

Elucidating the dual physiological induced effect of gliotoxin on plants

J.J. BEZUIDENHOUT

Thesis submitted for the degree *Philosophiae Doctor* in Microbiology at the Potchefstroom Campus of the North-West University

Promotor: Prof. L. van Rensburg

Assistant promoter: Mr. P.J. Jansen van Rensburg

May 2012

Contents

DECLARATION	iii
SUMMARY	iv
OPSOMMING	vi
ACKNOWLEDGEMENTS	viii
LIST OF FIGURES	ix
LIST OF TABLES	xii
ABBREVIATIONS	xiv
CHAPTER 1: INTRODUCTION AND RATIONALE	1
1.1 <i>Trichoderma</i>	1
1.2 Biological control aspects of <i>Trichoderma</i>	1
1.3 Growth promotion and crop enhancement aspects	3
1.4 Specific aims and objectives	3
CHAPTER 2 – LITERATURE REVIEW	5
2.1 Fungi as plant pathogens	5
2.2 Fungi as plant pathogens - various treatment options	6
2.2.1 Physical and chemical control	6
2.2.2 Biological control	7
2.3 <i>Trichoderma</i>	7
2.3.1 <i>Trichoderma harzianum</i> T39 as biological control agent (BCA)	8
2.3.2 <i>Trichoderma</i> – biological control - aspects	8
2.3.2.1 <i>Trichoderma</i> – biological control - enzymes	9
2.3.2.2 <i>Trichoderma</i> – biological control - gliotoxin	9
2.4 Gliotoxin	9
2.4.1 Gliotoxin risk – immunomodulation effects	10
2.4.2 Apoptosis and gliotoxin	10
2.5 Plant Hormones	12
2.5.1 Gibberellic acid	12
2.5.1.1 Gibberellic acid and germination	13
2.5.1.2 Gibberellic acid and germination – alpha-amylases	14
2.5.2 Gibberellic acid signalling	14
2.5.2.1 Gibberellin Insensitive Dwarf 1 (GID1)	14

2.5.2.2 Gibberellin Insensitive Dwarf 1 (GID1) and signalling	15
2.5.2.3 Gibberellin Insensitive Dwarf 1 (GID1) GA binding	16
2.6 Effect of treatments on plants – study methods.....	16
2.6.1 Transcript analysis.....	17
2.6.2 Protein analysis	17
2.6.3 Metabolite analysis	18
2.6.4 Bioinformatics	18
2.6.5 Molecular modelling	19
2.6.5.1 Docking	19
2.6.5.2 Pharmacophore description	20
2.6.6 Germination and plant height	21
2.6.7 Chlorophyll <i>a</i> fluorescence F ₀ -J-I-P transient test (JIP test)	21
2.7 Gibberellic acid gliotoxin (GA GT) hypothesis	24
CHAPTER 3 – MATERIALS AND METHODS	27
3.1 Molecular modelling and docking	27
3.2 Molecular similarity	27
3.3 Molecular docking.....	27
3.4 Germination: Plant height and emergence	27
3.5 Germination: Zymogram	28
3.6 Chlorophyll fluorescence transient (OJIP)	31
CHAPTER 4 – RESULTS AND DISCUSSION.....	34
4.1 Results from common pharmacophore generation.....	34
4.3 Comparison of plant height and emergence.....	41
4.4. Comparison of α -amylase induction.....	43
4.5 Comparison of JIP-fluorescence responses	45
CHAPTER 5 - CONCLUSIONS.....	65
REFERENCES.....	68
APPENDIX A.....	80

DECLARATION

I declare that the dissertation submitted by me for the degree of *Philosophiae Doctor* in Natural Sciences at the North-West University (Potchefstroom Campus), Potchefstroom, North West, South Africa, is my own independent work and has not previously been submitted by me at another university.

Signed in Potchefstroom, South Africa

Signature:

Johannes Jacobus Bezuidenhout

SUMMARY

Fungi and Oomycetes represent the two most important groups of eukaryotic plant pathogens. Besides chemical and physical control of these pathogens, biological control is an approach enjoying increasingly more focus. One of the biological agents increasingly employed in biological control of plant pathogenic fungi is ironically the fungus *Trichoderma*, more specifically *Trichoderma harzianum*. Besides control of the fungal plant pathogens, another interesting aspect observed when plants are treated with *Trichoderma harzianum* are effects such as complete and even stand of plants, faster seed germination, increases in plant height and overall enhanced plant growth. Though there have been various studies on this effect, almost no research has yet been conducted to elucidate the mechanism by which these effects occur. In particular, effects such as faster seed germination suggest that *Trichoderma harzianum* produces a metabolite that may mimic the plant growth hormone gibberellic acid. Through an evaluation of the various metabolites produced by *Trichoderma harzianum*; gliotoxin seemed structurally most similar to gibberellic acid. To verify that gliotoxin can indeed serve as an analogue for gibberellic acid and elicit similar physiological responses in plants, a two-pronged approach was followed.

Firstly, molecular similarity evaluation through common pharmacophore evaluation was conducted, followed by docking simulations into the recently discovered receptor for gibberellic acid. Common pharmacophore evaluation between gibberellic acid and gliotoxin showed successful alignment of gliotoxin into the gibberellic acid based pharmacophore space. Furthermore, docking simulations further strengthened this by the similarity in docking scores calculated and the similar poses of the ligands (gliotoxin and gibberellic acid) in the receptor space. However, similarity in pharmacophore alignment and docking simulation results only suggest that gliotoxin should be able to occupy the receptor space, but it is not a guarantee that similar physiological responses will be elicited.

In the second part of the project, the ability of gliotoxin to elicit similar physiological responses in plants to gibberellic acid was investigated. For this, α -amylase induction; plant emergence and height; and chlorophyll fluorescence were compared for both gliotoxin and gibberellic acid treatments. In terms of α -amylase induction, gliotoxin was able to induce production of the enzyme as visualised by starch-containing native gel electrophoresis (zymograms). Gliotoxin induced the strongest response at a 10^{-6} M dilution which is

typically the range expected for hormones in biological systems in de-embryonated seeds of *Phaseolus vulgaris*. Gibberellic acid was able to induce the strongest response at a 10^{-7} M dilution. In essence, similar physiological responses were observed. In terms of plant emergence and plant height, treatment with gliotoxin or gibberellic acid resulted in plant emergence a day earlier than the untreated control. However, even though there were slight differences in plant height favouring the gliotoxin or gibberellic acid treated plants, the differences were not statistically significant. Thus, in this regard similar responses were again observed for both gliotoxin and gibberellic acid treatments. In the final evaluation the effect of gliotoxin and gibberellic acid treatments on the chlorophyll fluorescence of mature plants was investigated. Overall, both gliotoxin and gibberellic acid elicited beneficial effects on plant vitality, expressed through $PI_{(Abs)}$ with the gliotoxin treatment performing better than the equivalent gibberellic acid treatment.

Overall, the physiological tests demonstrated that gliotoxin can indeed elicit similar positive physiological responses to gibberellic acid in *Phaseolus vulgaris*. Furthermore the test used in this project can serve as a standard evaluation bench for screening for gibberellic acid analogues on a laboratory scale before larger scale field trials are considered.

Keywords: gibberellic acid, gliotoxin, GID1, molecular similarity, *Trichoderma harzianum*

OPSOMMING

Fungi en Oomycetes verteenwoordig die twee mees belangrike groepe van eukariotiese plantpatogene. Buiten chemiese en fisiese beheer van hierdie plantpatogene geniet veral biologiese beheer van plantpatogene toenemend meer fokus. Een van die biologiese agente wat vir die biologiese beheer van plantpatogeniese fungi toegepas word, is ironies genoeg die fungus *Trichoderma*, meer spesifiek *Trichoderma harzianum*. Buiten die beheer van die plantpatogene is 'n ander interessante verskynsel wat waargeneem word in plante wat met *Trichoderma harzianum* behandel word, soos gelyke stand van plante, vinniger saadontkieming, toename in plant hoogte en algeheel verbeterde plant groei. Alhoewel daar verskeie studies oor hierdie effek bestaan, is daar bykans geen navorsing oor die meganisme verantwoordelik vir hierdie waarnemings. Veral effekte soos vinniger saadontkieming lei tot die gevolgtrekking dat *Trichoderma harzianum* 'n metaboliet produseer wat die planthormoon gibberelliensuur naboots. Deur 'n evaluasie van die verskeie metaboliete wat deur *Trichoderma harzianum* geproduseer word, is gliotoksien geïdentifiseer as die verbinding wat die grootste strukturele ooreenkomste met gibberelliensuur toon. Om te bevestig dat gliotoksien inderdaad as 'n analoog vir gibberelliensuur kan dien en soortgelyke fisiologiese response in plante kan uitlok, is 'n tweeledige benadering gevolg.

Eerstens is die molekulêre ooreenkomstigheid ondersoek deur 'n gemeenskaplike farmakofoor-evaluasie, gevolg deur molekulêre passingsimulasies in die nuut-ontdekte reseptor vir gibberelliensuur. Gemeenskaplike farmakofoor-evaluasie van gliotoksien en gibberelliensuur het suksesvolle belyning in die gibberelliensuur-gebaseerde farmakoforiese ruimte. Verder het die passingsimulasies die hipotese versterk deur berekening van soortgelyke posisies vir gliotoksien en gibberelliensuur. Alhoewel ooreenkomste in farmakofoorbelyning en passingsimulasies inderdaad toon dat gliotoksien dieselfde reseptorspasia kan beset, is dit steeds nie 'n waarborg dat soortgelyke fisiologiese response uitgelok sal word nie.

In die tweede deel van die projek is die vermoë van gliotoksien om soortgelyke fisiologiese response aan gibberelliensuur uit te lok, ondersoek. Vir hierdie evaluasies is response soos α -amilase induksie, plant-uitkoms en hoogte, en chlorofilfluoresensie vergelyk vir beide gliotoksien- en gibberelliensuurbehandelings. Met betrekking tot α -amilase induksie, was gliotoksien in staat om produksie van die ensiem te induseer soos gevisualiseer deur

styselbevattende gel-elektroforese (“zymograms”). Gliotoksien het die sterkste respons geïnduseer by ‘n 10^{-6} M verdunning, wat die tipiese konsentrasie is van hormone in biologiese stelsels in gede-embrioneerde sade van *Phaseolus vulgaris*. Gibberelliensuur het die sterkste induksie van α -amilase getoon by ‘n 10^{-7} M verdunning. In beginsel is daar dus soortgelyke fisiologiese response waargeneem vir hierdie aspek. In terme van plant-uitkoms en hoogte het behandeling met gliotoksien of gibberelliensuur veroorsaak dat die behandelde plante ‘n dag vroeër as die kontrole uitgekome het. Verder, alhoewel daar geringe verskille in die gliotoksien- en gibberelliensuurbehandeling was in terme van plant hoogte, was die verskille nie statisties betekenisvol nie. Weereens ook in hierdie aspek is daar dus soortgelyke response tussen gliotoksien en gibberelliensuur waargeneem. Met die evaluasie van die effekte van gliotoksien en gibberelliensuur behandelings op die chlorofilfluoresensie van volwasse plante is die volgende waargeneem. Beide gliotoksien en gibberelliensuur het voordelige effekte in die plante uitgelok, soos uitgedruk deur die $PI_{(Abs)}$, met gliotoksienbehandelings wat beter presteer het as die ooreenstemmende gibberelliensuurbehandelings.

Opsommend kan gesê word dat gliotoksien wel in staat is om soortgelyke positiewe fisiologiese response as gibberelliensuur in *Phaseolus vulgaris* teweeg te bring. Verder kan die reeks toetste wat in die studie gebruik is as ‘n toetsreeks gebruik word vir die evaluasie van gibberelliensuuranaloe in die laboratorium alvorens daar oorgegaan word na veldproewe.

Sleutelwoorde: gibberelliensuur, gliotoksien, GID1, molekule ooreenkoms, *Trichoderma harzianum*

ACKNOWLEDGEMENTS

Prof. Leon van Rensburg, my project supervisor, for all those think-tank sessions and unfailing help and guidance throughout the entire project. Also many thanks for the proof-reading of this dissertation.

Riaan Strauss and Misha de Beer for always being willing to help and advise me when I felt out of my depth and familiarising me with the plant physiology-based techniques.

Dr. Sandra Barnard and Dr. Jacques Berner for always being willing to assist me with a critical second opinion.

Dr. Sarina Claassens for proof-reading this dissertation.

Prof. Carlos Bezuidenhout for always being available when advice was needed.

Karen Jordaan for all the advice and assistance with the molecular techniques.

Bennie Repsold from Pharmaceutical Chemistry, for all his assistance in aspects of molecular modelling.

And last, but not least, my friends and family for their encouragement and support.

LIST OF FIGURES

	Page number
Figure 2.1: Structure of gliotoxin (Grovel <i>et al.</i> , 2006).	9
Figure 2.2: Proposed gliotoxin-induced apoptosis pathway (Adapted from Waring, 1990).	11
Figure 2.3: Structure of gibberellic acid 3 (GA ₃) from Murase <i>et al.</i> , 2008.	13
Figure 2.4a: Ball and stick representation of the structures of GA ₃ (left) and gliotoxin (right).	25
Figure 2.4b: Space filling sphere representation of the structures of GA ₃ (left) and gliotoxin (right).	25
Figure 4.1.1 a (left, side view) and b (right, top view): Structure of GA ₃ as aligned to the common pharmacophore model. (Colour coding employed by CATALYST: Magenta = hydrogen bond donor, Light Blue = hydrophobic region, Green = hydrogen bond acceptor).	34
Figure 4.1.2 a (left, side view) and b (right, top view): Structure of gliotoxin as aligned to the common pharmacophore model. (Colour coding employed by CATALYST: Magenta = hydrogen bond donor, Light Blue = hydrophobic region, Green = hydrogen bond acceptor).	35
Figure 4.1.3 a (left) and b (right): Numbered structures for gibberellic acid (left) and gliotoxin (right).	35
Figure 4.1.4 a (left, side view) and b (right, top view): Stick representation of GA ₃ (red) and gliotoxin (green) as aligned to common pharmacophore model.	36
Figure 4.2.1 a (left) and b (right): GA ₃ binding within the binding site of 3ED1. Stick representation - left, and the sphere representation – right.	37
Figure 4.2.2 a (left) and b (right): GA ₃ (Generated with CORINA) binding within the binding site of 3ED1. Stick representation – left and sphere representation – right.	38
Figure 4.2.3 a (left) and b (right): Comparison of the docking poses for the GA ₃ molecule crystallised with 3ED1 (blue) and the GA ₃ molecule from CORINA. Docking poses with the binding site displayed (a) and docking poses with the binding site removed (b) for greater clarity. In Figure 4.2.3 b, the GA ₃ molecule within 3ED1 is represented as wireframe and the GA ₃ from CORINA is represented by a stick representation.	38
Figure 4.2.4 a (left) and b (right): GT (Generated with CORINA) binding within the binding site of 3ED1. Stick representation – left and sphere representation – right.	39
Figure 4.2.5 a (left) and b (right): Comparison of the docking poses for the GA ₃ molecule crystallised with 3ED1 (red) and the GT molecule from CORINA (green). Docking poses with the binding site displayed (a) and docking poses with binding site not displayed (b) for greater clarity. In Figure 4.2.5 b, the GA ₃ molecule from CORINA is represented as wireframe and the GT from Corina is represented by a stick representation.	39

- Figure 4.2.6 a (left) and b (right):** Comparison of the docking poses for the GA₃ molecule crystallised with 3ED1 (blue) and the GT molecule from CORINA (green). Docking poses with the binding site displayed (a) and docking poses with binding site not displayed (b) for greater clarity. The GA₃ molecule within 3ED1 (Figure 4.2.6 b) is represented as wireframe and the GT from CORINA is represented as a stick representation. **40**
- Figure 4.3.1 a (left) and Figure 4.3.1 b (right):** Plant height for gibberellic acid (GA) treatment and gliotoxin (GT) treatment. GA4 through GA8 represents a dilution series of GA₃ starting at 10⁻⁴ M GA (GA4) through 10⁻⁸ M GA (GA8); GT4 through GT8 represents a dilution series of gliotoxin starting at 10⁻⁴ M GT (GT4) through 10⁻⁸ M GT (GT8). **42**
- Figure 4.4.1:** Zymogram of de-embryonated seeds treated with gibberellic acid. NC = negative control, PC = positive control; GA1 through GA6 represents a dilution series starting at 10⁻³ M GA (GA1) through 10⁻⁸ M GA (GA6). **44**
- Figure 4.4.2:** Zymogram of de-embryonated seeds treated with gliotoxin. NC = negative control, PC = positive control; GT1 through GT6 represents a dilution series starting at 10⁻³ M GT (GT1) through 10⁻⁸ M GT (GT6). **44**
- Figure 4.5.1 a (top) and b (bottom):** Spider plot summary of key Kautsky transient parameters at day 8 after treatment with growth regulators. Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin in a dilution series range from 10⁻⁴ M through 10⁻⁸ M. **47**
- Figure 4.5.2 a (top) and b (bottom):** Summary of percentage change in PI_(Abs) for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to a serial dilution series of GT of 10⁻⁴ M through 10⁻⁸ M. **49**
- Figure 4.5.3 a (top) and b (bottom):** Summary of percentage change in ET₀/RC for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to a serial dilution series of GT of 10⁻⁴ M through 10⁻⁸ M. **51**
- Figure 4.5.4 a (top) and b (bottom):** Summary of percentage change in ET₀/CS₀ for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to a serial dilution series of GT of 10⁻⁴ M through 10⁻⁸ M. **53**
- Figure 4.5.5 a (top) and b (bottom):** Summary of percentage change in ABS/RC for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to a serial dilution series of GT of 10⁻⁴ M through 10⁻⁸ M. **55**

- Figure 4.5.6 a (top) and b (bottom):** Summary of percentage change in ΦE_0 for gibberellic acid 3 (GA_3) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA_3 of 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to a serial dilution series of GT of 10^{-4} M through 10^{-8} M. **57**
- Figure 4.5.7 a (top) and b (bottom):** Summary of percentage change in Ψ_0 for gibberellic acid 3 (GA_3) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA_3 of 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to a serial dilution series of GT of 10^{-4} M through 10^{-8} M. **59**
- Figure 4.5.8 a (top) and b (bottom):** Summary of percentage change in F_v/F_0 for gibberellic acid 3 (GA_3) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA_3 of 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to a serial dilution series of GT of 10^{-4} M through 10^{-8} M. **61**

LIST OF TABLES

	Page number
Table 1.1: Summary of plant pathogens controlled by <i>Trichoderma</i> -based formulations.	2
Table 2.1: Summary of JIP-test parameters.	23
Table 4.3.1: Summary of plant heights per treatment (n = 10). (Statistical means ± standard error). GA4 through GA8 represents a dilution series of GA ₃ starting at 10 ⁻⁴ M GA (GA4) through 10 ⁻⁸ M GA (GA8); GT4 through GT8 represents a dilution series of gliotoxin starting at 10 ⁻⁴ M GT (GT4) through 10 ⁻⁸ M GT (GT8).	42
Table 4.3.1: Summary of plant height per treatment (n = 10) (Continued).	43
Table 4.5.1: Summary of Performance Index (PI _(Abs)) results (Statistical means ± standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA ₃) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M.	45
Table 4.5.2: Summary of normalised PI _(Abs) data with Day 0 (D0) serving as baseline. (Statistical means ± standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA ₃) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M.	46
Table 4.5.3: Summary of normalised ET ₀ /RC data with Day 0 (D0) serving as baseline. (Statistical means ± standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA ₃) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M.	52
Table 4.5.4: Summary of normalised ET ₀ /CS ₀ data with Day 0 (D0) serving as baseline. (Statistical means ± standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA ₃) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10 ⁻⁴ M through 10 ⁻⁸ M.	54

- Table 4.5.5:** Summary of normalised ABS/RC data with Day 0 (D0) serving as baseline. **56**
 (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.
- Table 4.5.6:** Summary of normalised ΦE_0 data with Day 0 (D0) serving as baseline. **58**
 (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.
- Table 4.5.7:** Summary of normalised Ψ_0 data with Day 0 (D0) serving as baseline. **60**
 (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.
- Table 4.5.8:** Summary of normalised F_v/F_0 data with Day 0 (D0) serving as baseline. **62**
 (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.

ABBREVIATIONS

µg	Microgram
3D	Three dimensional
6PP	6- <i>n</i> -pentyl-6 <i>H</i> -pyran-2-one or 6-pentyl- α -pyrone
ABA	Abscisic acid
Abs	Absorption energy flux
ANOVA	Analysis Of Variance
BCA	Biological control agent
CS	Excited cross section of leaf sample
DNA	Deoxyribonucleic acid
g	Gram
GA(s)	Gibberellic acid(s) or Gibberellin(s)
GID1	Gibberellin-Insensitive Dwarf1
GT	Gliotoxin
JIP Test	Chlorophyll <i>a</i> fluorescence F ₀ -J-I-P transient test
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
NC	Negative control
PAGE	Polyacrylamide gel electrophoresis
PC	Positive control
PEA	Plant Efficiency Analyser
pI	Isoelectric point
PSI	Photosystem I
PSII	Photosystem II
RC	Reaction centre
SCF	SKP1-CULLIN-F-Box
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
v/v	Volume per volume

w/v

α -amylase

Weight per volume

Alpha-amylase

CHAPTER 1: INTRODUCTION AND RATIONALE

1.1 *Trichoderma*

Trichoderma is a fungal genus that occurs worldwide (ubiquitous) and can easily be isolated from soil, wood and other forms of decaying plant organic matter. This genus is classified as *fungi imperfecti* due to the absence of a sexual stage in the reproduction of this fungus. *Trichoderma* exhibit a high growth rate in culture and production of numerous spores (conidia) that are various shades of green. The underside of the colonies is often uncoloured, buff, yellow, amber, or yellow-green and many species produce prodigious quantities of thick-walled spores (chlamydo spores) in submerged culture (Howell, 2003).

As early as 1930 the potential of *Trichoderma* to serve as a biological control agent was recognised and research is increasing the list of diseases controlled by this genus of fungus. This has led to the commercial production of several *Trichoderma* species and *Trichoderma*-based products in countries such as Israel, New Zealand, India, Sweden and South Africa for crop-protection and growth enhancement (Howell, 2003).

In order to effectively apply *Trichoderma* it is essential to study the mechanism involved in both the crop protection aspects and growth enhancement. This will also assist in the registration of the product to ensure compliance with the relevant guidelines for safety and responsible use (Howell, 2003).

1.2 Biological control aspects of *Trichoderma*

Several studies reported the production of an antibiotic compound by *Trichoderma* species and of particular interest is the compound gliotoxin. Research into fungal-fungal antibiotic compounds is however not as far advanced as those for antibiotics targeting bacteria. Literature indicates that gliotoxin exposure results in cytoplasmic leakage of the pathogenic fungi. Another aspect which must however also be considered is the toxicity of gliotoxin to humans and animals (Waring & Beaver, 1996; Lewis *et al.*, 2005; Grovel *et al.*, 2006).

Another mechanism by which *Trichoderma* antagonises pathogenic fungi is through the secretion of extracellular enzymes, glucanases and particularly exochitinase. It is thought that these enzymes attack and disrupt or weaken the pathogenic fungi's cell walls thereby destroying cell wall integrity (Vey *et al.*, 2001).

A rather outstanding characteristic of the genus *Trichoderma* is their ability to parasitise other fungi. This ability of mycoparasitism has been applied as biocontrol for various fungal plant diseases, with several studies done on *Rhizoctonia solani*. Refer to Table 1.1 for a summary of plant pathogens managed by *Trichoderma*-based formulations and products. During this process, the hyphae of the biocontrol agent coils around the target pathogen and penetrates the cell wall, resulting in the dissolution of the host cytoplasm. This phenomenon occurs regardless of the supply of external nutrients to either the host or the mycoparasite (Vey *et al.*, 2001).

Table 1.1: Summary of plant pathogens controlled by *Trichoderma*-based formulations.

Control agent	Pathogen	Crop Disease	Reference
<i>T. harzianum</i> (T39) (Trichodex)	<i>Botrytis cinerea</i>	Grey mould (lettuce)	Card <i>et al.</i> , 2002
<i>T. virens</i>	<i>Pythium ultimum</i> <i>Rhizoctonia solani</i>	Damping of cotton seedlings	Hanson, 2000
<i>T. virens</i>	<i>Verticillium dahliae</i>	Verticillium wilt	Hanson, 2000
<i>T. harzianum</i> <i>T. aureoviride</i>	<i>Pyrenophora tritici-repentis</i> (anamorph= <i>Drechslera</i> <i>tritici-repentis</i>)	Tan spot of wheat	Perello <i>et al.</i> , 2003
<i>T. harzianum</i> (T39) (Trichodex)	<i>Botrytis cinerea</i> <i>Pseuoperonospora cubensis</i> <i>Sclerotinia sclerotiorum</i> <i>Sphaerotheca fusca</i> (syn. <i>S.</i> <i>fuliginea</i>)	Various foliar pathogens	Elad, 2000.

1.3 Growth promotion and crop enhancement aspects

Comparison of the structures of gliotoxin and gibberellic acid indicate a high degree of similarity, leading to the possibility that gliotoxin may act as an analogue molecule to gibberellic acid in plants. Gibberellic acid, also referred to as gibberellin, serves various developmental functions in plants:

- Stimulates stem elongation (especially marked in dwarf and rosette plants)
- Stimulates seed germination
- Enhances digestion of storage reserves during germination of cereal grasses
- Stimulates parthenocarpy (fruit set)
- Stimulates trichome development
- Action often antagonised by abscisic acid (ABA)

However, these characteristics have also been observed where *Trichoderma* based products, has been applied (Howell, 2003 and references therein), leading to the hypothesis that gliotoxin may serve as a structural analogue for gibberellic acid in plants.

1.4 Specific aims and objectives

The project was envisioned to serve as a case study for the methodology employed. Two stages were envisioned for this project. The first stage was a screening stage during which molecular similarity between gliotoxin and gibberellic acid were evaluated using common pharmacophore modelling and docking to a receptor protein. During the second stage the physiological responses were elucidated. We investigated the effect of gliotoxin treatments in comparison with gibberellic acid treatments on germination, growth and chlorophyll a fluorescence.

Specific aims identified were as follows:

- Evaluation of the molecular similarity between gliotoxin and gibberellic acid using molecular similarity software;
- Evaluation of the molecular similarity between gliotoxin by docking simulations to a selected ligand;
- Comparison between gliotoxin and gibberellic acid in α -amylase expression in de-embryonated seeds using zymograms;
- Comparison between gliotoxin and gibberellic acid in enhancing seed germination using seedling emergence data; and
- Comparison between gliotoxin and gibberellic acid by comparison of effects on mature plants using fluorescence data.

CHAPTER 2 – LITERATURE REVIEW

2.1 Fungi as plant pathogens

Fungi and Oomycetes represent the two most important groups of eukaryotic plant pathogens (Latijnhouwers *et al.*, 2003). Overall, fungi represent a significant threat to plants, for example, in the United States, 12 out of the 19 most threatening plant pathogens are fungi (Maor & Shirasu, 2005). The incidence of fungal diseases of plants can be a severe limiting factor in the production of various crops and plants of interest for various applications. Crops affected include, amongst others, the following:

- Cacao (Krauss & Soberanis, 2001);
- Grapes (Harman *et al.*, 1996; Latorre *et al.*, 1997);
- Tomatoes (Datnof *et al.*, 1995);
- Tea (*Camellia sinensis*), coffee (*Coffea arabica*), avocado (*Persia americana*), banana (*Musa acuminata*), pine (*Pinus* spp.), eucalyptus (*Eucalyptus* spp.) and cypress (*Cupressus* spp.) (Otieno *et al.*, 2003a)

The plant pathogenic fungi can basically be divided into two groups based on their nutrition strategy, namely necrotrophs and biotrophs. The necrotrophs can also be referred to as perthotrophs to emphasise the fact that they first kill the host cells before colonisation. This killing of the host cells can be accomplished by the secretion of toxins and extracellular enzymes by the attacking fungi. Biotrophs on the other hand, depend on the metabolism of the host cells and surrounding tissues. Some biotrophs will delay host cell death until the completion of reproduction, while others will simply switch over to a saprophytic phase following the collapse and death of the host cells. Even if a biotrophic fungus does not kill cells or tissues, the burden placed on the plant metabolism may render the plant more susceptible to various stresses. Plant death can thus still occur during biotrophic infection by the continuous withdrawal of nutrients and secretion of waste products by the pathogenic fungi (Prell & Day, 2001).

