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EVALUATION OF BIOACTIVITY OF PHYTOTOXINS FROM PATHOGENIC FUNGI OF OROBANCHE SP

SUH CHRISTOPHER

Ph.D THESIS

Athens
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DEDICATION

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LIST OF ABBREVIATIONS

ANOVA.	Analysis of variance
2, 4 D	2, 4-Dichlorophenoxyacetic acid
DNA	DeoxyriboNucleic Acid.
EPTC	Ethyl dipropylthiocarbamate
GA_3	Gibberellic acid
GC/MS	Gas chromatography/mass spectrometry
HPLC	High Pressure Liquid Chromatography
IAA	Indole-3-acetic acid
LC-MS	Liquid Chromatography-Mass Spectrometry
MPLC	Medium Preesure Liquid Chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PDA	Potato dextrose agar
PEB	Polyethylene bags
SEM	Standard error mean.
SPE	Solid-phase extraction
TLC	Thin layer chromatography
TTC	2, 3, 5-triphenyl tetrazolium chloride
U.S.	United States

Περίληψη Διδακτορικής Διατριβής του
Christopher Suh

Τίτλος: Αξιολόγηση βιοδραστικότητας φυτοτοξινών από παθογόνους μύκητες της *Orobanche* spp.

ΕΙΣΑΓΩΓΗ

1. Γενικά περί Οροβάγχης

Είδη του γένους *Orobanche* spp. είναι υποχρεωτικά φανερόγαμα παράσιτα και ανήκουν στη οικογένεια Orobanchaceae. Υπάρχουν περίπου 150 είδη και 17 γένη. Είναι ποώδη δικοτυλήδονα φυτά χωρίς χλωροφύλλη . Τα είδη της Οροβάγχης παράγουν μεγάλο αριθμό μικρού μεγέθους σπόρους που εύκολα διασπείρονται και μολύνουν τα συγκομιζόμενα προϊόντα και τους αγρούς. Επίσης τα είδη του γένους *Orobanche* είναι παράσιτα χωρίς εξειδίκευση ξενιστού και προσβάλλουν πολλά καλλιεργούμενα φυτά. Οι συνήθεις ξενιστές ανήκουν στις οικογένειες Solanaceae και Fabaceae. Γενικά οι μεσογειακές χώρες θεωρούνται ως περιοχές προέλευσης του παρασίτου. Η Οροβάγχη είναι παράσιτο με παγκόσμια εξάπλωση σε εύρος κλιματικών συνθηκών, εκτός από την *Orobanche crenata* η οποία εντοπίζεται σε μεσογειακές χώρες, Μέση Ανατολή και Ανατολική Αφρική. Οι ζημιές που προκαλούνται στην παραγωγή των καλλιεργειών που προσβάλλονται από τα είδη της οροβάγχης κυμαίνεται σε ποσοστό από 5 μέχρι και 100% (με μέσο όρο 34%).

2. Βιολογία της *Orobanche crenata*

Η σχέση ξενιστού-*Orobanche crenata* είναι υψηλής εκλεκτικότητας. Το παράσιτο παράγει 5000 σπόρους /κάψα και το κάθε φυτό μπορεί να παράγει περισσότερες από 100 κάψες. Οι σπόροι μπορεί να διατηρήσουν τη ζωτικότητά τους στο έδαφος μέχρι και 10 χρόνια. Η επίδραση στο φυτο-ξενιστή γίνεται εμφανής όταν το παράσιτο εμφανίζεται πάνω από την επιφάνεια του εδάφους. Για την ολοκλήρωση του βιολογικού κύκλου του παρασίτου απαιτούνται 3-5 μήνες. Η υπόγεια όμως φάση του παρασίτου διαρκεί 30-100 ημέρες, ανάλογα με τις καιρικές συνθήκες. Για την βλάστηση των σπόρων απαιτείται η έκθεση τους σε συνθήκες υψηλής θερμοκρασίας και υγρασίας για αρκετές ημέρες, σε συνδυασμό με την παρουσία ειδικών χημικών ενώσεων εξωγενούς προέλευσης.

3. Μέθοδοι αντιμετώπισης οροβάγχης

Οι προτεινόμενοι μέθοδοι αντιμετώπισης περιλαμβάνουν προληπτικά, καλλιεργητικά, φυσικά , χημικά, βιολογικά μέτρα. Η πιο αποτελεσματική μέθοδος είναι η πρόληψη με τη χρήση μη μολυσμένου σπόρου. Η προμήθεια εγγυημένου σπόρου, επιτόπιοι έλεγχοι αγρών και ενημέρωση των παραγωγών συμπεριλαμβάνονται στα προληπτικά μέτρα. Επίσης εφαρμογή ζιζανιοκτόνων στα φυτά της καλλιέργειας που παραμένουν στο χωράφι και η χρήση χημικών ενώσεων που διεγείρουν το φύτρωμα των σπόρων της οροβάγχης συμβάλλουν στη μείωση του μολύσματος και κατά συνέπεια μπορεί να θεωρηθούν ως προληπτικά μέτρα.

Τα καλλιεργητικα μέτρα που έχουν προταθεί συμπεριλαμβάνουν βαθύ όργωμα, σπορά της καλλιέργειας σε εποχή μη-ευνοϊκή για το παράσιτο, αμειψισπορά, χρήση φυτών –παγίδων (π.χ. τριφύλλι), εφαρμογή λιπασμάτων που δεν ευνοούν το παράσιτο, κατάκλυση του εδάφους για εβδομάδες.

Τα χημικά μέσα που επιτυχώς έχουν χρησιμοποιηθεί για την αντιμετώπιση της οροβάγχης είναι ζιζανιοκτόνα που πρέπει πάντα να εφαρμόζονται μεταφυτρωτικά για το παράσιτο. Οι παράμετροι που καθορίζουν τη χρήση ζιζανιοκτόνων είναι το επίπεδο εκλεκτικότητας σε επίπεδο φυτού και το κρίσιμο του χρόνου εφαρμογής . Το glyphosate είναι το πιο αποτελεσματικό ζιζανιοκτόνο για την αντιμετώπιση της *Orobanche crenata* στα κουκιά και χρησιμοποιείται στη πράξη. Άλλα ζιζανιοκτόνα που έχουν αποδειχθεί αποτελεσματικά ενάντιον διαφόρων ειδών οροβάγχης ανήκουν στη κατηγορία των σουλφονουριών και ιμιδαζολινών.
Η αντιμετώπιση της οροβάγχης με βιολογικά μέσα σχετίζεται με την εφαρμογή ζωντανών οργανισμών (έντομα, μικροοργανισμοί, μύκητες) για τον περιορισμό του πληθυσμού του παρασίτου. Ανάμεσα στους οργανισμούς με προοπτική χρήσης ως βιολογικά παρασκευάσματα συμπεριλαμβάνονται περίπου 30 είδη μυκήτων με εξέχοντα θέση στελέχη ειδών του γένους *Fusarium* όπως το *Fusarium oxysporum* και το *Fusarium arthrosporiodes* τα οποία προσβάλλουν αρκετά είδη οροβάγχης. Εκτός από τη χρήση των παθογόνων μυκήτων της οροβάγχης, η διερεύνηση της δραστικότητας των μεταβολιτών που παράγονται απο τους μύκητες αυτούς παρουσιάζει ενδιαφέρον. Στελέχη του *Fusarium oxysporum* παράγουν ένα μεγάλο αριθμό βιοδραστικών μεταβολιτών όπως fusaric acid, fumonisins, beauvericin, enniatin, moniliformin, trichothecenes. Κατά συνέπεια μεταβολίτες με φυτοτοξική δράση στην οροβάγχη μπορεί να χρησιμοποιηθούν per se ή ως χημικά πρότυπα για την ανάπτυξη νέων ζιζανιοκτόνων αποτελεσματικά εναντίον του παρασίτου. Επίσης η μελέτη του τρόπου δρασης σε υποκυτταρικό επίπεδο μπορεί να οδηγήσει στην ανακάλυψη νέων βιοχημικών στόχων, τομέας σημαντικός για ανάπτυξη νέων ζιζανιοκτόνων.
Λαμβάνοντας υπόψη τη πολυπλοκότητα του παρασιτισμού ανωτέρων φυτών από την οροβάγχη που είναι και αυτή ανώτερο (δικότυλο) φυτό, η επιλογή κατάλληλης μεθόδου ή βιοδοκιμής αξιολόγησης της εκλεκτικής βιοδραστικότητας χημικών ενώσεων είναι σημαντική παράμετρος για αξιοποιήσιμα αποτελέσματα. Μεταξύ των μεθόδων που μπορεί να χρησιμοποιηθούν σε τέτοιου είδους πειραματισμό συμπεριλαμβάνονται: Βιοδοκιμή βλάστησης σπόρων, βιοδοκιμή σε φύλλα με έκθεση των φύλλων σε χημικές ενώσεις μετά από πρόκληση πληγής, βιοδοκιμή με τη χρήση ιστοκαλλιεργειών της οροβάγχης, αλλά και πρωτόκολλα πειραματισμού στο χωράφι.

4. Σκοπός της παρούσης έρευνας:

Μεταβολίτες που παράγονται μύκητες έχουν φυτοτοξική δραστικότητα σε αρκετά φυτά και έχουν μελετηθεί για περαιτέρω αξιοποίησή τους στη φυτοπροστασία. Επίσης μερικές από τις φυτοτοξίνες αυτές έχουν εκλεκτικότητα ως προς ένα είδος φυτού. Οι πληροφορίες αυτές και το ότι η οροβάγχη προσβάλεται από μύκητες οδήγησε στην παρούσα έρευνα με στόχο την απομόνωση και ταυτοποίηση μεταβολιτών από παθογόνα της οροβάγχης με σκοπό την αξιοποίησή τους στην αντιμετώπιση του παρασίτου με χημικές ενώσεις με εκλεκτική δράση μόνο στην οροβάγχη και όχι στο ξενιστή. Ο εντοπισμός κηλίδων σε βλαστούς *Orobanche crenata* πάνω σε φυτά κουκιάς οδήγησε στην απομόνωση στελεχών του μύκητα *Fusarium oxysporum* και στην περαιτέρω διερεύνηση της βιοδραστικότητας και χημικής δομής των μεταβολιτών τους που παράγονται σε θρεπτικό υλικό.

5.ΥΛΙΚΑ ΚΑΙ ΜΕΘΟΔΟΙ:

Υλικά

Σπόροι από *Orobanche crenata, Orobanche cumana* και *Orobanche aegyptiaca* είχαν συλλεχθεί από φυτά στην ελλάδα και στην Κύπρο. Ως ξενιστές χρησιμοποιήθηκαν φυτά

Vicia faba , *Vicia sativa* και *Helianthus annuus*. Τα στελέχη ΟΚ7 και C11 του *Fusarium oxysporum* που χρησιμοποιήθηκαν στην μελέτη είχαν απομονωθεί από φυτά *Orobanche crenata* από καλλιέργεια κουκιάς στον Μαραθώνα Αττικής.

Μέθοδοι

Καλλιέργεια μυκήτων και οροβάγχης – θρεπτικά υλικά

Τα στελέχη του *Fusarium oxysporum* αναπτύχθηκαν και διατηρήθηκαν σε υλικό PDA. Για την παραγωγή αιωρήματος σπορίων πυκνότητας $3x10^3$ ή $3x10^4$ σπόρια/ml για τις δοκιμές παθογένεσης τα στελέχη αναπτύχθηκαν στο ίδιο θρεπτικό υλικό για 3 εβδομάδες.

Για την ανάπτυξη του *Fusarium oxysporum* για την απομόνωση μεταβολιτών χρησιμοποιήθηκαν θρεπτικά υλικά PDA και αποστειρωμένοι σπόροι ρυζιού (100 g σπόροι βρασμένοι σε 30 ml απιονισμένο νερό).

Για τα πειράματα στις σακκούλες πολυαιθυλενίου χρησιμοποιήθηκε το θρεπτικό διάλυμα Hoagland και Arnon. Για την παρασκευή ιστοκαλλιέργειας οροβάγχης χρησιμοποιήθηκε τροποποιημένο θρεπτικό υλικό Gamborg B5 που περιείχε ΙΑΑ ή 2,4-D (ανάλογα με το είδος των κάλλων).

Δοκιμές παθογένεσης

Για τις δοκιμές παθογενετικής ικανόπτητας των απομονωθέντων στελεχών του *Fusarium oxysporum* χρησιμοποιήθηκε η μέθοδος με σακκούλες πολυαιθυλενίου (PEB) σύμφωνα με τους Parker και Dixon (1983), Goldwasser et al. (1997) και Linke et al. (2001). Με τη μέθοδο αυτή ήταν δυνατή η μακροσκοπική παρακολούθηση των σταδίων ανάπτυξης της οροβάγχης σε σχέση με το παθογόνο. Αποστειρωμένοι σπόροι οροβάγχης (0.5 g περίπου 100 σπόροι) τοποθετήθηκαν σε ειδικά φίλτρα ινών υάλου και τοποθετήθηκαν σε διαφανείς σακκούλες πολυαιθυλενίου (25x35 cm). Ένα σπορόφυτο ηλίανθου ή βίκου σε στάδιο κοτυληδόνων στερεώθηκε πάνω στο φίλτρο που είχε τους σπόρους της οροβάγχης. Θρεπτικό διάλυμα Hoagland (20 ml) σε 1:1 αραίωση προσετέθηκε σε κάθε σακκούλα στην αρχή και δυο φορές την εβδομάδα με΄χρι το τέλος του πειράματος. Οι σακκούλες τοποθετήθηκαν σε θάλαμο ανάπτυξης (20 ± 20^oC, 175μmoles^{-2} s^{-1} έντασης φωτός και 12 ώρες φωτοπερίοδο). Δύο εβδομάδες μετά την τοποθέτηση των σπόρων στις σακκούλες προστέθηκε ο διεγέρτης βλάστησης GR-24. Το ποσοστό των σπόρων που βλάστησαν και προσκόλησαν στις ρίζες των ξενιστών υπολογίσθηκε μετά από εξέταση κάτω από στερεοσκόπιο 3 εβδομάδες μετά την προσθήκη του GR-24. Το αιώρημα σπορίων του μύκητα ψεκάστηκε όταν είχε γίνει προσκόλληση του παρασίτου πάνω στον ξενιστή.

Επιπλέον έγιναν πειράμματα παθογένεσης και σε φυτά που αναπτύχθηκαν σε γλάστρες (12x12x12cm) σε συνθήκες θερμοκηπίου. Στα πειραματα στις γλάστρες χρησιμοποιήθηκαν οι παρακάτω συνδυασμοί παρασίτου-ξενιστού: *Vicia faba – Orobanche crenata*, *Vicia sativa-Orobanche crenata, Helianthus annuus-Orobanche cumana.* Περίπου 100 σπόροι οροβάγχης διασκορπίστηκαν στην επιφάνεια του αποστειρωμένου (δυο φορές) χώματος (0.8 Kg) στην κάθε γλάστρα.Το αιώρημα σπορίων του *Fusarium oxysporum* ψεκάστηκε στην επιφάνεια του χώματος πριν ή μετά την εμφάνιση της οροβάγχης, προσετέθηκε επιπλέον χώμα και ποτίστηκε. Η αξιολόγηση έγινε όταν σταμάτησε η ανάπτυξη των ξενιστών φυτών παρουσία της αντίστοιχης οροβάγχης και απουσία του μύκητα. Ως βλαστήσαντες βλαστοί θεωρούντο όσοι είχαν 0.5cm ύψος πάνω από την επιφάνεια του εδάφους. Στα πειράματα μετά την εμφάνιση του παρασίτου το αιώρημα κονιδίων του *Fusarium oxysporum* ψεκάστηκε πάνω στα φυτά.

Εκχύλιση και χημικός προσδιορισμός των μεταβολιτών

Για την παραγωγή δευτερογενών μεταβολιτών τα στελέχη του μύκητα *Fusarium oxysporum* αναπτύχθηκαν σε θρεπτικό υπόστρωμα με βρασμένους σπόρους ρυζιού. Αιώρημα κονιδίων προσετέθηκε στους σπόρους που επωάστηκαν σε 20^{o} C στο σκοτάδι για 1 μήνα. Στη συνέχεια οι καλλιέργειες εκχυλίστηκαν με ακετόνη και το εκχύλισμα διηθήθηκε πάνω από άνυδρο θειϊκό νάτριο σε διηθητικό χαρτί. Το εκχύλισμα εξατμίστηκε υπό κενό και το υπόλειμμα παραλήφθηκε σε 3 ml ακετόνης και υπεβλήθηκε σε TLC (silica 60 F_{254}) και τα κλάσματα αναλύθηκαν περαιτέρω με HPLC, MPLC, GCMS, NMR. Ο διαχωρισμός με HPLC έγινε με ένα Varian ProStar System (ProStar Solvent Delivery Module 230, injector Rheodyne 7125, Prostar 330 UV-VIS photo diode array detector). Ο διαχωρισμός με MPLC έγινε με ένα preparative Buchi system, silica gel υπό πίεση 200 mbar. Η ανάλυση με GC-MS έγινε με τη χρήση Hewlett Packard 5973-6890 GC-MS (EI mode a t 70eV). Ως φέρον αέριο χρησιμοποιήθηκε ήλιο με ροή 0.8 ml/min και τριχοειοδείς στήλες HP 5MS, HP Innowax. Τα φάσματα NMR ελήφθησαν με ένα Bruker 600MHz με $CDCl_3$. Η ταυτοποίηση έγινε με βάση τους χρόνους κατακράτησης και με τα φάσματα μάζας σε συσχέτιση με δεδομένα από βάσεις δεδομένων και σχετική βιβλιογραφία.

