subsequently triggers inducible defence responses to produce a primed phenotype.

Smoke water and lumichrome-treated plants showed that these compounds, although effective growth promoting agents, were unable to reduce fungal infection.

Positive growth profiles of tobacco and grapevine plants were obtained when exposed to varying concentrations of Nijmegen-1 and (±)-GR24.

The fungal pathogen, *B. cinerea*, is sensitive to the strigolactone analogues, (±)-GR24 and Nijmegen-1. More specifically, our results suggest that strigolactones mediate this response by influencing ROS homeostasis in *B. cinerea.*

The strigolactones (±)-GR24 and Nijmegen-1 increased the induction of defence mechanisms, whereby the extent of these responses appeared to be associated with a single and transient burst of ROS (reactive oxygen species) *in planta*.

1x10-9M for both strigolactone analogues proved to be the most effective in reducing symptom development in own rooted tobacco and grapevine plantlets.

(±)-GR24 and Nijmegen-1 applied to grafted 1-year old Cabernet Sauvignon and Sauvignon Blanc vines did not (even after repeated applications) cause a decrease in susceptibility to *B. cinerea* when challenged using detached leaf assays.

Recommendation: we have only been able to do 'proof-of-concept' trials. It is recommended that small-scale nursery trials should be conducted by industry to assess efficacy of (±)-GR24 and Nijmegen-1 as potential anti-fungal agents.

9. PLANNED OUTPUTS

a) TECHNOLOGY DEVELOPMENT, PRODUCTS AND PATENTS

The thesis of Ms Dominic Vogel (MSc) (2019) will be embargoed for a limited period i.e. protected (not placed in the public domain) until it is determined if the work (method and approach) has sufficient value or not for final transfer to the industry.
Strigolactones are in the public domain e.g. Strigolab (https://strigolab.eu/) and the compounds are not patentable, it is uncertain if the application can be protected.

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

Evaluating the strigolactone analogues in a nursery trial will determine if these compounds could be used by industry.

c) HUMAN RESOURCES DEVELOPMENT / TRAINING (STUDENTS)

Student Name and Surname	Student Nationality	Degree (eg Hons, MSc)	Level of studies in final year of project	Total Bursary Cost for Industry for entire project
Honours				
Kerry Ann Jordaan	South African	BSc (Hons)	1	R 0
Masters				
Dominic Vogel	South African	MSc	3	R 180000
Yolani Furunek	South African	MSc	1	R 45000
Brock Kuhlman	American	MSc	2	R 16853
PhD				
Florent Weiller	French	PhD	5	R 92000
Postdocs				

d) LIKELY PUBLICATIONS (POPULAR, PRESS RELEASES, SCIENTIFIC)

Dominic Vogel, Kerry Ann Jordaan, Florent Weiller, Paul N. Hills, John P. Moore. 2021. A role for strigolactones in protecting Nicotiana tabacum and Vitis vinifera plants against the necrotrophic fungal pathogen Botrytis cinerea. Journal of Plant Physiology. (in preparation)

Kerry Ann Jordaan, Paul Hills, John P. Moore. 2021. Investigating the role of strigolactones in plant defence against biotic stresses. South African Journal of Botany (or) South African Journal of Enology and Viticulture (in preparation).

John P. Moore, Dominic Vogel, Kerry Ann Jordaan, Florent Weiller, Paul N. Hills. 2021. Towards protecting grapevine using natural bioprotectants: Priming for protection. Wineland (in preparation).

e) PRESENTATIONS/PAPERS THAT COULD BE DELIVERED

Dominic Vogel, Paul N. Hills, John P. Moore. 2017. Evaluating the role of natural plant derived compounds in modifying disease defence mechanisms in Nicotiana tabacum and Vitis vinifera plantlets in South African Journal of Botany, South African Association of Botanists Conference, 8-11th January, Cape Town, South Africa

Dominic Vogel. 2019. (MSc in Wine Biotechnology) Evaluating the role of natural plant derived compounds in modifying disease defence mechanisms in Nicotiana tabacum and Vitis vinifera plantlets (Supervisor with Dr Paul Hills) Stellenbosch University, MSc thesis (online open access, embargoed)

Florent Weiller. 2020. (PhD in Wine Biotechnology) Cell wall profiling of tobacco and grapevine in the context of Botrytis cinerea infection. Stellenbosch University, PhD thesis in preparation

(online open access)

10. PROJECT OUTCOME AND IMPACT

New Knowledge	Benefits Supply Chain	Direct Grower Application	Direct Packhouse/Winery/ Cellar Application	Other
X		X		

Other is:

The Value of the project to industry

Creates new knowledge on strigolactone usage for the industry in enhancing disease resistance of plant material and potentially provides a method to protect nursery vines for growers.

11. PERSONS PARTICIPATING IN THE PROJECT:

INITIALS AND SURNAME	HIGHEST QUALIFICATION	RACE (M,W)	GENDER (M,F)	INSTITUTE DEPARTM	POSITION	TOTAL COST TO PROJECT
RESEARCH PERSONNEL						R 20000
Charmaine Stander	MSc	W	F	Stellenbosch University, Institute for Wine Biotechnology T/DVO	TA	R 20000
Paul Hills	PhD	W	M	Stellenbosch University, IPB (Plant Biotechnology)	Coll	R 0
Melane A. Vivier	PhD	W	F	Stellenbosch University, SA Grape and Wine Research Institute/DVO	Coll	R 0
SUPPORT PERSONNEL						R 47409
Leigh Hendricks	NQF 5 International Certificate in Computer Studies	B	F	Stellenbosch University, SA Grape and Wine Research Institute/DVO	Finance Officer	R 47409.42

POSITION: Co = Co-worker (other researcher at your institution)

Coll = Collaborator (participating researcher that does not receive funding for this project from industry)
PF = Post-doctoral fellow
PL = Project leader
RA = Research assistant
TA = Technical assistant/ technician

12. TOTAL COST OF PROJECT

TOTAL ANNUAL COSTS (ALL YEARS)	CFPA	Raisin SA	HORTGRO	SATI	WINETECH	ARC	OTHER	TOTAL
2015	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0
2016	R 0	R 0	R 0	R 0	R 200000	R 0	R 0	R 200000
2017	R 0	R 0	R 0	R 0	R 216000	R 0	R 0	R 216000
2018	R 0	R 0	R 0	R 0	R 240700	R 0	R 0	R 240700
2019	R 0	R 0	R 0	R 0	R 202142	R 0	R 0	R 202142
2020	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0
TOTAL	R 0	R 0	R 0	R 0	R 858842	R 0	R 0	R 858842