2.2 Fungi as plant pathogens - various treatment options

The main approaches for the control of fungal pathogens of plants are usually chemical, biological, or integrated management, where both the biological and chemical control measures are combined.

2.2.1 Physical and chemical control

Physical control of fungal plant pathogens involves approaches such as cold treatment, heat treatment, radiation and physical removal of the infected plants. Though successfully applied against a variety of plant pathogens, these approaches do suffer from certain limitations (Otieno *et al.*, 2003b).

This is particularly the case with *Armillaria*. *Armillaria* affects a variety of plants and crops such as tea (*Camellia sinensis*), coffee (*Coffea arabica*), avocado (*Persia americana*), banana (*Musa acuminata*), pine (*Pinus* spp.), eucalyptus (*Eucalyptus* spp.) and cypress (*Cupressus* spp.) To control this disease, physical removal of root and stump remnants is in essence the single most effective way to minimise the incidence of *Armillaria*. This approach is however difficult to perform over large areas of land when the site needs to be prepared for the next planting (Otieno *et al.*, 2003a). Also approaches such as heat treatment and chemical fumigation does not guarantee complete control of the pathogen as soil depth and structure can limit the efficacy of the selected treatment (Otieno *et al.*, 2003b).

In some cases the chemical control of fungal diseases in crop plants can be completely unsuccessful. This has been demonstrated in the case of cacao, particularly in the Latin America region (Krauss & Soberanis, 2001).

Besides growing concern about the health effects of synthetic chemical pesticides on consumers, another factor against chemical control of fungal pathogens in plants is that increasing resistance against the fungicides are being observed (Datnof *et al.*, 1995; Nemeč *et al.*, 1996). These factors serve as driving factors for the development of more environmentally friendly technologies.

2.2.2 Biological control

Biological control can be defined as "The action of parasites, predators or pathogens in maintaining another organism's population density at a lower average than would occur in their absence" (De Bach, 1964: cited in Siddiqui & Mahmood, 1996).

Biological control can usually be implemented during the active production phase, as well as post-harvest to protect both plant and product (Nemec *et al.*, 1996; Batta, 2004). Further discussion will focus mainly on protection of the plant itself.

During biological control, antagonistic organisms such as bacteria and fungi can be applied (Nemec *et al.*, 1996; Gracia-Garza *et al.*, 1997; Hervás *et al.*, 1998). Biological control of fungal pathogens can even combine either bacteria or fungi with other organisms such as insects. One example of this is the use of fungus gnats combined with *Trichoderma* spp. to control *Sclerotinia sclerotiorum* (Gracia-Garza *et al.*, 1997). Furthermore, non-pathogenic strains of the usual pathogen can also be employed (Hervás *et al.*, 1998). Fungal biocontrol agents can also be employed against crop threats such as nematodes (Siddiqui & Mahmood, 1996).

2.3 *Trichoderma*

As early as 1930 the potential of *Trichoderma* to serve as a biological control agent was recognised and research is increasing the list of diseases controlled by this genus of fungus. This has led to the commercial production of several *Trichoderma* species and *Trichoderma*-based products in countries such as Israel, New Zealand, India, Sweden and South Africa for crop-protection and growth enhancement (Howell, 2003).

Literature reports that certain *Trichoderma* strains are known to produce a variety of classes of bioactive metabolites such as antibiotics of the peptaibols class, as well as inhibitors of fungal growth of a mainly terpenic nature (Mannina *et al.*, 1997). Another compound of interest from *Trichoderma* is 6-pentyl- α -pyrone (Landreau *et al.*, 2002; Vinale *et al.*, 2008a). Overall, the production of secondary metabolites in *Trichoderma* species is strain dependent and includes volatile and non-volatile antifungal substances such as 6-*n*-pentyl-6*H*-pyran-2-

one (6PP or 6-pentyl- α -pyrone), gliotoxin, viridin, harziaopyridone, harziandione and peptaobols (Vinale *et al.*, 2008a).

The compound 6PP is a compound produced by many *Trichoderma* species (Dod *et al.*, 2000; Landreau *et al.*, 2002; Vinale *et al.*, 2008a). The compound exhibits no phytotoxic effects against crops and is thought to play a role in biocontrol of fungal pathogens by *Trichoderma*. Previous research has demonstrated control of *Athelia rolfsii* (Curzi) (= *Sclerotium rolfsii* Sacc.) by this compound (Dod *et al.*, 2000).

2.3.1 *Trichoderma harzianum* T39 as biological control agent (BCA)

Trichoderma harzianum, particularly isolate T39, is regarded as the model for illustration of biocontrol of pathogens and the mechanisms involved. The biocontrol activity of this particular strain is multi-faceted as it involves both the fungus itself as well as excretions (enzymes and secondary metabolites) that can function independently of the producing fungus (Elad, 2000).

A table containing examples of fungal pathogens and biological control agents applied against them for various plants and crops are contained in Table A1 in Appendix A.

2.3.2 *Trichoderma* – biological control - aspects

One of the most striking aspects of the genus *Trichoderma* is its mycoparasitism. This led Weindling (1934) to attribute biocontrol of *Rhizoctonia solani* to mycoparasitism by *Trichoderma* and describe the process in detail. The process involves coiling of *Trichoderma* around the pathogen hyphae, penetration of the hyphae and finally dissolution of the host cytoplasm. What is of particular interest is that this process occurs regardless of a sufficient supply of external nutrients. Other mechanisms employed by *Trichoderma* in the biocontrol of fungal plant pathogens include the secreting of various hydrolytic enzymes (chitinases and/or glucanases, proteases) and secondary metabolites such as gliotoxin (Howell, 2003). There seems to be a degree of redundancy in the ability of *Trichoderma* to control plant pathogenic fungi as several studies in which parts of the biocontrol systems have been

disabled, still resulted in biocontrol of the plant pathogens (Howell, 2003). This concert of mechanisms may explain the ability of *Trichoderma* to control a wide range of pathogens.

2.3.2.1 *Trichoderma* – biological control - enzymes

Trichoderma strains have been reported in various studies to produce a variety of hydrolytic enzymes. These include chitinase, N-acetylglucosaminidase, β -1,3-glucanase, protease, cellulase and amylase in the presence of the appropriate substrate (De Marco *et al.*, 2003).

2.3.2.2 *Trichoderma* – biological control - gliotoxin

Gliotoxin was first described in 1934 and initially the compound was described as a “lethal principle” produced by *Trichoderma lignorum* (Weindling, 1934). By 1941 this “lethal principle” was characterised further and demonstrated to be toxic to both *R. solani* and *Sclerotinia americana* and named gliotoxin. Resultantly, the fungus producing the gliotoxin has been identified as *Gliocladium virens* and not *Trichoderma lignorum*. Recently *Gliocladium virens* has been renamed to *Trichoderma virens* (Howell, 2003).

2.4 Gliotoxin

Gliotoxin is a fungal metabolite belonging to the epipolythiodioxopiperazine group of compounds, some of which are toxic (Council for Agricultural Science and Technology, 2003; Grovel *et al.*, 2006). Refer to Figure 2.1 for the structure of gliotoxin. One of the distinguishing properties of gliotoxin is the disulphide bridge between C1 and C11. There is also a carbonyl group at C2 and C12, and a hydroxyl group at C5.

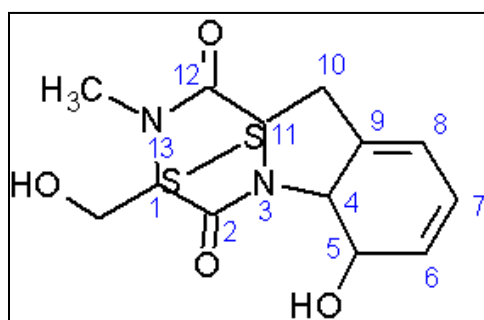


Figure 2.1: Structure of gliotoxin (Grovel *et al.*, 2006).

Fungi other than *Trichoderma* also produce gliotoxin. It is produced by *Aspergillus fumigatus* during its pathogenic state as the causative agent of aspergillosis in turkeys (Council for Agricultural Science and Technology, 2003). Some research also suggests that gliotoxin may play a role in human yeast infections caused by *Candida albicans* (Council for Agricultural Science and Technology, 2003).

Currently, the status of gliotoxin and its risk as an agent involved in mycotoxicoses established through contaminated feeds, is relatively unknown (Council for Agricultural Science and Technology, 2003). Gliotoxin has been reported to exhibit numerous activities in biological systems. The oxidised form of gliotoxin, with an intact disulphide bridge, appears to be the main mechanism through which these biological activities occur, specifically through interaction of the polysulphide link with sulphur nucleophiles in a thiol-disulphide exchange. The reduced dithiol form of gliotoxin is biologically inactive (Waring & Beaver, 1996; Grovel *et al.*, 2006).

2.4.1 Gliotoxin risk – immunomodulation effects

One particularly interesting property of gliotoxin is the fact that it exhibits immunomodulating properties, a factor that plays a role in the virulence of certain mycotoxicoses (Council for Agricultural Science and Technology, 2003). Current research suggests that this immunosuppression involves specific cellular immunity phenomena and non-specific humoral factors associated with immunity (Council for Agricultural Science and Technology, 2003).

2.4.2 Apoptosis and gliotoxin

Several studies have demonstrated the ability of gliotoxin to induce apoptosis in a variety of cells (Waring *et al.*, 1997). Waring (1990) proposed the path outline in Figure 2.2 as a possible mechanism for the induction of apoptosis by gliotoxin.

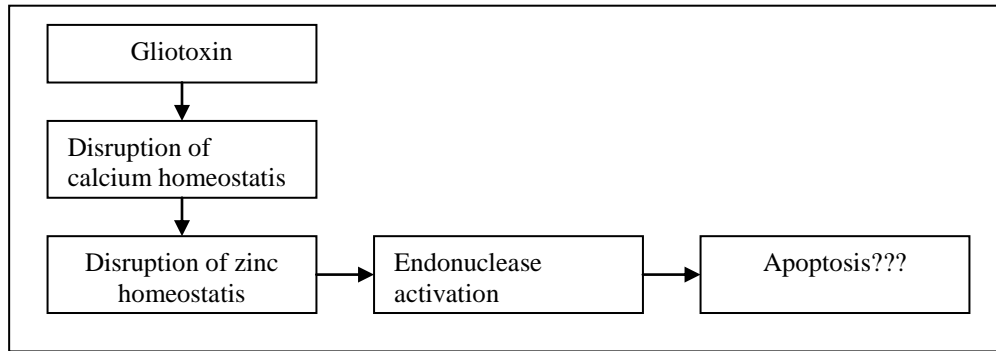


Figure 2.2: Proposed gliotoxin-induced apoptosis pathway (Adapted from Waring, 1990).

Very noticeable in this pathway is that the exposure of the cell(s) to gliotoxin results in disruption in calcium and zinc homeostasis. This cascade continues and leads to endonuclease activation that ends in apoptosis of the exposed cell (Waring, 1990: cited Council for Agricultural Science and Technology, 2003)

In thymocytes, apoptosis seem to occur by a calcium independent mechanism and is not affected by protein synthesis inhibitors. Gliotoxin results in increased phosphorylation of the protein Histone H3 in the thymocyte cells. Furthermore, exposure to gliotoxin also results in increased cyclic adenosine-monophosphate levels and increased protein kinase A activity (Waring *et al.*, 1997).

Gliotoxin has also been shown to cause cells to enter the cell cycle at an inappropriate stage. This failed entry results in an abortion of the cell cycle that leads to apoptosis. The aborted cell cycle entry has been shown to be a feature in various cells undergoing apoptosis. In summary the mechanism proposed suggests that the increased phosphorylation of the chromatin material may trigger deoxyribonucleic acid (DNA) dissolution resulting in apoptosis (Waring *et al.*, 1997). Phosphorylation of the histone protein play an important role during the condensing of the genetic material during the cell cycle, but hyperphosphorylation render the chromatin more susceptible to the action of nucleases. These findings regarding the effect of protein phosphorylation may be of particular significance in elucidating the mechanism of gliotoxin-induced apoptosis as phosphorylation of proteins play an important role in receptor-mediated signal transduction, e.g. phosphorylation of tyrosine is an early step in the transduction of receptor signals in cells.

Most of the studies mentioned above however, relate to the effect of gliotoxin on animal cell systems, no comparable studies have been reported for plant cells.

2.5 Plant Hormones

The mechanisms by which plants control growth are many faceted and complex. One of these mechanisms entails the so-called “plant growth substances” or plant hormones (Hill, 1973). According to Hill (1973), a plant growth substance can be defined as: “an organic substance which is produced within a plant and which will at low concentrations promote, inhibit or qualitatively modify growth, usually at a site other than its place of origin”. A further group of compounds is possible with this definition as a base. They are the “plant regulators” and they can be defined as compounds whose effects, when applied to plants, closely resemble that of the plant hormone. A variety of these compounds are known and some of them are chemical analogues of the endogenous plant hormones, though not all.

2.5.1 Gibberellic acid

Gibberellic acid was first isolated from the fungus *Gibberella fujikuroi*, which causes a disease in rice known as “bakanae” or “foolish seedling”. In the period 1926 to 1950, a lot of research went into describing the physiological responses in plants to this compound, as well as determining the structure of gibberellic acid. Though originally isolated from a fungal culture, research suggested that this compound may also be found in higher plants. Since then gibberellic acid has been isolated from various fungi and higher plants (Hill, 1973; Murase *et al.*, 2008).

Gibberellic acids (GAs) (also referred to as gibberellins) are classified as tetracyclic diterpenoid plant growth regulators. According to current studies 126 GAs have been identified in higher plants, fungi and bacteria. GA regulates various developmental and growth processes in plants such as:

- Stimulates seed germination
 - Enhances digestion of storage reserves during germination of cereal grasses
- Stimulates stem elongation (especially marked in dwarf and rosette plants)
- Flowering

- Stimulates parthenocarpy (fruit development)
- Regulation of gene expression
- Stimulates trichome development
- Action often antagonised by abscisic acid (ABA)

Of the various known gibberellins only a few are biologically active in plants, these are GA₁, GA₃, GA₄ and GA₇ (Komatsu *et al.*, 1996; MacMillan & Gaskin, 1996; Bethke & Jones, 1998; Gomi & Matsuko, 2003; Murase *et al.*, 2008). The bioactive GAs are characterised by hydroxylation at C3, a lactone ring between C4 and C10 and a carboxyl group at C6 (Refer to Figure 2.3). Hydroxylation at C2 however, inactivates bioactive gibberellins (Murase *et al.*, 2008).

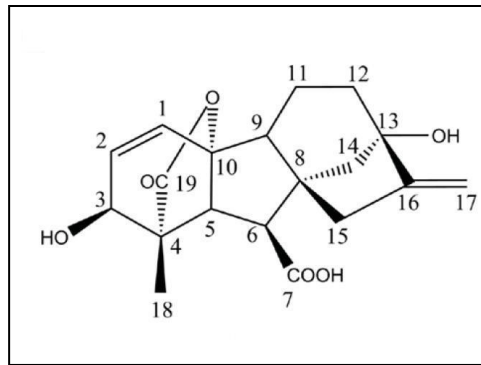


Figure 2.3: Structure of gibberellic acid 3 (GA₃) from Murase *et al.*, 2008.

Gibberellic acid itself is a hydrophobic carboxylic acid. This would render it soluble in the intracellular and intercellular compartments of plant cells as a carboxylate anion. It may also be able to cross the plasmamembrane of the plant cell in its protonated acid form through passive diffusion (Ueguchi-Tanaka *et al.*, 2005).

2.5.1.1 Gibberellic acid and germination

The germination of seeds is one of the areas where GA plays an important role. Several studies on cereal grains (rice, wheat, barley in particular) showed that the aleurone cells respond to gibberellic acid by synthesising and secreting a variety of hydrolytic enzymes from the scutellum and aleurone layer. The hydrolytic enzymes then proceed to hydrolyse

and mobilise the storage molecules. The enzyme α -amylase is one of the best-studied enzymes that are activated by gibberellic acid (Sargeant, 1980; Cejudo *et al.*, 1995; Washio, 2001). The gibberellic acid, GA₃, is primarily involved in these responses. While most studies demonstrated the effect of GA₃ in the aleurone layer to induce the synthesis of hydrolytic enzymes such as α -amylase, GA₃ also affects the scutella and results in the synthesis and secretion of α -amylases and β -glucanases from this cell layer (Cejudo *et al.*, 1995).

2.5.1.2 Gibberellic acid and germination – alpha-amylases

Alpha-amylases (α -amylases, E.C. 3.2.1.1), also known as 1,4- α -D-glucan glucanohydrolase, hydrolyse starch to produce the component monosaccharides (Cejudo *et al.*, 1995). In wheat, three groups of α -amylases have been identified. Group I is controlled by the loci *α -Amy1* and is characterised by a basic isoelectric point (pI), typically 6.3-7.5 while group II is controlled by the loci *α -Amy2* and is characterised by a more acidic pI, typically 4.9-6.0 (Lazarus *et al.*, 1985). Little information is currently available of Group III, under control of the loci *α -Amy3* (Baulcombe *et al.*, 1987). These various form of α -amylase serve different functions during the life of the plant as observed through differential expression of the respective genes. Literature reports that the *α -Amy1* form is found predominantly during seed germination, while it is absent during development of the grain, where *α -Amy2* appears to be the dominant form (Sargeant, 1980).

2.5.2 Gibberellic acid signalling

GAs have been shown to affect cellulair processes though gibberellic acid receptors and a few candidate GA-binding proteins for GA receptors have been identified through a variety of techniques in various studies (Komatsu *et al.*, 1996).

2.5.2.1 Gibberellin Insensitive Dwarf 1 (GID1)

Due to the hydrophobic properties of GA, it has been postulated that GA may have both membrane-bound and soluble receptors in plant cells (Ueguchi-Tanaka *et al.*, 2005). Until recently research has as yet not completely homed in on the specific receptors for GA,

however the list of intracellular GA signal transduction elements has been expanded to include guanine nucleotide-binding proteins (G-proteins) and protein kinases (Bethke & Jones, 1998). However, in the past decade various factors have been identified through studies of rice (*Oryza sativa*) and *Arabidopsis* mutants (Hirano *et al.*, 2007a,b). Recently, Gibberellin-Insensitive Dwarf1 (GID1) has been identified as a soluble receptor for GA in both rice and *Arabidopsis* (Ueguchi-Tanaka *et al.*, 2005).

The GID1 proteins display a close structural similarity to hormone sensitive lipases such as those found in higher animals, being a globular protein and containing a pocket for the substrate. Unlike the hormone sensitive lipases in animals, GID1 is not involved in lipid metabolism due to a change in a critical amino acid. Additionally, GID1 possesses a loose strand at the amino terminal. This functions similarly to a lid closing the pocket the GA has bound to the protein. GA functions as an allosteric activator in GID1, allowing structural changes that enables the receptor to associate with DELLA proteins, however GA does not interact with DELLA proteins by itself (Ueguchi-Tanaka *et al.*, 2007; Hedden, 2008; Murase *et al.*, 2008; Shimada *et al.*, 2008). During binding of GA₄ to GID1, the ent-gibberellane skeleton of the gibberellic acid molecules, contributes to keeping the ligand firmly in the pocket by non-polar interactions (Shimada *et al.*, 2008).

2.5.2.2 Gibberellin Insensitive Dwarf 1 (GID1) and signalling

Following the discovery of gibberellins in the 1950's, the question of gibberellin perception and interpretation has remained unsolved (Murase *et al.*, 2008). In recent years several breakthroughs shed light on the mechanism of gibberellin perception and interpretation. These breakthroughs include the discovery of the soluble GID1 gibberellin receptors, the transcriptional regulatory DELLA proteins and the F-Box proteins (Murase *et al.*, 2008). In summary gibberellin perception proceeds as follows:

- GID1 is activated on gibberellic acid binding;
- Recognition of DELLA proteins, though mechanism is still not fully elucidated, the end result is binding of GID1 and DELLA proteins;
- After GID1-DELLA binding the DELLA proteins can be recruited for a polyubiquitylation through a ubiquitin E3 SKP1-CULLIN-F-Box (SCF) complex;
- Degradation of the complex occurs through a 26S proteasome; and

- The GA-GID1 interactions with the DELLA allows for transcriptional reprogramming of gibberellic acid responsive genes.

Research has shown that overexpression of GID1 results in a GA hypersensitive phenotype (Ueguchi-Tanaka *et al.*, 2005).

2.5.2.3 Gibberellin Insensitive Dwarf 1 (GID1) GA binding

In a study by Murase *et al.* (2008), gibberellic acid perception by GID1 was described. Within the GID molecule studies, the embedded GA₃ exhibited a large contact area by directing its hydrophilic carboxylate group towards the bottom of the pocket and its hydrophobic aliphatic rings towards the entrance of the pocket and anchoring it there. The negative charge of the carboxylate group is neutralised by the oxyanion hole and by forming a salt bridge with Arg244. Also the C7 carboxylate anchors GA₃ to Ser116 and Ser191 (from the bottom of the pocket) and one water molecule through multiple hydrogen bonds. At the pocket the non-polar residues Ile126, Leu323, Val239 and Val319 forms a hydrophobic wall with which the aliphatic rings of GA₃ makes contact. Also the N-terminal extension helices possess non-polar residue projections (Ile24, Phe27 and Tyr31). These residues along with His119 are strictly adjusted for gibberellic acid ring recognitions. Another important bond position is Tyr127 which bonds to the C3 hydroxyl group and a bridging water molecule (Murase *et al.*, 2008). The other hydroxyl group at C13 (present in GA₁ and GA₃, but absent in GA₄) forms a weak bond with Phe238 and a bridging water molecule. Interestingly, the C13 hydroxylation overall results in a weaker binding affinity than that observed for GA₄ due to the group's close proximity to the negatively charged Asp243. Overall differences between these molecules are minimised when overlapped thus resulting in ensured bioactivity within GID1 (Murase *et al.*, 2008).

2.6 Effect of treatments on plants – study methods

Until recently, evaluation of stress or treatment modalities on plants have focused on an agronomic approach, which combines the genetic and environmental effects on plant growth. When evaluating a treatment from an agronomic view point, fungal strains were selected based on yield, plant survival, plant height, leaf area, leaf injury, relative growth rate and relative growth reduction (Ashraf & Harris, 2004). However, these criteria do not give any

indication on effects of a treatment on the molecular scale. Several methods do exist to investigate the effect of a specific treatment on a plant and include transcript analysis and protein analysis. Information gathered from these approaches are further refined through the use of bioinformatics (Fiehn *et al.*, 2001).

2.6.1 Transcript analysis

Among the approaches available for transcript analysis are micro-arrays, sequencing-based approaches, differential display-based approaches (Fiehn *et al.*, 2001; Leader, 2005). The analysis of these transcripts can serve as valuable information to determine responses of plants to various stimuli as gene expression are usually the result of the activity of various regulatory networks inside plants beside a specific gene in question (Ganesan *et al.*, 2008). Some examples of studies using this approach include:

- Studies on plant development, physiology and metabolism (Genoud, & Métraux, 1999; Wu *et al.*, 2001; Buckhout & Thimm, 2003; Schnable *et al.*, 2004;) Studies on the effect of environment stresses such as salinity (Jebara *et al.*, 2005; Ganesan *et al.* 2008);
- Studies on the effect of ultraviolet radiation (Zinser *et al.*, 2007);
- Studies on plant and plant pathogen interactions, including plant defence responses (Reymond, 2001; Fraire-Velázquez & Lozoya-Gloria, 2003);
- Studies on plant beneficial interactions (such as mycorrhiza) (Wiemkin & Boller, 2002); and
- Studies on the effect of herbicides and fungicides on plants (Ronchi *et al.*, 1997).

2.6.2 Protein analysis

Protein analysis represents the next level of analysis following transcript profiling. Two-dimensional gel electrophoresis is currently the best approach to achieve efficient separation. During two-dimensional electrophoresis, separation of the various proteins occurs by means of the physical properties of the proteins in a sample such as iso-electric points and molecular masses. This approach is highly popular in plant proteomic research. Two-dimensional gel electrophoresis however, can be problematic if proteome-wide identification or accurate quantification is required (Fiehn *et al.*, 2001).

Capillary iso-electric focusing represents an alternative to two-dimensional gel electrophoresis and can even handle crude protein extracts. This approach is also useful when non-sequenced plants are used in studies (Fiehn *et al.*, 2001).

In essence, these protein analysis techniques can be applied to the same research questions where transcriptional analyses were applied. Some examples of studies using this approach include:

- Studies on plant development, physiology and metabolism (Williamson *et al.*, 1985; Acevedo & Cardemil, 1997; Bethke *et al.*, 2001; Kopyra & Gwózdź, 2003).
- Studies on the effect of environment stresses such as salinity (Kopyra & Gwózdź, 2003; Jebara *et al.*, 2005).
- Studies on plant and plant pathogen interactions, including plant defence responses (Kwon & Anderson, 2001; Cho & Muehlbauer, 2004; Rozhnova *et al.*, 2007).
- Studies on the effect of herbicides and fungicides on plants (Broughton *et al.*, 2003; Sumner *et al.*, 2003).

2.6.3 Metabolite analysis

In essence, metabolites represent the ultimate result of gene expression. Metabolite profiling has to date been successfully applied in medical research when comparing diseased tissues to healthy tissues. In plant physiology it is only recently where the scope of metabolite analysis has been expanded to include a larger range of compounds. However, the sheer magnitude and complexity of plant metabolites remains a critical factor when pursuing this approach (Fiehn *et al.*, 2001; Sumner *et al.*, 2003).

2.6.4 Bioinformatics

An enormous amount of data can be generated by approaches such as expression and protein and metabolic profiling and the handling and processing of the data becomes the next critical step. In this regard, bioinformatics is a valuable tool for analysing the data and gaining insight into the interplaying processes of plant physiology (Fiehn *et al.*, 2001). In principle, bioinformatics represents a field of science where biology, computer science, statistics and

information technology combine into a single discipline with the goal to discover biological insights from which unifying principles in biology can be discerned. From this definition and scope, it is hardly surprising that bioinformatics provide a unified conceptual framework for fields such as molecular biology, biochemistry, molecular evolution, statistics, computer science and information technology (Blanchard, 2004).

2.6.5 Molecular modelling

Tools such as X-ray crystallography, nuclear magnetic resonance and computational chemistry and modelling are providing researchers with valuable data to design and study ligand/substrate and protein interactions in the fields of chemistry, biochemistry and pharmacology (Esposito *et al.*, 2000). Parallel to this, there has been a great increase in the number of high-resolution protein structures deposited in the Brookhaven Protein Databank (PDB). This has enabled successful drug design and evaluation in particularly the field of pharmacology (Hendlich *et al.*, 1997). In pharmaceutical research, structure-based drug design focuses on two main approaches: firstly, receptor-based docking techniques and secondly, pharmacophore-based virtual screening (Zhang *et al.*, 2005).