Βιοδοκιμές φυτοτοξικότητας

Για τον έλεγχο της βιοδραστικότητας κάθε παρασκευάσματος από καλλιέργεια του *Fusarium oxysporum* χρησιμοποιήθηκαν άνθη (από το επάνω ακραίο τμήμα του στελέχους) και τεμάχια βλαστών (1 cm από την κορυφή) οροβάγχης. Τα φυτικά μέρη εμβαπτίστηκαν σε υδατικά διαλύματα παρασκευασμάτων που προήλθαν από τις διάφορες ζώνες (Rf 0.06, 0.13, 0.23, 0.35, 0.42, 0.62, 0.77, 0.87) από τη χρωματογραφία λεπτής στοιβάδος. Επίσης χρησιμοποιήθηκαν και τμήματα από ιστοκαλλιέργειες οροβάγχης που τοποθετήθηκαν σε εκχυλίσματα καλλιεργειών του *Fusarium oxysporum* σε κελία σε πλάκες ELISA και η επίδραση αξιολογήθηκε με τη μέτρηση νωπού βάρους ιστών. Το ζιζανιοκτόνο glyphosate χρησιμοποιήθηκε ως φυτοτοξική ένωση αναφοράς σε όλες τις βιοδοκιμές.

Μια άλλη βιοδοκιμή που χρησιμοποιήθηκε ήταν η δοκιμή βλαστικότητας σπόρων της οροβάγχης. Σπόροι του παρασίτου απολυμάνθηκαν σε αιθανόλη για 1.5 min και μετά σε 5% υποχλωριώδες νάτριο παρουσία 0.1% Tween για 10 λεπτά και στη συνέχεια ξεπλύθηκαν με αποστειρωμένο νερό. Η ζωτικότητα των σπόρων ελέγχθκε με τη δοκιμή με tetrazolium. Μετά τον έλεγχο βλαστικότητας οι σπόροι τοποθετήθηκαν σε δίσκους διηθητικού χαρτιού και μετά από 10 ημέρες μεταφέρθηκαν σε νέους δίσκους χαρτιού παρουσία των διεγερτών βλάστησης (GR24 και Nijmegen-1). Μετά από επώαση μιας εβδομάδας εξετάστηκαν με στερεοσκόπιο για την έκπτυξη ριζιδίου.

Η ποσοτική εκτίμηση φυτοτοξικής δράσης των υπό εξέταση παρασκευασμάτων (εκχυλίσματα καλλιεργειών και καθαρές ουσίες) έγινε με τον προσδιορισμό χλωροφύλλης σε ιστούς φύλλων κουκιάς . Η εκχύλιση της χλωροφύλλης έγινε με DMSO και μετρήθηκε η απορρόφηση των διαλυμάτων στα 665nm Beckman DBG και ο υπολογισμός έγινε με την εξίσωση Chla(mg/g)=0.0127xA665-0.00269xA645

6.ΑΠΟΤΕΛΕΣΜΑΤΑ ΚΑΙ ΣΥΖΗΤΗΣΗ

Δοκιμές παθογένεσης

Η δοκιμή PEB έδειξε ότι *Orobanche crenata* και *Orobanche cumana* απουσία του *Fusarium oxysporum* εμφάνισαν τρία διαφορετικά υπόγεια όργανα του παρασίτου

Με αποτέλεσμα τον παρασιτισμό των ξενιστών τους. Η εφαρμογή αιωρήματος *Fusarium oxysporum* περιόρισε τον παρασιτισμό και ο περιορισμός ήταν ανάλογος της πυκνότητας του μολύσματος. Στα πειράματα παθογένεσης σε φυτά που αναπτύχθηκαν σε γλάστρες τα αποτελέσματα ήταν ανάλογα . Και σ' αυτή την περίπτωση η εφαρμογή αιωρήματος προφυτρωτικά και από τα δύο στελέχη του *Fusarium oxysporum* περιόρισε την προσβολή από *Orobanche crenata* , αλλά για την *Orobanche cumana* το ένα στέλεχος του μύκητα ήταν πιο παθογόνο από το άλλο. Στα πειράματα με μεταφυτρωτική εφαρμογή του αιωρήματος σπορίων του *Fusarium oxysporum* ο περιορισμός της προσβολής ήταν εντονότερος στις 14 ημέρες από τις 7 ημέρες μετά την μόλυνση.

Εκχύλιση και χημικός προσδιορισμός των μεταβολιτών
Το πρώτο εκχύλισμα από καλλιέργειες του *Fusarium oxysporum* ήταν φυτοτοξικό σε όλα τα είδη οροβάγχης με δραστικότητα καλύτερη από το glyphosate. Στη συνέχεις με διαδοχικές κλασματώσεις παραλήφθησαν παρασκευάσματα των οποίων η φυτοτοξική δράση ελέγχθηκε με τις διάφορες βιοδοκιμές και μόνο εκείνα τα παρασκευάσματα με ενδιαφέρουσα φυτοτοξικότητα αναλύθηκαν περαιτέρω με χρωματογραφία λεπτής στοιβάδας, στήλης και MPLC.
Με βάση τα αποτελέσματα από τις αναλύσεις απομονώθηκαν και ταυτοποιήθηκαν χημικά οι παρακάτω ενώσεις: glycerol, 1H-indole-3-carboxaldehyde, oleic acid methylester, oleic acid, tyrosol, parahydroxybenzoic acid.

Βιοδοκιμές φυτοτοξικότητας
Από τις ενώσεις που απομονώθηκαν μόνο η 1H-indole-3-carboxaldehyde και η tyrosol ήταν φυτοτοξική στην *Orobanche crenata* . Οι ενώσεις αυτές δοκιμάστηκαν σε συγκεντρώσεις από 2,5 έως 50 μg/ml. Όμως το glyphosate σε αντίστοιχες συγκεντρώσεις αποδείχθηκε πιο φυτοτξικό σε τεμάχια ιστοκαλλιέργειας *Orobanche crenata.* Η μελέτη της εκλεκτικής δραστικότητας σε τεμάχια φύλλων κουκιάς έδειξε ότι η tyrosol σε συγκεντρώσεις μικρότερες των 20 μg/ml δεν είχε επίδραση στους ιστούς κουκιάς. Ανάλογη ήταν και η επίδραση του 1H-indole-3-carboxaldehyde. Τα αποτελέσματα από τις μετρήσεις χλωροφύλλης σε ιστούς κουκιάς έδειξαν ότι η δράση των δυο αυτών ενώσεων ήταν ανάλογη με εκείνη του glyphosate και ότι η επίδραση της 1H-indole-3-carboxaldehyde στη μείωση της χλωροφύλλης ήταν μεγαλύτερη από εκείνη της tyrosol.

Επίδραση των GR24 και Nijmegen-1 και των αυξητικών ορμονών στην οροβάγχη.
Η επίδραση των ουσιών GR24 και Nijmegen-1 στη βλάστηση σπόρων της οροβάγχης δεν ήταν ίδια στα τρία είδη παρασίτου που χρησιμοποιήθηκαν (*Orobanche crenata, Orobanche cumana* και *Orobanche aegyptiaca).* Το μεγαλύτερο ποσοστό βλάστησης (70%) παρατηρήθηκε στην *Orobanche aegyptiaca.* Το ίδιο είδος επίσης αντέδρασε θετικότερα από τα άλλα δύο στο σχηματισμό κάλλων σε ιστοκαλλιέργεια μετά από προσθήκη αυξητικών ουσιών (IAA 2,4-D) στο θρεπτικό υλικό. Επίσης παρατηρήθηκε διαφοροποίηση στο είδος των ιστών μεταξύ των ειδών της οροβάγχης σε σχέση με το είδος των αυξητικών ουσιών που δοκιμάσθηκαν.

Στην μελέτη αυτή
1. Απομονώθηκαν στελέχη του *Fusarium oxysporum* παθογόνα στην *Orobanche crenata* από καλλιέργεια κουκιάς.

2. Αξιολογήθηκε η in planta επίδραση των στελεχών του *Fusarium oxysporum* στην παρεμπόδιση παρασιτισμού ειδών οροβάγχης στους ξενιστές τους.
3. Μελετήθηκε η επίδραση φυτορρυθμιστικών ουσιών και διεγερτών βλάστησης σπόρων οροβάγχης στην *in vitro* ανάπτυξη των *Orobanche crenata, Orobanche cumana* και *Orobanche aegyptiaca.*
4. Μελετήθηκε η βιοδραστικότητα παρασκευασμάτων από καλλιέργειες των παθογόνων στελεχών του *Fusarium oxysporum* σε είδη οροβάγχης και σε ιστούς κουκιάς.
5. Απομονώθηκαν οι μεταβολίτες glycerol, 1H-indole-3-carboxaldehyde, oleic acid methylester, oleic acid, tyrosol, parahydroxybenzoic acid υτοτοξικά παρασκευάσματα καλλιεργειών του από *Fusarium oxysporum*
6. Αξιολογήθηκε η φυτοτοξικότητα των ουσιών αυτών και η εκλεκτική φυτοτοξικότητα των 1H-indole-3-carboxaldehyd

SUMMARY

Weeds and Orobanche species in particular cause great loss to crop farmers worldwide.The devastating effect that *Orobanche crenata* attack has on legumes forces many farmers to delay or abandon their cultivation. Many efforts have been devoted to control this parasitic weed, but to date there are no completely satisfactory control measures.When comparing the control of Orobanche with non-parasitic weeds, it is realised that the control of Orobanche is exceptionally difficult. The ability of the parasite to produce a tremendously high number of seeds, which can remain viable in the soil for more several years, and the intimate physiological interaction that exists with their host plants, are the main difficulties that limit the development of successful control measures that can be accepted and used by all farmers. However, several control methods have been tried and recommended for the control of Orobanche. These include cultural and mechanical (crop rotation, trap and catch cropping, fallowing, hand-pulling, nitrogen fertilization, time and method of planting, intercropping and mixed cropping), physical (solarisation), chemical (herbicides, artificial seed germination stimulants), use of resistant varieties, and biological. At the field level, the management of Orobanche is still unsatisfactory since - with the exception of the use of glyphosate in faba bean to control *Orobanche crenata*, present control methods are not efficient enough to control the underground development stages of the parasite. Considering the increasing awareness of herbicide resistance, and the restriction of the use of chemical pesticides in agriculture, novel compounds from micro-organisms and plants may provide new alternatives for the control of weeds that may otherwise be difficult to control, e.g. parasitic weeds. Pathogenic fungi and bacteria often damage their host plants by producing toxins, which cause various symptoms including necrosis, chlorosis, wilting, water soaking and eventually the death of plants.

Since it is known that toxins produced by *Fusarium* spp. are also phytotoxic to several plants, this provided an impetus to investigate firstly whether potential *Fusarium* isolates from infected *Orobanche crenata* plants obtained in Greece are pathogenic to *Orobanche crenata* itself and other related species. Secondly, this study aimed at investigating if isolated *Fusarium oxysporum* is able to produce phytotoxic metabolites that have bioherbicidal effects against different developmental stages of *Orobanche sp* and selective effects on various *Orobanche spp* and other plants. To attain the objective of the work, green house and laboratory studies were conducted to determine the pathogenicity and phytotoxicity of *Fusarium oxysporum* on *Orobanche crenata* and related *Orobanche* species. When the results obtained proved that the *Fusarium* specie is pathogenic and phytotoxic to *Orobanche crenata*, identification of the possible metabolites were done using TLC, HPLC, MPLC, GCMS and NMR techniques. The following metabolites were identified: 1H-indole-3-carboxaldehyde , oleic acid methylester , oleic acid , glycerol, tyrosol and P-hydroxybenzoic acid**.** Preliminary phytotoxicity tests conducted with the identified metabiltes proved that only 1H-indole-3-carboxaldehyde and tyrosol were of interest to this research and so bioassays were conducted to acertain their effectivenes as phytotoxins.

CHAPTER 1.

GENERAL INTRODUCTION

1.1. DESCRIPTION OF OROBANCHE SPECIES

There are several species of angiosperms utilizing a parasitic mode of nutrition but not much has been studied on all these species. These parasitic flowering plants could be separated into the two broad groups of holoparasites and hemiparasites. Holoparasitic species are always obligate parasites which lack chlorophyll and have little independent capacity to assimilate or fix carbon and/or inorganic nitrogen (Stewart and Press, 1990). Hemiparasitic species on the other hand, may be facultative or obligate i.e. contain chlorophyll, and are generally thought to rely on their host only for water and minerals. It has been suggested that the extent to which parasitic plants depend on their hosts must somehow be related to their own photosynthesizing abilities (Tuohy, et. al., 1986).

Parasitic flowering plants could be further subdivided on the basis of their site of attachment to the host. We could thus have stem parasites, such as the holoparasitic dodders and the hemiparasitic mistletoes and root parasites, such as the holoparasitic broomrapes and hemiparasitic witchweeds. The distinguishing feature of all parasitic plants is the haustorium, the principal organ that functions in attachment, penetration, and solute transfer (Kuijt, 1977; Visser and Dorr, 1986; Stewart and Press, 1990).

Broomrapes (Orobanche spp.) are classified in the family Orobanchaceae belonging to the obligate parasitic group. It is also worth noting that these herbaceous root parasites without chlorophyll are annual. Orobanche spp produce large number of seeds that are widely distributed in harvested products and in the fields. Because of their size, they are easily distributed by water and wind (Jurado-Exposito, et. al., 1996).

Orobanche spp commonly parasitize *Solanaceae* and *Fabaceae (Leguminoceae)* hosts such as tomato, pepper, bean, pea and tobacco, reducing crop yields or totally destroying the crop. *Orobanche spp* are usually not specific to a single host with most species parasitizing a range of hosts, although among *Orobanche* species host ranges may differ. The genus comprises over 150 species in 17 genera (Thieret, 1971)

The main stem emerges after a considerable period of growth underground. Stem is erect and large or thin depending on specie. It is cylindrical (5-10mm in diameter) and thick at the base having short hairs and begins just below the inflorescence. The straw colour ranges from white, pale brown, reddish amber to black-brown depending on specie. The straw is glandular pubescent with a height of 10 to 100cm depending on specie. The stems terminate in flowering spikes (Holm *et al*. 1997). Surface reticulations are prominent, intermediate to large in size, often high-walled. The walls are thick, thin, coarse or moniliforme and sometimes nearly transparent or wavy. The leaves are in the form of scales (Holm *et al*. 1997).

The In-florescence is about 5-25 cm long, moderately- to few-flowered and not particularly dense; The bracts are 12-25 mm long; Flowers are about 20–40, loosely spiked on upper two-thirds of stem, distant throughout about 1cm apart, presenting a sub-decussate appearance with the floral bract as long as corolla. It has a 4-lobed calyx, not divided, with

triangular teeth, acuminate and shorter than corolla tube .Bracteoles are present at base of calyx been subtended by one ovate bract and 2 linear bracteole .The corolla is about 15 mm long, tubular, curved and may be constricted above ovary. The stamens are four, two long and two short, inserted on corolla tube. It has a pistil superior ovary, which is ovoid, 2-celled with a long style and large stigma. The bracts are scattered, few at base and very distant below on stem. The Stamens are inserted near the base of the corolla and are stout, glabrous and flexuous. Anthers are light umber-brown. Style is thick and dilated at top, glabrous, with a few (5–6) scattered microscopic glandular hairs near top. Stigma is large and bilobed. The lobes are globular, spreading, purple and finely papillose. (Holm *et al*. 1997) Some species may produce flowers within a week of emergence from the soil.

The fruit is usually a loculicidal or septicidal capsule with numerous seeds; Seeds commonly narrowly to broadly wedge shaped, irregularly wedge shaped, or teardrop-shaped, also elliptic, obovate, or oblong; tiny, dustlike, 0.2-0.6 mm long, 0.1-0.5 mm wide and thick. The embryo is very small to minute with endosperm present.

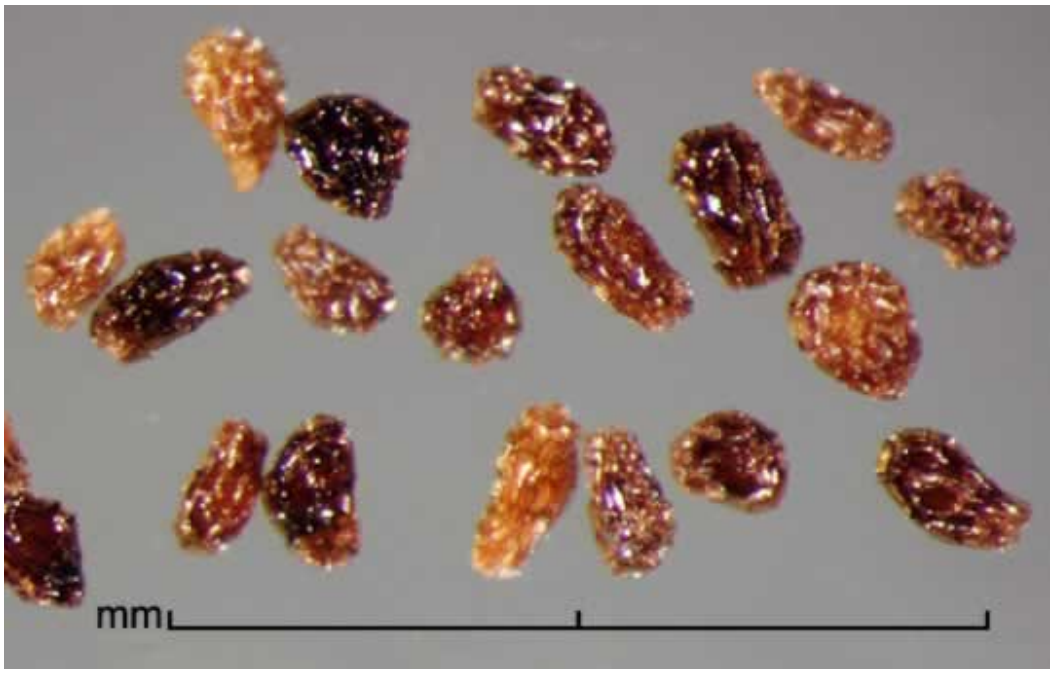


Figure 1. Seeds of *Orobanche crenata.*

Source: photograph is copied from the website www.lucidcentral.org of Federal Noxious weed disseminules of the U.S. by Julia Scher.

Figure 2. *Orobanche crenata* seed.
source : photograph is copied from the website www.lucidcentral.org
of Federal Noxious weed disseminules of the U.S. by Julia Scher.