2.6.5.1 Docking

In the field of computer aided drug design, ligand-protein interactions are a useful tool to design and evaluate potential ligands against a protein of interest (Chen *et al.*, 2002; Zhang *et al.*, 2005). Docking-ligand studies can be described as a target-based method (Taminau *et al.*, 2008). During docking, various interactions between the ligand and the protein must be considered such as shape complementarity, charge-charge interactions, solvation-desolvation interactions, hydrophobic interactions and hydrogen bonding. However, to compute or evaluate all of these interactions requires significant computational costs. As a result, some or many of these interactions are either simplified or omitted to reduce computational load (Esposito *et al.*, 2000). Potentially suitable ligands are usually selected based on a molecular binding scoring function (Chen *et al.*, 2002). In general, lower energy scores indicate better protein-ligand bindings when compared to higher energy scores. As a result, in most cases the docking is an attempt to optimise the computations to find the lowest binding energy (Thomsen, 2003).

Studies indicate that a scoring strategy based on the interaction energy between the protein and the ligand is useful for finding ligands and binding conformations close to experimentally determined structures (Chen *et al.*, 2002). Ligand or drug binding is competitive by nature (McIlwain, 1986 cited in Chen *et al.*, 2002). As a result, a drug or ligand would be considered less effective if it displays non-competitive binding against its natural ligand. In theory, a ligand can be considered to be competitive to the natural ligand if its binding scoring function is at least comparable to that of the ligand in an available protein-ligand 3D structure. This approach in particular has been very useful in the design and screening of potential pharmaceutical targets (Chen *et al.*, 2002). The drawback of this technique is that it can only be practically applied to small sets of compounds and it can be very time-consuming (Taminau *et al.*, 2008).

2.6.5.2 Pharmacophore description

In essence, a pharmacophore represents a qualitative prediction of binding by specifying the spatial arrangement of a small number of atoms of functional groups or in other words a 3D arrangement of atoms or functional groups necessary to bind to a given receptor (Wermuth *et al.*, 1998; Zhang *et al.*, 2005; Taminau *et al.*, 2008). Various critical interactions, relating to chemical features of the compound, include hydrogen bonding, charge transfer, steric and electrosteric properties, as well as lipophilic interactions (Taminau *et al.*, 2008). The advantage of using this approach is that prediction and screening can be performed on large databases as the pharmacophore serves as a guide for searching for compounds or the synthesis of new compounds and has been successfully applied to a multitude of drug development programs (Zhang *et al.*, 2005). In contrast to docking methods, ligand based methods will attempt to rank small molecules according to their similarity to one or more reference structures. To describe this similarity various concepts have been described such as molecular topology (in essence a fingerprint), molecular shape, molecular field descriptors and pharmacophores (Taminau *et al.*, 2008).

2.6.6 Germination and plant height

Since plant growth regulators affect the rate of emergence and percentage emergence of seeds, it has been applied in studies to investigate the effect of plant growth regulators in plants (Wang *et al.*, 1996). In a study by Wang *et al.* (1996) this property of plant growth regulators was applied to improve seedling emergence in low rhizosphere temperature conditions. The study demonstrated that GA₃ in particular was very effective in promoting seedling germination and emergence, with 0.1 mM GA₃ being described as the most effective. In another study, GA₃ was specifically applied to *Lesquerella* seeds to assist in germination (Puppala & Fowler, 2002). Using these and similar studies as a guideline, utilisation of the rate of emergence and percentage emergence can be considered as a method to evaluate novel plant growth regulators.

2.6.7 Chlorophyll *a* fluorescence F₀-J-I-P transient test (JIP test)

One of the tools available to measure the response of a plant to a specific treatment, is by studying the efficiency of photosystem II (PSII) through measurements of chlorophyll *a* fluorescence (Strasser *et al.*, 2000; Strasser *et al.*, 2004; De Beer, 2005). This technique has become one of the empirical diagnostic tools in the study of plant stress physiology.

The base for the diagnostic fluorescence procedure lies in the Kautsky effect (Kautsky & Hirsch, 1931: cited in Strasser *et al.*, 2000, 2004; De Beer, 2005). The Kautsky effect can be described as the characteristic changes in chlorophyll *a* fluorescence observed when a dark-adapted leaf is illuminated. The Kautsky transient observed after illumination of a dark-adapted leaf consists of a fast rise, completed within less than 1 second, followed by a slower decline to the steady state. Within the rising phase of the transient most of the primary reaction within photosynthesis are thought to occur (Krause & Weiss, 1991; Strasser *et al.*, 1995). The polyphasic kinetics observed when plotting the transient on a logarithmic scale consists of several steps:

- Initial O
- J (also called I₁)
- I (also referred to as I₂)
- P, maximum P level, (F_P)

The O-J phase occurs within 1-2 milliseconds after illumination. During this phase, photochemical reduction of the primary quinone acceptor (Q_A) occurs in the PSII reaction centres. During the J-I and I-P phases electron transport to secondary plastoquinones occur, but the reason for the existence of the two phases is not clear, though it has been suggested that they reflect heterogeneous rates of plastoquinone reduction by two PSII populations (Barthélemy *et al.*, 1997).

From these observed kinetics Strasser and Strasser (1995) developed the O-J-I-P test as a tool to translate the fluorescence measurements of the transients into expressions that quantify PSII function. Parameters that can be derived from the JIP-test data is summarized Table 2.1.

The OJIP test is a very useful tool and has been applied, under well-defined experimental conditions, as a biosensing tool to evaluate a plant in terms of vitality, productivity and sensitivity and resistance to stress. The Performance Index, $PI_{(Abs)}$, is in essence an indicator of plant vitality, a summarising expression of the plant's ability to resist constraints from the outside environment (Van Rensburg *et al.*, 1996; Strasser *et al.*, 2000; Strasser *et al.*, 2004).

As the transient responds to treatments and environmental conditions, it can also supply information about the structure, conformation and function of the photosynthetic apparatus (De Beer, 2005). As such, the Kautsky transient curve has been used in various studies as a screening tool of physiological parameters of plants against factors such as herbicides, cold acclimation and air pollution (van Rensburg *et al.*, 1996; Tyystjärvi *et al.*, 1999; Strauss *et al.*, 2006).

Table 2.1: Summary of JIP-test parameters.

Extracted and technical fluorescence parameters	
F_0 (also described as $F_{50\mu s}$)	Fluorescence intensity at 50 μs
$F_{100\mu s}$	Fluorescence intensity at 100 μs
$F_{300\mu s}$	Fluorescence intensity at 300 μs
F_J	Fluorescence intensity at the J step (at 2 ms)
F_I	Fluorescence intensity at the I step (at 30 ms)
F_M	Maximal fluorescence intensity
tF_M	Time to reach F_M in ms
$V_J = (F_{2ms} - F_0)/(F_M - F_0)$	
$(dV/dt)_0 = M_0 = 4 \cdot (F_{300} - F_0)/(F_M - F_0)$	
F_V/F_0	Energy per reaction centre of the light reaction
Quantum efficiencies or flux ratios	
$\phi_{P0} = TR_0/ABS = [1 - (F_0 - F_M)] = F_V/F_M$	Maximum quantum yield of primary photochemistry
$\phi_{E0} = ET_0/ABS = [1 - (F_0/F_M)] \cdot \Psi_0$	Quantum yield of electron transport
$\Psi_0 = ET_0/TR_0 = (1 - V_J)$	Efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-
Specific fluxes or specific activities	
$ABS/RC = M_0 \cdot (1/V_J) \cdot (1/\phi_{P0})$	Specific energy fluxes per reaction centre for absorption
$TR_0/RC = M_0 \cdot (1/V_J)$	Specific energy fluxes per reaction centre for trapping
$ET_0/RC = M_0 \cdot (1/V_J) \cdot \Psi_0$	Specific energy fluxes per reaction centre for electron transport
$DI_0/RC = (ABS/RC) - TR_0/RC$	Specific energy fluxes per reaction centre for dissipation at the level of the antenna chlorophylls
Phenomenological fluxes or phenomenological activities	
$ABS/CS = ABS/CS_{Chl} = Chl/CS$ or $ABS/CS_0 = F_0$ or $ABS/CS_M = F_M$	Phenomenological activity fluxes per excited cross section for absorption
$TR_0/CS = \phi_{P0} \cdot (ABS/CS)$	Phenomenological activity fluxes per excited cross section for trapping
$ET_0/CS = \phi_{P0} \cdot \Psi_0 \cdot (ABS/CS)$	Phenomenological activity fluxes per excited cross section for electron transport
$DI_0/CS = (ABS/CS) - (TR_0/CS)$	Phenomenological activity fluxes per excited cross section for dissipation
Performance index	
$PI_{(Abs)} = (RC/ABS) \cdot [\phi_{P0}/(1 - \phi_{P0})] \cdot [\Psi_0/(1 - \Psi_0)]$	Indicator of sample vitality, ability of sample (plant) to resist constraints from the outside
Density of reaction centres	
$RS/CS = \phi_{P0} \cdot V_J/M_0 \cdot (ABS/CS)$	Fraction of active PSII reaction centres per excited cross section

Abs = absorption energy flux; CS = excited cross section of leaf sample; RC = Reaction centre

2.7 Gibberellic acid gliotoxin (GA GT) hypothesis

One particularly interesting phenomenon observed when seeds or crops are treated with *Trichoderma* species, is the complete and even stand of the treated plots compared to the uneven and random stand of untreated plots (Howell, 2003). In a study of seed vigour of peas in potting soil, Zheng and Shetty (1999) showed that treatment with various *Trichoderma* species resulted in increased and faster seed germination, increases in plant height, an increase in phenolic compound content in the seedlings and overall enhancement of plant growth (Zheng and Shetty, 1999; Shoresh and Harman, 2008; Vinale *et al.*, 2008b). During this particular study *Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma pseudokoningii* were compared. During the particular study *Trichoderma harzianum* treatment of the peas resulted in the highest average plant height after 5 days, highest average weight of fresh seedlings and highest phenolic compounds in the seedlings relative to the other strains evaluated (Zheng and Shetty, 1999).

In a more recent study, the molecular basis for plant responses in maize following root inoculation with *Trichoderma harzianum* reported the following results. Following root inoculation, 114 proteins were upregulated (91 were identified) and 50 proteins were downregulated (31 were identified). Upregulated proteins included those involved in carbohydrate metabolism and some photosynthesis or stress related, as well as amino acid metabolism, cell wall metabolism and genetic information processing. Induced proteins included mainly proteins involved in stress and defence responses. Unaffected proteins included those involved in secondary metabolism and protein biosynthesis (Shoresh & Harman, 2008). Another study by Vinale *et al.* (2008b) also demonstrated that secondary metabolites from *Trichoderma* have a role in activation of plant defences as well as plant growth regulation.

This suggests that treatment with *Trichoderma* affect not only plant pathogens, but at the same time the plant itself is also affected. Of all the mechanisms employed by *Trichoderma*, the secretion of antibiotic substances seems the most probable route. It is suggested that some of the secondary metabolites excreted by *Trichoderma* may function as a homologue for a plant growth controlling substance or hormone. In a review by Vinale *et al.* (2008b), studies are cited where koniginin A and 6-*n*-pentyl-6*H*-pyran-2-one (6PP or 6-pentyl- α -

pyrone) were evaluated for plant growth promotion. At high concentrations (10^{-3} M) growth inhibitory effects were observed, however at concentrations in the range of 10^{-5} M and 10^{-6} M these compounds exhibited optimal auxin-like effects.

However, the auxin-like effects observed for koniginin A and 6PP does not account for all the effects observed from *Trichoderma* treatments. In particular, the complete and even stand, but more indicatively, the earlier germination of treated seeds following *Trichoderma* treatment, suggests that the effect of the plant hormone gibberellic acid, or gibberellin, is being mimicked. Refer to Figure 2.4 a and b for comparison between structure of gliotoxin and GA_3 .

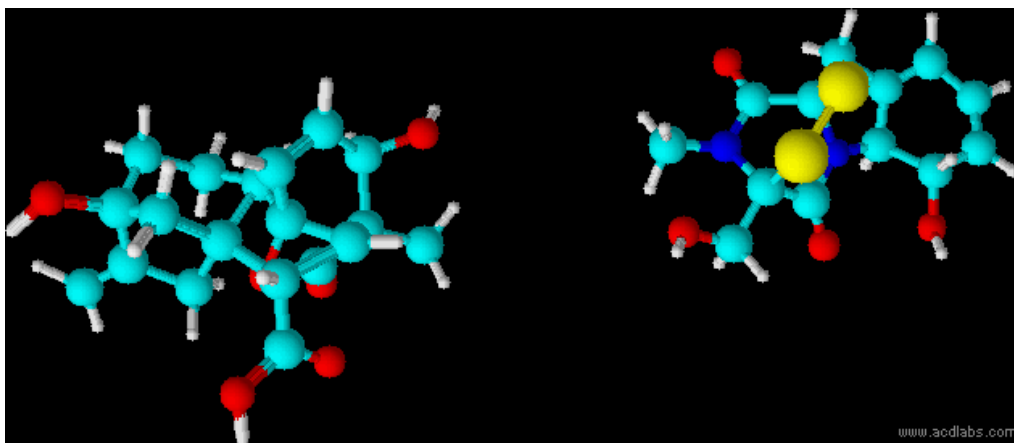


Figure 2.4a: Ball and stick representation of the structures of GA_3 (left) and gliotoxin (right).

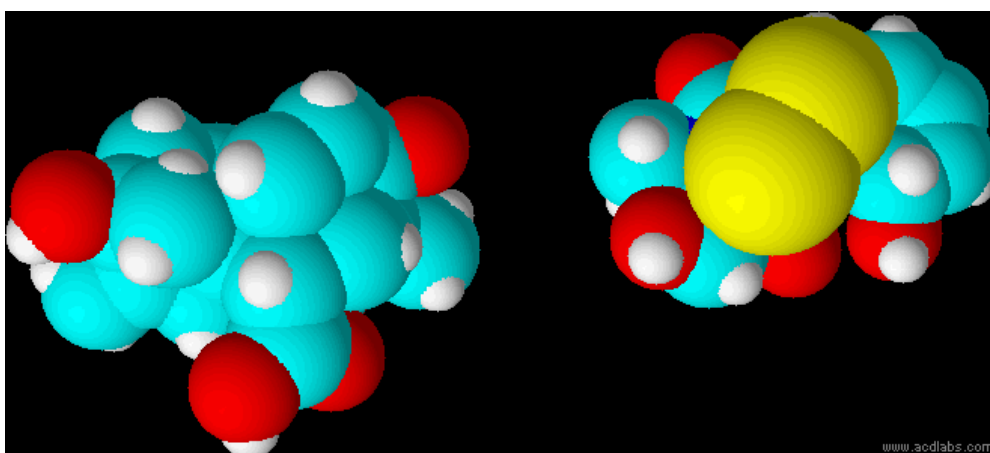


Figure 2.4b: Space filling sphere representation of the structures of GA_3 (left) and gliotoxin (right).

A more thorough evaluation between GA_3 and gliotoxin (GT) will be presented in Chapter 4.

This study was based on the hypothesis that gliotoxin can indeed elicit similar responses in plants to that of the natural plant growth regulator gibberellic acid.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Molecular modelling and docking

All the proteins used for this study were acquired from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Databank (www.rcsb.org). The Protein Data Bank (pdb) file 3ED1 (Crystal structure of rice GID1 complexed with GA₃) was used in the docking studies for GID1 and GA₃ and gliotoxin (Shimada *et al.*, 2008). The structure files for GT, GA₁, GA₃, GA₄, GA₅, GA₇ and GA₈ created online using CORINA (CORINA, 2008) from its SMILES (simplified molecular-input line-entry specification) notation and saved as a pdb file.

3.2 Molecular similarity

The molecular similarity evaluation was only conducted between gliotoxin and GA₃, the primary molecules of interest in this study. Common feature pharmacophore generation using the HipHop algorithm of the Catalyst molecular modelling software suite version 4.8 (Accelrys, 2006) was applied to evaluate similarity between the molecules. During the pharmacophore model generation gliotoxin was tested against a model generated by using GA₁, GA₃, GA₄, GA₅, GA₇ and GA₈ as input.

3.3 Molecular docking

For molecular docking the pdb file 3ED1 (Crystal structure of rice GID1 complexed with GA₃) (Shimada *et al.*, 2008) served as the target with GA₃ and gliotoxin serving as ligands. The software package ArgusLab version 4.0.0 (Mark Thompson and Planaria Software LLC, 2004) was used for the molecular docking and scorings. The ArgusDock exhaustive search docking engine was used with a grid resolution of 0.25 Ångstrom, docking precision set to high precision and flexible ligand docking mode enabled.

3.4 Germination: Plant height and emergence

Chemicals

All chemicals used in this study were obtained from Sigma Aldrich or Fluka BioChemika, depending on availability. GA₃ was used in this study as a reference for the natural plant

growth regulator. Gliotoxin from *Gliocladium fimbriatum* (Fluka BioChemika) was used as the source of gliotoxin for this study.

Plant material

Bean seeds (*Phaseolus vulgaris* L., supplied by the Agricultural Research Council, South Africa) were surface sterilised in 2.5 % sodium hypochlorite for 20 minutes, rinsed thoroughly, immersed in water containing 20 mM CaCl₂, 10 µg/ml chloramphenicol, 10 µg/ml ampicillin, and 25 units/ml nystatin (Washio, 2001). *Phaseolus vulgaris* was selected as a model plant system for this study due to the ease the seeds could be handled.

Treatment with growth regulators

The growth regulator treatment was performed by incubation of the sterilised seeds on filter paper soaked with an imbibition solution (10 mM CaCl₂, 20 mM Na-Acetate, pH 5.2) with the various growth regulators at selected concentrations, at 25 °C overnight in the dark (Cejudo *et al.*, 1995). The seeds were then transferred to pots containing vermiculite as a growth medium in a greenhouse. The pots were monitored for plant emergence and plant height until no further emergence occurred for 5 days. Air temperature was maintained at 25 °C and the day/night ratio of the season was approximately 16:8. Treatment with the growth hormones were conducted within a serial dilution series of 10⁻⁴ M through 10⁻⁸ M, designated as GA for gibberellic acid and GT for gliotoxin (Wang *et al.*, 1996). The gliotoxin was dissolved in methanol, then diluted to the application dilutions. Methanol (1µl in 15 ml distilled water) served as a control (Vinale *et al.*, 2008a).

Statistical analyses

Statistical analyses was performed on the data sets using STATISTICA 10 (StatSoft Inc ©, 2011). A one-way breakdown analysis of variance, ANOVA, (repeated measurements ANOVA) was performed, after which a Tukey's Honest significance test was performed to determine statistical significance between the various treatments.

3.5 Germination: Zymogram

Chemicals

All chemicals used in this study were obtained from Sigma Aldrich. GA₃ was used in this study as a reference for the natural plant growth regulator.

Plant material

Bean seeds (*Phaseolus vulgaris* L., supplied by the Agricultural Research Council, South Africa) were de-embryonated and the de-embryonated half seeds were surface sterilised in 2.5% sodium hypochlorite for 20 minutes, rinsed thoroughly, immersed in water containing 20 mM CaCl₂, 10 µg/ml chloramphenicol, 10 µg/ml ampicillin, and 25 units/ml nystatin (Washio, 2001). *Phaseolus vulgaris* was selected as a model plant system for this study due to the ease the seeds could be handled and the embryos removed. For this method the testa of the seeds were also removed.

Treatment with growth regulators

The growth regulator treatment was performed by incubation of sterilised de-embryonated half seeds (aleurone) on filter paper soaked with an imbibition solution (10 mM CaCl₂, 20 mM Na-Acetate, pH 5.2) with the various growth regulators at selected concentrations, at 25°C in the dark for 48 hours (Cejudo *et al.*, 1995). De-embryonated half seeds treated with only the imbibition solution containing no growth regulator served as the negative control. Non-de-embryonated half seeds treated with only the imbibition solution containing no growth regulator served as the positive control. Treatment with the growth hormones were conducted within a serial dilution series of 10⁻⁴ M through 10⁻⁸ M, designated as GA for gibberellic acid and GT for gliotoxin (Wang *et al.*, 1996). Three seeds were used per treatment and the experiment was repeated four times. The gliotoxin was dissolved in methanol, then diluted to the application dilutions. Methanol (1 µl in 15 ml distilled water) served as a control (Vinale *et al.*, 2008a).

Enzyme extraction

For enzyme extraction a Tris-HCl extraction buffer was used according to Mayol and Rosello (2001). The extraction buffer consisted of 0.1 % 2-β-mercaptoethanol, 0.5 mM EDTA, 0.010 M MgCl₂.6H₂O, 4% PVP-40, 0.1 M Tris-HCl, pH 8.5. Pooled seeds (totalling 0.1 g) were homogenised in a pre-cooled mortar and pestle over ice with 1.5 ml extraction buffer. The resultant homogenate was centrifuged at 12 000 rpm for 5 min at 4 °C and frozen and stored at -68 °C (Mayol & Rosello, 2001). The supernatant was used for subsequent zymograms.

Zymograms

To preserve enzyme activity for amylase activity screening, electrophoresis was performed under native conditions according to Davis (1964). The samples were diluted 1:1 in sample buffer (0.125 M Tris, 20 % v/v glycerol, 0.04 % v/v bromophenol blue, without mercaptoethanol) and were not boiled (Martinez *et al.* 2000). The native substrate-containing gel was prepared as described by Martinez *et al.*, (2000), utilising a 4 % stacking gel and a 12 % resolving gel. The makeup of the PAGE gel was as follows:

- Stacking gel (4 %)
 - 0.65 ml Polyacrylamide
 - 1.25 ml of 0.5 M Tris-HCl buffer (pH 6.8)
 - 3.10 ml distilled water
 - 5 µl TEMED
 - 10 % w/v Ammonium persulphate
- Resolving gel (12 %)
 - 4.0 ml Polyacrylamide
 - 2.5 ml of 1.5 M Tris-HCl buffer (pH 8.8)
 - 2.75 ml distilled water
 - 1.25 ml of 2 % w/v soluble starch stock solution
 - 2.35 ml distilled water
 - 5 µl TEMED
 - 50 µl 10 % w/v Ammonium persulphate

Note that SDS is replaced by additional distilled water in the stacking and resolving gel. A Tris-glycine running buffer (0.025 M Tris, 0.2 M glycine, pH 8.3) was used during electrophoresis. Running conditions were constant voltage of 30 V per gel until tracking dye enters the resolving gel, after which gels were subjected to a constant voltage of 100 V per gel at constant low temperature (0 – 2 °C) to avoid starch hydrolysis. Upon completion of electrophoresis the gels were washed in distilled water and in 0.1 M phosphate-citrate buffer and 0.05 M NaCl buffer, pH 6, for 2 – 3 hours and stained with Lugol solution (6.7 mg/ml KI and 3.3 mg/ml I₂) (Martinez *et al.*, 2000). The gel images were captured using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap software (Version 6.00.22).

3.6 Chlorophyll fluorescence transient (OJIP)

Plant material

Bean seeds (*Phaseolus vulgaris* L., supplied by the Agricultural Research Council, South Africa) were planted to pots containing vermiculite as a growth medium in a greenhouse. The plants were then allowed to grow for 10 days. Four seeds were planted to a pot and after ten days reduced to three plants per pot of similar height. Air temperature was maintained at 25 °C, and the day night ratio of the season was approximately 16:8. Hoagland's solution was used to supply plant nutrients to eliminate nutrient deficiencies as an experimental factor. Plants were watered with the Hoagland's solution every second day until the end of the trial. Treatment with the growth hormones were conducted within a serial dilution series of 10^{-4} M through 10^{-8} M, designated as GA for gibberellic acid and GT for gliotoxin (Wang *et al.*, 1996). The gliotoxin was dissolved in methanol, then diluted to the application dilutions. Methanol (1 μ l in 15 ml distilled water) served as a control (Vinale *et al.*, 2008a). Growth regulators were applied as a spray onto the leaves of the plants.

OJIP Test

Prior to treatment with the growth regulators the JIP test was conducted on the plants to establish the baseline. The OJIP test was performed 2, 6 and 8 days after treatment. The experiment was terminated after day 9 as the pots were becoming limiting to the plants' growth. A Plant Efficiency Analyser (PEA, Hansatech, Kingslynn, UK) was used for the JIP test. This specific analyser has a high time resolution, in addition to a large capacity for data acquisition (Strasser *et al.*, 1995; De Beer, 2005). OJIP transient induction occurred by a homogenous red light (peak 650nm) of $3200 \mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by an array of six light-emitting diodes.

Analysis of the Chlorophyll a Fluorescence Transient by the JIP test

The JIP test, as developed by Strasser and Strasser (1995) is a useful tool to translate the fluorescence measurements obtained from the O-J-I-P transients into phenomenological and biophysical expressions to enable quantification of PS II function by deriving several parameters from the data (Refer to Table 2.1 for derived parameters and how they are calculated) (De Beer, 2005). Specifically the following data from the original O-J-I-P fluorescence transients are used in the JIP test:

- F_M – maximal fluorescence intensity

- F_0 – fluorescence intensity at 50 μs
- $F_{300\mu\text{s}}$ – fluorescence intensity at 300 μs (This is required for the calculation of the initial slope (M_0))
- M_0 – Initial slope of the variable fluorescence kinetics (V)
- V_J – Fluorescence kinetics
- F_J – fluorescence intensity at 2 ms (the J step)

As stated before the JIP test can serve as a tool to translate the O-J-I-P fluorescence transients into biophysical and phenomenological parameters. The biophysical parameters can be used to quantify the stepwise flow of energy through PS II at both the reaction centre (RC) and excited cross-section (CS) level. The following parameters refer to time zero, in other words the onset of fluorescence induction (Refer to Table 2.1 for derived parameters and how they are calculated):

- Parameters related to specific fluxes
 - ABS/RC – specific energy fluxes per reaction centre for absorption
 - TR_0/RC – Specific energy fluxes per reaction centre for trapping
 - DI_0/RC – Specific energy fluxes per reaction centre for dissipation at the level of the antenna chlorophylls
 - ET_0/RC – Specific energy fluxes per reaction centre for electron transport
 - F_V/F_0 – Energy per reaction centre of the light reaction
- Quantum efficiencies of flux ratios
 - ϕ_{P0} – maximum quantum yield of primary photochemistry
 - Ψ_0 – the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-
 - ϕ_{E0} – the quantum yield of electron transport
- Phenomenological fluxes or phenomenological activities
 - ABS/CS – Phenomenological activity fluxes per excited cross section for absorption
 - TR_0/CS – Phenomenological activity fluxes per excited cross section for trapping
 - DI_0/CS – Phenomenological activity fluxes per excited cross section for dissipation

- ET_0/CS – Phenomenological activity fluxes per excited cross section for electron transport

The fraction of active PS II reaction centres per excited cross section (RC/CS) can also be calculated giving an indication of the density of the reaction centres (Strasser & Strasser, 1995).

Statistical analyses

Statistical analyses was performed on the data sets using STATISTICA 10 (StatSoft Inc ©, 2011). A one-way breakdown analysis of variance, ANOVA, (repeated measurements ANOVA) was performed, after which a Tukey's Honest significance test was performed to determine statistical significance between the various treatments.

CHAPTER 4 – RESULTS AND DISCUSSION

4.1 Results from common pharmacophore generation

As the operating hypothesis for this study entails that the action of gliotoxin is very similar to gibberellic acid, we did not screen available databases for other similar pharmacophores. As such we only focused on GA and GT for this evaluation. In this study the pharmacophore evaluation was only used to evaluate possible similar orientations of the molecules, and not to the extensive degree in other studies when pharmacophore generation generates and ranks candidate molecules.