Figure 3. *Orobanche crenata* inflorescence at maturity. Magnification 1X

Figure 4. *Orobanche crenata* flower 1 week after emergence-magnification 2X

1.2 ORIGIN & DISTRIBUTION OF OROBANCHE

Orobanche species like other parasitic weeds have evolved specificity to crops and plants in the natural vegetation and do not or only occasionally occur on crop species. Some of them, however, have more or less abandoned their natural hosts and become very noxious parasitic weeds causing serious losses in yields of economically important crops (Roman et al 2007).
The Mediterranean region is considered to be one of the centres of origin of *Orobanche* species. The species are distributed worldwide from temperate climates to the semi-arid tropics except *Orobanche crenata* Forsk whose distribution is restricted to the Mediterranean regions, the Middle East and East Africa (Parker and Riches, 1993). Though the main centre of distribution is the Mediterranean region, where large areas are heavily infested, other regions with similar climatic conditions (California, Western Australia, Cuba) have also been invaded (Musselman, 1986). Some species can be found in arid or semiarid environments, and others are found as far north as Sweden (Linke, et. al., 1989). Of the 100 or more species in the genus Orobanche, only a few are of economic importance as weeds in cropping systems. These parasitic plants vary greatly with respect to host range, and parasitize a wide range of plant families (Asteraceae, Fabaceae, Solanaceae, Apiaceae, and Cucurbitaceae).

Table 1 : Economic important orobanche species and their main hosts

OROBANCHE SPECIES	MAIN HOSTS
O. crenata	Faba bean, pea, lentil and chickpea
O. ramosa	Tomato, tobacco, hemp, eggplant, lentil and cucurbit
O. aegyptiaca	Tomato, tobacco, hemp, eggplant, lentil and cucurbit
O. cumana	Sunflower, tobacco, tomato and potato
O. cernua	Sunflower, tobacco, tomato and potato
O. minor	Alfalfa, clover and trefoil (lotus)
O. foetida	Faba bean, alfalfa and trefoil (lotus)

Source (Kroschel 2001)

1.3 BIOLOGY OF OROBANCHE CRENATA.

The host-parasite relationship of *Orobanche crenata* is of a highly specialized nature. A diagrammatic representation of the complexity of this relationship is illustrated below in Figure 1. This relationship however could be modified by climate and man.
Factors like host, soil or predators can play an additional role (Linke, et. al., 1989). The seeds of *Orobanche crenata* are among the smallest in the genus and plant kingdom as a whole.
Seed weight is approximately 4-9 x 10^{-3}mg (Miller 1994). The position of the seeds within the inflorescence also affects germinability, dormancy and seed size, with smaller seeds being found nearer the top of the spike. The seed coat shows characteristic thickenings at the surface which may help in dispersal by wind and water. Seeds are produced in large quantities, approximately 5000 per capsule, with more than 100 capsules per plant seldomly found. Plants of *O. crenata* can produce several hundred thousand seeds, while the smaller species (e.g., *O. ramosa*) may produce only 5,000-20,000 seeds per plant. The seeds can remain viable in the soil for more than 10 years (Linke, et. al., 1989). This feature alone makes the parasitic plants an extremely difficult intruder in agro-ecosystems. The duration of shoot development and emergence bears a close relation to the soil temperature, the nutritional status of parasite and crop, and to a lesser extent the identity of the host plant itself. The growth of the parasite occurs at the expense of water, mineral and organic compounds from the host. The tubercle and underground shoot accumulate carbohydrates and thereby become a strong sink for all plant nutrients. Effect on host plant growth becomes noticeable when the parasite emerges from the soil. Growth of Orobanche shoots is most rapid during that period and it induces a lack of carbohydrate in the host roots. Osmotic pressure in the host is reduced to an extent that symptoms like wilting and drought stress can occur.
Depending on environmental conditions the underground phase of the life-cycle of *Orobanche crenata* ranges from 30 to over 100 days. The whole life cycle from seed germination to seed production requires about 3-5 months (Kroschel 2001).

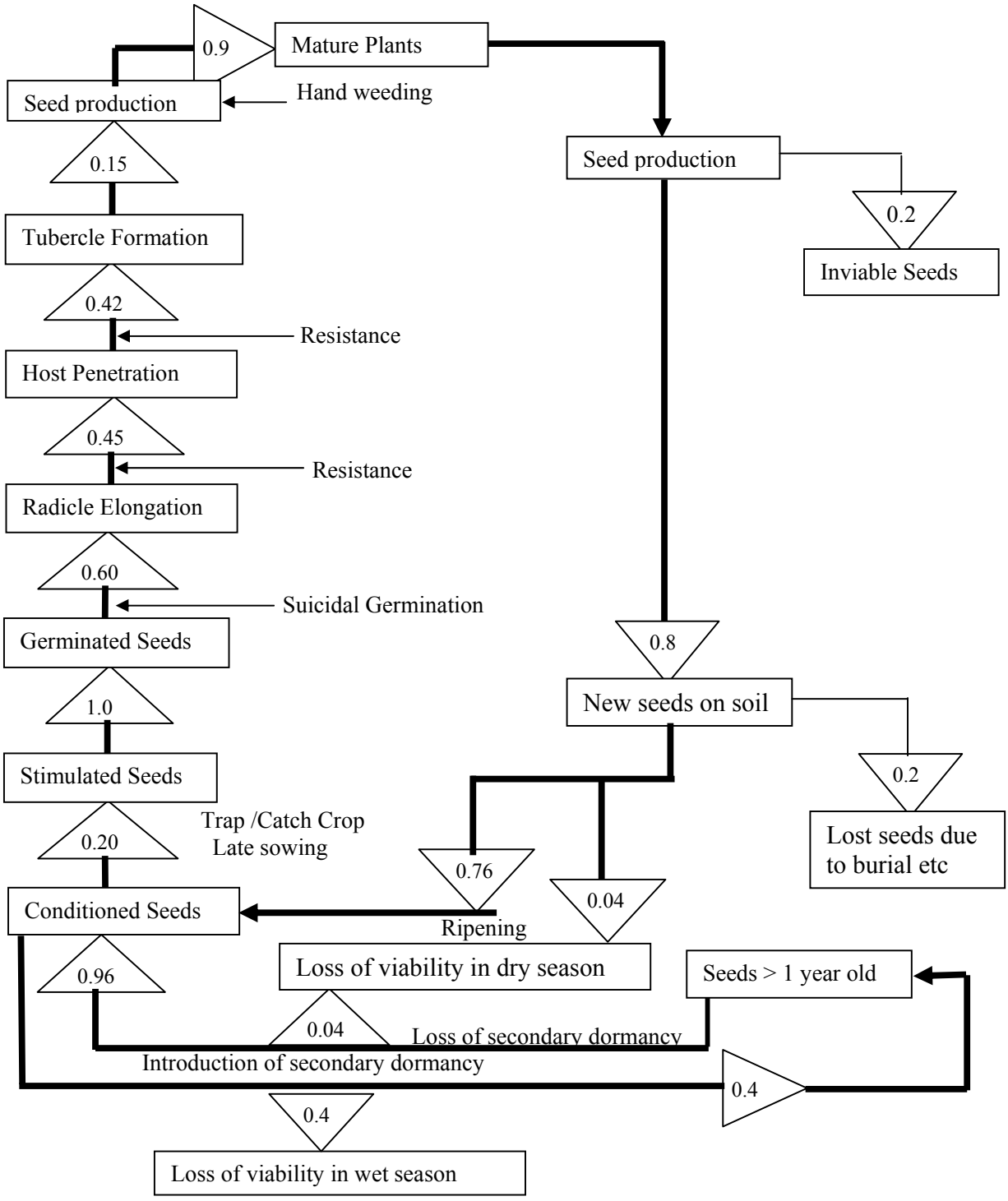


Figure 5. Life cycle of *Orobanche species*.
Thick lines represent the major route of the cycle and the numbers surrounded by a triangle indicate the probability of transition from one stage of the life-cycle to the next. Annotations describe stages where integrated control measures may act. Adapted from Kebreab & Murdoch 2001.

The seeds of the root-parasitic weeds vary in their ability to germinate immediately after they have reached maturity. Seeds of Orobanche are dormant and require a period of after-ripening or so called post-harvest ripening period (Riches, 1989). Seed germination occurs when ripened seeds are preconditioned by exposure to warm moist conditions for several days followed by exogenous chemical signals produced by host roots and some non-hosts (germination stimulant) (Worsham, 1987). Upon germination, a germ tube, which is in close proximity to the host roots, elongates towards the root of the host, develops an organ of attachment, the haustorium, which serves as a bridge between the parasite and its host, and deprives it of water, mineral nutrients and carbohydrates, causing drought stress and wilting of the host. Stunted shoot growth, leaf chlorosis and reduced photosynthesis are also phenomena that can be observed on susceptible host plants, which contribute to reduction of grain yield (Frost *et al.* 1997). Most of the seeds in the soil will not be reached by the stimulant, but will remain viable for up to 10 years, forming a seed reservoir for the next cropping seasons. The penetration of haustorial cells into host tissue (xylem and/or phloem system) is carried out mechanically by pressure on the host endodermal cells and by hydrolytic enzymes. Conditioning, germination, parasitic contact (attachment) and penetration are mediated by elegant systems of chemical communication between host and parasite-allelopathy (Maass, 1999). After several weeks of underground development the parasite emerges above the soil surface and starts to flower and produce seeds after another short period of time. Seed production is prodigious, up to 100 000 seeds or even more can be produced by a single plant and lead to a re-infestation of the field. Thus, if host plants are frequently cultivated, the seed population in the soil increases tremendously and cropping of host plants becomes more and more uneconomical (Kroschel, 2001).

1.4 ECONOMIC ASPECTS OF OROBANCHE INFESTATION

Weeds and Orobanche species in particular cause great loss to crop farmers worldwide. Yield reduction is dependent on the timing and severity of infestation. These weeds compete with crop plants for nutrition, water and space and may serve as alternate hosts for insect pests or disease agents.

Yield losses vary and generally range from 5 to 100% (Stewart and Press, 1990). The loss averaged across all broomrape species, is approximately 34%. *O. cernua* probably causes the most widespread damage of all *Orobanche* species, as it affects about 7 million hectares of sunflower in Eastern Europe and Near East. *O. crenata* and *O. aegyptiaca* occur in legume fields of West Asia and North Africa, threatening production of these crops in an area of nearly 4.4 million hectares.). For example, in Morocco, the infestation of *O. crenata* in food legumes caused yield losses of 32.7 percent on an average in five provinces in the year 1994, which was equal to a production loss of 14 389 tonnes (US$8.6 million. (Geipert *et al.* 1996). As a result of the complete devastation caused by *Orobanche* in many areas, production methods had to be modified and/or cultivation of some susceptible hosts had to be abandoned.

Another 2.6 million hectares of solanaceous crops like tomato, potato, tobacco and eggplant are threatened by *O. ramosa* and *O. aegyptiaca* in the same region (Linke, et. al., 1989).
Damage to an infested host crop is through reduction in yield of both seed and straw. Yield reduction occurs after any infestation as the parasites' dry matter is produced from the assimilates that should have been used by the host itself. Reduction in quality occurs in several crops such as tobacco, sunflower, tomatoes, carrots, cabbage and eggplants.

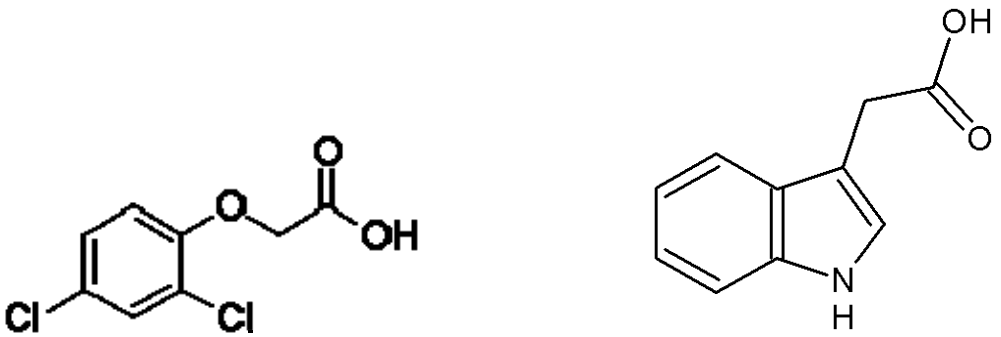
1.5 *In vitro* GROWTH OF OROBANCHE SPECIES

Tissue culture or plant regeneration is conducted *in vitro* so that the environment and growth medium can be manipulated to ensure a high frequency of regeneration. The primary aim of tissue culture is therefore to produce, as easily and as quickly as possible, a large number of regenerable cells that are available for bioassays. When plant cells and tissues are cultured *in vitro*, they initiate cell division from almost any tissue. In this way, whole plants could be subsequently regenerated. When cultured *in vitro*, all the needs, both chemical and physical of the plant cells have to be met by the culture media. i.e. the growth medium has to supply all the essential mineral ions required for growth and development. Besides the basic chemical necessities for plant cell culture media i.e. macro elements, microelements and source of carbon, other additions (plant growth regulators) are made in order to manipulate the pattern of growth and development of the plant cell culture. IAA (Indole-3-acetic acid) and 2, 4 D (2, 4-Dichlorophenoxyacetic acid) are the most commonly used in Orobanche tissue culture. Callus culture is often performed in the dark as light can encourage differentiation of the callus (Gamborg 2002).
In vitro growth of Orobanche is a difficult procedure especially as the seeds of Orobanche are usually contaminated with bacteria and fungal spores. To overcome this problem of contamination from bacteria and fungi, disinfection of the seeds is necessary. Different methods of disinfection have been reported (Kumar & Rangaswamy, 1977; Ben-Hod et al., 1991; Lane et al., 1991; Wolf & Timko, 1991; Hess et al., 1992; van Hezewijk et al., 1993; Joel & Losner-Goshen, 1994 and Batchvarova *et al*. 1999) which proved to be successful. This includes the use of sodium hypochlorite, formaldehyde, antibiotics or fungicides. Before proceeding to the growth media, a viability test is usually conducted using any of the known methods. The most common method for testing viability of Orobanche seeds is by use of TTC (2, 3, 5-triphenyl tetrazolium chloride) as reported by López Granados and García Torres, 1999;Aalders and Pieters, 1985.This method involves spreading seeds evenly on filter paper discs and immersion in a solution of 1% TTC . Filter paper discs are then placed in sealed Petri dishes (0.90 mm) wrapped in aluminium foil, and incubated at 30°C for 12 days. Seeds are then soaked in 6% sodium hypochlorite solution for 5 min. Seeds are then observed under a binocular dissecting microscope; seeds that have stained red or pink are considered to be viable, and those without pigmentation are considered as non-viable.Seed sterilisation, seed conditioning, seed germination, and attachment-organ formation are the major aspects of *in vitro* growth of Orobanche. Previous studies have shown that a period of several days of moist environment at permissive temperatures is required in order to render the ripe seeds of *Orobanche* responsive to germination stimuli (Joel *et al*., 1995). The exact physiological role of this preparatory phase, which has been termed conditioning or pre-conditioning, is not well known. In this respect, three distinct but not mutually exclusive hypotheses have been considered by Gonzalez-Verdejo et al., 2005. (1) Conditioning induces metabolic changes that are necessary for the response to

germination stimulants. During conditioning gibberellin, which is required for seed germination, is synthesized but is not yet present in the seeds. Indeed, inhibitors of gibberellin synthesis were shown to prevent germination after GR24 treatment and this effect could be reversed by exogenous GA_3 (Zehhar *et al*., 2002). (2) During the conditioning period the receptor proteins for GR24 or other natural stimulants are synthesized. (3) Structural modifications occurring in the seeds allow the stimulant to access its putative cellular target. In any case, the effects of conditioning would be maintained during a subsequent dry period. Seed germination in *Orobanche* has been shown to be stimulated by host root exudate or by synthetic strigol analogues such as GR24 (Joel and Losner-Goshen, 1994; van Woerden *et al*., 1994). Nijmegen-1 has also been reported as a broad-band germination stimulant, which probably is active to all *Orobanche* species, similar to the natural strigol or the synthetic GR 24.

To study germination and early development in the in *vitro* system, conditioned seeds of *Orobanche* are stimulated by adding a germination stimulant, and seed germination and development are followed daily by microscopic observation. The conical tip of the radicle gradually became enlarged and differentiated into a structure reminiscent of the attachment organ formed during root infection (Joel and Losner-Goshen, 1994). The attachment organ-like structures produced exhibit a considerable variability in size and morphology.

These attachment organ-like structures are called calluses and their growth is influenced by growth hormones like IAA and 2, 4-D. Slavov *et al.* (2004); Batchvarova *et al.* (1999) and Zhou *et al.* (2004) have reported the influences exhibited by these hormones on the growth of the calluses.



IAA **2,4 D**

Figure 6. Structures of IAA (Indole-3-acetic acid) and 2, 4 D (2, Dichlorophenoxyacetic acid)

The level of influence of these hormones also depends on the growth medium in which they are applied. Zhou et al., 2004 reported that better callus induction is obtained from Gamborg`s B5 culture medium-see materials and method (Gamborg et al., 1968) compared with the MS medium (Murashige and Skoog, 1962). 2, 4 D has been recognized as the most

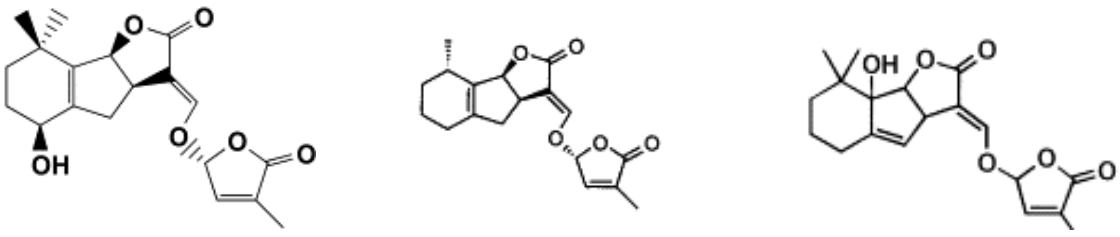
effective auxin for callus induction particularly in graminaceous plants (Subba Rao & Nitzsche 1984). However, induction depends on variety of plants used and the concentration of the hormones. Initially, the rate of callus growth is slow in the first few weeks but increases subsequently upon sub culturing.