The common pharmacophore model generated from the various gibberellic acid molecules identified three region types of interest for the pharmacophore alignment, namely the hydrogen bond donor regions (represented as magenta), hydrophobic regions (represented as light blue, and hydrogen acceptor regions (represented as green). When aligning the GA₃ into this pharmacophore model it was observed that a hydrophobic region (light blue) is located to the C2 and C3 side of the structure and was situated in close proximity to the lactone ring between C4 and C10. Hydrogen bond acceptor areas (green) lies between C9 and C10, as well as C10 through C11. In relative close proximity to this, the hydrogen bond donor areas (magenta) are located in the vicinity of C13 through C14 (Figure 4.1.1).

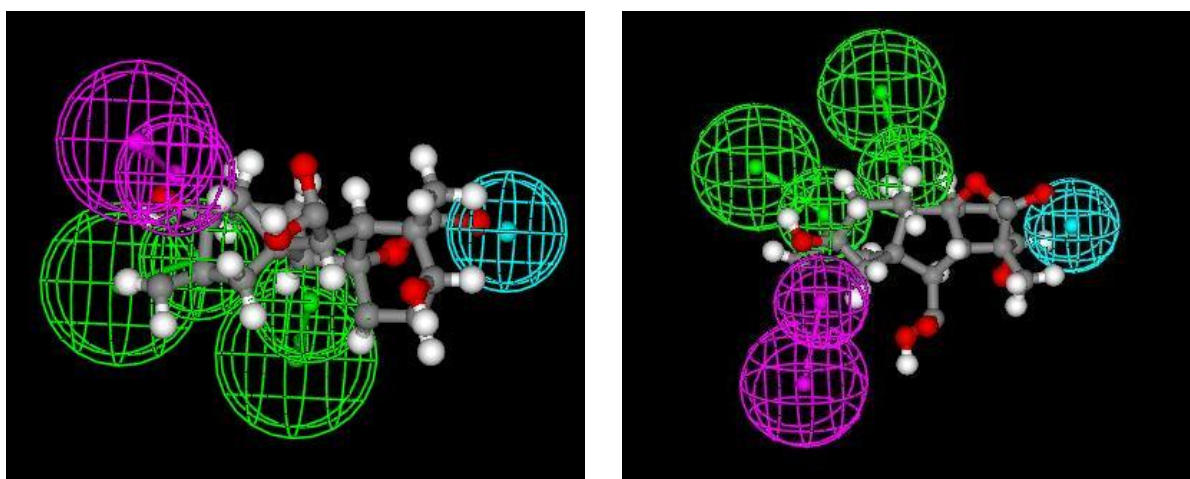


Figure 4.1.1 a (left, side view) and b (right, top view): Structure of GA₃ as aligned to the common pharmacophore model. (Colour coding employed by CATALYST: Magenta = hydrogen bond donor, Light Blue = hydrophobic region, Green = hydrogen bond acceptor).

As is apparent from Figure 4.1.2, gliotoxin was observed to successfully align in the common pharmacophore model that has been generated with the hydrogen bond acceptor regions (green) being located in the proximity of the carboxamide group and C12, while the hydrogen bond donor regions (magenta) were located in the proximity of C1 to C2 in relative close proximity the disulphide bridge. The hydrophobic region (light blue) was observed to align in the proximity of C7 to C8.

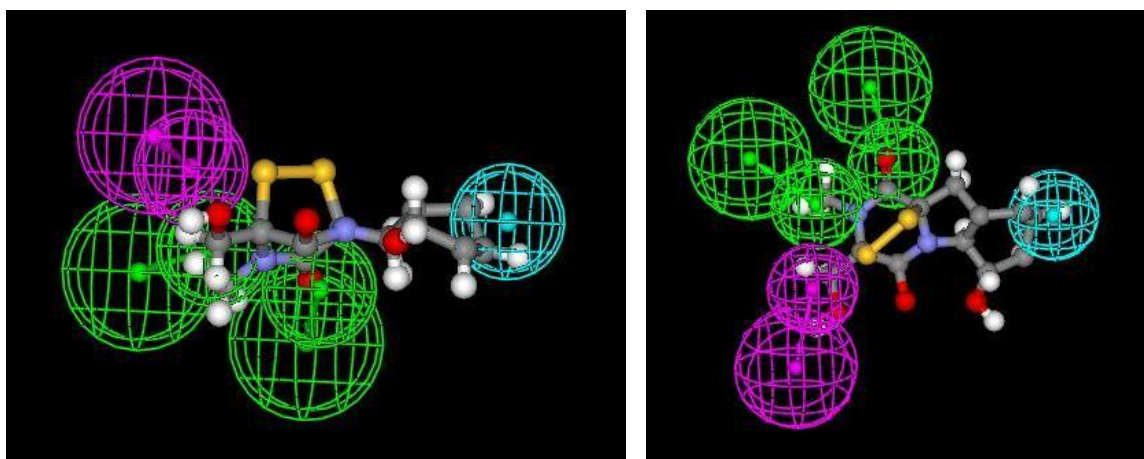


Figure 4.1.2 a (left, side view) and b (right, top view): Structure of gliotoxin as aligned to the common pharmacophore model. (Colour coding employed by CATALYST: Magenta = hydrogen bond donor, Light Blue = hydrophobic region, Green = hydrogen bond acceptor).

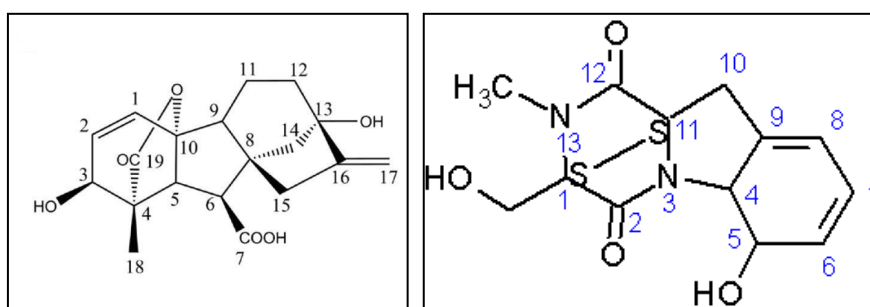


Figure 4.1.3 a (left) and b (right): Numbered structures for gibberellic acid (left) and gliotoxin (right).

The structural analysis of GA₃ and GT separately within the common pharmacophore model suggests that GT and GA₃ might be perceived as being similar in a plant system. This perception in plant systems is significantly strengthened when the structures are overlaid (Figure 4.1.4) onto each other within the pharmacophore model. The lactone ring of GA₃

overlays with the ring structure at C5 for GT, while the disulphide bridge at C1 and C11 overlays with the ring structure at C8-C13 in GA₃ (Refer to Figure 4.1.3 for numbering scheme employed). The spatial occupation of the molecules were also observed to be strikingly similar. Thus, from common pharmacophore modelling, the match between the molecules appear quite convincing, but it is important to remember that during common pharmacophore modelling only the functional characteristics are considered for model generation and alignment. In essence, gliotoxin should be able to bind to the same receptors as GA₃ does based on functional group interactions, but this does not guarantee that similar plant responses may be evoked.

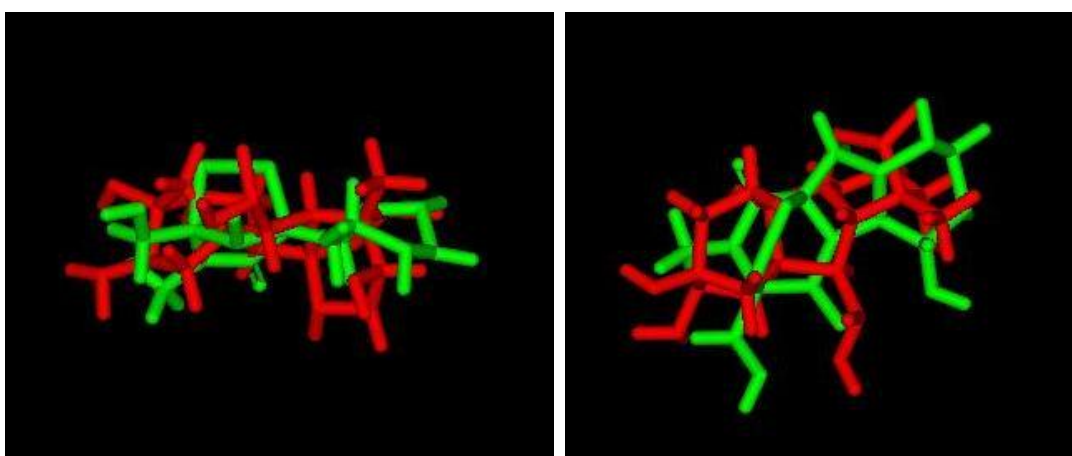


Figure 4.1.4 a (left, side view) and b (right, top view): Stick representation of GA₃ (red) and gliotoxin (green) as aligned to common pharmacophore model.

4.2 Docking simulation of GA₃ and GT into GID1

For reference purposes the structural basis of gibberellin (GA₃)-induced DELLA recognition by the gibberellin receptor (GID1) from Shimada *et al.* (2008) are presented first (Protein file 3ED1 from RCSB). The crystallised structure of GA₃ is shown in Figure 4.2.1 a through b.

As stated in the study by Murase *et al.* (2008) the following apparent features were observed:

- Arg244 and Ser116 and Ser191 and its orientation relative to the carboxylate group.
- Ile126, Val239 and Val319 (Leu323 not included in the binding site defined by ArgusLab) and its orientation relative to the aliphatic rings of GA₃.

- Ile24, Phe27 and Tyr31 (His119 not included in the binding site defined by ArgusLab) are the N-terminal extension helices which are adjusted for GA ring recognition.
- Tyr127 bond with the C3 hydroxyl group of GA₃.
- Phe238 form a weak bond with C13 hydroxyl group of GA₃.

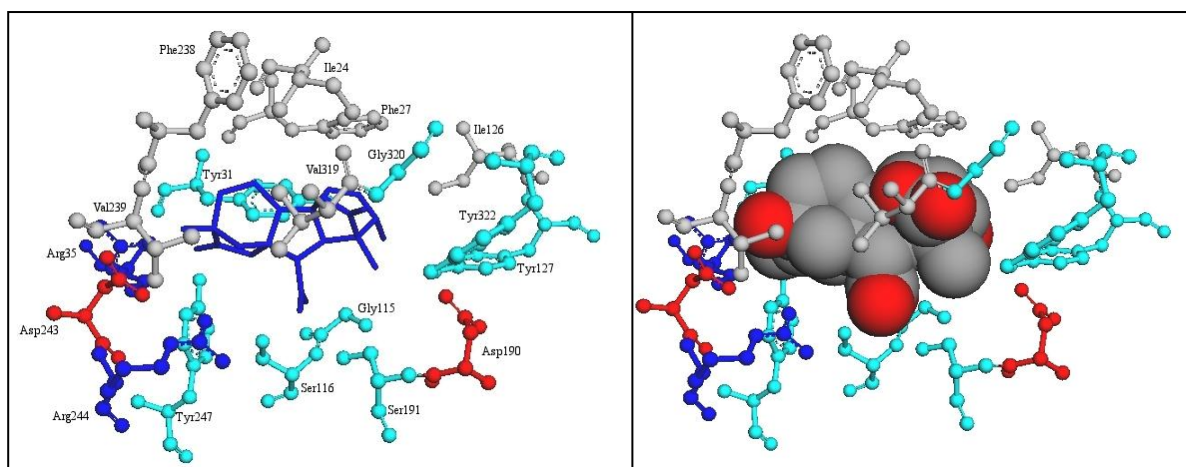


Figure 4.2.1 a (left) and b (right): GA₃ binding within the binding site of 3ED1. Stick representation - left, and the sphere representation - right.

For further comparison of the possible structural similarity, a docking simulation was performed in ArgusLab, using a GA₃ molecule constructed from SMILES notation and optimised using CORINA.

The docking pose for the optimised GA₃ molecule obtained with CORINA was observed to be situated relatively higher than that for the GA₃ molecule crystallised within 3ED1 (Figure 4.2.2 a and b as well as Figure 4.2.3 a and b). The orientation of the carboxylate group was similar. However, the orientation in terms of the lactone ring and hydroxyl groups appears to be flipped. A docking score of -10.78 kcal/mol was the optimal pose calculated. The change in the docking pose might be a result of the GA₃ from CORINA having a more sterical hindrance than that of the GA₃ molecule within 3ED1. The differences in the optimal pose calculated may also be due to differences between the optimal configuration calculated for the generated GA₃ and the structure of the GA₃ molecule within the receptor protein, a factor CORINA does not account for in the optimisation of the structure.

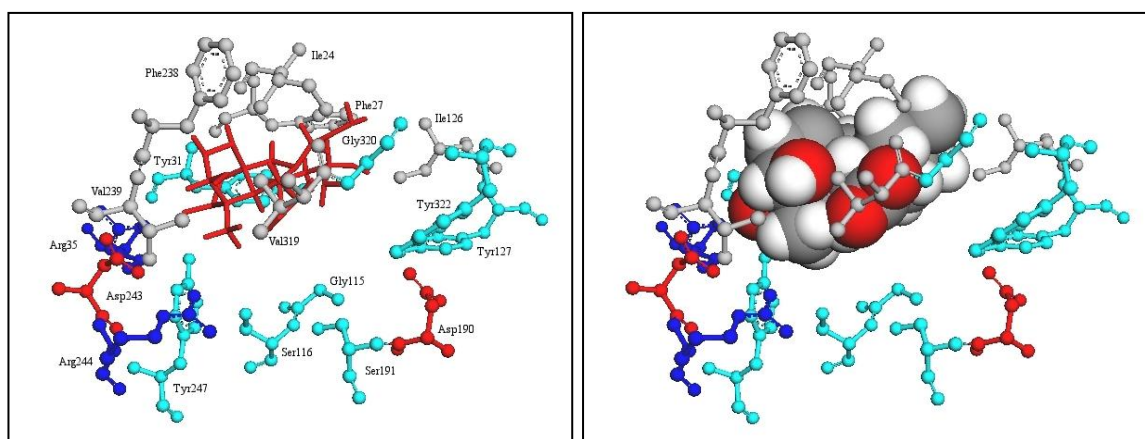


Figure 4.2.2 a (left) and b (right): GA_3 (Generated with CORINA) binding within the binding site of 3ED1. Stick representation – left and sphere representation – right.

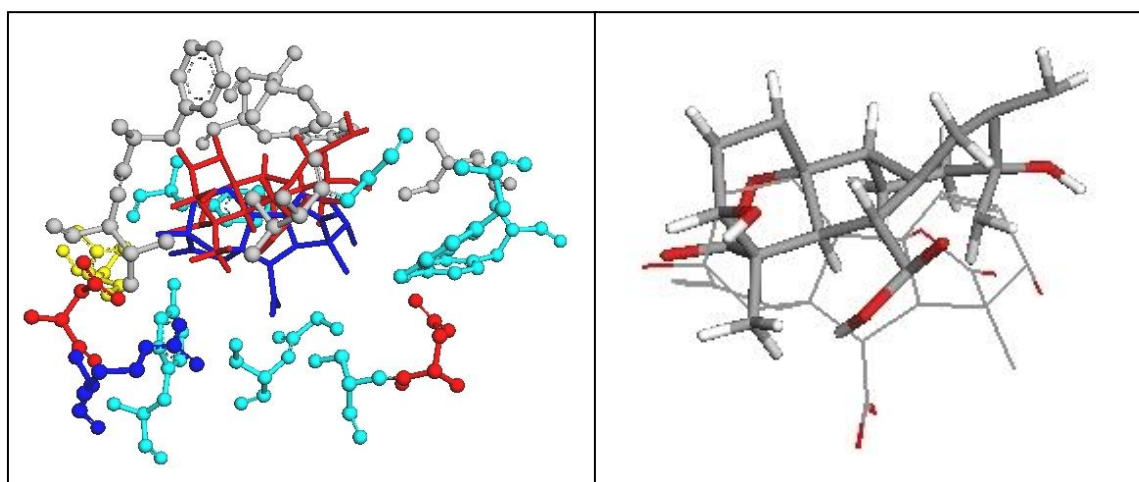


Figure 4.2.3 a (left) and b (right): Comparison of the docking poses for the GA_3 molecule crystallised with 3ED1 (blue) and the GA_3 molecule from CORINA. Docking poses with the binding site displayed (a) and docking poses with the binding site removed (b) for greater clarity. In Figure 4.2.3 b, the GA_3 molecule within 3ED1 is represented as wireframe and the GA_3 from CORINA is represented by a stick representation.

In the next docking simulation gliotoxin was evaluated. In this docking, the lactone ring of GA_3 was found to align with the ring structure at C5 (Refer to Figure 4.1.3), while the disulphide bridge at C1 and C11 aligned with the ring structure at C8-C13 in GA_3 . Furthermore, the COH group at C1 of GT aligned with the carboxylate group at C6 from GA_3 and with Arg244, Ser116 and Ser191 of the protein. The disulphide bridge of GT orientates

with Val319. A docking score of -10.18 kcal/mol was calculated by ArgusLab for the optimal pose between gliotoxin and GID1.

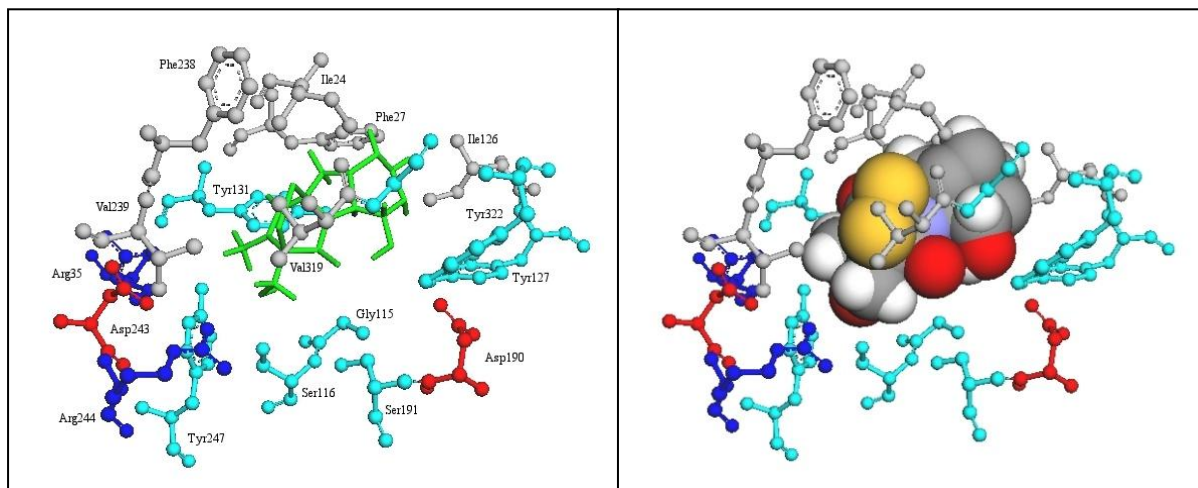


Figure 4.2.4 a (left) and b (right): GT (Generated with CORINA) binding within the binding site of 3ED1. Stick representation – left and sphere representation – right.

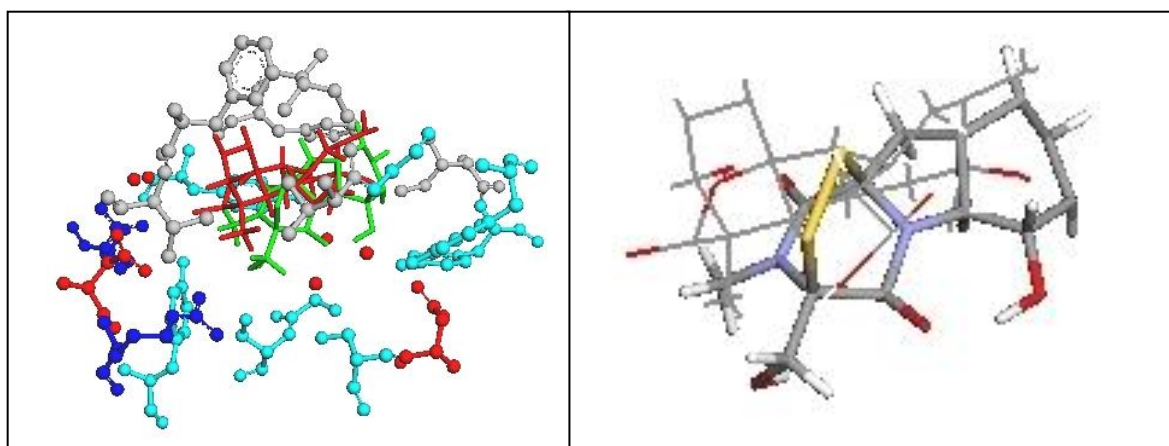


Figure 4.2.5 a (left) and b (right): Comparison of the docking poses for the GA₃ molecule crystallised with 3ED1 (red) and the GT molecule from CORINA (green). Docking poses with the binding site displayed (a) and docking poses with binding site not displayed (b) for greater clarity. In Figure 4.2.5 b, the GA₃ molecule from CORINA is represented as wireframe and the GT from CORINA is represented by a stick representation.

When comparing the docking of gliotoxin and the GA₃ molecule crystallised into the structure of GID1, the results appear to be more in line with the orientation calculated from pharmacophore generation. When the docking poses of both GA₃ (from the GID1 structure

itself) and GT are examined, the docking pose for GT appears very close to that of the GA₃ within the GID1 protein structure (Figure 4.2.6 a). Also, when the molecules' docking poses were compared without the docking cage (Figure 4.2.6 b), the orientation seemed to match that of the results obtained in Section 4.1. In this docking, the lactone ring of GA₃ aligned with the ring structure at C5 (Refer to Figure 4.1.3), while the disulphide bridge at C1 and C11 aligned with the ring structure at C8-C13 in GA₃. Furthermore, the COH group at C1 of GT aligned with the carboxylate group at C6 from GA₃ as well as with Arg244, Ser116 and Ser191 of the protein. The disulphide bridge of GT orientated with Val319 and a resulting docking score of -10.18 kcal/mol, was calculated by ArgusLab for the optimal pose.

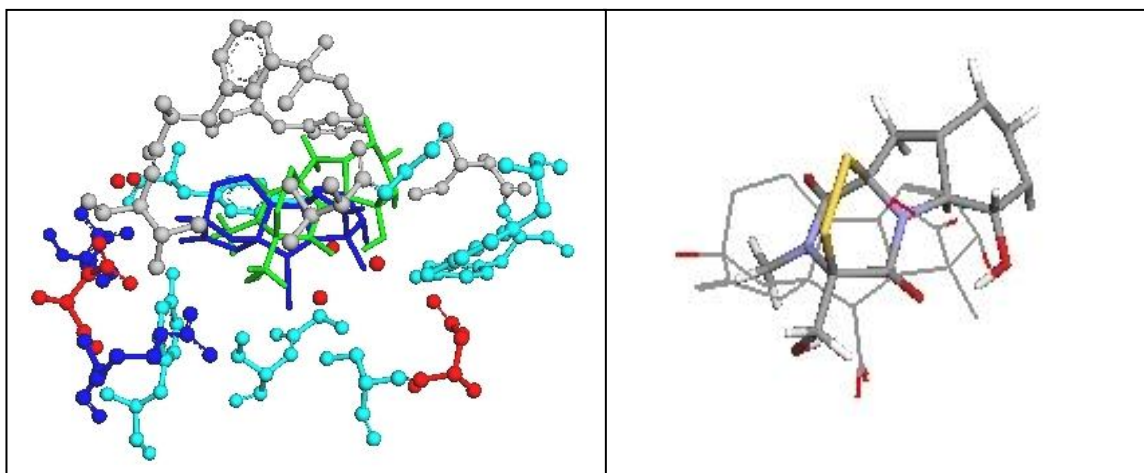


Figure 4.2.6 a (left) and b (right): Comparison of the docking poses for the GA₃ molecule crystallised with 3ED1 (blue) and the GT molecule from CORINA (green). Docking poses with the binding site displayed (a) and docking poses with binding site not displayed (b) for greater clarity. The GA₃ molecule within 3ED1 (Figure 4.2.6 b) is represented as wireframe and the GT from CORINA is represented as a stick representation.

Overall, with GT docked within 3ED1, gliotoxin does appear to dock in a pose that is quite similar to that predicted by the common pharmacophore alignment. The COH group at C1 of GT appear to orientate in a similar manner to the carboxylate group of GA₃. Alignment to the other functional groups appear similar to that predicted from common pharmacophore alignment. Again, sterical hindrance might play a role in the alignment not being an exact overlay match. Another reason for the differences between the docking results obtained with regard to molecule poses and the common pharmacophore poses, might be that with the common pharmacophore modelling only the characteristics of the molecules are considered

and aligned, whereas with docking, molecular charges and various interactions between the ligand and the binding site are allowed to affect the final calculated orientation. In terms of docking with the aid of ArgusLab, a docking score of -10.78 kcal/mol for the GA₃ molecule from CORINA (2008) and a docking score of -10.17 kcal/mol for GT was calculated. Though the docking scores suggest good similarity between the molecules, differences in the structure and shape of the GA₃ within GID1 and the external GA₃ (constructed using CORINA), might contribute to the differences in docking poses observed. Thus, based on the data presented above and the docking scores obtained, it seems reasonable to deduce that GT will dock to the GID receptor protein and may elicit physiological responses similar to that of the natural GA₃ molecule.

Even though similarities between docking poses and docking scores may strongly suggest similar affinities for the ligands to the receptor, it still does not guarantee that similar physiological responses will be evoked. For this reason it was deemed necessary to compare the physiological responses elicited in a plant by the two compounds. Subsequently, the effect of gibberellic acid treatment and gliotoxin treatment on germination and plant height, α -amylase expression and OJIP-fluorescence was also evaluated and is presented in the following sections.

4.3 Comparison of plant height and emergence

When comparing the treatments (Table 4.3.1 and Figure 4.3.1 a and b), it became clear that with the exception of treatments GA4 and GA5, the gibberellic acid treatment yielded improved plant heights on day 12 relative to the control (untreated) plants. All the gliotoxin treated plants were characterised by higher plant heights from day 8 to day 12, relative to the control, despite GT7 not performing better than the untreated controls. Although none of the observed differences in plant height across the various treatments over the entire time period were found to be statistically significant, it is important to note that for the treatments GA4, GA7, GA8, GT4, and GT5 plant emergence occurred one day earlier than for the control. Furthermore, for the treatments GA7, GA8, GT7 and GT8, the overall plant height seemed to be similar throughout the period, suggesting that these concentrations represent the optimal treatment concentrations.

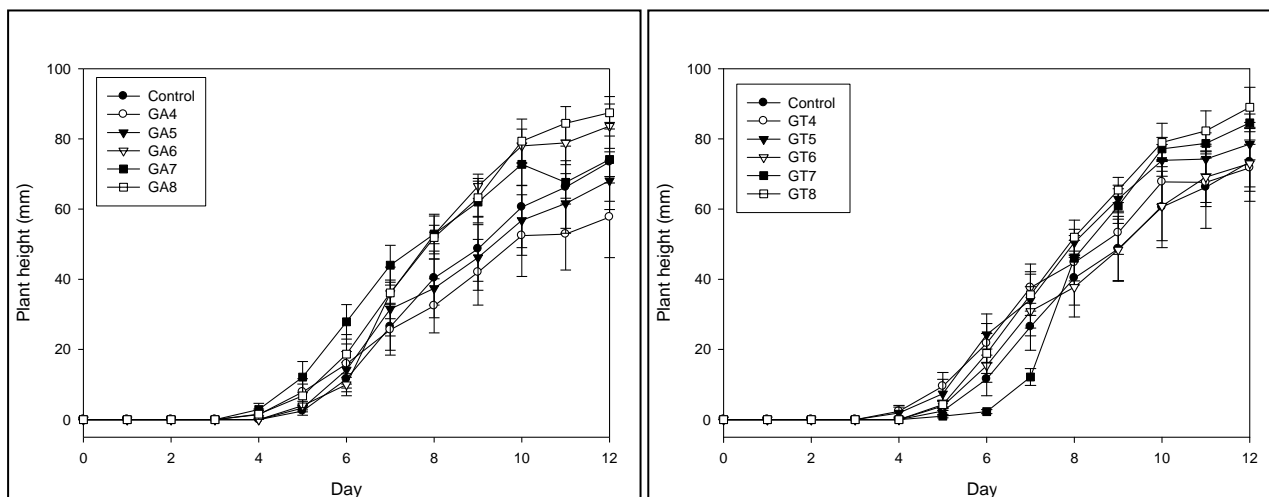


Figure 4.3.1 a (left) and Figure 4.3.1 b (right): Plant height for gibberellic acid (GA) treatment and gliotoxin (GT) treatment. GA4 through GA8 represents a dilution series of GA₃ starting at 10⁻⁴ M GA (GA4) through 10⁻⁸ M GA (GA8); GT4 through GT8 represents a dilution series of gliotoxin starting at 10⁻⁴ M GT (GT4) through 10⁻⁸ M GT (GT8).