1.6 GERMINATION STIMULATION AND ATTACHMENT

Germination stimulation is an area in parasitic plant research generally receiving the most attention. This is presumably, in an attempt to isolate an active analog which can be used as a control measure i.e. forcing germination of parasitic plants so as to easily control it. Germination stimulants have however, proved to be elusive for three major reasons viz: they are active at extremely low concentrations, their presence in a complex media such as soil makes their isolation difficult and finally, the structures which have been successfully isolated and identified are quite fragile or transitory in the soil environment (Stewart and Press, 1990).

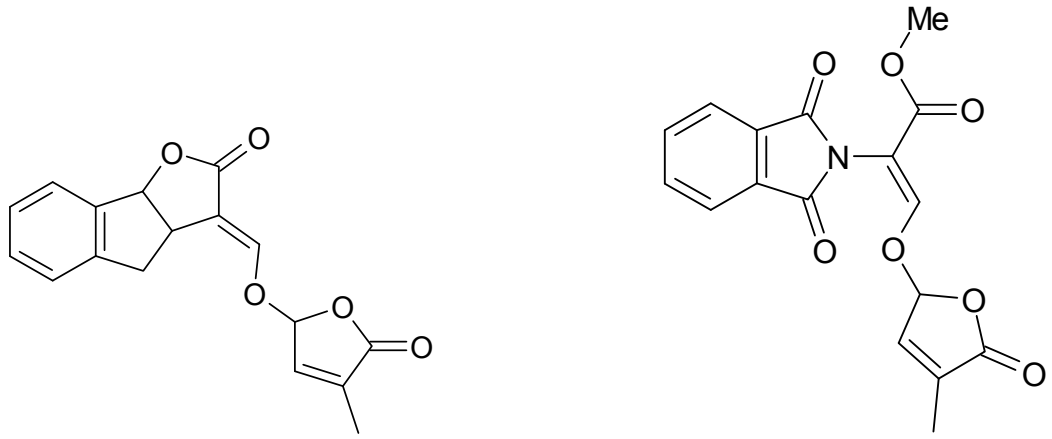
Nun and Mayer (1993) recently indicated that freshly harvested seeds remain dormant for several days or months depending on species and environment. In addition, these researchers indicated, that even under favourable conditions, germination takes place only in the presence of a germination stimulant released by roots of the host plant. Spread of stimulant in the rhizosphere depends on soil water content. This view has also been projected by Musselman (1987) and Parker and Riches (1993). Thus far, three naturally occurring germination stimulants, belonging to the class of the strigolactones (Butler,1995), viz : strigol (Cook et al., 1996), sorgolactone (Hauck et al.,1992), and alectrol(Muller et al.,1992) have been isolated.

Synthetic germination stimulants GR24 and Nijmegen 1 have also been reported by Wigchert et al., 1999; Johnson et al., 1981; Mangnus et al., 1992; Kranz et al., 1996 and Thuring et al., 1997.While multigram scale production of GR24 is not yet economically feasible, Nefkens et al., (1997) reported that Nijmegen 1 can easily be prepared in multigram quantities probably because it contains only one stereogenic centre.



Strigol Sorgolactone Alectrol

Figure 7: Structures of naturally occurring germination stimulants.



GR-24 Nijmegen-1

Figure 8: Structures of the synthetic germination stimulants GR24 and Nijmegen 1

Temperature seems to be a key factor for Orobanche germination and development. Temperatures within the range of 15-25^{o}C are optimal for *O. crenata* and *O. ramosa*, while below 5^{o}C and above 30^{o}C only a few seeds will germinate and develop. Upon germination a hyaline, root-like structure, the germ tube, expands out of the testa. It can reach a length of 3-4mm with a diameter of 0.15mm. For this reason, only seeds in the immediate vicinity of the host roots (3-4mm) can lead to parasitism. The germ tube shows a positive chemotropy in the vicinity of the host root, (i.e., it grows in the direction of the host root).
With respect to seed preconditioning (imbibition), Nun and Mayer (1993) clearly illustrated that seeds which are not exposed to a germination stimulus are able to remain viable for long periods without damage. However, they also showed that seeds which start to germinate, but do not reach a host show indications of stored nutrient exhaustion and the likelihood of death. If the germ tube reaches the host root its tip thickens and attaches itself to the root surface. The thickening is known as the appressorium.
The appressorium connects itself by means of enzymatic degradation and mechanical penetration of the host root vessels. Both of these phenomena have been documented in recent experimental procedures, however, the preponderance of evidence at this time supports the involvement of enzymatically mediated degradation (pectin methylesterase, Ben-Hod, et. al., 1993; and cellulase, xylanase, and poly-galacturonase, Singh and Singh, 1993) in the infection of host plants by Orobanche. This connecting tissue is the haustorium (Linke, et. al., 1989). After contact between the appressorium and the host vessels is made,

the former is henceforth known as the tubercle. This organ, yellow to orange in color, now starts to enlarge. The mature tubercle is 0.5-2.5cm thick in most species, though thicker in some (up to 5cm). With this organ the parasite withdraws water, mineral and organic compounds from the host (Kuijt, 1977; Visser and Dorr, 1986; Stewart and Press, 1990).
After the formation of crown roots on the tubercle a bud develops (bud stage) which later forms a shoot. The main shoot is sparsely covered with scaly leaves. In some species branching of the shoot is normal (e.g., *O. aegyptiaca*, *O. ramosa*), whereas in others it is rare (e.g. *O.minor*, *O. crenata*, *O. cernua*). During the underground stage of its life-cycle Orobanche accumulates carbohydrates; visible growths being comparatively slow. But the reserve of carbohydrate enables the parasite to elongate its shoot, emerge from the ground, and produce an aerial shoot and flowers within a very short period.

1.7. CONTROL METHODS OF OROBANCHE CRENATA AND THE CONSTRAINTS.

Orobanche crenata infestation has a major economic and agronomic impact, because legumes play an important role in crop rotation (for soil improvement) in Mediterranean Agriculture, and are an essential component of a sustainable agricultural system. However, several control methods have been tried and recommended for the control of Orobanche. These include cultural and mechanical (crop rotation, trap and catch cropping, fallowing, hand-pulling, nitrogen fertilization, time and method of planting, intercropping and mixed cropping), physical (solarisation), chemical (herbicides and artificial seed germination stimulants), use of resistant varieties, and biological. These methods of control have been well reviewed by Parker and Riches (1993), Jurado-Expósito *et al.*, (1996), Cubero & Hernández (1991), Cubero & Moreno (1999), Mesa-García & García-Torres (1991), and recently summarized in Kroschel (2001), Omanya (2001) and Vurro &Gressel (2007). At the moment, the restoration of infested fields can only succeed through the improvement of existing farming systems based on analysis of the parasitic weed problem and the development of a sustainable long-term integrated control programme consisting of the more applicable control approaches that are compatible with existing farming systems and with farmer preference and income (Kroschel, 1999). The success of cultural measures becomes evident only in the long run and will not improve yields in the present crop, because of the long underground developmental phase as well as the high seed production and longevity (Parker and Riches, 1993). One other technique is delayed sowing, which has a strong effect on *Orobanche crenata* attack in legumes. Delayed sowing is recommended (Moreno-Márquez, 1947; Mesa-García & García-Torres, 1991; Zaitoun & Ibrahim, 1998) in order to prevent severe attacks of the parasite. However, some legumes have a long life cycle and delayed sowing can decrease yield because of the shortened cropping season (Saxena *et al.*, 1994; Zaitoun & Ibrahim, 1998).
As reported by Duke and Lydon (1993) about two-third, by volume, of the chemicals used in agricultural production are herbicides. The present emphasis on reduced-or no-tillage agriculture will depend heavily on herbicides for weed control. On the other hand, the increasing incidence of herbicide resistance is creating a demand for new herbicides with unexploited mechanism of action. The potential for undesirable environmental contamination from herbicides is relatively high, and there is a need for environmentally

safe herbicides that are equally or more effective and selective than currently available synthetic herbicides. Thus, the need for new herbicides with known mode of actions becomes obvious to solve the dilemma of the continued demand for herbicides while older herbicides are being removed from the production fields for environmental purposes.
The income of most farmers is usually too low to justify the use of highly sophisticated technical inputs such as ethylene to trigger ineffective seed germination, as used by some farmers to eradicate the parasite, or with soil solarization. In addition to the cost, selectivity, low persistence and availability are major constraints that limit the successful usage of herbicides. In addition, the use of synthetic germination stimulants and application of high dosage of nitrogen fertilizer (more than 80 kg N ha^{-1}, mainly as ammonium sulphate or urea), are not readily applicable in African farming systems (Kroschel *et al.* 1997). Few resistant lines for some host-parasite associations were reported (Lane *et al.* 1997) but resistance is often interfered by the large genetic diversity of the parasites. Recent successes have been achieved in biological control, but it has not led to practical field application owing to the difficulties associated with mass rearing, release, formulation and delivery systems.

1.7 .a. Preventive control.

Since Orobanche has not yet infested all potential cultivation fields and regions of the world, the spread of this economic weed could be avoided. The seeds of Orobanche are disseminated by wind, water, animals, machines and contaminated crop seeds. The best control method is preventive i.e. sowing clean seeds. Because Orobanche seeds are too tiny, they are easily removed from large seeded crops through washing. Also bags used in transporting crops from orobanche infested fields to new fields could be thoroughly washed or discarded to avoid contamination of the new field. Other methods of prevention include: Buying certified seed only, buying seeds from regions known to be non-infested, conduction of visual surveys of endangered areas from time to time to ensure that there are no Orobanche that could spread, testing of crop seeds for contamination with Orobanche, regular field inspections and timely information to farmers from time to time about the morphological features of this parasite to enable them recognize an early infestation. The eradication by hand of single parasitic plants in new infested fields to prevent the build up of a large seed bank could also be a good preventive measure. Post harvest preventive measures could also be beneficial. They include: tilling of the soil to expose Orobanche seeds to harsh environmental conditions which are detrimental to their survival; Burning of the post harvest remains using herbicides or germination stimulant or fire to destroy any Orobanche seeds that could be found in the field before the next planting season.

1.7. b. Biological control.

The widespread use of synthetic chemical herbicides that are not naturally occurring compounds has caused societal and environmental concern. Thus, biological control which is more environmentally benign is preferred because of their natural origin. Biological control of weeds refers to the utilization of living organisms to manipulate (suppress, reduce, or eradicate) weed population. It could also be described as agricultural management using a biological agent to reduce the impact of a specific pest or pathogen. Biological control, especially using insects and fungal antagonists against parasitic weeds, has gained

considerable attention in recent years and appears to be promising as a viable supplement to other control methods. Biological control includes insect control, breeding for resistant crop varieties, allelopathy and microbial agents.

1.7. b.1. Insect as control agents.

Many phytophagous insects have been collected on *Orobanche* species but most are polyphagous and the target weed species are often not their principal host plants. The fly *Phytomyza orobanchia* Kalt. (Diptera: Agromyzidae) and *Smicronyx cyaneus* Gyll. are of great interest in the biological control of *Orobanche* spp. (Klein and Kroschel, 2002). As a single method, biological control with herbivores will hardly be fully successful in tackling the parasitic weed problem. But in combination with other methods in an integrated control package, herbivores could play a role in lowering the parasitic weed population and reducing its reproductive capacity and spread. Most promising are inundative mass releases of *P. orobanchia*. However, also classical biological control might be an option by introducing *P. orobanchia* into countries where *Orobanche* spp. are not endemic and have been unintentionally introduced, such as Chile (Norambuena *et al.* 1999, 2001; Klein and Kroschel, 2002) where *P. orobanchia* (Diptera, Agromyzidae) has co-evolved with *Orobanche* species. Therefore, its distribution is related to the natural occurrence of plants of the genus *Orobanche.* The damage on *Orobanche* is caused by the larvae, which mine in shoots and seed capsules. Thereby *P. orobanchia* has an enormous impact on the seed production of *Orobanche* spp. Depending on the site, the host plant and the *Orobanche* species, up to 95 percent of the seed capsules can be infested by *P. orobanchia*. A natural reduction of the seed production between 11-79 percent is reported from several countries of the Mediterranean region, North and East Africa and the Near East (Klein and Kroschel, 2002). However, the efficacy of *P. orobanchia* to reduce the *Orobanche* population is mainly limited by cultural practices (by destroying pupae in soil through soil cultivation methods or by using insecticides) and the occurrence of natural enemies. Most important are larval and pupal parasitoids of the order Hymenoptera (Klein and Kroschel, 2002). The natural population of *P. orobanchia* is too low to be able to reduce sufficiently the *Orobanche* population to the point that no economic losses occur. The natural equilibrium between *Orobanche* and *P. orobanchia* as exists in natural vegetation is disturbed by the extensive cropping of *Orobanche* host plants. Therefore, an increase in the efficacy of *P. orobanchia* can only be achieved by augmentation of the natural population.
Biocontrol of *Orobanche* spp. by *P. orobanchia* is based on the prerequisite that *Orobanche* shoots are loaded with *P. orobanchia* pupae at the time of harvest of the *Orobanche* host plants and that no rearing methods on artificial diets have been successfully developed. This means that *Orobanche* shoots have to be collected and stored until the next season. By doing so, the mechanical destruction of pupae by tillage or other field operations can be avoided. Storage can preferably be done in a so-called 'Phytomyzarium', which offers special advantages for the collection and release of hatched adults (Klein and Kroschel, 2002; Kroschel and Klein, 2003). However, the hatching rate of adults from collected and stored pupae is only 4 percent because a high proportion of the pupae are in diapause, which can last for three years. If applicable, storage under colder conditions in the refrigerator for a certain period of time can increase the hatching rate to 10 percent.

In calculating the number of flies to be released in the field, the expected *Orobanche* infestation level as well as the reproductive capacity of the fly has to be taken into consideration. The efficacy of inundative releases of *P. orobanchia* to reduce the seed production of *O. cernua* or *O. aegyptiaca* parasitizing sunflower and other crops has already been demonstrated in the 1960s and 1970s in different regions of the former Soviet Union. Releases of 500-1 000 adults per ha resulted in a reduction of up to 96 percent of the *Orobanche* seed production (Natalenko, 1969; Bronstejn, 1971; Kapralov, 1974). In Morocco, inundative releases significantly reduced the reproduction of *O. crenata* in faba bean. During the 3-year research period, only 3.7 percent to 6.2 percent of the seeds were viable after releases, in comparison to 94.9 percent (1996), 57.1 percent (1997) and 36.5 percent (1998) in the control plots without inundative releases (Kroschel and Klein, 2003).
Inundative releases require an efficient mass rearing method or in the case of *P. orobanchia* a sufficient provision of flies from collected *Orobanche* shoots. In Morocco, the number of hatched flies from stored shoots in a Phytomyzarium with a hatching rate of only 4 percent will still be sufficient even for the treatment of highly infested fields. However, more effective methods should be developed to manipulate diapause and hatching of adults, which would make the collection and storage of shoots more efficient and practical.
Taking the enormous seed bank and the longevity of *Orobanche* seeds into consideration, inundative releases of *P. orobanchia* will not be sufficient as a single control method in heavily infested fields. On the contrary, in weakly infested areas it could prevent further dissemination. However, especially for the control of parasitic weeds, a successful and sustainable weed management system must be based on combinations of different techniques. Biological control with *P. orobanchia* could be an important part in an integrated *Orobanche* management system and, therefore, especially part of Farmers' Field Schools, e.g. the combination of hand-weeding of *Orobanche* shoots before seed ripening and the storage of the collected shoots to rear *P. orobanchia* could be very effective. Furthermore, the bioagent could limit the seed production of escaping *Orobanche* shoots which develop when resistant varieties are used and may prevent the development of more aggressive *Orobanche* races.

1.7. b.2. Breeding for resistant crop varieties.

Host plant resistance and tolerance.

Host plant resistance would probably be the most feasible and potential method for parasitic weed control. Resistant host plants offer a potentially durable tool for resourceful farming. Unfortunately, research on crop resistance to Orobanche has not received much attention (Cubero, 1991). Using biotechnological approaches (including biochemistry, tissue culture, plant genetics and breeding, and molecular biology) significant progress has been made in developing screening methodologies and new laboratory assays, leading to the identification of better sources of parasitic weed host resistance (Ejeta *et al.* 2000; Haussmann *et al,.* 2000; Omanya, 2001; Mohamed *et al.* 2001).However, good levels of resistance have been found in several host/parasite systems including broad bean to *O.crenata* and common vetch to *O.crenata*. Crop genotypes which interfere with one or several stages of the parasite's life cycle could be resistant. A study by Zaitoun and Sabra (2000) confirmed that the new broad bean resistant varieties (Giza 667 and Giza 843) were less infected by *O. crenata*. Full immunity of host plants to Orobanche has not yet been

found. However, several resistant crop varieties are used nowadays in various parts of Africa, Europe and Asia. As the reported resistant or tolerant cultivars are often not accepted by farmers because of their low yield, low seed and storing quality, poor adaptation to a wide range of agro-ecological zones and their sensitivity to pest and diseases, the newly developed techniques significantly contribute to overcoming these problems by permitting transfer of resistance genes into adapted cultivars with high-yielding potential. This will lead to a lower parasite infestation and to a higher crop yield.