Table 4.3.1: Summary of plant heights per treatment (n = 10). (Statistical means ± standard error). GA4 through GA8 represents a dilution series of GA₃ starting at 10⁻⁴ M GA (GA4) through 10⁻⁸ M GA (GA8); GT4 through GT8 represents a dilution series of gliotoxin starting at 10⁻⁴ M GT (GT4) through 10⁻⁸ M GT (GT8).

Day	Control	GA4	GA5	GA6	GA7	GA8
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4	0.00 ± 0.00	1.43 ± 1.20	0.00 ± 0.00	0.00 ± 0.00	2.88 ± 1.78	1.56 ± 1.48
5	2.44 ± 1.18	7.86 ± 3.81	3.30 ± 1.09	4.00 ± 1.22	12.13 ± 4.44	6.78 ± 3.36
6	11.56 ± 4.75	15.86 ± 5.71	14.20 ± 5.19	10.00 ± 2.06	27.88 ± 4.92	18.67 ± 5.58
7	26.44 ± 6.69	25.57 ± 7.18	31.50 ± 7.66	36.00 ± 3.76	44.00 ± 5.68	36.11 ± 7.35
8	40.33 ± 7.69	32.43 ± 7.71	37.40 ± 8.36	52.75 ± 2.62	52.88 ± 5.64	51.89 ± 6.12
9	48.67 ± 9.25	42.00 ± 9.35	46.20 ± 9.34	66.50 ± 3.46	62.00 ± 5.93	63.22 ± 5.50
10	60.56 ± 11.53	52.43 ± 11.64	56.80 ± 9.97	78.00 ± 4.78	72.75 ± 6.07	79.33 ± 6.30
11	66.22 ± 11.70	52.86 ± 10.24	61.60 ± 8.52	78.88 ± 6.23	67.63 ± 6.18	84.44 ± 4.76
12	73.44 ± 11.23	57.71 ± 11.55	68.10 ± 8.21	83.63 ± 6.31	74.13 ± 6.70	87.44 ± 4.62

Table 4.3.1: Summary of plant height per treatment (n = 10) (Continued)

Day	Control	GT4	GT5	GT6	GT7	GT8
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4	0.00 ± 0.00	2.38 ± 1.67	1.88 ± 1.68	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	2.44 ± 1.18	9.50 ± 3.95	7.38 ± 4.12	3.88 ± 1.27	1.00 ± 0.48	4.29 ± 1.18
6	11.56 ± 4.75	21.75 ± 5.63	24.25 ± 5.86	15.38 ± 4.74	2.29 ± 0.57	18.86 ± 5.68
7	26.44 ± 6.69	37.63 ± 6.71	34.13 ± 8.02	30.88 ± 7.00	12.14 ± 2.39	35.57 ± 5.88
8	40.33 ± 7.69	44.75 ± 6.34	50.50 ± 3.73	37.75 ± 8.49	46.00 ± 6.45	52.00 ± 4.83
9	48.67 ± 9.25	53.25 ± 6.15	62.88 ± 3.93	48.38 ± 8.75	60.86 ± 4.93	65.43 ± 3.58
10	60.56 ± 11.53	67.75 ± 6.37	73.88 ± 4.53	60.88 ± 9.89	77.14 ± 3.27	79.00 ± 5.43
11	66.22 ± 11.70	67.63 ± 6.90	74.25 ± 4.21	69.13 ± 7.23	78.71 ± 2.99	82.29 ± 5.70
12	73.44 ± 11.23	71.75 ± 6.70	78.50 ± 4.46	73.00 ± 6.68	84.57 ± 2.52	89.00 ± 5.71

It is important to bear in mind that the envisaged application concentration for gliotoxin and gibberellic acid are within the 10^{-6} and 10^{-8} M range. This range corresponded to the concentration ranges where α -amylase expression were detected in de-embryonated seeds in a related study (Cejudo *et al.*, 1995) (Section 4.4). Further discussion will focus on this concentration range in the data. Another aspect to consider in the interpretation of these results, is that gliotoxin was evaluated in isolation from other known secondary metabolites of *Trichoderma*. In particular, the gibberellic acid type effects such as seed germination, are of interest here. Other studies where more significant differences in plant height due to *Trichoderma* treatment were observed, can be attributed to the auxin-type effect that secondary metabolites part of a *Trichoderma* treatment, such as koniginin A and 6PP, can elicit (Vinale *et al.* 2008b) in conjunction with the earlier germination effect of gliotoxin demonstrated here.

4.4. Comparison of α -amylase induction

The purpose of this gel was solely to detect the presence of amylase activity indicating gibberellins-like activity. Amylase activity bands could be observed for the positive control (PC) and all the gibberellic acid lanes, GA1 through GA6 (Figure 4.4.1). As expected, amylase activity was absent for the negative control (NC) lane. The highest activity was apparent in lane GA5, which represented a concentration of 10^{-7} M gibberellic acid. Amylase activity could be observed for the PC lane and the lanes GT2 through GT6, while the amylase activity observed in the NC lane (Figure 4.4.2) was due to a transfer from PC during the

loading of the gel (Figure 4.4.2). The highest amylase activity was apparent in lane GT4 corresponding to a gliotoxin concentration of 10^{-6} M gliotoxin.

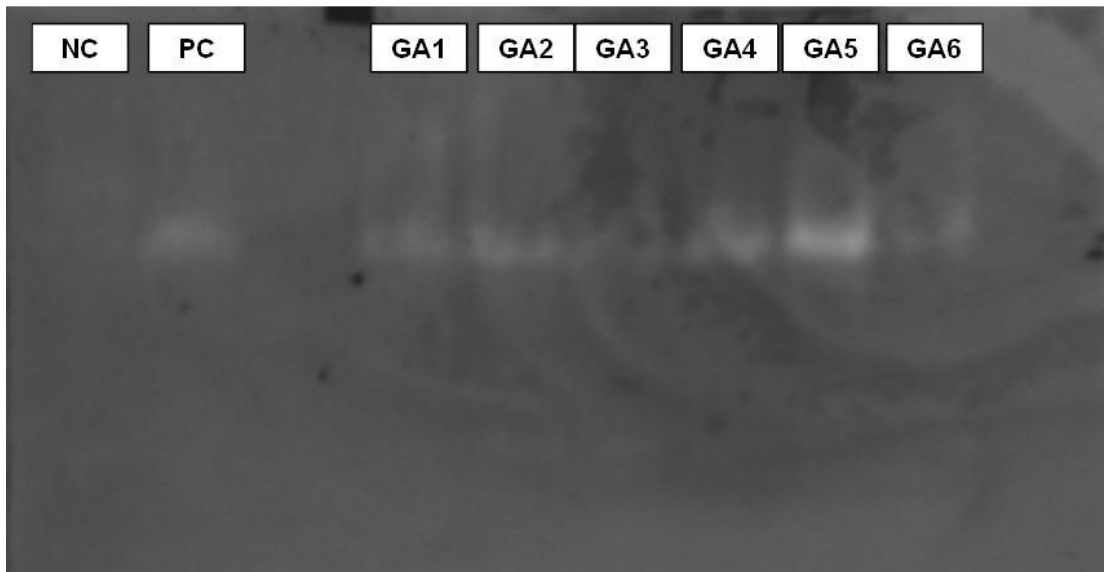


Figure 4.4.1: Zymogram of de-embryonated seeds treated with gibberellic acid. NC = negative control, PC = positive control; GA1 through GA6 represents a dilution series starting at 10^{-3} M GA (GA1) through 10^{-8} M GA (GA6).

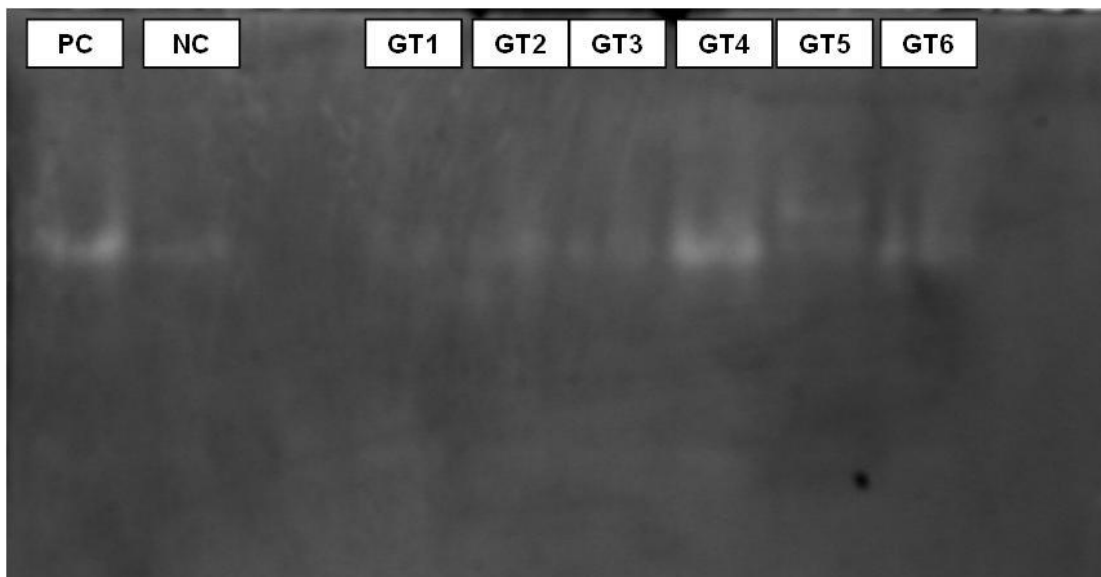


Figure 4.4.2: Zymogram of de-embryonated seeds treated with gliotoxin. NC = negative control, PC = positive control; GT1 through GT6 represents a dilution series starting at 10^{-3} M GT (GT1) through 10^{-8} M GT (GT6).

From the zymogram data, it is interesting to note that for both gliotoxin and GA₃, the concentration of highest amylase induction occurred in the range expected for hormones, i.e. 10⁻⁶ M or less. However, gibberellic acid was able to elicit the physiological response at a lower concentration than gliotoxin (Figure 4.4.1 and Figure 4.4.2).

4.5 Comparison of JIP-fluorescence responses

A summary of the photosynthetic performance index (PI_(Abs)) results is shown in Table 4.5.1. In this table day 0 represents the PI_(Abs) results for the plants prior to treatment. Treatment with the growth hormones were conducted within a series of 10⁻⁴ M through 10⁻⁸ M, designated as GA for gibberellic acid and GT for gliotoxin (Wang *et al*, 1996). It was evident that there was some heterogeneity in the data. However, statistically significant differences were also observed between the various treatments.

Table 4.5.1: Summary of Performance Index (PI_(Abs)) results (Statistical means ± standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.

Code	Treatment	PI Day 0	PI Day 2	PI Day 6	PI Day 8
C	Control	1.640 ± 0.079 ^{a,b}	1.442 ± 0.067 ^a	1.589 ± 0.064 ^{b,f}	1.364 ± 0.130 ^a
GA4	10 ⁻⁴ M GA	1.258 ± 0.077 ^{a,c,d}	1.159 ± 0.142 ^{a,c}	1.403 ± 0.080 ^{a,b}	1.206 ± 0.309 ^a
GA5	10 ⁻⁵ M GA	1.288 ± 0.072 ^{a,c,d}	1.269 ± 0.069 ^a	1.160 ± 0.098 ^{a,d,e}	1.549 ± 0.168 ^a
GA6	10 ⁻⁶ M GA	1.521 ± 0.079 ^{a,b}	1.364 ± 0.083 ^a	1.303 ± 0.056 ^{a,b,e}	1.276 ± 0.160 ^a
GA7	10 ⁻⁷ M GA	2.082 ± 0.068 ^f	2.059 ± 0.081 ^d	2.035 ± 0.103 ^g	1.204 ± 0.047 ^a
GA8	10 ⁻⁸ M GA	0.691 ± 0.095 ^e	0.640 ± 0.087 ^b	0.712 ± 0.043 ^c	0.838 ± 0.292 ^a
GT4	10 ⁻⁴ M GT	1.058 ± 0.071 ^{c,d,e}	0.717 ± 0.138 ^{b,c}	0.761 ± 0.142 ^{c,d}	1.209 ± 0.100 ^a
GT5	10 ⁻⁵ M GT	1.487 ± 0.075 ^{a,b,d}	1.250 ± 0.060 ^a	1.833 ± 0.117 ^{f,g}	1.225 ± 0.244 ^a
GT6	10 ⁻⁶ M GT	1.834 ± 0.138 ^{b,f}	1.430 ± 0.139 ^a	1.510 ± 0.070 ^{a,b,f}	1.357 ± 0.199 ^a
GT7	10 ⁻⁷ M GT	1.529 ± 0.116 ^{a,b}	1.173 ± 0.073 ^{a,c}	1.397 ± 0.062 ^{a,b}	1.346 ± 0.228 ^a
GT8	10 ⁻⁸ M GT	0.959 ± 0.100 ^{c,e}	0.524 ± 0.112 ^b	0.902 ± 0.057 ^{c,d,e}	1.280 ± 0.096 ^a

The heterogeneity of the data makes a direct evaluation of the effects of the treatments rather problematic. To overcome this problem, the data was normalised by calculating a percentage

change over time from the Day 0 (pre-treatment) data, with the 100% value indicating no change and serving as a reference point for comparison (Strauss *et al.*, 2006).

The following could be observed from the data. On day 2 (D2) a decrease in the $PI_{(Abs)}$ was observed for all treatments, but the effect was quite marked in treatment GT4 and GT8 (Table 4.5.2). By day 6 (D6), most of the gliotoxin (GT4 through GT8) treatments showed increases in $PI_{(Abs)}$ relative to day 2, while slight decreases were observed for the gibberellic acid treatments (GA4 through GA8) with the exception of treatment GA8. By day 8 (D8), treatment GT8 displayed the highest change in $PI_{(Abs)}$ relative to the baseline for the gliotoxin treatments and treatment GA5 for the gibberellic acid treatments. This suggested that the best stimulation of $PI_{(Abs)}$ was observed for the 10^{-5} M and 10^{-8} M treatments of gibberellic acid and for the 10^{-4} M and 10^{-8} M treatments for gliotoxin. In practice, for agricultural purposes, gliotoxin treatments of 10^{-8} M is suggested solely from an economical point of view and either 10^{-7} M or 10^{-8} M for gibberellic acid based on the data obtained. When comparing the 10^{-8} M treatments of both gliotoxin and gibberellic acid on an equivalent concentration base, the gliotoxin treatment had a far greater effect on $PI_{(Abs)}$ than gibberellic acid. However, both these concentrations resulted in an increase in $PI_{(Abs)}$, supporting the hypothesis that gliotoxin can elicit similar responses in plants relative to gibberellic acid.

Table 4.5.2: Summary of normalised $PI_{(Abs)}$ data with Day 0 (D0) serving as baseline. (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA_3) in a dilution series range from 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to treatment with gliotoxin in a dilution series range from 10^{-4} M through 10^{-8} M.

Code	Treatment	$PI_{(Abs)}$ D0	$PI_{(Abs)}$ D2	$PI_{(Abs)}$ D6	$PI_{(Abs)}$ D8
C	Control	100.00	$89.02 \pm 5.99^{a,b}$	$97.76 \pm 5.03^{a,b,c}$	84.34 ± 9.67^a
GA4	10^{-4} M GA	100.00	91.97 ± 8.56^a	$113.30 \pm 8.82^{a,b}$	90.93 ± 19.51^a
GA5	10^{-5} M GA	100.00	99.36 ± 5.76^a	$89.91 \pm 5.07^{a,b,c}$	123.91 ± 17.91^a
GA6	10^{-6} M GA	100.00	90.27 ± 5.02^a	$86.30 \pm 3.67^{a,b,c}$	85.97 ± 12.70^a
GA7	10^{-7} M GA	100.00	99.39 ± 5.07^a	$98.63 \pm 7.15^{a,b,c}$	58.13 ± 2.84^a
GA8	10^{-8} M GA	100.00	95.61 ± 8.72^a	$113.14 \pm 14.77^{a,b}$	107.26 ± 29.48^a
GT4	10^{-4} M GT	100.00	$66.27 \pm 11.90^{a,b}$	70.60 ± 10.38^c	115.92 ± 11.27^a
GT5	10^{-5} M GT	100.00	$86.08 \pm 8.41^{a,b}$	125.41 ± 11.83^b	85.77 ± 17.61^a
GT6	10^{-6} M GT	100.00	$77.22 \pm 3.40^{a,b}$	$84.02 \pm 5.01^{a,c}$	82.52 ± 23.11^a
GT7	10^{-7} M GT	100.00	$78.12 \pm 6.08^{a,b}$	$92.81 \pm 4.37^{a,b,c}$	91.88 ± 17.86^a
GT8	10^{-8} M GT	100.00	53.00 ± 10.91^b	$97.30 \pm 7.85^{a,b,c}$	136.02 ± 5.90^a

Analysis of the data reported a statistically significant effect of the various treatments on the $PI_{(Abs)}$ ($p = 0.00001$). Presented in Figure 4.5.1 a (top) and b (bottom) is a summary of the key Kautsky transient parameters at day 8 after treatment with gibberellic acid or gliotoxin.

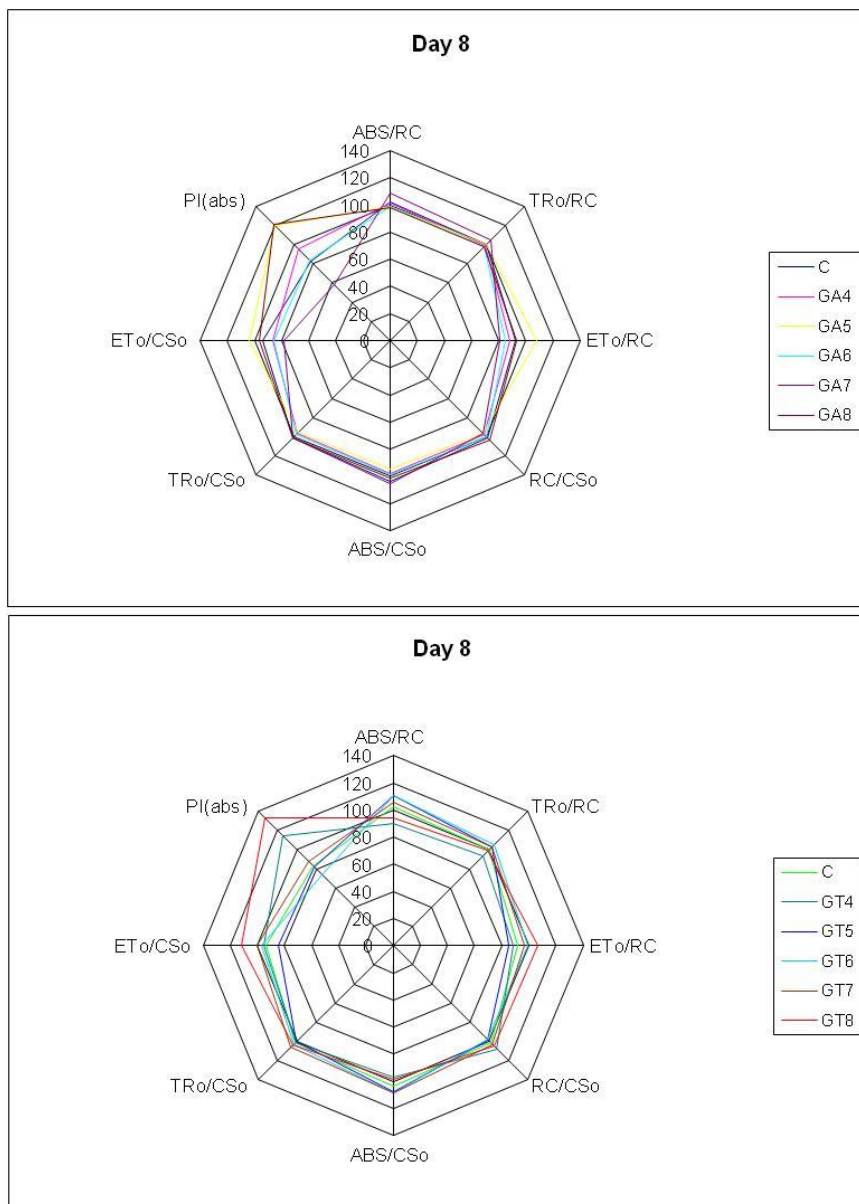


Figure 4.5.1 a (top) and b (bottom): Spider plot summary of key Kautsky transient parameters at day 8 after treatment with growth regulators. Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA_3) in a dilution series range from 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10^{-4} M through 10^{-8} M.

From the spider plot (Figure 4.5.1 a and b), variations in the responses to the various treatments were observed and the following parameters were selected for more detailed discussion:

- $PI_{(Abs)}$ – Performance index
- ET_0/RC – Specific energy fluxes per reaction centre for electron transport
- ET_0/CS_0 – Phenomenological activity fluxes per excited cross section for trapping
- ABS/RC – Specific energy fluxed per reaction centre for absorption

$PI_{(Abs)}$

The performance index $PI_{(Abs)}$ serves as an indicator of sample (plant) vitality and by extension of this definition, the ability of the sample (plant) to resist constraints from the outside (Strasser & Strasser, 1995). As was expected, the control plants showed no change in $PI_{(Abs)}$ over the 8 days the plants were monitored (Figure 4.5.2 a and b; Table 4.5.2). However, when comparing the $PI_{(Abs)}$ results for GA_3 treated plants relative to the control plants, the following was observed. A decline in $PI_{(Abs)}$ was observed during days 0 through 2 for treatment GA4. This was followed by an increase during days 2 through 6 and a decline during days 6 through 8. With treatment GA5 a decline in $PI_{(Abs)}$ was observed during days 0 through 6, followed by a marked increase during days 6 through 8. For treatment GA6 a decline was observed during the entire period post treatment. Treatment GA7 displayed a slight decline in $PI_{(Abs)}$ during days 0 through 6, followed by a marked decline during days 6 through 8. Despite an initial decline in $PI_{(Abs)}$ during day 0 through 2, similar to GA6, the decline was followed by an increase in $PI_{(Abs)}$ during days 2 through 6, followed by a marked increase during days 6 through 8. Treatment GA8 was also characterised by the largest increase in percentage change in $PI_{(Abs)}$ after the 8 day period.

The gliotoxin treated plants were all typified by a decline in $PI_{(Abs)}$ during days 0 through 2. Overall, the declines observed during this period were larger than for their equivalent GA_3 treated counterparts. During days 2 through 6 all treated plants displayed an increase in percentage change in $PI_{(Abs)}$ to varying degrees with treatments GT4, GT6 and GT7 showing a slighter increase than compared to the more marked increases observed for treatments for treatments GT5 and GT8. Though treatment GT5 showed a higher percentage change compared to GT8, during days 6 through 8 treatment GT5 showed a marked decline while treatment GT8 continued an increase in $PI_{(Abs)}$.

Overall, after 8 days treatment GT8 resulted in a higher percentage change in $PI_{(Abs)}$ compared to its GA_3 equivalent GA8, though overall the trends observed were similar.

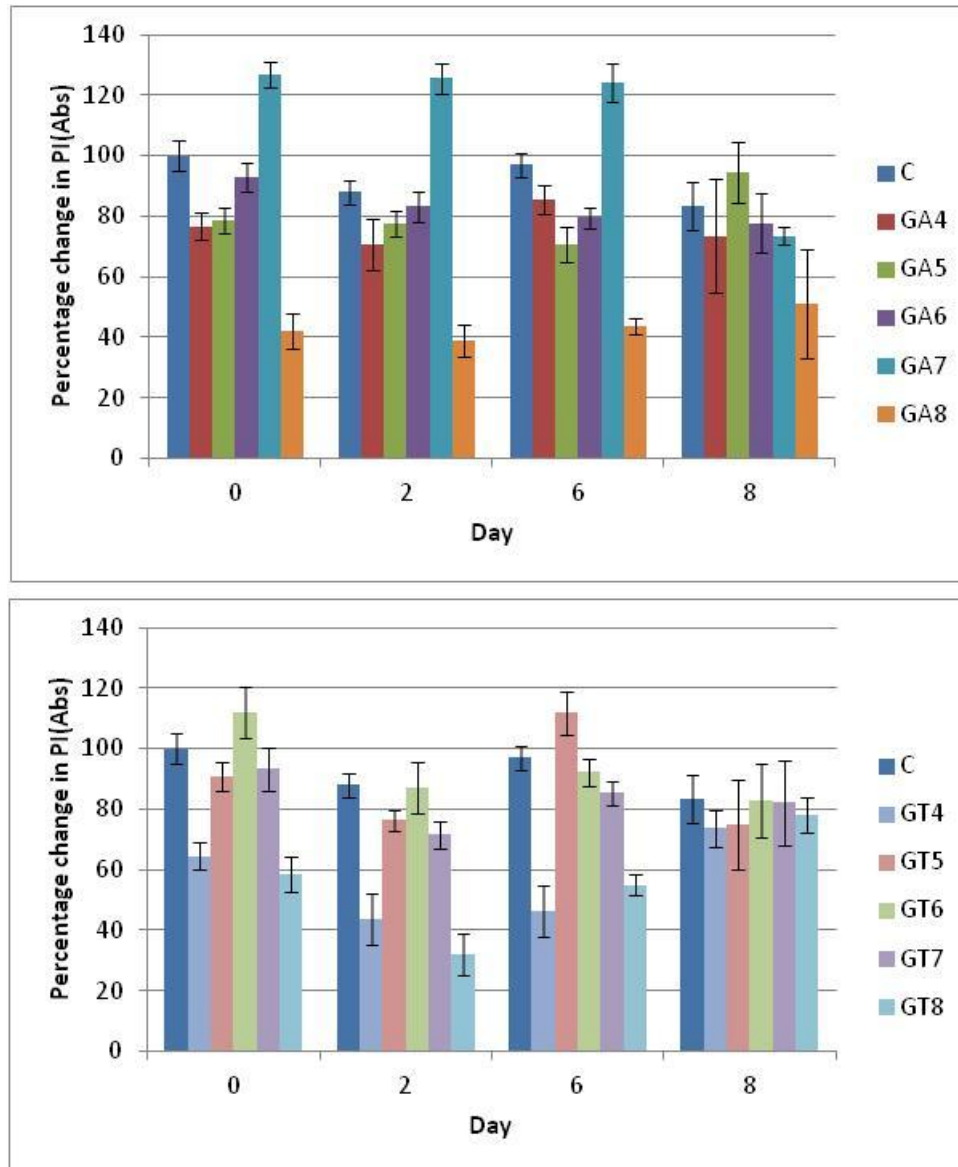


Figure 4.5.2 a (top) and b (bottom): Summary of percentage change in $PI_{(Abs)}$ for gibberellic acid 3 (GA_3) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA_3 of 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to a serial dilution series of GT of 10^{-4} M through 10^{-8} M.

ET₀/RC

The parameter ET₀/RC quantifies the specific energy fluxes per reaction centre for electron transport (Strasser & Strasser, 1995). When evaluating the ET₀/RC data (Figure 4.5.3 a and b; Table 4.5.3), statistically significant differences were observed between the various treatments from day 0 through 6, with no statistically significant differences between the treatments observed at Day 8. In the control plants, a decline in percentage change in ET₀/RC was observed during days 0 through 2, followed by a slight increase during days 2 through 6 and a slightly more pronounced increase during days 6 through 8. A similar pattern to that of the control was observed for treatments GA4 through GA8, but in all gibberellic acid treatments the ET₀/RC ratio was higher than the control. During days 6 through 8, all treatments showed a decline in the ET₀/RC ratio, with the exception of GA5. In essence, treatment GA5 appeared to follow a similar trend as the control.

The ET₀/RC ratio for all gliotoxin treated plants followed a similar pattern of decline and increase relative to what was observed for the control, with the exception of GT5, which showed a decline during days 6 through 8. During days 0 through 2, all treatments showed declines to ET₀/RC ratios lower than the control treatment. Treatment GT8 showed the highest amount of increase at the end of the 8 day period.

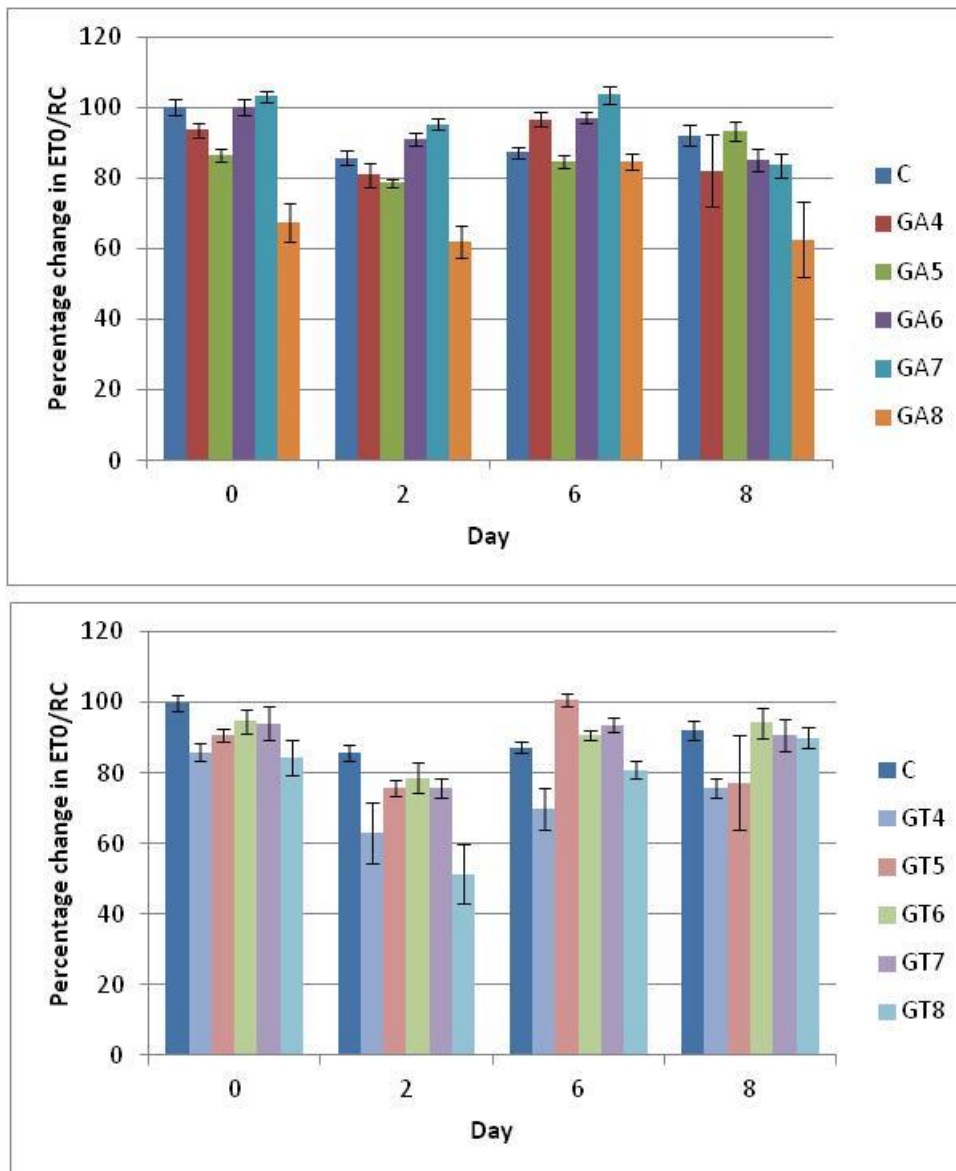


Figure 4.5.3 a (top) and b (bottom): Summary of percentage change in ET₀/RC for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to a serial dilution series of GT of 10⁻⁴ M through 10⁻⁸ M.

Table 4.5.3: Summary of normalised ET_0/RC data with Day 0 (D0) serving as baseline. (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA_3) in a dilution series range from 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to treatment with gliotoxin in a dilution series range from 10^{-4} M through 10^{-8} M.

Code	Treatment	ET_0/RC D0	ET_0/RC D2	ET_0/RC D6	ET_0/RC D8
C	Control	100.00 \pm 2.30 ^{a,b}	85.82 \pm 2.14 ^a	87.25 \pm 1.65 ^{a,b,c}	92.20 \pm 2.75 ^a
GA4	10^{-4} M GA	93.70 \pm 1.95 ^{a,b,c}	80.95 \pm 3.29 ^{a,b}	96.70 \pm 2.00 ^{a,c,d,e}	82.18 \pm 10.22 ^a
GA5	10^{-5} M GA	86.48 \pm 1.85 ^{a,b}	78.81 \pm 1.13 ^{a,b}	84.63 \pm 1.93 ^{a,b}	93.31 \pm 2.87 ^a
GA6	10^{-6} M GA	100.22 \pm 2.14 ^{a,c}	91.07 \pm 1.85 ^a	97.19 \pm 1.63 ^{c,d,e}	85.19 \pm 3.22 ^a
GA7	10^{-7} M GA	103.15 \pm 1.57 ^c	95.35 \pm 1.79 ^a	103.58 \pm 2.36 ^e	83.66 \pm 3.46 ^a
GA8	10^{-8} M GA	67.47 \pm 5.37 ^d	61.93 \pm 4.42 ^{b,c}	84.74 \pm 2.13 ^{a,b}	62.53 \pm 10.69 ^a
GT4	10^{-4} M GT	85.96 \pm 2.53 ^{a,c}	63.00 \pm 8.67 ^{b,c}	70.06 \pm 5.91 ^f	75.76 \pm 2.53 ^a
GT5	10^{-5} M GT	90.76 \pm 1.96 ^{a,b,c}	75.69 \pm 2.42 ^{a,b}	100.81 \pm 1.82 ^{d,e}	77.26 \pm 13.39 ^a
GT6	10^{-6} M GT	94.75 \pm 3.40 ^{a,b,c}	78.58 \pm 4.35 ^{a,b}	90.85 \pm 1.32 ^{a,b,c,d}	94.15 \pm 4.25 ^a
GT7	10^{-7} M GT	94.13 \pm 4.80 ^{a,b,c}	75.63 \pm 2.77 ^{a,b}	93.57 \pm 2.11 ^{a,c,d,e}	90.76 \pm 4.71 ^a
GT8	10^{-8} M GT	84.25 \pm 4.95 ^b	51.39 \pm 8.39 ^c	80.90 \pm 2.60 ^{b,f}	89.99 \pm 2.97 ^a

ET_0/CS_0

The parameter ET_0/CS_0 quantifies the phenomenological activity fluxes per excited cross section for electron transport (Strasser & Strasser, 1995). When evaluating the ET_0/CS_0 data, almost no change in the ET_0/CS_0 ratio was observed for the control plants during the entire period (Figure 4.5.4 a through b; Table 4.5.4). Statistically significant differences were observed for the various treatments between day 0 until day 6, with no statistically significant differences between the various treatments at day 8. With treatments GA4 through GA8 during days 0 through 2, a decline in the ET_0/CS_0 ratio was observed to a level lower than the control, followed by an increase during days 2 through 6 and a decline during days 6 through 8. Only treatment GA5 continued to show an increase in the ET_0/CS_0 ratio. Treatment GA8 peaked at day 6, but overall after the 8 day monitoring period treatment GA5 had the highest percentage change in ET_0/CS_0 ratio.

The gliotoxin treated plants displayed similar trends in the ET_0/CS_0 ratio as those of the gibberellic acid treated plants during days 0 through 6. Treatment GT5 showed a decline in the ET_0/CS_0 ratio during days 6 through 8, while the rest of the treatments continued to show increases throughout after day 2. At the end of the monitoring period, the highest percentage change in the ET_0/CS_0 ratio was observed at treatment GT8.

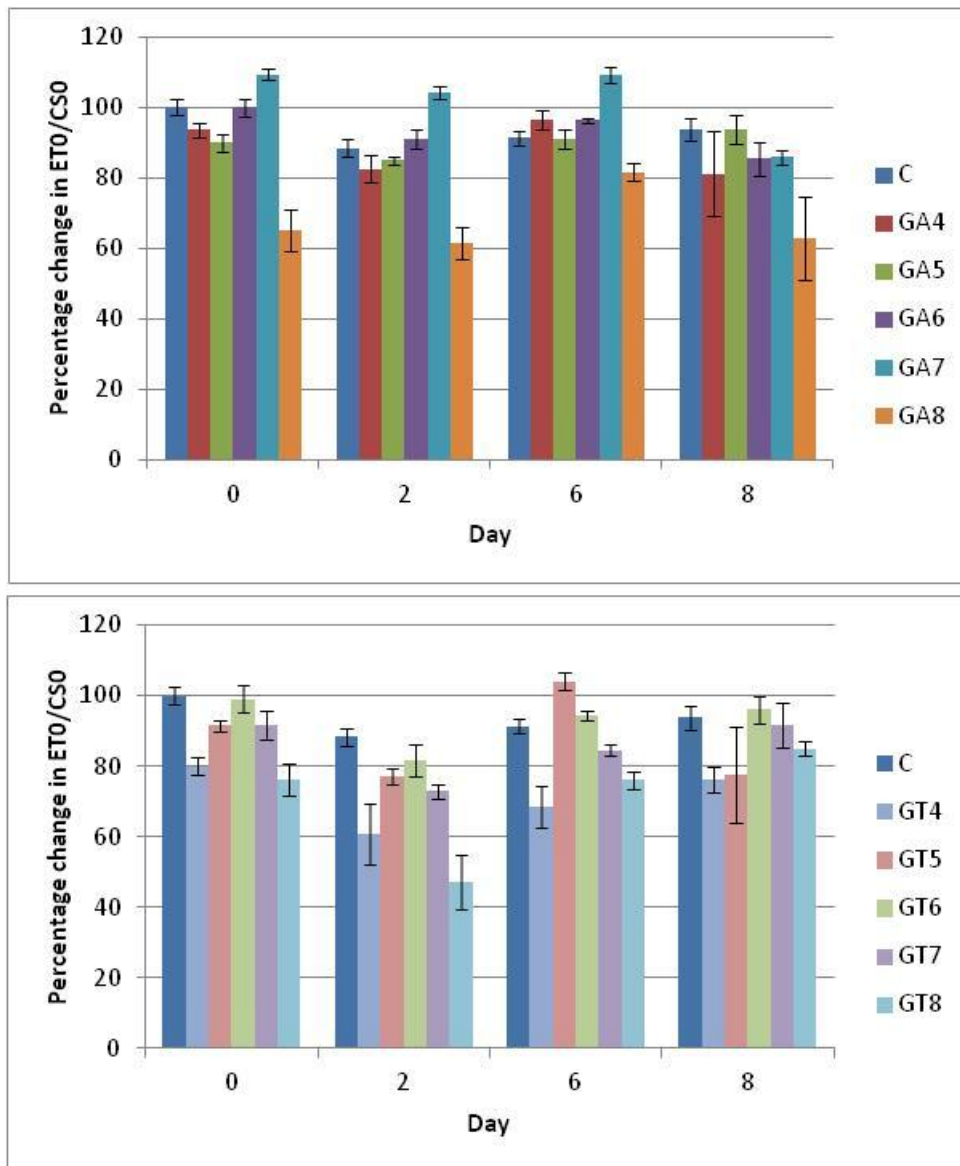


Figure 4.5.4 a (top) and b (bottom): Summary of percentage change in ET_0/CS_0 for gibberellic acid 3 (GA_3) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA_3 of 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to a serial dilution series of GT of 10^{-4} M through 10^{-8} M.

Table 4.5.4: Summary of normalised ET_0/CS_0 data with Day 0 (D0) serving as baseline. (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA_3) in a dilution series range from 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to treatment with gliotoxin in a dilution series range from 10^{-4} M through 10^{-8} M.

Code	Treatment	ET_0/CS_0 D0	ET_0/CS_0 D2	ET_0/CS_0 D6	ET_0/CS_0 D8
C	Control	100.00 \pm 2.31 ^{a,d}	88.33 \pm 2.41 ^{a,d}	91.24 \pm 2.04 ^{a,b,c}	93.75 \pm 3.30 ^a
GA4	10^{-4} M GA	93.54 \pm 1.93 ^{a,c,d}	82.53 \pm 3.84 ^{a,d}	96.60 \pm 2.71 ^{a,c,e}	81.13 \pm 11.94 ^a
GA5	10^{-5} M GA	89.86 \pm 2.50 ^{a,b,c}	84.94 \pm 1.25 ^{a,d}	90.88 \pm 2.62 ^{a,b,c}	93.47 \pm 4.07 ^a
GA6	10^{-6} M GA	99.81 \pm 2.66 ^{a,d}	91.06 \pm 2.82 ^{a,d}	96.19 \pm 0.85 ^{a,c}	85.32 \pm 4.61 ^a
GA7	10^{-7} M GA	109.24 \pm 1.59 ^d	104.03 \pm 1.71 ^d	109.32 \pm 2.26 ^e	85.80 \pm 1.96 ^a
GA8	10^{-8} M GA	64.87 \pm 5.89 ^e	61.30 \pm 4.48 ^{b,c,e}	81.56 \pm 2.44 ^{b,d}	62.67 \pm 11.84 ^a
GT4	10^{-4} M GT	80.04 \pm 2.56 ^{b,c,e}	60.64 \pm 8.57 ^{b,e}	68.62 \pm 5.85 ^f	76.20 \pm 3.52 ^a
GT5	10^{-5} M GT	91.58 \pm 1.55 ^{a,b,c}	77.21 \pm 2.22 ^{a,b,c}	104.05 \pm 2.37 ^{c,e}	77.67 \pm 13.58 ^a
GT6	10^{-6} M GT	99.05 \pm 3.73 ^{a,d}	81.67 \pm 4.45 ^{a,c}	94.36 \pm 1.31 ^{a,b,c}	96.14 \pm 3.89 ^a
GT7	10^{-7} M GT	91.62 \pm 4.22 ^{a,b,c}	72.86 \pm 1.99 ^{a,b,c}	84.58 \pm 1.77 ^{a,b,d}	91.54 \pm 6.23 ^a
GT8	10^{-8} M GT	76.05 \pm 4.54 ^{b,e}	47.09 \pm 7.73 ^e	76.02 \pm 2.42 ^{d,f}	85.05 \pm 2.12 ^a

ABS/RC

The parameter ABS/RC quantifies the specific energy fluxes per reaction centre for absorption (Strasser & Strasser, 1995). When evaluating the ABS/RC data (Figure 4.5.5 a and b; Table 4.5.5) almost no change in the ABS/RC ratio were observed for the control plants during the entire period the plants were monitored. There were statistically significant differences in the ABS/RC ratio between the various treatments from day 0 through 6, with no statistically significant differences between the treatments observed at Day 8. All gibberellic acid treatments showed a decline during days 0 through 2, followed by an increase during days 2 through 6. Treatment GA7 and GA5 showed increases during days 6 through 8, while the remaining treatments all showed a decline in the ABS/RC ratio. At the end of the monitoring period, treatment GA7 had the highest percentage change for this parameter.

For the gliotoxin treatments the following were observed. Treatments GT4 and GT8 showed an increase in the ABS/RC ratio during days 0 through 2, while the remaining treatments all displayed decreases. Treatment GT8 continued to show a decline in the ABS/RC ratio until day 8, while treatment GT4 only showed declines in the ABS/RC ratio during day 6 through 8. The remaining treatments displayed an initial decrease during days 0 through 2, followed

by a gradual increase for the remainder of the period. Only treatments GT5 and GT6 showed marked increases in the ABS/RC ratio during days 6 through 8. At the end of the monitoring period treatments GT5 and GT6 showed the same level of percentage change in the ABS/RC ratio.

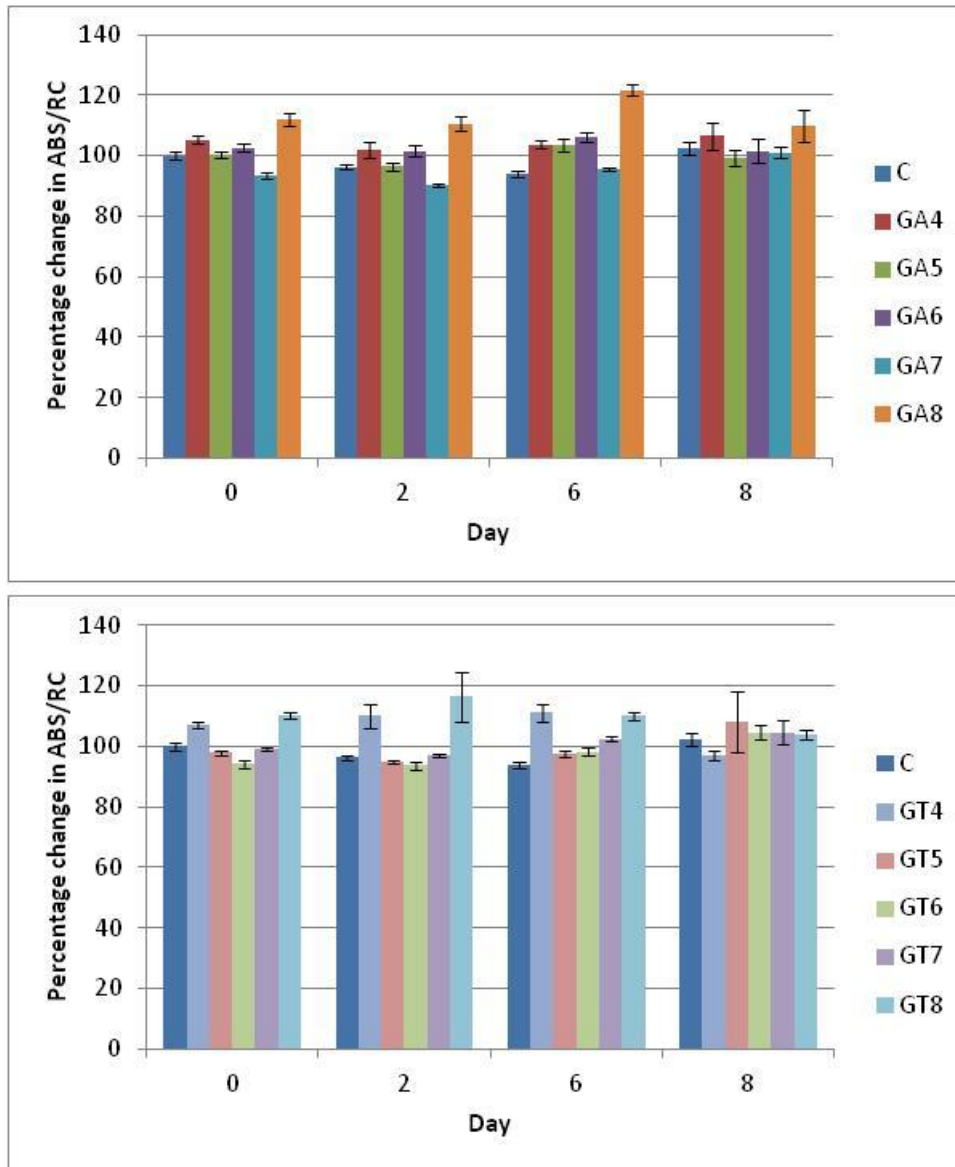


Figure 4.5.5 a (top) and b (bottom): Summary of percentage change in ABS/RC for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to a serial dilution series of GT of 10⁻⁴ M through 10⁻⁸ M.

Table 4.5.5: Summary of normalised ABS/RC data with Day 0 (D0) serving as baseline. (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.

Code	Treatment	ABS/RC D0	ABS/RC D2	ABS/RC D6	ABS/RC D8
C	Control	100.00 \pm 1.31 ^{a,c,d}	96.28 \pm 0.63 ^{a,b}	94.05 \pm 1.08 ^b	102.32 \pm 2.00 ^a
GA4	10 ⁻⁴ M GA	105.31 \pm 1.13 ^{d,e,f}	101.92 \pm 2.57 ^{a,b}	103.63 \pm 1.41 ^{a,d,e}	106.39 \pm 4.36 ^a
GA5	10 ⁻⁵ M GA	100.30 \pm 0.95 ^{a,d}	96.40 \pm 1.26 ^{a,b}	103.49 \pm 1.88 ^{a,d,e}	99.01 \pm 2.66 ^a
GA6	10 ⁻⁶ M GA	102.62 \pm 1.50 ^{a,d,e}	101.56 \pm 1.87 ^{a,b}	106.31 \pm 1.56 ^{d,e}	101.57 \pm 3.78 ^a
GA7	10 ⁻⁷ M GA	93.28 \pm 0.96 ^b	90.36 \pm 0.54 ^a	95.42 \pm 0.52 ^{b,c}	101.06 \pm 2.00 ^a
GA8	10 ⁻⁸ M GA	111.85 \pm 2.23 ^g	110.56 \pm 2.54 ^{b,c}	121.73 \pm 1.92 ^g	109.65 \pm 5.38 ^a
GT4	10 ⁻⁴ M GT	107.26 \pm 1.02 ^{e,f,g}	110.10 \pm 3.96 ^{b,c}	111.20 \pm 2.85 ^f	96.81 \pm 1.63 ^a
GT5	10 ⁻⁵ M GT	97.78 \pm 0.73 ^{a,b,c}	94.90 \pm 0.70 ^a	97.51 \pm 1.20 ^{a,b,c}	108.14 \pm 9.87 ^a
GT6	10 ⁻⁶ M GT	94.30 \pm 1.32 ^{b,c}	93.59 \pm 1.30 ^a	98.28 \pm 1.34 ^{a,b,c}	104.52 \pm 2.28 ^a
GT7	10 ⁻⁷ M GT	98.98 \pm 0.54 ^{a,b,c}	97.19 \pm 0.51 ^{a,b}	102.46 \pm 0.94 ^{a,c,d}	104.44 \pm 3.96 ^a
GT8	10 ⁻⁸ M GT	110.26 \pm 1.06 ^{f,g}	116.36 \pm 8.04 ^c	110.11 \pm 1.24 ^{e,f}	103.80 \pm 1.56 ^a

ΦE_0

The parameter ΦE_0 quantifies the quantum yield of electron transport or electron transport capabilities of the photosystem (Strasser & Strasser, 1995). When evaluating the ΦE_0 data (Figure 4.5.6 a and b; Table 4.5.6), statistically significant differences were observed between the various treatments from day 0 through 6, with no statistically significant differences between the treatments observed at Day 8. In the control plants almost no change in the ΦE_0 is observed during the experiment. When comparing the results for the GA₃ treated plants the following were observed. For the GA4, GA5 and GA6 treatments a decrease in the ΦE_0 was observed on day 0 through 2, and is particularly apparent at the higher GA₃ concentrations and becomes less pronounced as the concentration of GA₃ decreases. During day 6 GA4 and GA6 the ΦE_0 approach levels comparable to the control while GA5 showed an increase in ΦE_0 . The GA7 treatment exhibited an increase in ΦE_0 on day 0 and levelled off during days 2 through 6, and decreased during day 8. Only the GA8 treatment showed a decrease in ΦE_0 relative to the control during days 0 through 8 with a slight increase in ΦE_0 between days 6 through 8.

The gliotoxin treated plants all exhibited a decrease in ΦE_0 during days 0 through 2 relative to the control, with the effect particularly evident for the GT4 and GT7. Between days 6 through 8 increases in the ΦE_0 were observed with ΦE_0 approaching that of the control for the GT6, GT7 and GT8 treatments. Only the GT5 treatment on day 6 showed ΦE_0 exceeding that of the control.

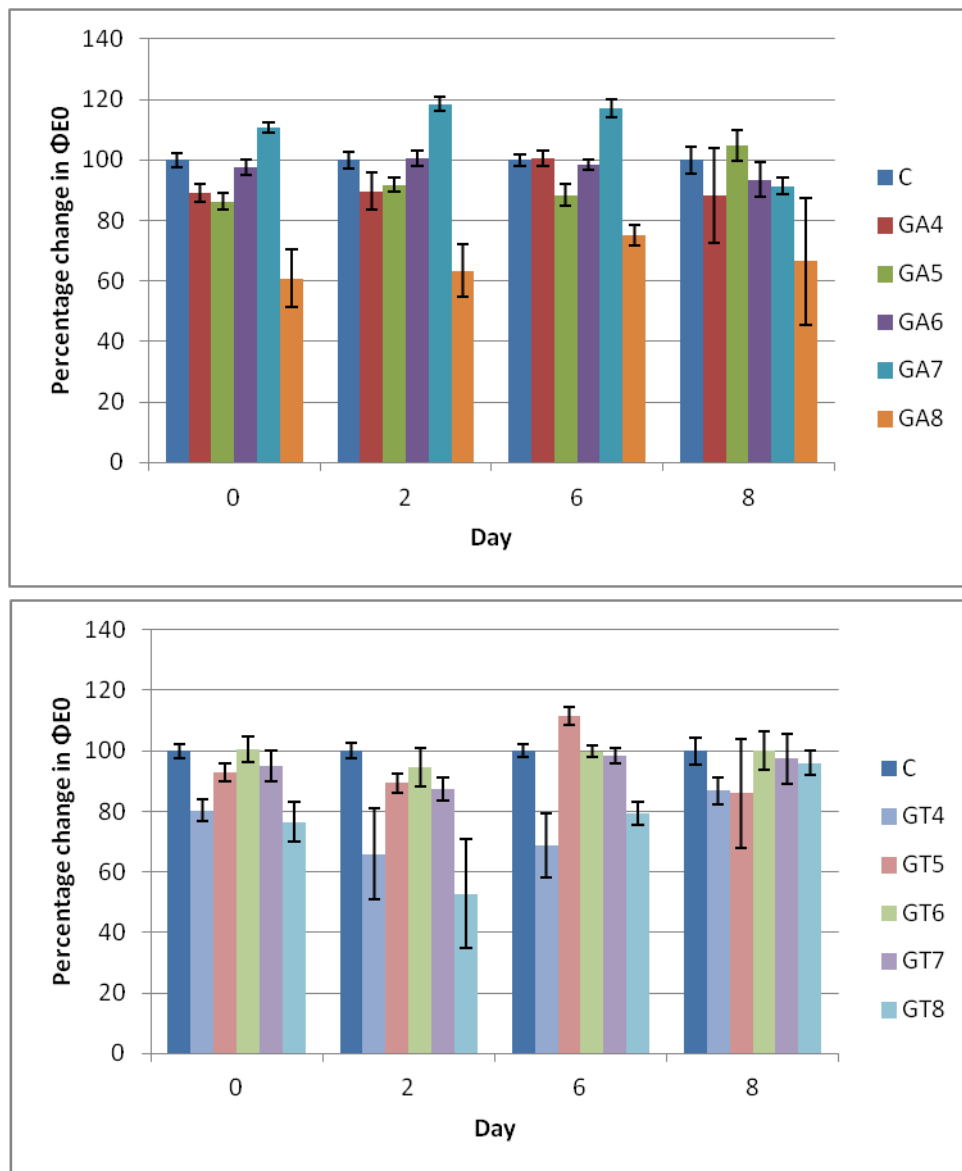


Figure 4.5.6 a (top) and b (bottom): Summary of percentage change in ΦE_0 for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to a serial dilution series of GT of 10^{-4} M through 10^{-8} M.