Resistant/tolerant varieties to *Orobanche* have been developed in several crops and used for some years. The most outstanding example has been the development of sunflower varieties resistant to *O. cernua*/*cumana*. Unfortunately, this resistance has often been overcome by new, more virulent 'races' of the parasite in many countries across the Mediterranean region, eastern Europe and the former Soviet Union (Rodriguez-Ojeda *et al.* 2001). Two cultivars of faba been (Giza 429 and Giza 674) with a good level of resistance to *O. crenata* have been released in middle- and upper-Egypt (Khalil *et al.* 1993). Furthermore, a new faba bean genotype (X-843) resistance to *O. crenata,* derived from Giza 402, was reported to have a good yield performance and was recommended for release in north Egypt (Saber *et al.* 1999). A well-adapted, high-yielding faba bean cultivar 'Baraca' has been developed in Spain under field conditions, with a high level of resistance to *O. crenata* (Cubero 1994). So far, seeds of the released and recommended faba bean cultivars are not commercialized and are thus not available for farmers. Alonso (1998) has intensively reviewed the most significant results achieved in the breeding for resistance to other *Orobanche* spp. Some cases of complete resistance under field conditions of some vetch lines (473A) (Gil *et al.* 1987; Goldwasser *et al.* 1997), and tomato lines (PZU-11) (Foy *et al.* 1988) have been confirmed. Nevertheless, under artificial inoculation with a very high dose of *Orobanche,* even the field resistant lines were attacked by broomrape. More recently, Sauerborn *et al.* (2002) induced resistance in sunflower against *O. cumana* by using the synthetic chemical benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), the active ingredient of Bion®, under controlled growth camber conditions.

1.7.b.3. Allelopathy.

Allelopathy could be defined as a chemical interaction or communication between plants and the organisms living in their environment (Rice, 1995; IAS, 1996). Molisch (1937) coined the term allelopathy from two Greek words 'Allelo' and 'Pathy' meaning 'mutual harm'. In this sense, the parasitic weed-host recognition mechanism is a good example of allelopathy, i.e. based on the actual definition and hypothesis. Parasitic weeds use specific compounds exuded by their host to recognize their presence in the surroundings (Joel et al, 1995). The nature of allelopathic compounds could be described as secondary plant products released into the environment through volatilisation, leaching, root exudation and decomposition of plant residues in soil. These metabolites such as phenolics, flavonoids, alkaloids, terpenoids and cyanogenic glycosides have always attracted scientists to elucidate their structure and biological function (Cutler and Cutler 1999; Rice 1995). Similarly, Putnam (1988) listed 6 classes of allelochemicals isolated from over 30 families of terrestrial and aquatic plants. These classes are alkaloids, benzoxazinones, cinnamic acid derivatives, cyanogenic compounds, ethylene and other seed germination stimulants, and flavonoids. All these chemicals possess actual or potential phytotoxicity. These compounds induce the

germination of dormant seeds and also help them to locate the host roots for attachment following an increasing concentration direction (chemotropism) (Fate et al, 1990; Chang et al, 1986). However, it is unlikely that these compounds were synthesized and exuded by the plant with this purpose; rather they should be considered as defence products. Co-evolution of parasitic plants and their host led to a situation in which parasites take advantage of these compounds to recognize a viable host by which they could complete their life cycle.
Plants differ in the production of allelochemicals depending upon the environment in which they are grown and particularly in response to stresses they encounter. Nutrient deficiencies also affect the quantity of allelochemicals produced by plants (Rice 1983).The type and age of plant tissues also affects the production of allelochemicals.
To date, few compounds have been isolated and characterized as inductors from natural hosts, all of them belonging to plants being parasitized by members of the *Orobanchaceae* and *Striga* families. Many of these host plants have been researched upon, several inductors characterized, and their structures reviewed recently (Galindo et al, 2004). Interestingly, all of these "natural inductors" belong to the same skeletal type, named strigolactones. Other natural products and synthetic derivatives have also been found to induce broomrape or witchweed germination. However, none of them have been isolated from their typical hosts. The mode of actions of allelopathic compounds in plants are not well understood but could include effects on interference with division and elongation of cells; involvement in hormone induced growth; cell membrane permeability; mineral uptake and nucleic acid synthesis.

1.7.b.4. Microbial agents (in general).

A number of biological, environmental and technological limitations, variability of field performance, cost of production and market size are the major constraints that potentially affect the economic feasibility of any given biological control product (Auld and Morin, 1995). Considering the increasing awareness of herbicide resistance, and the restriction of the use of chemical pesticides in agriculture, novel compounds from micro-organisms may provide new alternatives for the control of weeds that may otherwise be difficult to control, e.g. parasitic weeds. Microbially-derived compounds may be pursued either as templates for new synthetic chemical herbicides or as pathogens applied directly to the target weed (Boyetchko 1999). Pathogenic fungi and bacteria often damage their host plants by producing toxins, which cause various symptoms including necrosis, chlorosis, wilting, water soaking and eventually the death of plants (Scheffer, 1983). One criterion of the importance of a toxin in a disease syndrome caused by a pathogen is that toxicity is often related to pathogenicity or virulence (Scheffer, 1983).
Plant pathogens are proposed for use in a non-classical inundative approach as 'bio herbicides' for biological control of parasitic weeds. The protocol for their use involves: surveying the weed with pathogens; isolation; identification and classification; inoculum production; screening for efficacy (pathogenicity testing); host specificity and safety testing; inoculum mass production; preliminary field testing; formulation and delivery to target weed. According to Abuelgasim Elzein and Jürgen Kroschel (2003), the type of formulation used for delivering a bioherbicide depends on the biology of the target weed, the type, biology and mode of action of the pathogen as well as on the available application technology. For fungal pathogens, the simplest method is the use of a spore or mycelial suspension in water, which can be used as a soil drench or a post-emergence spray application. For parasitic weeds like

Orobanche, this formulation was used in the early stages for the evaluation of the efficacy of potential fungal antagonists either in greenhouse or field conditions. Solid or granular formulations are more suitable for pathogens that infect their target weeds at or below the soil surface (i.e. attacking weed seedlings as they emerge from the soil), a system more appropriate for root parasitic weeds and best suited for pre-emergence application (Elzein & Kroschel 2003). Hence, several solid substrates have often been used as carriers to deliver mycoherbicides of parasitic weeds. These include infested cereal grains or straw (barely, wheat, sorghum), soil/maize feed mixtures, a mixture of straw and maize flour, and river sand and maize meal. Although these simple granular (solid) formulations have proven to be very effective under greenhouse and field conditions in both control of the parasites and improvement of the performance of host plants, it was found that very high levels of fungal inoculum (approximately 800 kg ha^{-1}) were required for successful parasite control. In addition, many undesirable characteristics are encountered with the use of cereal grains and straw as substrates for delivering pathogenic fungi for parasitic weed control, especially under large-scale heavy infestation, including:

- efficient production of biomass
- storage ability and method of application
- difficulty in controlling the production of the appropriate (desired) fungal propagules;
- high cost;
- Not suitable for commercial use.
- Basic aspects of strain selection.
- a better understanding of mechanisms of action, nutrition and ecology of the biocontrol agent
- Avoidance of unintentional adverse effects on non-target cultivated and wild plant species, related to the target weed or grown within the range of dissemination of that pathogen, so called 'host specificity' needs to be ascertained.
- The potential for genetic manipulation of microbial agents to create genetically superior strains or hybrids that can perform better than the wild types needs to be considered.

However, it should be kept in mind that discovery of a pathogen is only one step in the long process of development of a bioherbicide. The final biocontrol product is one where all processes in production and formulation, delivery, and information on the pathogenicity, mode of action and host specificity have been evaluated. The progress in biological control of parasitic weeds microbially is intended to be addressed with regard to the achievements in overcoming the above mentioned obstacles.

Of course, the odds against finding a truly useful agent are daunting. On average, it requires approximately 12 years and is estimated to cost over US$800 million to develop and bring to market a new drug, and thousands of candidate compounds are dropped for every one that ultimately reaches the market (Anonymous 1995; DiMasi et al. 2003; Barton and Emanuel 2005). Even so, the potential payoff, and the track record of fungi as sources of useful compounds have fostered continued industrial interest in fungal natural products chemistry within many screening programs. In recent years, it has become increasingly difficult to find new bioactive natural products from microbial sources because of the extensive screening efforts that have already taken place. In fact, the need to dereplicate cultures, i.e., to weed out

well-known metabolites that are responsible for positive results in a bioassay (Harris 2005; Dinan 2005; Hansen et al. 2005), is a source of tremendous expense and frustration (Corley and Durley 1994), and is viewed by many as a significant negative feature of continued screening efforts. Part of this problem stems from long-term reliance on screening of sheer numbers of actinomycetes and common fungi isolated mainly from soil samples as sources of bioactive metabolites, while other, less widely studied niche groups have been largely neglected. Inattention to the specific types of organisms chosen for screening and the habitats from which they are isolated, together with disinterest in fungi that are slow-growing, more difficult to isolate, and/or difficult to adapt to standard liquid fermentation protocols, combine to exacerbate the problem. Thus, many taxonomic and ecological groups of fungi have not been systematically explored for useful secondary metabolites, despite literature evidence that directly or indirectly indicates their potential in this area.

The concept of using biological control strategies in crop protection has been studied intensively for many years (Gardner and McCoy 1992; Powell1993). These strategies involve the use of certain fungi as mycoherbicides (Boyette 2000; Ghorbani et al. 2005), mycoinsecticides, or mycoparasites (Harman 2006) to control weeds, insects, or fungal pathogens, respectively. Such approaches involve the deployment of a microbial disease agent effective against the pest, rather than (or as an adjunct to) the application of a chemical pesticide of some sort. Ultimately, damage to the pest is often caused by toxin(s) produced by the pathogen, and the use of measured and properly formulated quantities of the natural products themselves could provide an alternative control strategy. Use of the microorganism itself is appealing because it serves to selectively direct the toxin(s) to the target. Some toxin producing microorganisms, most notably the bacterium *Bacillus thuringiensis*, have proven to be particularly effective in such applications. However, there are many specialized hurdles that must be overcome to implement an effective biocontrol strategy (Powell 1993), and the use of biocontrol agents will not necessarily eliminate the problem of resistance (Gardner and McCoy 1992). From a chemistry standpoint, knowledge of any metabolites involved in biocontrol effects is important as a means of avoiding unwanted side-effects, and precedents indicate that studies of fungi with mycopesticidal properties are likely to lead to discovery of pesticidal compounds. Some examples of such results are provided below. 1. Plant Pathogens Plant pathogenic fungi are well known as producers of diverse compounds with phytotoxic effects on host plants (Turner and Aldridge 1983; Harborne 1993; D'Mello and MacDonald 1998). The effects of the toxins are often principal causes of symptoms associated with the corresponding plant disease. In many cases, fungal phytotoxins produce damage that fosters fungal invasion and colonization of the plant. Typically, the compounds are general phytotoxins (Ballio 1991), but in some cases, a co evolutionary process has led to at least some degree of host selectivity (Walton and Panaccione 1993;Wolpert et al. 2002). The host-selective toxins are particularly interesting from a chemical standpoint because they tend to have structural features that distinguish them from commonly encountered fungal metabolites that might show more general toxicity. Notable examples include victorin, HCtoxin, and certain *Alternaria* toxins (Walton and Panaccione 1993; Wolpert et al. 2002; Masunaka 270 J.B. Gloer et al. 2005). Plant pathogenic fungi as a group could be viewed as logical sources to explore in search of biologically active metabolites in a general sense, since they have already demonstrated the capacity to produce bioactive compounds with distinctive chemical structures. Some fungal metabolites with phytotoxic effects are known to exhibit medically relevant activities as well, including antitumor and antibiotic effects. It is interesting to note that mevinolin is a rather potent herbicide (Hoagland 1990). From a more specific viewpoint,

fungi pathogenic to weeds have been proposed as rational sources of herbicides. Indeed, phytotoxins with novel and unusual structures have been isolated from weed pathogens, and subsequently shown to display herbicidal activity toward weeds (Hoagland 1990, 2001; Amalfitano et al. 2002; Evidente et al. 2004). This would seem to be a particularly worthy avenue of investigation in view of the fact that other microbial natural products, such as bialaphos (produced by a *Streptomyces* sp.), have already been used successfully as commercial herbicides (Hoagland 2001).

1.7.b.5. Microbial agents (Orobanche species).

Accordingly, a variety of fungal and very few bacterial agents applied to the seeds, foliage and/or soil have been explored as potential candidates for parasitic weeds of the genus *Orobanche* since the early 1990s (well reviewed in Kroschel and Müller-Stöver, 2003). Promising fungal isolates with potentials for the biocontrol of *Orobanche* have been found to have a different host range (Bedi 1994; Thomas *et al.* 1998). Müller-Stöver, (2001) observed a reduced germination rate of *O. crenata* seeds caused by Fungal isolates, although no pathogenicity was observed towards later developmental stages of the parasitic weeds. Fusarium strains isolated in Israel have been observed to attack *O. aegyptiaca, O. cernua* and *O. ramosa*, but are avirulent against *O. cumana* (Amsellem *et al*. 2001). This advantage and the ability of the potential fungal isolates to control more than one *Orobanche* species provides an opportunity to control more parasites simultaneously in those regions where they are co-existing, which may encourage the regulatory authorities to accept and implement inundative biological control of parasitic weeds. It is very important that host-specificity testing and risk assessment methodologies should both lead to prevention of the release of any organism that is likely to have detrimental impacts on non-target plants and/or on the environment. As reported by Bedi, 1994; Amsellem *et al.* 2001; Müller-Stöver 2001, Most *Orobanche*-pathogenic *Fusarium* strains do not attack any of various tested crop plants.

1.7.c. Cultural control method.
Cultural and mechanical.

- Land preparation: It has been realised that zero and minimum tillage increased broad bean infestation by O.crenata (Kukula and Masri, 1984) while deep ploughing of 40-50cm deep reduced infestation by 80-90% (Kasasian, 1971).
- Planting or sowing date: Though delaying crop sowing date can reduce crop yield, however in the presence of a certain level of parasitic plant infestation, a shift of the sowing date may be more adverse to the parasite than to the host crop.
- Crop rotation with trap/catch crop:
- Trap crops are plants that stimulate germination of the parasite seed but do not become infected. They therefore reduce the seed bank of the parasite in the soil and cause a decrease in infestation. Depending upon the agricultural system, different crops can be used as trap species. Crop rotation with trap and catch crops has long been proposed and practised as control measure for parasitic weeds. The most favourable rotation as far as *Orobanche* control is concerned is a rotation with a trap crop. The most promising trap crops reported for *Striga* and *Orobanche* species as recently reported by Kroschel (2001) are those grown for fodder production(e.g. *Trifolium alexandrinum*) as these crops are planted densely and extensively

penetrate the soil with their roots.. It is obvious that farmers from countries or regions with possibilities for marketing *Striga* trap crops like cotton and soybean have an overall advantage in adopting such farming systems (Kroschel and Sauerborn, 1996). It has been concluded that large differences between cultivars in their 'trapping' ability exists and that the characterization and recommendation of the best cultivars should be a routine activity for researchers focusing on *Striga* and *Orobanche* infested areas (Berner *et al.* 1995). It should also be borne in mind, however, that farmers prefer many traits in a particular cultivar such as high yield potential, earliness, colour, cooking time, etc., which need to be considered when making recommendations to farmers (Ransom, 1999).

Table 2. Trap crops for *Orobanche* spp.

Scientific name	Reference
Orobanche ramosa	
Allium sativum	http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm
Arabidopsis thaliana	Goldwasser et al. 2000
Brassica sp.	Cited by Kasasian, 1973
Brassica napus	Cited by Kasasian, 1971
Brassica rapa	Al-Menoufi & Adam, 1996
Capsicum spp.	Cited by Kasasian, 1971; Sand, 1983
Carum ajowan	http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm
Coriandrum sativum	Al-Menoufi & Adam, 1996
Cucumis sativus	Labrada & Perez, 1988
Glycine max	http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm
Lablab purpureus	http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm
Lathyrus ochrus	http://www.uni-hohenheim.de/~www380/parasite/oro_path.htm
Linum usitatissimum	Eplee, 1984; Khalaf, 1992
Lupinus termis	Al-Menoufi & Adam, 1996

Mediacago sativa	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Pennisetum sp.	Cited by Kasasian, 1971
Phaseolus aureus	Kleifeld, 1996
Phaseolus	*vulgaris* Labrada & Perez, 1988; http://www.unihohenheim. de/~www380/parasite/oro_path.htm
Pisum sativum.	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Ricinus communis	Cited by Kasasian, 1971; http://www.unihohenheim. de/~www380/parasite/oro_path.htm
Sesamum indicum	Cited by Kasasian, 1971; http://www.unihohenheim. de/~www380/parasite/oro_path.htm
Setaria sp.	Cited by Kasasian, 1971
Sorghum bicolor	Labrada & Perez, 1988
Trifolium sp.	Cited by Kasasian, 1971
Trigonella foenum graecum	Al-Menoufi & Adam, 1996
Vigna radiata	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Vigna unguiculata spp. *unguiculata*	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Zea mays	Cited by Kasasian, 1971; Labrada & Perez, 1988
Orobanche aegyptiaca	
Allium sativum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Arabidopsis thaliana	Goldwasser et al. 2000
Capsicum annuum	Hershenhorn et al. 1996
Carum ajowan	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Glycine max	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Lablab purpureus	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Lathyrus ochrus	http://www.uni-hohenheim.de/

	~www380/parasite/oro_path.htm
Linum usitatissimum	Kleifeld et al. 1994
Medicago sativa	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Phaseolus vulgaris	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Pisum sativum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Ricinus communis	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Sesamum indicum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Vigna radiata	Kleifeld et al. 1994
Vigna unguiculata spp. *unguiculata*	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Orobanche cernua	
Amaranthus spp.	Rao, 1955
Bidens pilosa	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Cajanus cajan	Krishnamurthy & Chandwani, 1975
Capsicum annuum	Cited by Kasasian, 1971; http://www.unihohenheim. de/~www380/parasite/oro_path.htm
Cicer arientinum	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
Cichorium intybus	http://www.uni-hohenheim.de/ www380/parasite/oro_path.htm
Colocasia sp.	Rao, 1955
Crotalaria juncea	Dhanapal & Struik, 1996; Dhanapal, Mallory-Smith & Ter Borg, 2001
Curcuma domestica	Rao, 1955
Glycine max	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
Gossypium spp.	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Guizotia abyssinica	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Hibiscus sabdariffa	Rao, 1955

Illacrotvloma uniflorum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Linum usitatissimum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Medicago sativa	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Pennisetum typhoides	Rao, 1955
Phaseolus aureus	Dhanapal, Mallory-Smith & Ter Borg, 2001
Phaseolus aconitifolius	Rao, 1955
Ricinus communis	Rao, 1955;http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Sesamum indicum	Rao, 1955
Setaria indica	Rao, 1955
Sinapis alba	Cited by Kasasian, 1971
Solanum melongena	Rao, 1955
Sorghum sp.	Krishnamurthy & Chandwani, 1975; Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
Tridax procumbens	http://www.unihohenheim. de/~www380/parasite/oro_path.htm
Vigna mungo	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Vigna radiatia	Dhanapal & Struik, 1996
Cannabis sativa	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
Vigna radiata	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
Medicago sativa	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
Vigna acontifolia	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Vigna unguiculata	Krishnamurthy & Rao, 1976; Krishnamurthy et al. 1977
Vicia dasycarpa spp. *villosa*	Linke et al. 1991
Orobanche cumana	
Bidens pilosa	http://www.uni-hohenheim.de/

	~www380/parasite/oro_path.htm
Capsicum annuum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Cichorium intybus	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Crotalaria juncea	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
*Gossypium*spp.	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Guizotia abyssinica	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Illacrotvloma uniflorum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Linum usitatissimum	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Medicago sativa	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Ricinus communis	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Tridax procumbens	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Vigna acontifolia	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Vigna mungo	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Vigna radiatia	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Orobanche crenata	
Allium sativum	Hassan, 1998
Astragalus boeticus	Schnell et al. 1994
Brassica rapa	Al-Menoufi & Adam, 1996
Capsicum annuum	Al-Menoufi & Adam, 1996
Coriandrum sativum	Al-Menoufi & Adam, 1996; Zemrag & Bajja,2001
Glycine max	Schnell et al. 1994
Hedysarum coronarium	Schnell et al. 1994
Helianthus annuus	http://www.uni-hohenheim.de/ ~www380/parasite/oro_path.htm
Hordeum vulgare	Kasasian, 1973; Linke et al. 1991;http://www.uni-hohenheim.de/

	~www380/parasite/oro_path.htm
Lablab purpureus	Schnell et al. 1994
Lathyrus ochrus	Schnell et al. 1994
Linum usitatissimum	Khalaf, 1992; Abou-Salama, 1995
Lupinus termis	Al-Menoufi & Adam, 1996
Phaseolus vulgaris	Schnell et al. 1994
Pisum sativum	Hassan, 1998
Saccharum officinarum	Abou-Salama, 1995
Sesamum indicum	Al-Menoufi, 1991
Trifolium alexandrinum	Schnell et al. 1994; Al-Menoufi & Adam, 1996
Trigonella foenum-graecum	Al-Menoufi & Adam, 1996; Zemrag & Bajja, 2001
Vicia dasycarpa spp. *villosa*	Kasasian, 1973; Linke et al. 1991
Vicia narbonensis	Schnell et al. 1994
Vigna radiata	Schnell et al. 1994
Vigna unguiculata	Schnell et al. 1994
Orobanche minor	
Allium sativum	Hassan, 1998
Arabidopsis thaliana	Goldwasser et al. 2000
Linum usitatissimum	Brown et al. 1952
Pisum sativum	Hassan, 1998
Sorghum bicolor	Brown et al. 1952
Zea mays	Brown et al. 1952

Catch crops of *Orobanche* are host plants of parasitic weeds that induce germination and can be parasitized. They are true hosts infected or enhanced germination and attachment but hinder parasite development by mechanical, physiological or chemical factors. However, true

hosts should be harvested, converted into the soil or destroyed after 6-8 weeks, before the parasite appears above the soil, or at least before starts flowering and seeding. This method can exhaust the parasite seed bank in the soil and may be effective on the long run term. Acharya et al. (2002) evaluated the effectiveness of toria (*Brassica campestris* var. *toria*) plant as a catch crop for the reduction of *O. aegyptiaca* seed bank. They found that two successive crops of toria reduced *O. aegyptiaca* seed bank by 20.9% and 26.2% for both crops, respectively. Optimum density of toria plants required for significant reduction of *O. aegyptiaca* seed bank was about 140/m2. However, catch crops or plant species are likely to be used as preceding crops and have to be closely planted or sown at high density. This method of control however, is coasty due to additional labor, not usable if growing season is short, and needs good mechanization because of possible loss of a growing period. On the other hand, cultivation of all hosts should be easy and inexpensive, germination stimulation should be high, their elimination is not problematic, and should be high yielding as fodder or green manure.

- Mineral fertiliser and organic material: Since different plants are sensitive to different nutrients (Nandula, Foy and Westwood, 1996), the application of mineral fertilizers at considerable level could be useful in controlling Orobanche parasites. Ammonium fertilisers are more inhibitory to *Orobanche* spp. than nitrate fertilisers (Westwood and Foy, 1999).Foliar spray of 0.5% boric acid 60 days after *vicia faba* sowing could completely control *O.crenata* (Hassan and Farrag, 1982).
- Flooding: If flooding is done for an extended period of weeks, it could result in the killing of parasitic weeds because they are deprived of oxygen. However, in areas with water shortage problem, this method is not feasible.

1.7. d. Chemical methods.

Although weed control has been achieved through physical and cultural practices, herbicides remain as a main component of a weed management system. In order to be effective and beneficial within the growing season, herbicides for the control of root parasitic weeds, which are characterized by long underground developmental stages, may be applied pre- or post-emergence of the crop but should always be pre-emergence of the parasites. During the last decades, some potential useful chemical interventions have become available for parasitic weed control (Garcia-Torres, 1998). However, lack of application technology, marginal crop selectivity, low persistence and availability are major constraints that limit the successful usage of herbicides in developing countries where the income of subsistence farmers is usually too low to afford them. Several attempts and intensive research have been made in different countries to screen for potential herbicides against *Orobanche*. In general, the most limiting factor in the use of the promising herbicides is their degree of selectivity among the crops at the required rate for parasite control, and the critical time of application, especially of the foliarly applied systemic herbicides. However, glyphosate is the first promising herbicide developed for *Orobanche crenata* control in faba bean (Schmitt *et al.* 1979), and is still the most important herbicide used. Since then, a considerable number of studies have been performed in trying to clarify the selectivity and use of glyphosate in other

legume and non-legume crops that are susceptible *Orobanche* hosts other than faba bean. Some degree of success has been achieved in some crops under specific conditions such as time and rate of application. Hence, the crops in which the use of glyphosate is well confirmed, so far, include faba bean, carrot (*Daucus carota* L.) and celery (*Apium graveolens* L.). However, the corresponding yield increase is inconsistent especially under heavy infestation. In addition to glyphosate, other herbicides, e.g. sulfonylureas, and imidazolinones proved to be effective in many host crops, both at the demonstration or commercial levels used in many locations in the Near East (Israel, Jacobson, 2002) and the Mediterranean region (Spain, Garcia-Torres *et al.* 1999).Imazapic and glyphosate have been tested and proven by Goldwasser et al (2002) to be very effective against *O.crenata* and *O.aegyptiaca.* It has been shown that the phytotoxicity of some post-emergence herbicides on crops can be avoided or reduced by altering their application and delivery technique. For example, by systematically applying chlorsulfuron and triasulfuron into the soil, *O. aegyptiaca* can be successfully controlled and the yield of tomato increased without damaging the crop (Kleifeld *et al.* 1999). In Spain, promising results were also obtained (60-80 percent *O. crenata* control) by soaking or coating faba bean and pea (*Pisum sativum* L.) seeds, or lentil seeds in low concentration of imazethapyr or imazapyr, respectively (Jurado Exposito *et al.* 1996, 1997; Garcia-Torres *et al.* 1999). The seed germination and crop growth were not affected by the phytotoxicity of both herbicides. However, this technology has not yet been developed or optimized for application at the field level.

1.8 FUNGI IN BIOLOGICAL CONTROL

Fungi are lower plants without chlorophyll, and consequently cannot utilize carbon dioxide from the air. As a result they are unable to manufacture their own carbohydrates, and have to live on organic matter (heterotrophic). The fungi provide almost limitless potential for metabolic variation. Fungi rank second only to the insects in estimated species biodiversity. Fungi such as *Alternaria*, *Fusarium, Colletotrichum* etc. can produce phytotoxins. Ohra and Morita (1995) found that phytotoxins from *Colletotrichum gloeosporioides* have weed control activity. An experiment to control 7 different kinds of weeds indicated that joint vetch (*Aeschynomene virginica*) was extremely damaged, pigweed (*Amaranthus retroflexus*) and florida beggarweed (*Desmodium tortuosum*) were severely burned and would not grow out of the damage, and Johnson grass (*Sorghum halepense*) was stunted but not killed by its extract. Conservative estimates suggest that there are likely to be over 1.5 million fungal species, of which only approximately 5% have been described (Hawksworth 1991, 2001). This is over five times the number of predicted plant species, and 50 times the estimated number of bacterial species (Hawksworth 1991). Some researchers have conferred an element of urgency upon studies of the chemistry of certain fungi to rationalize appeals for accelerated studies of plant chemistry, i.e., concerns about the loss of biodiversity (Balandrinet al.1993). Many endangered plant and insect species are associated with specific fungal flora, and loss of those species would also result in a concomitant loss of fungal species.

Some are parasitic getting nourishment from living organisms, but most rely on rotting plant substance. A fungus is composed of hyphae and mycelium. A large number of Fungi reproduce by releasing airborne spores, which have different structures and dimensions.

Spores present in the air settle on surfaces. When conditions are favourable, spores start to germinate.

The virulence of parasitic weeds, their regional occurrence and their parasitic life-style make them suitable targets for biocontrol using the bioherbicidal approach where multiplication and periodic release of indigenous microbial agents for sustained control of the target species is employed. Periodic usage will be necessary because these indigenous biological control agents have indigenous antagonists that limit their growth and development. Because of their frequently occurring high host-specificity, pathogens can distinguish between a crop and a closely linked parasitic weed where chemical herbicides are considered unsafe. Approximately 30 fungal genera were reported to occur on *Orobanche* spp. (Kott, 1969; Stankevich, 1971; Timchenko and Dovgal, 1972; Taslakh'Yan and Grigoryan, 1978; Hódosy, 1981; Al-Menoufi, 1986; Bedi and Donchev, 1991; Amsellem et al., 1999; Thomas et al., 1999a; Boari and Vurro, 2004). Also about 16 fungal genera have been reported on other parasitic plants e.g *Striga* spp. (Meister and Eplee, 1971; Zummo, 1977; Greathead, 1983; Abbasher and Sauerborn, 1992; Kirk, 1993; Ciotola et al.,1995; Abbasher et al., 1995 and Abbasher et al., 1998; Kroschel et al., 1996; Marley et al., 1999; Hess et al., 2002). Results of surveys for fungal pathogens of *Orobanche* and *Striga* revealed that *Fusarium* species were the most prominent ones associated with diseased broomrapes and witchweeds. Of these, *F. oxysporum* was the predominant species.

Among the most fascinating and important properties of fungi is their ability to produce a tremendous variety of so-called secondary metabolites that display a broad range of biological activities (Demain et al. 2005).
Fungi are widely known for the production of compounds that have a deservedly negative reputation due to their activities as toxins. Such compounds include aflatoxins, ochratoxins, citreoviridin, trichothecenes, fumonisins, and various indole-derived tremorgenics (Miller and Trenholm 1994; Cole and Schweikert 2003).
On the other hand, numerous important pharmaceuticals have also been discovered through studies of fungal chemistry (Masurekar 2005; Demain et al. 2005). This dichotomy is indicative of the diversity of bioactive compounds that fungi can produce. Fungi are particularly prolific sources of biologically active natural products (Pel´aez 2005).Antibacterial agents such as penicillins and cephalosporins are perhaps the best known examples, but a variety of other compounds with distinctive pharmacological activities have also been discovered as fungal metabolites. A survey of the literature on bioactive fungal metabolites during the period from1993–2001 (718 references) revealed that antifungal activity was the most common biological effect reported (Pel´aez 2005). Fungal products also show considerable potential as natural agrochemicals (Gardner and McCoy 1992; Anke and Sterner 2002; Pel´aez 2005; Liu and Li 2005), with important examples including nodulisporic acids (insecticides/ antiparasitics), strobilurins (fungicides), and various phytotoxins (herbicides).

Table 3: Fungal pathogens useful for the biocontrol of Orobanche species.

Fungus	**Country**	**Target weed**	**Reference**
Fusarium oxysporum	Iran	*O.cumana, O.ramosa*	Amsellem *et al.* (2001), Mazaheri *et al*. (1991
Fusarium oxysporum	Morocco	*O. crenata*	Kroschel *et al.* (1996b), Elzein and Kroschel (2003)
Fusarium oxysporum f.sp. orthoceras	India	*O. cumana, O. cernua, O. aegyptiaca*	Bedi and Donchev (1991)
Fusarium oxysporum	Israel	*O. aegyptiaca, O. ramosa, O. cernua*	Amsellem *et al.* (2001)
Fusarium arthrosporioides	Israel	*O. aegyptiaca, O. ramosa, O. cernua*	Amsellem *et al.* (2001)
Fusarium spp	Nepal		Thomas *et al*., 1999
Fusarium oxysporum,	Hungary	*O.ramosa*	Hodosy, 1981).
Fusarium solani	Hungary	*O.ramosa*	Hodosy, 1981).
Fusarium oxysporum	India	*O.cumana*	Bedi and Donchev, 1995
Fusarium oxysporum	Egypt	*O.crenata*	Al-Menoufi, 1986).
Fusarium arthrosporioides	Israel	*O.ramosa, O.aegyptiaca and O.cernua*	Amsellem *et al.* (2001),
Fusarium oxysporum	Italy	*O.ramosa*	Bruno et al , 2005
Fusarium lateritium	Bulgaria	*O. ramosa* and *O. mutelii*	Bozoukov and Kouzmanova (1994)
Botrytis cinerea	U.S.A	*O. fasciculata*	(Shaw, 1973; Farr *et al*., 1989
Thielaviopsis basicola	Ukraine	*O.ramosa*	Popova, 1929
Colletotrichum lagenarium	Eastern Europe	*O. aegyptiaca*	Stankevich, 1971; Prokudina, 1973
Sclerotium rolfsii	India	*O. cernua*	(Raju *et al*., 1995
Ulocladium atrum	Syria, Morocco, France	*O.crenata , O.minor*	Linke et al.,1992
Alternaria sp	Syria, Morocco, France	*O.crenata,* *O.minor*	Linke et al.,1992
Fusarium spp	Hungary	*Orobanche spp*	Beres et al 2000

1.9 FUSARIUM IN OROBANCHE CONTROL

Two very promising species of Fusarium: *Fusarium arthrosporioides* and *Fusarium oxysporum* attack *O. aegyptiaca, O. ramosa* and *O. cernua* (Amsellem *et al*., 2001*;* Boari & Vurro, 2004). These species can be formulated as mycelia, reducing the dew period and the expense of spore production (Amsellem *et al.,* 1999).
It is also possible to enhance their activity two fold by engineering in genes for overproduction of auxin (Cohen *et al*., 2002), but more than a doubling of virulence is needed.
The fungus *F. nygamai* Burgess & Trimboli has been found to be very promising for use as a biological control agent since it reduces the emergence of parasitic weeds by up to 97% (Abbasher & Sauerborn, 1992). More recently, Ciotola *et al*. (1995) found an isolate of *F. oxysporum* (Schlecht) to be particularly promising for parasitic weed biocontrol, being able to reduce seed germination and attachment of the parasite to the host roots. In addition, laboratory trials have shown that the preconditioning of parasitic weeds with a conidial suspension of four *Fusarium* species (*F. nygamai, F. semitectum* Berk & Rav. var. *majus, F. solani* (Mart.) Appel & Wollenw. and *F. oxysporum*) has a suppressive effect on seed germination, depending on spore concentration, species and nutrient amendment (Abbasher *et al*., 1996). *Fusarium* species as soil-borne fungi possess several advantages which render them suitable for the bioherbicide approach. In the soil they are relatively protected from environmental stress of drought and heat, frequently occurring in the area of distribution of Orobanche. The saprophytic nature of *Fusarium* spp. allows them to be cultured in liquid as well as solid media; and particularly the *formae speciales* of *F. oxysporum* are highly host-specific. Because most of the damage to host crops occurs while the parasitic weed is still underground, use of soil-borne bio control agents such as *Fusarium* spp. can add to improving crop yield by destroying the parasite at its early developmental stages. Presently about 17 *Fusarium* species are reported to be associated with Orobanche spp. Six of these *Fusarium* species (*F. arthrosporioides*, *F. nygamai*, *F. oxysporum*, *F. oxysporum* f.sp. *orthoceras*, *F. semitectum* var. *majus*, *F. solani*) have shown significant disease development in selected species of *Orobanche* when tested under controlled and/or field conditions(Kott, 1969; Nalepina, 1971; Timchenko and Dovgal, 1972; Panchenko, 1974; Hódosy, 1981; Al-Menoufi, 1986; Bedi and Donchev, 1991; Thomas et al., 1998 ;Thomas et al 1999; Amsellem et al., 2001; Müller-Stöver et al., 2002; Shabana et al., 2003) .All growth stages from un-germinated seeds to inflorescences can be attacked (Sauerborn et al., 1996; Thomas et al., 1999). Consequently, seeds of *Orobanche* may be infected by the application of *Fusarium* spp. even if no host plant for the parasite is present in the field. That means that the parasite seed bank could be lowered every season. A successful application of this strategy would add advantages to the bioherbicide approach. *Fusarium oxysporum* var. *orthoceras* gave some control of *O.aegyptiaca* (Panchenko 1981) and *O. cernua* (Bedi and Donchev 1991). *F. oxysporum*, along with a complex of fungi, controlled *O. ramosa* in tomato to some extent (Hodosy 1981). Amsellem et al. (2001) and Cohen et al. (2002) observed reduction in *O. aegyptiaca* attached to tomato in greenhouse experiments using host-specific strains of *F. oxysporum* and *F. arthrosporioides*. Charudattan (2001) reported that a novel approach to increase the

level of control of Orobanche is to use a multiple-pathogen strategy. In this strategy two or more pathogens are combined and applied before or after parasite emergence. The feasibility of this approach has been demonstrated in the control of *O. cumana* in sunflower (Dor et al., 2003). A bioherbicide system was based on two fungal pathogens *F. oxysporum* f. sp. *orthoceras* and *F. solani* which had been isolated from *O. cumana* on sunflower and *O. aegyptiaca* on tomato, respectively. In pot trials, the pathogens gave a low control level when used individually but when applied as a mixture, both fungi caused a significant reduction of the number of emerged *O. cumana* and of the parasite's dry weight .The inoculum density of each fungus when applied alone was 10^5 colony forming units (cfu) ml^{-1}. The same inoculum level of each fungus was used with the mixture, thus resulting in an inoculum density of 2×10^5 cfu ml^{-1}.