Table 4.5.6: Summary of normalised ΦE_0 data with Day 0 (D0) serving as baseline. (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.

Code	Treatment	ΦE_0 D0	ΦE_0 D 2	ΦE_0 D 6	ΦE_0 D 8
C	Control	100.00 \pm 2.33 ^{a,d}	100.00 \pm 2.67 ^{a,d}	100.00 \pm 1.99 ^{a,b}	100.00 \pm 4.47 ^a
GA4	10 ⁻⁴ M GA	89.06 \pm 2.93 ^{a,b,c}	89.68 \pm 5.98 ^{a,b}	100.64 \pm 2.54 ^{a,b}	88.18 \pm 15.70 ^a
GA5	10 ⁻⁵ M GA	86.27 \pm 2.84 ^{a,b,c}	91.83 \pm 2.51 ^{a,b}	88.37 \pm 3.70 ^{a,d}	104.81 \pm 5.05 ^a
GA6	10 ⁻⁶ M GA	97.73 \pm 2.51 ^{a,d}	100.71 \pm 2.54 ^{a,d}	98.55 \pm 1.63 ^{a,b}	93.52 \pm 5.74 ^a
GA7	10 ⁻⁷ M GA	110.56 \pm 1.65 ^d	118.42 \pm 2.27 ^d	117.05 \pm 2.76 ^e	91.39 \pm 2.74 ^a
GA8	10 ⁻⁸ M GA	60.84 \pm 9.40 ^e	63.39 \pm 8.72 ^{c,e}	75.14 \pm 3.31 ^{c,d}	66.47 \pm 21.10 ^a
GT4	10 ⁻⁴ M GT	80.20 \pm 3.63 ^{b,c}	65.95 \pm 15.21 ^{b,c,e}	68.73 \pm 10.69 ^c	86.78 \pm 4.46 ^a
GT5	10 ⁻⁵ M GT	92.85 \pm 2.79 ^{a,b,c}	89.46 \pm 3.17 ^{a,b,c}	111.59 \pm 2.92 ^{b,e}	85.89 \pm 18.16 ^a
GT6	10 ⁻⁶ M GT	100.63 \pm 4.24 ^{a,d}	94.55 \pm 6.40 ^{a,d}	99.69 \pm 1.91 ^{a,b}	100.10 \pm 6.27 ^a
GT7	10 ⁻⁷ M GT	95.04 \pm 5.04 ^{a,c,d}	87.34 \pm 3.96 ^{a,b,c}	98.41 \pm 2.38 ^{a,b}	97.44 \pm 8.28 ^a
GT8	10 ⁻⁸ M GT	76.57 \pm 6.63 ^{b,c}	52.75 \pm 17.95 ^e	79.27 \pm 3.69 ^{c,d}	96.03 \pm 4.03 ^a

Ψ_0

The parameter Ψ_0 quantifies the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A⁻ or Energy loss at the antenna level. When evaluating the Ψ_0 data (Figure 4.5.7 a and b; Table 4.5.7), statistically significant differences were observed between the various treatments from day 0 through 6, with no statistically significant differences between the treatments observed at day 8. The control exhibited a decrease in Ψ_0 during day 0 through 2, which increases slightly and levelled off during days 6 through 8. A similar trend has been observed in the GA treated plants, with the exception of the GA7 treatment where Ψ_0 exceeded that of the control during days 0 through 6, but decreased significantly during day 8. The decrease in Ψ_0 for the GA8 treatment was also more pronounced than the other GA treatments.

When comparing the Ψ_0 data for the gliotoxin treated plants the following were observed. An initial decrease in Ψ_0 is apparent for all gliotoxin treatments during day 0 through 2, but is particularly evident for the GT4 and GT8 treatment. During days 6 through 8 slight increases

in Ψ_0 was observed and levels of during this period for all the gliotoxin treatments. Only the GT5 treatment showed Ψ_0 approaching that of the control on day 6.

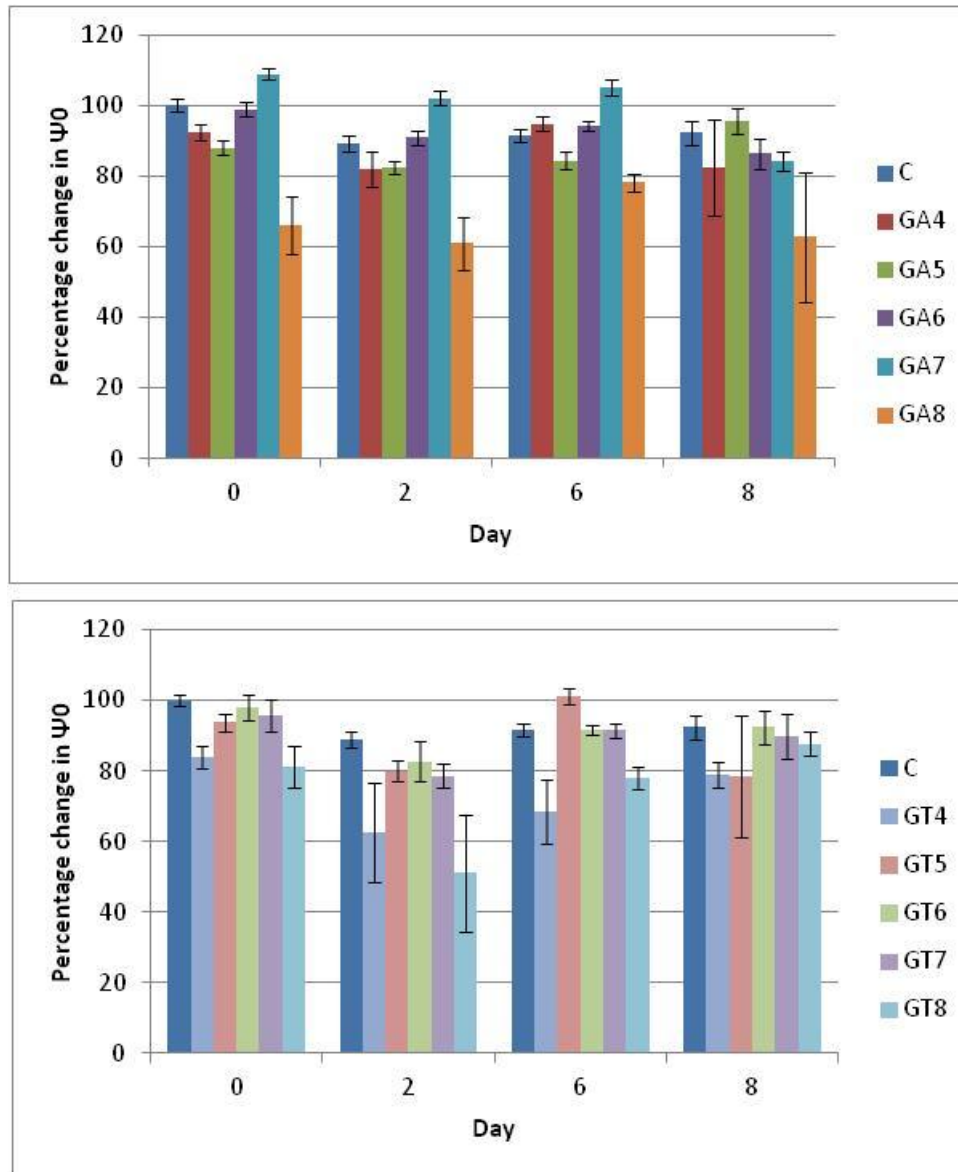


Figure 4.5.7 a (top) and b (bottom): Summary of percentage change in Ψ_0 for gibberellic acid 3 (GA₃) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA₃ of 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to a serial dilution series of GT of 10⁻⁴ M through 10⁻⁸ M.

Table 4.5.7: Summary of normalised Ψ_0 data with Day 0 (D0) serving as baseline. (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA₃) in a dilution series range from 10⁻⁴ M through 10⁻⁸ M; GT4 through GT8 corresponds to treatment with gliotoxin in a dilution series range from 10⁻⁴ M through 10⁻⁸ M.

Code	Treatment	Ψ_0 D0	Ψ_0 D2	Ψ_0 D6	Ψ_0 D8
C	Control	100.00 \pm 1.77 ^{b,d}	89.12 \pm 2.28 ^{b,c}	91.66 \pm 1.73 ^{a,b}	92.35 \pm 3.36 ^a
GA4	10 ⁻⁴ M GA	92.38 \pm 2.30 ^{a,b,c}	82.01 \pm 4.82 ^{a,b,c}	94.88 \pm 2.05 ^{a,b,c}	82.28 \pm 13.57 ^a
GA5	10 ⁻⁵ M GA	88.09 \pm 2.22 ^{a,b,c}	82.51 \pm 1.93 ^{a,b,c}	84.47 \pm 2.50 ^{a,e}	95.46 \pm 3.68 ^a
GA6	10 ⁻⁶ M GA	98.97 \pm 2.21 ^{a,b,d}	90.84 \pm 2.10 ^{b,c}	94.16 \pm 1.28 ^{a,b,c}	86.32 \pm 4.29 ^a
GA7	10 ⁻⁷ M GA	108.90 \pm 1.63 ^d	102.05 \pm 2.13 ^c	105.10 \pm 2.41 ^c	84.32 \pm 2.86 ^a
GA8	10 ⁻⁸ M GA	65.99 \pm 8.17 ^e	60.94 \pm 7.39 ^{a,e}	78.25 \pm 2.54 ^{d,e}	62.88 \pm 18.35 ^a
GT4	10 ⁻⁴ M GT	83.87 \pm 3.02 ^{a,c}	62.49 \pm 13.97 ^{a,e}	68.51 \pm 8.99 ^d	78.79 \pm 3.64 ^a
GT5	10 ⁻⁵ M GT	93.68 \pm 2.46 ^{a,b,c,d}	79.98 \pm 2.93 ^{a,b}	101.22 \pm 2.20 ^{b,c}	78.57 \pm 17.33 ^a
GT6	10 ⁻⁶ M GT	98.06 \pm 3.63 ^{a,b,d}	82.78 \pm 5.69 ^{a,b,c}	91.64 \pm 1.40 ^{a,b}	92.47 \pm 4.73 ^a
GT7	10 ⁻⁷ M GT	95.71 \pm 4.72 ^{a,b,c,d}	78.60 \pm 3.48 ^{a,b}	91.54 \pm 2.06 ^{a,b}	89.83 \pm 6.21 ^a
GT8	10 ⁻⁸ M GT	81.15 \pm 5.85 ^{c,e}	51.04 \pm 16.56 ^d	77.88 \pm 3.12 ^{d,e}	87.75 \pm 3.26 ^a

F_v/F₀

The parameter F_v/F₀ quantifies the energy per reaction centre of the light reaction. When evaluating the F_v/F₀ data (Figure 4.5.8 a and b; Table 4.5.8), statistically significant differences were observed between the various treatments from day 0 through 6, with no statistically significant differences between the treatments observed at Day 8. The control was exhibited very little change in F_v/F₀ during day 0 through 2, followed by an increase in F_v/F₀ at day 6 and a decrease in F_v/F₀ by day 8. When comparing the GA treated plants a decrease in F_v/F₀ was observed for the GA4, GA5, GA6 and GA8 treatments, which a much more pronounced decrease in F_v/F₀ for the GA8 treatment specifically. Only the GA7 treatment showed increases in F_v/F₀ during day 0 through 6, but decreased by day 8. Also apparent is the large decrease in F_v/F₀ for the GA8 treatment in particular.

When evaluating the F_v/F₀ data for the gliotoxin treated plants the following were observed. A marked decrease in F_v/F₀ is observed for GT4 and GT8 on day 0, but increased again by day 8. For treatment GT5 and GT7 the decrease in F_v/F₀ was not as pronounced relative to GT4 and GT8. Only the GT6 treatment showed an increase in F_v/F₀ during days 0 through 6 to levels higher than that of the control, but showed a decrease in F_v/F₀ by day 8.

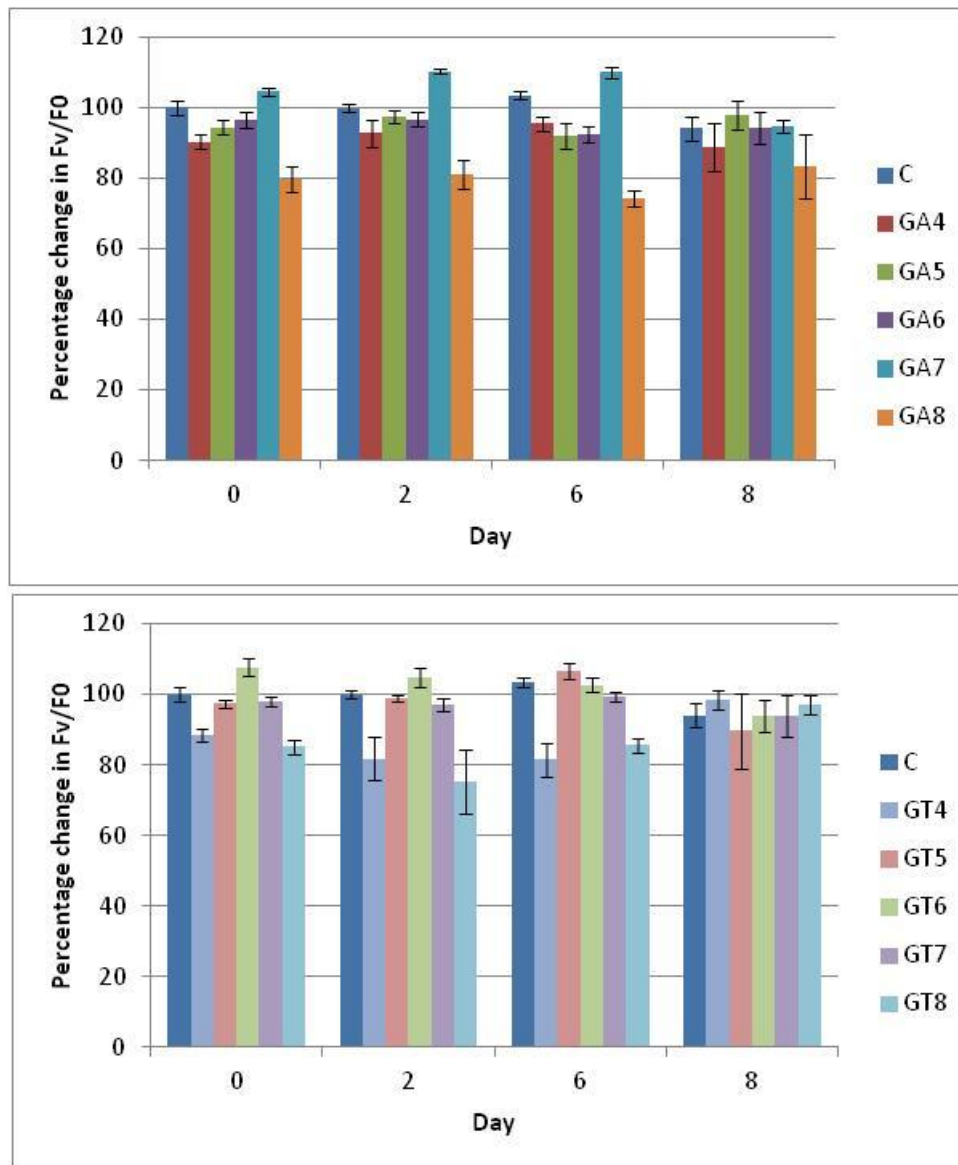


Figure 4.5.8 a (top) and b (bottom): Summary of percentage change in F_v/F_0 for gibberellic acid 3 (GA_3) treated plants (a; top) and gliotoxin treated plants (b; bottom). Key: C = control; GA4 through GA8 corresponds to a serial dilution series of GA_3 of 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to a serial dilution series of GT of 10^{-4} M through 10^{-8} M.

Table 4.5.8: Summary of normalised F_v/F_0 data with Day 0 (D0) serving as baseline. (Statistical means \pm standard error). Superscript lettering represents statistically significant differences in the data derived from repeated measurements ANOVA (95% confidence interval). Key: C = Control; GA4 through GA8 corresponds to treatment with gibberellic acid 3 (GA_3) in a dilution series range from 10^{-4} M through 10^{-8} M; GT4 through GT8 corresponds to treatment with gliotoxin) in a dilution series range from 10^{-4} M through 10^{-8} M.

Treat	Treatment	F_v/F_0 D0	F_v/F_0 D2	F_v/F_0 D6	F_v/F_0 D8
C	Control	100.00 \pm 2.08 ^{c,d,g}	99.89 \pm 1.15 ^d	103.48 \pm 1.28 ^{b,e}	94.13 \pm 3.40 ^a
GA4	10^{-4} M GA	90.33 \pm 1.98 ^{a,b,e}	92.79 \pm 3.90 ^{d,e}	95.55 \pm 2.10 ^{a,d,e}	88.69 \pm 6.91 ^a
GA5	10^{-5} M GA	94.31 \pm 1.99 ^{a,b,c,e}	97.63 \pm 1.82 ^{c,d,e}	92.06 \pm 3.58 ^{a,c,d}	97.81 \pm 4.19 ^a
GA6	10^{-6} M GA	96.57 \pm 2.16 ^{a,b,c,d}	96.73 \pm 2.18 ^{a,c,e}	92.28 \pm 2.22 ^{a,c,d}	94.24 \pm 4.67 ^a
GA7	10^{-7} M GA	104.53 \pm 1.10 ^{d,g}	110.28 \pm 0.74 ^{a,b,c}	109.96 \pm 1.50 ^{b,e}	94.66 \pm 1.95 ^a
GA8	10^{-8} M GA	79.71 \pm 3.79 ^f	80.93 \pm 4.06 ^{a,b,c}	74.16 \pm 2.34 ^f	83.27 \pm 9.08 ^a
GT4	10^{-4} M GT	88.42 \pm 1.95 ^{a,e,f}	81.75 \pm 6.18 ^{a,b}	81.53 \pm 4.84 ^{c,f}	98.59 \pm 2.67 ^a
GT5	10^{-5} M GT	97.43 \pm 1.13 ^{a,b,c,d}	99.04 \pm 0.83 ^{a,b}	106.66 \pm 2.27 ^b	89.61 \pm 10.83 ^a
GT6	10^{-6} M GT	107.62 \pm 2.41 ^g	104.68 \pm 2.72 ^{a,b}	102.66 \pm 1.96 ^{a,b,e}	93.96 \pm 4.54 ^a
GT7	10^{-7} M GT	97.87 \pm 1.41 ^{b,c,d}	97.11 \pm 1.72 ^{a,b}	99.25 \pm 1.47 ^{a,b,e}	94.12 \pm 5.91 ^a
GT8	10^{-8} M GT	85.08 \pm 2.19 ^{e,f}	75.44 \pm 9.12 ^b	85.54 \pm 2.21 ^{c,d}	96.94 \pm 2.65 ^a

General discussion of OJIP data

An aspect which requires further investigation, is the observation that the energy flux parameters ET_0/RC and ET/CS_0 did not show gradients across the treatments as $PI_{(Abs)}$ and ABS/RC did. The responses for the GA treated plants with regards to ET_0/CS_0 and ET_0/RC looks similar in the trends observed (Figure 4.5.3 a and Figure 4.5.4 a), and the same applies when ET_0/CS_0 and ET_0/RC for the gliotoxin treated plants are observed. Comparison of the responses in ET_0/CS_0 and ET_0/RC per treatment suggest that the gliotoxin treatment (Figure 4.5.3 b and Figure 4.5.4 b) at 10^{-8} M resulted in increased energy fluxes of electron transport (from day 2 to day 8) after the initial decrease in energy fluxes from day 0 to day 2, while the equivalent gibberellic acid treatment resulted in varying increases in energy fluxes from day 2 to day 6, and a decrease in energy fluxes from day 6 to day 8. In essence, increasing energy fluxes of electron transport were observed for the gliotoxin treatments at day 8 while gibberellic acid treatments resulted in decreased energy fluxes at day 8. This suggests a possible key difference in the mechanism by which gliotoxin affects a mature plant which will require further study to clarify. Also, the increase in the ET_0/CS_0 suggested an increase in the photochemistry of the gliotoxin treated plants which in turn would contribute to the

increase observed in $PI_{(Abs)}$, while the same effect was not observed in the gibberellic acid treated plants (Figure 4.5.4 a and b).

As the photosynthetic performance index, $PI_{(Abs)}$, is essentially an indicator of plant vitality (Strasser *et al.*, 2000; Strasser *et al.*, 2004), treatment with either gibberellic acid or gliotoxin appeared to have a beneficial effect of plant vitality. Furthermore, both gliotoxin and gibberellic acid appear to have beneficial effects on plant vitality, with gliotoxin treatment performing better than its equivalent gibberellic acid concentration. This further supports the hypothesis that gliotoxin can mimic the effects/functions of gibberellic acid in plants. A study performed by Shores and Hamman (2008) on the response of maize seedlings to *Trichoderma harzianum*, reported amongst other findings an upregulation of proteins involved in photosynthesis. In particular, two forms of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) large subunit, Rubisco, and PSII oxygen-evolving protein complex showed increased levels. In principle this might translate into increased photosynthesis and the resultant increased starch accumulation (Shores & Harman, 2008). This may tentatively be supported by the observation that for both gliotoxin and gibberellic acid treated plants, overall $PI_{(Abs)}$ showed increases (Figure 4.5.2 a and b) despite the fact that decreases in ABS/RC (Figure 4.5.5 a and b) were observed. In essence, more reaction centres would result in a lower ABS/RC yet still yield a higher $PI_{(Abs)}$. This is further evidence of the similarity of physiological responses evoked by gliotoxin and gibberellic acid. Furthermore, another similarity which was observed is that for both gibberellic acid and gliotoxin, higher concentrations of the compounds had an inhibitory effect, while at lower concentrations a stimulatory effect was observed. These findings provide evidence that gliotoxin production by *Trichoderma* is probably the mechanism by which the well documented responses such as enhanced plant growth (Zheng and Shetty, 1999; Shores and Harman, 2008; Vinale *et al.*, 2008b) is produced.

Overall treatment with either gibberellic acid and gliotoxin did not result in a positive response from the chlorophyll a fluorescence transients as was evident from ΦE_0 (quantum yield of electron transport), Ψ_0 (efficiency with which a trapped exciton can move an electron into the transport chain further than Q_A^- , relating to the dark reaction) and F_v/F_0 (the energy per reaction centre of the light reaction). The decreases in the mentioned parameters were not as severe with the gliotoxin treatment relative to the gibberellic acid treatment. Despite the observation that these compound affected the responses elicited was quite similar further

support the hypothesis that gliotoxin can elicit similar physiological responses to gibberellic acid in plants.

CHAPTER 5 - CONCLUSIONS

Overall gliotoxin was able to elicit similar physiological responses to gibberellic acid (See below detailed outcomes). As literature states that gliotoxin degrades relatively rapidly in the environment it makes the possibility of applying gliotoxin to plants more ecologically enticing. Furthermore application of gliotoxin represent a method of improving plant health and performance without directed genetic engineering of the plant, alleviating consumer concerns with regards to genetically modified plants. Applying a growth regulator such as gliotoxin could also prove to be financially more viable than genetic engineering and testing. Also the cascade of effects elicited with a single compound cannot easily be achieved with genetic engineering where single, specific traits are introduced at a time.

Molecular modelling, specifically common pharmacophore evaluation and molecular docking, proved to be valuable tools to evaluate molecular similarities between gibberellic acid and gliotoxin. Results from the common pharmacophore evaluation suggested good similarity between the molecules and enabled the next stage during which gliotoxin was successfully docked into the receptor for gibberellic acid. Though the molecular similarity was quite convincing and the molecular docking showed a good binding possibility for gliotoxin into the receptor for gibberellic acid, it is not a guarantee that similar, even favourable, physiological responses would be elicited in a plant system. To address this, physiological plant responses were evaluated to determine whether gliotoxin would mimic the action of gibberellic acid in a plant system.

The induction of amylase activity in de-embryonated seeds by gliotoxin suggested that gliotoxin can indeed elicit gibberellin-like activity. Furthermore results from a plant height and emergence study supported this with both the gliotoxin and gibberellic acid treated seeds emerging one day before the control. From the chlorophyll fluorescence transient analysis it was observed that gliotoxin and gibberellic acid have a beneficial effect on plant vitality, with gliotoxin performing better than gibberellic acid. However, in terms of other chlorophyll fluorescent transient parameters gliotoxin and gibberellic acid had a negative effect, but the observed patterns were similar. Also, the effect of gliotoxin was not as severe as those of gibberellic acid. These findings further support the hypothesis that gliotoxin can mimic gibberellic acid in a plant system. Also apparent from the data for all the plant physiological responses evaluated was that the best responses were observed at concentration

ranges of less than 10^{-6} M. This corresponds very well with literature regarding the typical concentrations of hormones in plant systems.

For this study the following goals were identified:

1. Evaluation of the molecular similarity between gliotoxin and gibberellic acid using molecular similarity software;
2. Evaluation of the molecular similarity between gliotoxin and gibberellic acid by docking to a selected ligand;
3. Comparison between gliotoxin and gibberellic acid in α -amylase expression in de-embryonated seeds using zymograms;
4. Comparison between gliotoxin and gibberellic acid in enhancing seed germination using seedling emergence data; and
5. Comparison between gliotoxin and gibberellic acid by comparison of effects on mature plants using fluorescence data.

In terms of the first objective, molecular similarity between gliotoxin and gibberellic acid was indeed observed using common pharmacophore generation as a tool. The results from this evaluation allowed the research to progress to the second objective: the evaluation of the molecular similarity using molecular docking simulations. Overall, the best results were obtained when comparing the pose for the GA₃ within the GID1 protein with gliotoxin. These results also corresponded the closest with the results predicted from common pharmacophore generation. However, using the GA₃ as constructed by an external program, in the docking simulation was problematic due to differences in the structure of the molecules.

In terms of objectives 3 through 5, the evaluation of gliotoxin as an analogue for gibberellic acid was successful on all counts. Similar responses were observed for germination and plant height, amylase induction and effects of plant fluorescence data.

Based on the outcomes of this project the following avenues of research are recommended:

- Molecular modelling provided a useful tool to verify that gliotoxin could indeed bind to the same receptor as gibberellic acid. In the pharmaceutical research field molecular modelling is used for screening of new and novel pharmaceutical

compounds. It is suggested that these practices and methods could be applied equally successfully to screen for novel plant growth regulators.

- Chlorophyll *a* fluorescence monitoring could not be extended beyond 8 days during this study so as to prevent the size of the pots becoming limiting to the plants. It is recommended that the experiment should progress to controlled field trials to determine how long the effects of treatment with the growth regulators last. To further clarify the physiological responses it is recommended that Photosystem I (PSI) responses should also be investigated.
- Germination emergence and plant height can also be considered for field trials to determine whether the same effects will be observed under field conditions with relation to plant emergence and increase in plant height. It will also be interesting to verify whether a complete and even stand in the treated plants will be verified as Howell (2003) reported occurring when plants are treated with *Trichoderma* in particular.
- Treatment of mature plants under field conditions will also supply sufficient plant material for additional experiments to determine whether treatment with the growth regulators will influence amino acid, carbohydrate levels in the plants, so as to further gain insight into the effect of the growth regulators on mature plants. (Plant material was limited during this study).