1.10 FUSARIUM PHYTOTOXINS

Microbial toxins are metabolites produced by plant pathogens (fungi, bacteria), and play a role in host pathogen interactions and in disease expression. Abbas and Duke, 1995 reported that plant pathogens are good sources of potent phytotoxins as they usually kill tissues before they consume them. They are in general low molecular weight substances produced by some pathogens which are capable of reproducing symptoms similar to those found in natural infections in plants (Bilgram and Dube, 1976). Some metabolites of pathogens with high molecular weight polysaccharides secreted by wilt inducing bacteria obstruct the flow of fluid in the xylem vessels and may result in death of plant are not toxins.

Berestetskiy (2008) reported that these metabolites are capable of deranging the vital activity of plant cells or causing their death at concentrations below 10Mm. Chrysayi-Tokousbalides et al.(2007) also reported that pyrenophorin (phytotoxin) is toxic on *V. fischeri* at a concentration of 3.57 M 10–5 .According to Scheffer (1983) phytotoxins are products of microbial pathogens, which should cause an obvious damage to plant tissue and must be known with some confidence to be involved in disease development.

The term phytotoxin could therefore be used for substances produced by fungi and bacteria, although sometimes it could be referred to toxic substances produced by higher plants. In the context of this research, phytotoxin is referred to a substance produced by (fungal) micro-organisms which is toxic to plants. Only those toxic substances that are toxic to cells of their host, i.e. phytotoxins, can be considered from the viewpoint of their role in plant pathogenesis. If a phytotoxin is produced at an early stage of plant disease development and causes some or even all of the symptoms of the disease, then it has a role in pathogenesis. The interference of phytotoxins with the functioning of plants consist simply of killing plant cells for the purpose of nutrient uptake or a more subtle redirecting of the cellular machinery (Keen, 1986) often it is achieved through the production of phytotoxins with varying degrees of specificity toward different plants. Some toxins are host selective where as others are active in a wide range of plant species. (Walton, 1996). Evidente and Motta 2001 also reported that, phytotoxins are bioactive compounds with different chemical structures, mechanism of action, host specificities and biological/ecological impacts.

Several characteristics have been used for the classification of toxins that affect plants. Such features include their chemistry. Based on this, some phytotoxins are regarded as low

molecular weight peptides, others have terpenoid structures and still others contain carbohydrates (Amusa, 1991). However, few other structures are known for toxins that play an unquestionable role in plant disease (Scheffer and Briggs, 1981). Another form of classification is based on the producing organism (fungi, bacteria). This is, however, of no predictive value since more than one type of phytotoxins can be produced by one organism. Phytotoxin classification has also been based on biological activities such as enzyme inhibitors, anti metabolites, membrane-affecting compounds (Scheffer and Briggs, 1981). The phytotoxic activity may also reside in the structurally simple secondary metabolites of fungi, such as organic acids (aliphatic, aromatic, etc.) and their derivatives (e.g., oxalic, β-nitropropionic, citric, and cinnamic acids). These substances are produced both by typical phytopathogens (e.g., *Septoria* and *Sclerotinia* spp.) and by soil fungi (Reino et al., 2004). Many fungal phytotoxins are not specific to one weed specie and may be considered potential natural herbicides in native forms or as derivatives and analogs. Either their direct application or their application with derivatives or/and analogs in combination with the pathogen may increase their herbicidal activity, so improving the efficacy in terms of pathogenesis, virulence and herbicidal selectivity (Duke et al.,2000;Bottiglieri et al., 2000).However, the widely accepted classification is that based on toxic selectivity to plant genotypes (host selective or non-host selective) (Rudolph, 1976; Scheffer, 1976) and on the general role in disease development (Wheeler and Luke, 1963; Scheffer and Pringle, 1967).

The ability of a pathogen to infect and invade a susceptible host may be facilitated by the production of toxins that induce cell death in the proximity of the invading organism (Baker et al., 1997; Dangl and Jones, 2001). These toxins were also reported to play important roles in inhibiting the physiological processes in cells surrounding the point of infection, enabling the spread of the disease (Feys and Parker, 2000; Staskawicz et al., 2001). Aliferis and Chrysayi-Tokousbalides (2006) reported that phytotoxic fungal metabolites could cause bleaching of leaf sections accompanied by increased electrolyte leakage, elevated superoxide dismutase activity, severe lipid peroxidation, rapid loss of photosynthetic pigments, and a decline in total protein.(Gaumann (1950) has earlier suggested that some pathogens would be unsuccessful if the toxin did not kill the cells in advance of the fungus and permit it to establish itself continually on dead or dying cells and produce more toxins. While Baker et al. (1997) reported that the virulence of an organism is sometimes enhanced by its ability to produce phytotoxins that kill cells in the tissue surrounding the point of infection.

Phytotoxins often act as the initiation factor for successful pathogenesis. Spores of some fungal pathogen have been associated with phytotoxin production, which probably kill cells of susceptible host paving way for the penetration of the germ tube. All known host specific toxins can be detected from the spore germinating fluids of each virulent pathogen but not from those of the avirulent ones (Nishimura and Kohomoto, 1983; Nutsugah et al., 1994; Otani et al., 1998; Quayyum et al., 2003).

Most of the phytotoxic metabolites act by modifying the metabolism of the host plants, while some are toxic to the plant tissues once accumulated in the plant tissues. A phytotoxin secreted by *Psuedomonas syringae* pv. *tabaci*, the pathogen inducing wild fire disease of tobacco, drastically modifies the amino acid metabolism of the plant with the eventual accumulation of ammonia in tobacco leaves, which causes extensive blighting. Interestingly, the pathogens that synthesize the phytotoxin remain unaffected by the toxin (Balasubramanian, 2003). The practical significance of the pathologically important toxins is that it can act as reliable surrogate for pathogen that produces them (Yoder, 1980).

Essential conditions for attributing the function of a disease determinant to a toxin are: 1) the demonstration that the toxin occurs in infected plants; and 2) the ability of the toxin to cause at least a portion of the syndrome when placed in healthy plants (Aducci et al.,1997). Phytotoxins of the groups: aflatoxins, trichothecenes, fumonisins, zearalenones, enniatins and beauvericins have been intensively studied over the past years (Desjardins and Hohn, 1997; Jestoi et al., 2004; Reynoso et al., 2004., Strobel *et al*., 1991; Duke & Lydon, 1993) and some Fusarium toxins, such as enniatin and fumonisin, have been evaluated for their herbicidal properties (Abbas *et al*., 1991; Hershenhorn *et al*., 1992).

Fusarium spp are the most frequently studied concerning their production of substances toxic to human, animal and plant cells (Zemankova and Lebeda, 2001; Abbas and Duke 1995).Members of the genus *Fusarium* produce a range of chemically different phytotoxic compounds, such as fusaric acid, fumonisins, beauvericin, enniatin, moniliformin and trichothecenes(Nelson *et al.* 1994). These possess a variety of biological activities and metabolic effects including necrosis, chlorosis, growth inhibition, wilting, inhibition of seed germination and effects on calli (Wakulinski, 1989; Van Asch *et al*., 1992; Desjardins & Hohn, 1997). Some of these compounds have a marked toxic effect on humans and animals and are probably carcinogenic (e.g. moniliformin and fumonisin B_1) (Nelson *et al.* 1993). Because of health concerns and potential risks associated with phytotoxin contamination, the use of bioherbicide candidates that produce carcinogenic phytotoxins cannot be recommended. For example, the use of *F. nygamai,* a potential candidate for the control of *S. hermonthica* (Abbasher and Suerborn, 1992) was rejected because of the production of fumonisin B_1 (Zonno *et al.* 1996). Fortunately, fusaric acid, 10-11-dehydrofusaric acid and their methyl esters, which do not present health risks, are metabolites produced by potential *F. oxysporum* isolates for biological control of *Striga* and *Orobanche* (Savard *et al.* 1997; Thomas, 1998; Amalfitano *et al.* 2002) making these isolates very interesting candidates for the biological control of parasitic weeds. Also a report by Capasso *et al*., 1996 showed that four phytotoxins produced by *F. nygamai* isolated from *S. hermonthica* (fusaric and dehydrofusaric acids and their respective methyl esters) were able to strongly inhibit *S. hermonthica* seed germination .

Once a fungal agent has shown potential for parasitic weed control in laboratory, greenhouse, and field tests, mass production of viable, infective and genetically stable propagules becomes a major concern for the development of a bioherbicide. For laboratory and greenhouse research and even small-scale field trials, production of a sufficient quantity of fungal inoculums may often be easily achieved. However, mass production methods and techniques must be developed for large-scale practical use. Some fungal biocontrol isolates perform best if their inoclum contain chlamydospores, while others cause a pathogenic effect with conidia and/or mycelia. Aimed at the development of economically feasible mass production methods and techniques, recent investigations emphasized the use of agricultural by-products as substrates, including sorghum straw, maize straw, cotton seed cake and wheat-based stillage, to mass produce *F. oxysporium* isolates from *Striga* and *Orobanche*. Hinojo et al., 2006 also reported that rice could be successfully used in growing fusarium species. These substrates are readily available, inexpensive and do not require any further processing prior to their use. Using sorghum straw (Ciotola *et al.* 2000), a mixture of sorghum straw and wheat-based stillage (Müller-Stöver, 2001) and maize straw, plus wheat stillage (Elzein, 2003), abundant chlamydospores of three *F. oxysporum* isolates (M12-4A, FOO and Foxy 2, respectively) were produced in liquid fermentation systems. On the other

hand, mass production of other propagules, e.g. conidia and mycelia and of all promising fungal antagonists of *Striga* or *Orobanche,* easily achieved using artificial culture media such as potato dextrose broth, malt extract agar or agricultural by-products (Abbasher, 1994; Bedi, 1994; Ciotola *et al.* 1995; Kroschel *et al.* 1996a; Thomas, 1998, Amsellem *et al.* 2001, Müller-Stöver, 2001; Elzein, 2003).

1.11. MODE OF ACTION OF PHYTOTOXINS

When pathogens attack plants, the spores penetrate the plant body and proliferate therein. While in the plant's body, they produce toxic metabolites called phytotoxins which inhibit metabolic processes in the plant's system. Toxins do not attack the structural integrity of the tissue but they affect the metabolism in a subtle manner (Buddenhanen and Kilman, 1964). Phytotoxins act directly on protoplast of the cell. Phytotoxins from other fungi have weed control activity such as AAL-toxin, cornexistin and tentoxin. AAL-toxin and its analog in structure can suppress cearmide synthetase and results in sphingol accumulation that makes membrane break. Cornexitin is metabolic inhibitor and action mechanism of this is similar to aminoacetic salt. It inhibits one isoenzyme of asparagines aminotransferases, but when acid from tricarboxylic acid cycle such as aspartic acid and glutamic acid is added the activity of toxin will disappear. Tentoxin has two different action mechanisms under different conditions. One is interrupting the formation of chloroplast by blocking synthesis of coding nucleo-cytoplasmic protein and the other is energy transfer inhibitor of ATPase's coupling factor for controlling photophosphorylation (Duke et al. 1996).

Several phytotoxins are now known, beyond reasonable doubt, to be the determinant factor in pathogenesis and some can even act as reliable surrogates for pathogen that produce them. Amusa (1994) reported that the partially purified metabolites of *Colletotrichum* spp. induced necrotic lesion of varying sizes on leaves and stems of susceptible hosts. While the phytotoxic metabolites of *Colletotrichum graminicola*, *C. truncatum* and *C. lindemutianum* inhibited seed germination in respective host crops.

One of the first physiologically detectable event induced by phytotoxin is an increased loss of electrolytes from susceptible leaves (Kohomoto et al., 1987). Nishimira and Kohomoto (1983) reported that *Alternaria kikuchiana* toxin released during spore germination on leaves caused an almost instantaneous increase in electrolyte loss from susceptible but not resistant tissue. Different phytotoxin are known to have different modes of action. Kimura et al. (1973) reported that a phytotoxin produced by *Colletotrichum lagenarium* was found to function as an anti-auxin. Sensitivity of sugarcane clones relating to electrolyte leakage caused by *Helminthosporium sacchari* toxin has been reported (Scheffer and Livingston, 1980).

The many mechanisms by which phytotoxins produce toxicity are varied, and their relative importance in producing illness is not fully understood (Coulombe, 1993).They include the following:

Inhibition of protein synthesis, thought to be the most important effect (Ueno, 1983; Ueno et al., 1984; Tutelyan and Kravchenko, 1981)

Inhibition of DNA synthesis (Thompson and Wannemacher, 1984), which might contribute to their radiomimetic properties.

Impairment of ribosome function (NAS, 1983; Coulombe, 1993; Tutelyan and Kravchenko, 1981)

Inhibition of mitochondrial protein synthesis (Pace et al ., 1985)

Induction of reparable single strand breaks in DNA

Immunosuppression , allowing secondary and opportunistic bacterial infections and possibly delayed hypersensitivity(Ueno,1983;Yarom et al .,1984;Jagadeesan et al .,1982)

Phytotoxins may act as an inducer of accessibility for fungal penetration, and/or a selective virulence factor (Kohmoto and Otani, 1991).

Phytotoxins react readily with thiol groups and, at low concentrations, inhibit thiol enzymes (e.g Creatin kinase) (Ueno et al., 1984; Tutelyan and Kravchenko, 1981).They can be incorporated into lipid or protein elements of cell membranes. Tissue culture studies show alteration of membrane function(Coulombe,1993;Pfeifer and Irons, 1985).An example of cell membrane injury is by T-2 toxin which induces cell membrane injury ,apparently via a free radical mechanism(Segal et al .,1983;Coulombe , 1993).

Inhibition of chloroplast development (Vaughn & Duke, 1984).

Toxins also cause rapid cellular leakage and loss of plasma membrane integrity (Abbas et al., 1992 and Tanaka et al., 1993).

As the main mechanism of action of Trichothecene, they inhibit protein biosynthesis by binding to the ribosome and inhibiting peptidyl transferase activity. They also inhibit DNA biosynthesis (Grove, 1996., Grove, 1993).

Crude undiluted culture filtrates of *F. oxysporum* and *F. arthro sporioides* inhibit ceramide synthase by about 50 % (Cohen, 2001).

They suppress elicitation of phenylalanine ammonia-lyase, one of the first defense mechanisms of many plants following pathogen attack (Vurro & Ellis 1997).

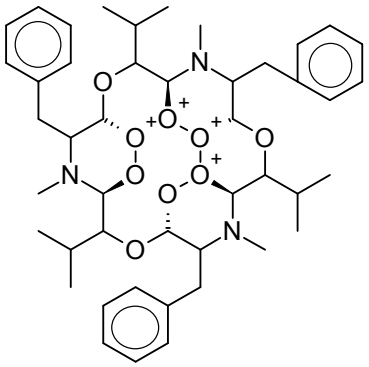
Fungal metabolites could cause bleaching of leaf sections accompanied by increased electrolyte leakage, elevated superoxide dismutase activity, severe lipid peroxidation, rapid loss of photosynthetic pigments, and a decline in total protein (Aliferis and Chrysayi-Tokousbalides 2006)

Some Fusarium Phytotoxins include:

1.Beauvericin.

It is produced by several *Fusarium species* e.g. *Fusarium proliferatum*. It has also been isolated from *Beauveria bassiana.* It is a bioactive cyclodepsipeptide, which contains three D-a-hydroxy-isovaleryl and N-methyl-L-phenylalanyl residues in alternating sequence. It was first reported as a compound produced by entomopathogenic fungi (Hamill et al, 1969;Bernardini et al 1975;Gupta et al ,1991), and subsequently was shown to be produced by phytopathogenic fungi(Logrieco et al , 1993).An investigation of the fusarium genus, showed that various species produced beauvericin, including some strains of *F. oxysporum* (Logrieco et al 1998).Sagakuchi et al (2000) provided data on the phytotoxic effect of beauvericin compared with other toxins (e.g. fusaric acid and fumonisin B1).It causes 20% inhibition of seed germination of Orobanche at a concentration of 100 μM (Vurro et al., 2009)

Structural formular:



The empirical formula of Beauvericin is CH $(CH_3)_2CH_3CH_2C_6H_5$, with isomers having various substitutions :

Compound	R_I	R_2	R_3
Beauvericin	CH $(CH_3)_2$	CH_3	$CH_2C_6H_5$
Beauvericin A	CH (CH_3) CH_2CH_3	CH_3	$CH_2C_6H_5$
Beauvericin D	CH $(CH_3)_2$	H	$CH_2C_6H_5$
Beauvericin E	CH $(CH_3)_2$	H	$CH_2CH(CH_3)_2$
Beauvericin F	CH_2 $CH(CH_3)_2$	CH_3	$CH_2C_6H_5$

The chemical characteristics are shown below:

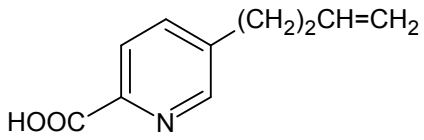
Compound	Melting point	molecular formula	molecular weight
Beauvericin			
Beauvericin A	93-97 ^{o}C	$C_{45}H_{57}N_3O_9$	783 g/mol
Beauvericin D	84-85 ^{o}C	$C_{44}H_{55}N_3O_9$	769 g/mol
Beauvericin E	73-75 ^{o}C	$C_{41}H_{57}N_3O_9$	735 g/mol
Beauvericin F	63-65 ^{o}C	$C_{46}H_{59}N_3O_9$	797 g/mol

References: Takashi et al 2004., Hamill et al 1994

2.Dehydrofusaric acid.