Overall the tools used in this study, i.e. molecular modelling, germination trials, chlorophyll *a* fluorescence and protein electrophoresis (zymogram in particular), could make up a valuable ensemble of screening tests for possible novel growth regulators in the laboratory environment before field trial evaluations are considered, especially where gibberellic acid and its effects are of interest.

REFERENCES

ACCELRYLS. 2006. Catalyst Software Suite (Molecular modelling software suite, version 4.8). Accelrys Software Inc. (<http://www.accelrys.com>)

ARGUSLAB Version 4.0.0. 2004. Mark Thompson and Planaria Software LLC

ASHRAF, M., & HARRIS, P.J.C. 2004. Review: Potential biochemical indicators of salinity tolerance in plants. *Plant Science* **166**:3-16

ACEVEDO, E. & CARDEMIL, L. 1997. Regulation of α -amylase isoenzyme expression in *Araucaria araucana* by gibberellic and abscisic acids. *Phytochemistry* **44**(8):1401-1405

BARTHÉLEMY, X., POPOVIC, R., & FRANCK, F. 1997. Studies on the O-J-I-P transient of chlorophyll fluorescence in relation to photosystem II assembly and heterogeneity in plastids of greening barley. *Journal of Photochemistry and Photobiology B*: **39**:213-218

BATTA, Y.A. 2004. Effect of treatment with *Trichoderma harzianum* Rifai formulated in invert emulsion on post harvest decay of apple blue mold. *International Journal of Food Microbiology* **96**:281-288

BAULCOMBE, D.C, HUTTLY, A.K., MARTIENSEN, R.A., BARKER, R.F., & JARVIS, M.G. 1987. A novel wheat α -amylase gene (α -Amy3). *Molecular & General Genetics* **209**:33-40

BETHKE, P.C. & JONES, R.L. 1998. Gibberellin signalling. *Current opinions in plant biology* **1**:440-446

BETHKE, P.C., FATH, A., & JONES, R.L. 2001. Regulation of viability and cell death by hormones in cereal aleurone. *Journal of Plant Physiology* **158**:429-438

BLANCHARD, J.L. 2004. Bioinformatics and systems biology, rapidly evolving tools for interpreting plant response to global change. *Field Crop Research* **90**:117-1313

- BROUGHTON, W.J., HERNANDEZ, G., BLAIR, M., BEEBE, S., GEPTS, P. & VANDERLEYDEN, J.** 2003. Beans (*Phaseolus* spp.) – model food legumes. Plant and soil **252**:55-128
- BUCKHOUT, T.J., & THIMM, O.** 2003. Insights into metabolism obtained from microarray analysis. Current Opinion in Plant Biology **6**:288-296
- CARD, S., JASPERS, M.V., WALTER, M. & STEWART, A.** 2002. Evaluation of micro-organisms for biocontrol of grey mould on lettuce. 55th Conference Proceedings (2002) of The New Zealand Plant Protection Society Incorporated 197-201
- CEJUDO, F.J., CUBO, M.T., & BAULCOMBE, D.C.** 1995. *Amyl* expression during wheat seed germination. Plant Science **106**:207-213
- CHEN, X., LI, Z.L., ZHI, D.G., & CHEN, Y.Z.** 2002. CLiBe: a database of computed ligand binding energy for ligand-receptor complexes. Computers and Chemistry **26**:661-666
- CHO, S. & MUEHLBAUER, F.J.** 2004. Genetic effect of differentially regulated fungal response genes on resistance to necrotrophic fungal pathogens in chickpea (*Cicer arietinum* L.) Physiological and Molecular Plant Physiology **64**:57-66
- CORINA.** 2008. (Structure generation for 3D models) Molecular Networks. (www.molecular-networks.com)
- COUNCIL FOR AGRICULTURAL SCIENCE AND TECHNOLOGY.** 2003. Task Force Report - Mycotoxins: Risks in Plant, Animal, and Human Systems. Council for Agricultural Science and Technology Ames, Iowa, USA No. 139
- DATNOFF, L.E., NEMEC, S. & PERNEZNY, K.** 1995. Biological control of Fusarium crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. Biological Control **5**:427-431

DAVIS, B.J. 1964. Disc electrophoresis: II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**:404–427

DE BACH, P. (1964). *Biological Control of Insect Pests and Weeds*, 1st edn. Chapman and Hall, London.

DE BEER, M. 2005. The assessment of soil microbial and plant physiological changes during the treatment of soil containing Bromacil, Tebuthiuron and Ethidimuron. Masters Dissertation, North West University, Potchefstroom, South Africa

DE MARCO, J.L., VALADARES-INGLIS, M.C., & FELIX, C.R. 2003. Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity against *Crinipellis pernicioso*, the causal agent of witches' broom of cocoa. *Brazilian Journal of Microbiology* **34**:33-38

DOD, S.L., HILL, R.A., & STEWARD, A. 2000. Control of *Athelia rolfsii* disease on lentil seedlings using 6-pentyl- α -pyrone. *Soil biology and biochemistry* **32**:1033-1034

ELAD, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Protection* **19**:709-714

ESPOSITO, E.X., BARAN, K., KELLY, K., & MADURA, J.D. 2000. Docking of sulfonamides to carbonic anhydrase II and IV. *Journal of Molecular Graphics and Modelling* **18**:283-289

FIEHN, O., KLOSKA, S., & ALTMAN, T. 2001. Integrated studies on plant biology using multiparallel techniques. *Current Opinion in Biotechnology* **12**(1):82-86

FRAIRE-VELÁZQUES, S., & LOZOYA-GLORIA, E. 2003. Differential early gene expression in *Phaseolus vulgaris* to Mexican isolates of *Colletotrichum lindemuthianum* in incompatible and compatible interactions. *Physiological and Molecular Plant Pathology* **63**:79-89

GANESAN, G., SANKARARAMASUBRAMANIAN, H.M., NARAYANAN, J.M., SIVAPRAKASH, K.R., & PARIDA, A. 2008. Transcript level characterization of a cDNA encoding stress regulated NAC transcription factor in the mangrove plant *Avicennia marina*. *Plant Physiology and Biochemistry* **46**:928-934

GENOUD, T., & MÉTRAUX, J.P. 1999. Crosstalk in plant cell signaling: structure and function of the genetic network. *Trends in Plant Science Perspectives* **4**(12):503-507

GOMI, K., & MATSUOKA, M. 2003. Gibberellin signalling pathway. *Current opinion in plant biology* **6**:489-493

GRACIA-GARZA, J.A. REELEDER, R.D., & PAULITZ, T.C. 1997. Degradation of sclerotia of *Sclerotinia sclerotium* by fungus gnats (*Bradysia coprophila*) and the biocontrol fungi *Trichoderma* spp. *Soil Biology & Biochemistry* **29**(2):123-129

GROVEL, O., KERZAON, I., PETIT, K., DU PONT T.R., & POUCHUS, Y. 2006. A new and rapid bioassay for the detection of gliotoxin and related epipolythiodioxopiperazines produced by fungi. *Journal of microbiological methods* **66**:286-293

HARMAN, G.E., LATORRE, B., AGOSIN, E., SAN MARTIN, R., RIEGEL, D.G., NIELSEN, P.A., TRONSMO, A., & PEARSON, R.C. 1996. Biological and Integrated Control of *Botrytis* Bunch Rot of Grape Using *Trichoderma* spp. *Biological Control* **7**:259-266

HANSON, L.E. 2000. Plant Pathology & Nematology- Reduction of *Verticillium* Wilt Symptoms in Cotton Following Seed Treatment with *Trichoderma virens*. *The Journal of Cotton Science* **4**:224-231

HENDLICH, M., RIPPMMANN, F., & BARNICKEL, G. 1997. LIGSITE: Automatic and efficient detection of potential small molecule-binding sites in proteins. *Journal of Molecular Graphics and Modelling*. **15**:359-363

HERVÁS, A., LANDA, B., DATNOFF, L.E., & JIMÉNEZ-DIAZ, R.M. 1998. Effects of commercial and indigenous microorganisms on *Fusarium* wilt development in chickpea. *Biological Control* **13**:166-176

HILL, T.A. 1973. *Studies in Biology no 40 – Endogenous plant growth substances.* William Clowes & Sons, Ltd. London. 68pp

HIRANO, K., UEGUCHI-TANAKA, M., & MATSUOKA, M. 2007a. *GID1-mediated gibberellin signalling in plants.* *Trends in Plant Science* **13**(4):192-199

HIRANO, K., NAKAJIMA, M., ASANO, K., NISHIYAMA, T., SAKIKIBARA, H., KOJIMA, M., KATOH, E., XIANG, H., TANAHASHI, T., HASEBE, M., BANKS, J., UEGUCHI-TANAKA, M., & MATSUOKAA, M. 2007b. *The GID1-Mediated Gibberellin Perception Mechanism Is Conserved in the Lycophyte *Selaginella moellendorffii* but Not in the Bryophyte *Physcomitrella patens*.* *The Plant cell* **19**:3058-3079

HOWELL, C.R. 2003. *Mechanisms Employed by *Trichoderma* Species in the Biological Control of Plant Diseases: The History and Evolution of Current Concepts.* *Plant Disease* **87**(1):1-10.

HEDDEN, P. 2008. *Gibberellins closes the lid.* *Nature* **456**:455-456

JEBARA, S., JEBARA, M., LIMAN, F., & ELARBI AOUANI, M. 2005. *Changes in ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide desmutase activities in common bean (*Phaseolus vulgaris*) nodules under salt stress.* *Journal of Plant Physiology* **162**:929-936

KAUTSKY, H. & HIRSCH, A. 1931. *Neue Versuche zur Kohlensäureassimilation,* *Naturwissenschaften,* **19**:964-964

KOMATSU, S., MASUDA, T., & HIRANO, H. 1996. *Rice gibberellin-binding phosphoprotein structurally related to ribulose-1,5-biphosphate carboxylase/oxygenase activase.* *FEBS Letters* **384**:167-171

KRAUSS, U. & SOBERANIS, W. 2001. Biocontrol of Cocoa Pod Diseases with Mycoparasite Mixtures. *Biological control* **22**:149-158

KRAUSE, G.H. & WEISS, E. 1991, 'Chlorophyll fluorescence and photosynthesis: the basics', *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 313-349

KOPYRA, M. & GWÓZDZ, E.A. 2003. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. *Plant Physiology and Biochemistry* **41**:1011-1017

KWON, S.-I. & ANDERSON, A.J. 2001. Differential production of superoxide dismutase and catalase isozymes during infection of wheat by a *Fusarium proliferatum*-like fungal isolate. *Physiology and Molecular Plant Pathology* **58**:73-81

LATORRE, B.A., AGOSIN, E., SAN MARTIN, R., & VASQUES, G.S. 1997. Effectiveness of conidia of *Trichoderma harzianum* produced by liquid fermentation against Botrytis bunch rot of table grape in Chile. *Crop Protection* **16**(3):209-214

LANDREAU, A., POUCHUS, Y.F., SALLENAVE-NAMONT, C., BIARD, J.-F., BOUMARD, M.-C., DU PONT, T.R., MONDEGUER, F., GOULARD, C., & VERBIST, J.-F. 2002. Combined use of LC/MS and a biological test for rapid identification of marine mycotoxins produced by *Trichoderma koningii*. *Journal of Microbiological Methods* **48**:181-194

LATIJNHOUWERS, M., DE WIT, P.J.G.M. & GOVERS, F. 2003. Oomycetes and fungi: similar weaponry to attack plants - Review. *TRENDS in Microbiology* **11**(10):462-469

LAZARUS, C.M., BAULCOMBE, D.C., & MARTIENSEN, R.A. 1985. α -Amylase genes of wheat are two multigene families which are differentially expressed. *Plant Molecular Biology* **5**:13-24

LEADER, D.J. 2005. Review – Transcriptional analysis and functional genomics in wheat. *Journal of Cereal Science* **41**:149-163

LEWIS, R.E., WIEDERHOLD, N.P., CHI, J., HAN, X.Y., KOMANDURI, K.V., KONTOYIANNIS, D.P. & PRINCE, R.A. 2005. Detection of Gliotoxin in Experimental and Human Aspergillosis. *Infection and Immunity*, **73**:635-637

MACMILLAN, J. & GASKIN, P. 1996. Gibberellins in endosperm and embryos of *Marah macrocarpus*. *Phytochemistry* **42**(5):1263-1266

MARTÍNEZ, T.F., ALARCÓN, F.J., DÍAZ-LÓPEZ, M., & MOYANO, F.J. 2000. Improved detection of amylase activity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with copolymerized starch. *Electrophoresis* **21**:2940-2943

MANNINA, L., SEGRE, A.L., RITIENI, A., FOGLIANO, V., VINALE, F., RANDAZZO, G., MADDAU, L., & BOTTALICO, A. 1997. A new fungal growth inhibitor from *Trichoderma viride*. *Tetrahedron* **53**(9):3135-3144

MAOR, R., & SHIRASU, K. 2005. The arms race continues: battle strategies between plants and fungal pathogens. *Current Opinion in Microbiology* **8**:1–6

MAYOL, M., & ROSSELLO, J.A. 2001. Seed isozyme variation in *Petrocoptis* A. Braun (Caryophyllaceae). *Biochemical Systematics and Ecology* **29**:379-392

MURASE, K., HIRANO, Y., SUN, T., & HAKOSHIMA, T. 2008. Gibberellin-induced DELLA recognition by the receptor GID1. *Nature* **456**:459-464

NEMEC, S., DATNOFF, L.E., & STRANDBERG, J. 1996. Efficacy of biocontrol agents in planting mixes to colonise plant roots and control root diseases of vegetables and citrus. *Corp Protection* **15**(8):735-742

OTIENO, W., JEGER, M., & TERMORSHUIZEN, A. 2003a. Effect of infesting soil with *Trichoderma harzianum* and amendment with coffee pulp on survival of *Armillaria*. *Biological Control* **26**:291-301

OTIENO, W., TERMORSHUIZEN, A., JEGER, M., & OTHIENO, C.O. 2003b. Efficacy of soil solarisation, *Trichoderma harzianum*, and coffee pulp amendment against *Arimillaria* sp. *Crop Protection* **22**:325-331.

PERELLO, A., C. MONACO, M.R., SIMON, M., SISTERNA, M. G. & DAL BELLO, G. 2003. Biocontrol efficacy of *Trichoderma* isolates for tan spot of wheat in Argentina. *Crop Protection* **22**:1099–1106

PRELL, H.H., & DAY, P.R. 2001. Plant-fungal pathogen interaction - A classical and Molecular view. Springer-Verlag, Heidelberg, Germany. 214pp

PUPPALA, N. & FOWLER, J.L. 2002. *Lesquerella* seed pretreatment to improve germination. *Industrial Crops and Products* **17**:61-69

REYMOND, P. 2001. DNA microarrays and plant defence. *Plant Physiology and Biochemistry* **39**:313-321

RONCHI, A., FARINA, G., GOZZO, F., & TONELLI, C. 1997. Effects of a triazolic fungicide on maize plant metabolism: modifications of transcript abundance in resistance related pathways. *Plant Science* **130**: 51-62

ROZHNOVA, N.A., ODINTSOVA, T.I. & GERASHCHENKOV, G.A. 2007. Protein Composition of Tobacco Leaves with Antiviral Resistance Induced by Activators of Defense Responses and TMV Infection. *Russian Journal of Plant Physiology* **54**(6):776-781

SARGEANT, J.G. 1980. α -Amylase isozymes and starch degradation. *Cereal Research Communications* **8**:77-86

SCHNABLE, P.S., HOCHHOLDINGER, F., & NAKAZONO, M. 2004. Global expression profiling applied to plant development. *Current opinion in Plant Biology* **7**:50-56

SIDDIQUI, Z.A., & MAHMOOD, I. 1996. Biological control of plant parasitic nematodes by fungi: A review. *Bioresource Technology* **58**:229-239.

SHIMADA, A., UEGUCHI-TANAKA, M., NAKATSU, T., NAKAJIMA, M., NAOE, Y., OHMIYA, H., KATO, H., & MATSUOKA, M. 2008. Structural basis for gibberellin recognition by its receptor GID1. *Nature* **456**:520-524

SHORESH, M., & HARMAN, G.E. 2008. The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root: A proteomic approach. *Plant Physiology* **147**:2147-2163

STATSOFT, INC. (2011). STATISTICA (data analysis software system), version 10. www.statsoft.com.

STRASSER, R.J., SRIVATAVA, A. & GOVINDJEE. 1995, 'Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria', *Photochem. Photobiol.* **61**, 32-42

STRASSER, B.J. & STRASSER, R.J. 1995, 'Measuring fast fluorescence transients to address environmental questions: The JIP test', Mathis, P ed., *Photosynthesis: Light to Biosphere*, Kluwer Academic Publishers, Dordrecht, pp. 977-980

STRASSER, R.J., SRIVASTAVA, A., & TSIMILLI-MICHAEL, M. 2000. The fluorescent transient as a tool to characterise and screen photosynthetic samples. In: Yunus, M., Pathre, U., Mohanty, P. (Eds.), *Probing Photosynthesis: Mechanisms, Regulation and Adaptation*. Taylor and Francis, London, pp. 445–483

STRASSER, R.J., SRIVASTAVA, A., & TSIMILLI-MICHAEL, M. 2004. Analysis of the Chlorophyll a fluorescence transient. In: Papageorgiou, G.C., Govindjee (Eds.), *Chlorophyll a Fluorescence: A Signature of Photosynthesis*. *Advances in Photosynthesis and Respiration*, vol. 19. Springer, Dordrecht, The Netherlands, pp. 321–362

STRAUSS, A.J., KRÜGER, G.H.J., STRASSER, R.J., & VAN HEERDEN, P.D.R. 2006. Ranking of dark chilling tolerance in soybean genotypes probed by the chlorophyll a fluorescence transient O-J-I-P. *Environmental and Experimental Botany* **56**:157-157

- SUMNER, L.W., MENDES, P. & DIXON, R.A.** 2003. Review - Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry* **62**:817-836
- TAMINAU, J., THIJS, G., & DE WINTER, H.** 2008. Pharao: Pharmacophore alignment and optimization. *Journal of Molecular Graphics and Modelling* **27**:161-169
- THOMSEN, R.** 2003. Flexible ligand docking using evolutionary algorithms: investigating the effects of variation operators and local search hybrids. *Biosystem* **72**:57-73
- TSROR (LAHKIM), L., BARAK, R., & SNEH, B.** 2001. Biological control of black scurf on potato under organic management. *Crop Protection* **20**:145-150
- TYYSTRJÄRVI, E., KOSKI, A., KERÄNEN, M., & NEVALAINEN, O.** 1999. The Kaustksy Curve Is a Built-in Barcode. *Biophysical Journal* **77**:1159-1167
- UEGUCHI-TANAKA, M., ASHIKARI, M., NAKAJIMA, M., ITOH, H., KATOH, E., KOBAYASHI, M., CHOW, T., HSING, Y., KITANO, H., YAMAGUCHI, I., & MATSUOKA, M.** 2005. GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature* **437**:693-698
- UEGUCHI-TANAKA, M., NAKAJIMA, M., KATOH, E., OHMIYA, H., ASANO, K., SAJI, S., HONGYU, X., ASHIKARI, M., KITANO, H., YAMAGUCHI, I., & MATSUOKA, M.** 2007. Molecular interactions of a soluble gibberellin receptor, *GID1*, with a rice *DELLA* Protein, *SLR1*, and gibberellin. *The Plant Cell* **19**:2140-2155
- VAN RENSBURG, L., KRÜGER, G.H.J., EGGENBERG, P. & STRASSER, R.J.** 1996. Can screening criteria for drought resistance in *Nicotiana tabacum* L. be derived from the polyphasic rise of the chlorophyll a fluorescence transient (OJIP)? *South African Journal of Botany* **62**(6):337-341
- VEY, A., HOAGLAND, R.W., & BUTT, T.M.** 2001. Toxic metabolites of fungal biocontrol agents. In "Fungi as Biocontrol Agents". T.M. Butt, C. Jackson, N. Magan, eds. CABI Publishing Co. Chapter 12, 311-345

VINALE, F., SIVASITHAMPARAM, K., GHISALBERTI, E.L., MARRA, R., BARBETTI, M.J., LI, H., WOO, S.L. & LORITO, M. 2008a. A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiological and Molecular Plant Pathology* **72**:80-86

VINALE, F., SIVASITHAMPARAM, K., GHISALBERTI, E.L., MARRA, R., WOO, S.L. & LORITO, M. 2008b. *Trichoderma*-plant-pathogen interactions – Review Article. *Soil Biology and Biochemistry* **40**:1-10

WANG, Q., ZHANG, F., & SMITH, D.L. 1996. Application of GA3 and kinetin to improve corn and soybean seedling emergence at low temperature. *Environmental and Experimental Botany* **36**(4):377-383

WARING, P. 1990. DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by raised inositol triphosphate levels. *Journal of Biological Chemistry* **265**:14476–14480

WARING, P., & BEAVER, J. 1996. Review: Gliotoxin and related Epipolythiodioxopiperazines. *General Pharmacology* **27**(8):1311-1316

WARING, P., KHAN, T., & SJAARDA, A. 1997. Apoptosis Induced by Gliotoxin Is Preceded by Phosphorylation of Histone H3 and Enhanced Sensitivity of Chromatin to Nuclease Digestion. *The journal of biological chemistry* **272**(29):17929-17936

WASHIO, K. 2001. Identification of Dof proteins with implication in the gibberellin-regulated expression of a peptidase gene following the germination of rice grains. *Biochimica et Biophysica Acta* **1520**:54-62

WEINDLING, R. 1934. Studies on a lethal principle effective in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other soil fungi. *Phytopathology* **24**:1153-1179

WERMUTH, C.G., GANELLIN, C.R., LINDENBURG, P. & MITSCHER, L.A. 1998. Glossary of terms used in medicinal chemistry (IUPAC Recommendations 1998). *Pure and Applied Chemistry* **70**(5):1129-1143

WIEMKIN, V., & BOLLER, T. 2002. Ectomycorrhiza: gene expression, metabolism and the wood-wide web. *Current Opinion in Plant Biology* **5**:1-7

WILLIAMSON, J.D., QUANTRANO, R.S. & CUMING, A.C. 1985. Em polypeptide and its messenger RNA levels are modulated by abscisic acid during embryogenesis in wheat. *European Journal of Biochemistry* **152**:501-507

WU, S.H., RAMONELL, K., GOLLUB, J., & SOMERVILLE, S. 2001. Plant gene expression profiling with DNA microarrays. *Plant Physiology and Biochemistry* **38**:917-926

ZHANG, X.W., YAP, Y.L., & ALTMAYER, R.M. 2005. Generation of predictive pharmacophore model for SARS-coronavirus main proteinase. *European Journal of Medicinal Chemistry* **40**:57-62

ZINSER, C., SEIDLITZ, H.K., WELZL, G., SANDERMAN, H., HELLER, W., ERNST, D., & RAU, W. 2007. Transcriptional profiling of summer wheat, grown under different realistic UV-B irradiation regimes. *Journal of Plant Physiology* **164**:913-922

ZHENG, Z., & SHETTY, K. 1999. Effect of apple pomace-based *Trichoderma* inoculants on seedling vigour in pea (*Pisum sativum*) germinated in potting soil. *Process Biochemistry* **34**:731-735

APPENDIX A

Table A1: Summary of various biological control agents for various fungal plant pathogens for a variety of crops

Control agent	Pathogen	Crop Disease	Reference
<i>Trichoderma harzianum</i> (T39) (Trichodex)	<i>Botrytis cinerea</i>	Grey mould (lettuce)	Card <i>et al.</i> , 2002
<i>Trichoderma virens</i>	<i>Pythium ultimum</i> <i>Rhizoctonia solani</i>	Damping of cotton seedlings	Hanson, 2000
<i>Trichoderma virens</i>	<i>Verticillium dahliae</i>	Verticillium wilt	Hanson, 2000
<i>Trichoderma harzianum</i> (T39) (Trichodex)	<i>Botrytis cinerea</i> <i>Pseuoperonospora cubensis</i> <i>Sclerotinia sclerotiorum</i> <i>Sphaerotheca fusca</i> (syn. <i>S. fuliginea</i>)	Various foliar pathogens	Elad, 2000.
<i>Trichoderma harzianum</i> (isolate Th15, Th11,Th2, Th81,Th7,Th13,Th8,Th5), <i>Trichoderma aureoviride</i> (isolate Ta1,Ta100) and <i>Trichoderma koningii</i> (isolate Tk11,Tk6)	<i>Pyrenophora tritici-repentis</i> (Died) Drechs. (anamorph= <i>Drechslera tritici-repentis</i> (Died) Shoem.	Tan spot of wheat	Perelló <i>et al.</i> , 2003
<i>Trichoderma</i> spp.; <i>Trichoderma virens</i> ; <i>Clonostachys rosea</i>	<i>Moniliophthora roreri</i> ; <i>Crinipellis perniciosa</i> ; <i>Phytophthora palmivora</i> .	Moniliasis; Witches broom; Black pod (respectively) (pod diseases of cacao)	Krauss & Soberanis, 2001
<i>Trichoderma harzianum</i> <i>Trichoderma</i> spp	<i>Botrytis cinerea</i>	<i>Botrytis</i> Bunch rot of grapes (<i>Vitis vinifera</i>)	Harman <i>et al.</i> , 1996
<i>Trichoderma harzianum</i>	<i>Rhizoctonia solani</i> Kuhn AG-3 [teleomorph: <i>Thanathephorus cucumeris</i> (Frank) Donk] – non-pathogenic strains	Black scurf of potatoes	Tsrer <i>et al.</i> , 2001
<i>Trichoderma harzianum</i> ; <i>Glomus intraradices</i> (VAM)	<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>	<i>Fusarium</i> crown rot of tomatoes	Datnof <i>et al.</i> , 1995

<i>Trichoderma</i> spp	<i>Sclerotinia sclerotium</i>		Gracia-Garza <i>et al.</i> , 1997
<i>Trichoderma harzianum</i>	<i>Armillaria</i>	Armillaria root rot various crops	Otieno <i>et al.</i> , 2003a,b
<i>Trichoderma harzianum</i>	<i>Penicillium expansum</i>	Apple blue mold	Batta, 2004
<i>Trichoderma harzianum</i>	<i>Botrytis cinerea</i>	Botrytis rot of grapes (<i>Vitis vinifera</i>)	Latorre <i>et al.</i> , 1997
<i>Trichoderma harzianum</i> ; <i>Bacillus subtilis</i> ; <i>Fusarium oxysporum</i> (non-pathogenic strain)	<i>Fusarium oxysporum</i>	Fusarium wilt in chickpea (<i>Cicer arietinum</i>)	Hervás <i>et al.</i> , 1998
<i>Trichoderma harzianum</i> , <i>Bacillus subtilis</i> , <i>Glomus intraradices</i> , <i>Gliocladium virens</i> , <i>Streptomyces griseovirdis</i>	<i>Phytophthora</i> <i>Thietaviopsis</i> <i>Pythium</i> spp. and <i>Fusarium</i> spp. <i>Phytophthora cuspici</i>	Phytophthora root rot on citrus Thietaviopsis root rot on citrus, Celery root rot caused by <i>Pythium</i> and <i>Fusarium</i> spp., Pepper crown and root rot caused by <i>Phytophthora cuspici</i>	Nemec <i>et al.</i> , 1996