This is produced by *Fusarium nygamai* and it is a plant growth inhibitor for *Striga hermonthica* as reported by Zonno and Vurro 1999. It causes 27% inhibition of seed germination of Orobanche at a concentration of 100 µM (Vurro et al., 2009).
The Structural formula of dehydrofusaric acid and its chemical characteristics are shown below.

Structural formula:



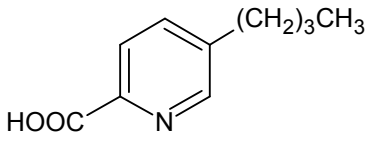
$C_{10}H_{11}NO_2$
Molecular weight 177 g/mol
Melting Point 121 ^{o}C

Ref: Stoll 1954

3. Fusaric acid.

This is produced by most *Fusarium species* e.g. *Fusarium nygamai.*
It weakly inhibits gram-positive and gram-negative bacteria. It causes 27% inhibition of seed germination of Orobanche at a concentration of 100 μM (Vurro et al., 2009). The structural formula and chemical characteristics are shown below.

Structural Formula:



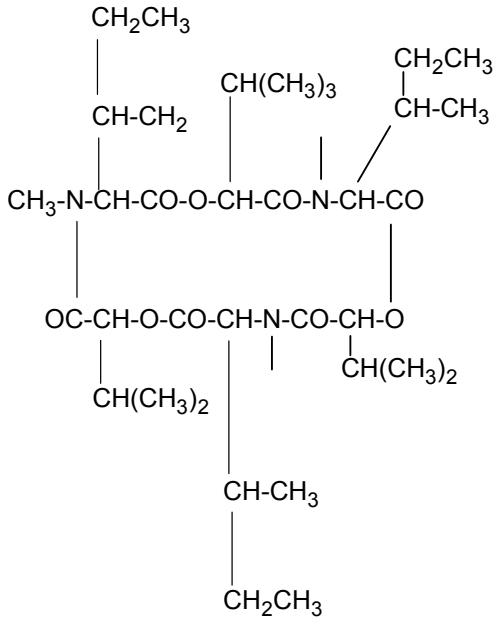
$C_{10}H_{13}NO_2$
Molecular weight 179 g/mol
Melting Point 108-109 ^oC
Ref. Hidaka, 1971

4. Enniatin.

This is also produced by fusarium . It is an antibiotic active against gram-positive, gram-negative and acid-fast bacteria. It inhibits phytopathogenic fungi. The activity of Enniatin A against bacteria and phytopathogenic fungi is stronger than Enniatin B and C. (Gaumann et al 1947., Plattner et al 1948). It causes 8% inhibition of seed germination of Orobanche at a concentration of 100 μM (Vurro et al., 2009). The structural formula and chemical characteristics of the various isomers are shown below.

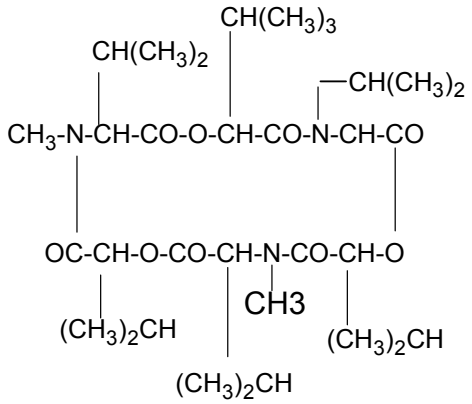
Structural Formula:

Enniatin A



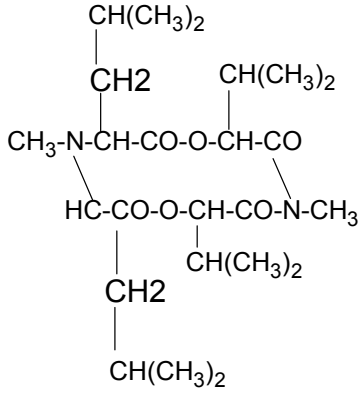
$C_{36}H_{63}N_3O_9$
Molecular weight 681 g/mol
Melting point 122 °C

Enniatin B



$C_{33}H_{57}N_3O_9$
Molecular weight 639 g/mol
Melting point 174-176 °C

Enniatin C



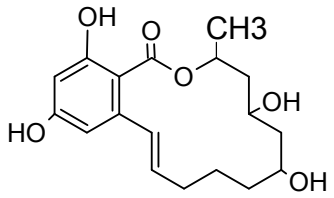
$C_{24}H_{42}N_2O_6$
Molecular weight 454 g/mol
Melting point 123 ^oC

Ref. Plattner et al 1948

5.Zearalenone derivatives.

This is produced by *Fusarium species*. Zearalenone (ZEA) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-beta-resorcyclic-acid-lactone].It is a secondary fungal metabolite that is primarily produced by *Fusarium graminearum* and *Fusarium culmorum* .Also produced by *Fusarium moniliforme*. The only naturally occurring ZEA derivative is *trans*-a-zearalenol [6-(10-hydroxy-6-R-hydroxy-*trans*-1-undecenyl)-beta-resorcyclic-acid-lactone] (a-ZOL). It causes 19% inhibition of seed germination of Orobanche at a concentration of 100 μM (Vurro et al., 2009).The structural formula and chemical characteristics of its derivatives are shown below.

a)6',8'-Dihydroxyzearalene.



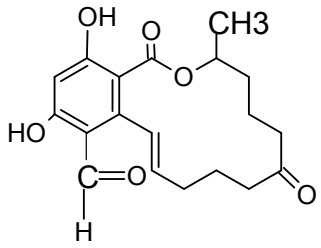
$C_{18}H_{24}O_6$
Molecular weight 336 g/mol

Ref: Steele et al 1976

b) 5-Formylzearalenone

$C_{19}H_{22}O_6$

Molecular weight 346 g/mol



Ref: Von Bolliger and Tamm (1972)

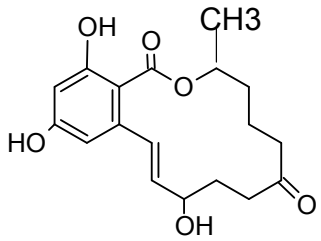
c)3'-Hydroxyzearalenone

$C_{18}H_{22}O_6$

Molecular weight 334 g/mol

Melting point 188-190 ^{o}C

Ref : Pathre et al 1978

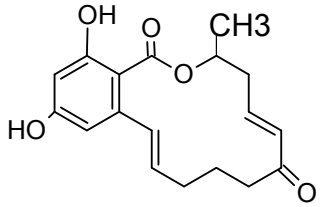


d)7'-Dehydrozearalenone

$C_{18}H_{20}O_5$

Molecular weight 316 g/mol

Melting point 197-200 ^{o}C



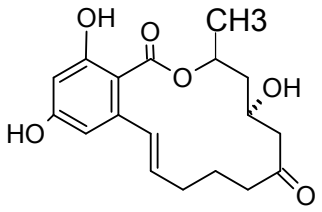
Ref: Von Bolliger and Tamm (1972).

e) 8'-epi-Hydroxyzearalenone

$C_{18}H_{22}O_6$

Molecular weight 334 g/mol

Melting point 172-174 °C



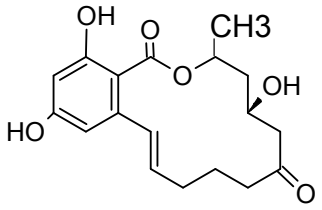
Ref: Von Bolliger and Tamm (1972)

f) 8'-Hydroxyzearalenone

$C_{18}H_{22}O_6$

Molecular weight 334 g/mol

Melting point 210-212 °C

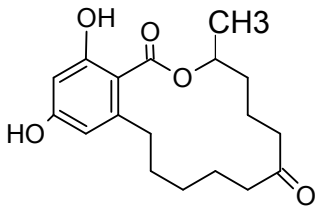


Ref: Von Bolliger and Tamm (1972)

g) Zearalenone.

$C_{18}H_{24}O_5$

Molecular weight 320 g/mol

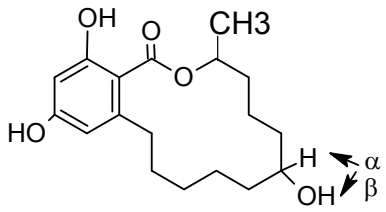


Ref: Richardson et al 1985

h) Zearalanol

$C_{18}H_{26}O_5$

Molecular weight 322 g/mol



Ref : Steele et al 1976. Hagler et al 1979., Jackson et al 1974

6.T-2 toxin derivatives.

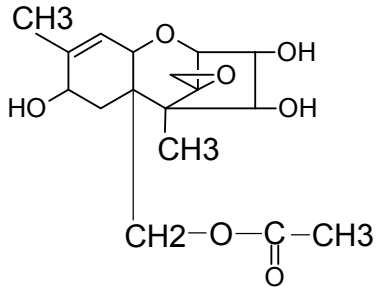
This is produced by *Fusarium acuminatum* (mostly when grown on oat and barley) and other fungi species . It causes 100% inhibition of seed germination of Orobanche at a concentration of 10 μM (Vurro et al., 2009).The structural formula and chemical characteristics of its derivatives are shown below. They Inhibit protein synthesis (Ishii and Ueno 1981). They are less cytotoxic than T-2 toxin by *In vitro* studies. (Corley et al., 1986).

Structural Formula

a) 15-Acetoxy-T-2-Tetraol

$C_{17}H_{24}O_7$

Molecular weight 340 g/mol

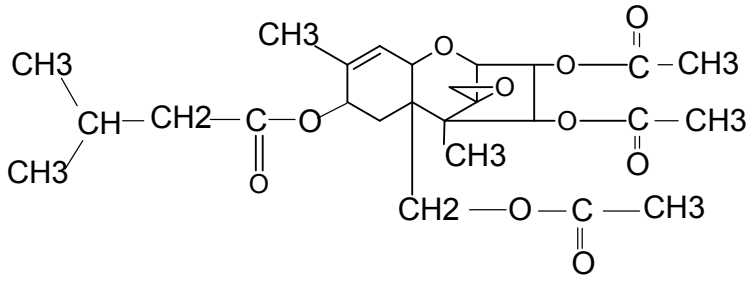


Ref: Cole et al 1981

b) Acetyl T-2 Toxin

$C_{26}H_{36}O_{10}$

Molecular weight 508 g/mol

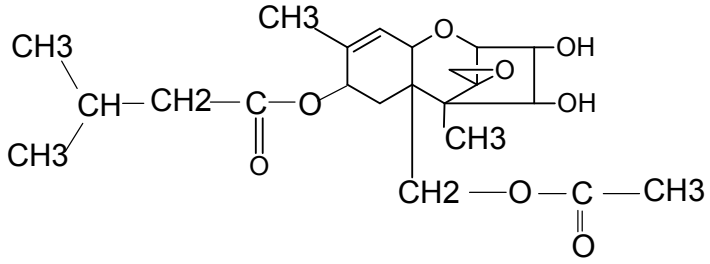


Ref: Kotsonis et al 1975

c) HT-2 Toxin

$C_{21}H_{32}O_8$

Molecular Weight 412 g/mol

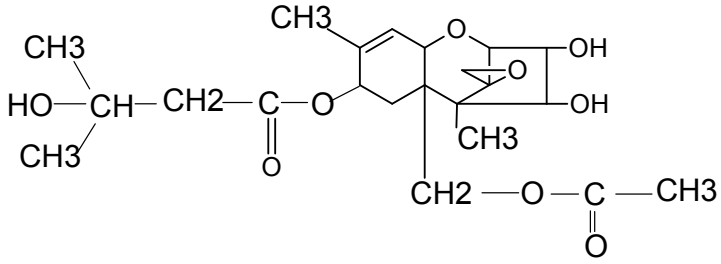


Ref: Ueno 1973

d)3'-Hydroxy-HT-2 toxin

$C_{22}H_{32}O_9$

Molecular weight 440 g/mol

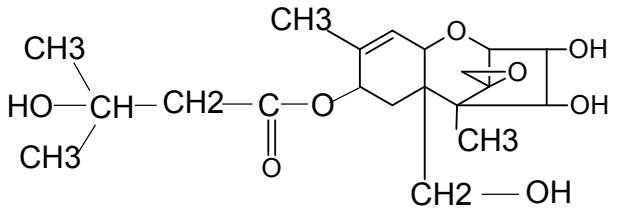


Ref : Cole et al 1981

e) 3'-Hydroxy-T-2-Triol

$C_{20}H_{30}O_8$

Molecular weight 398 g/mol



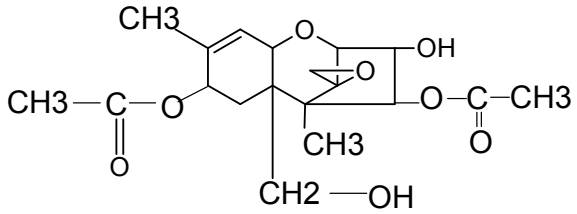
Ref: Cole et al 1981

f) NT-1 Toxin

$C_{19}H_{26}O_8$

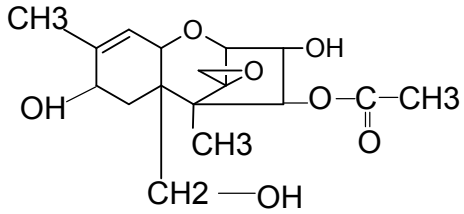
Molecular weight 382 g/mol

Melting point 172.5-173.5 ^oC



Ref : Ishii and Ueno 1981

g) NT-2 Toxin



$C_{17}H_{24}O_7$

Molecular weight 340 g/mol

Melting point 172-173 ^oC.

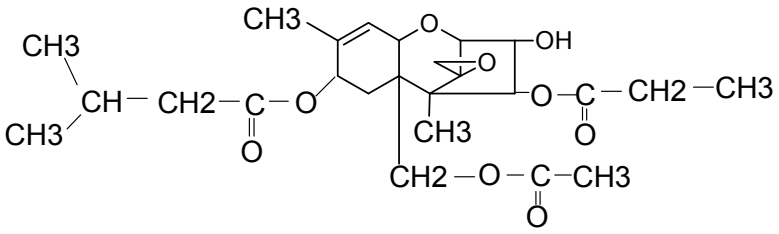
Ref: Ishii and Ueno 1981

h) 4- Propanoyl HT-2

$C_{25}H_{36}O_9$

Molecular weight 470 g/mol

Melting point 141-142 ^oC

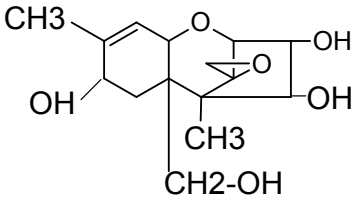


Ref: Corley et al 1986

i) T-2 Tetraol

$C_{15}H_{22}O_6$

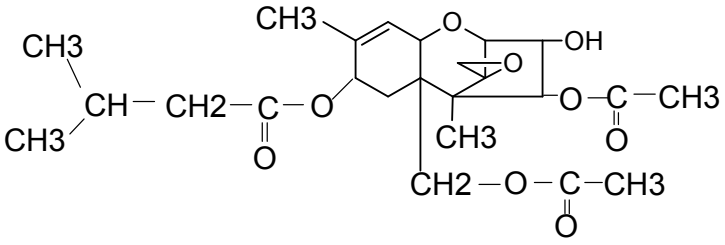
Molecular weight 298 g/mol



Ref : Mirocha and Pathre 1973

j) T-2 Toxin

$C_{24}H_{34}O_9$



Molecular weight 466 g/mol

Melting point 151-152 ^{O}C

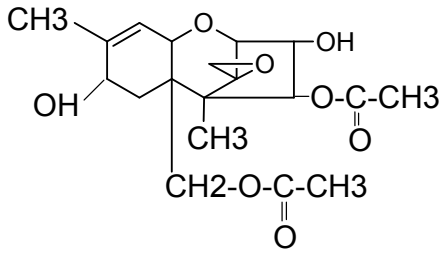
Ref: Ueno 1973

k) Neosolaniol

$C_{19}H_{26}O_8$

Molecular weight 382 g/mol

Melting point 171-172 ^{o}C

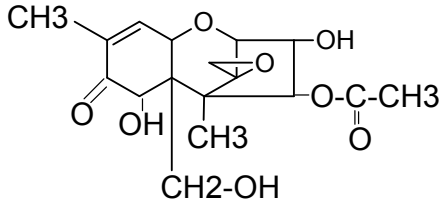


Ref : Ueno 1973

7.Nivalenol derivatives.

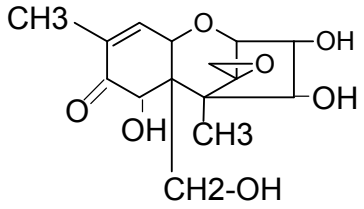
These are produced by *Fusarium species* e.g. *Fusarium nivale.* It causes 100% inhibition of seed germination of Orobanche at a concentration of 10 μM (Vurro et al., 2009).
The Structural Formula of Nivalenol (and its derivatives) and their chemical characteristics are shown below.

a) Fusarenone-x , Nivalenol-4-acetate



$C_{17}H_{22}O_8$
Molecular weight 354 g/mol
Melting point 91-92 ^oC
Ref : Ueno 1973

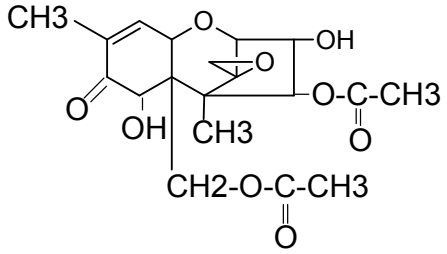
b) Nivalenol



$C_{15}H_{20}O_7$
Molecular weight 312 g/mol
Melting point 222-223 ^{o}C

Ref: Ueno 1973

C) Diacetylnivalenol Nivalenol diacetate



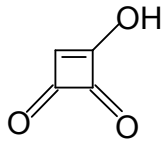
$C_{19}H_{24}O_9$
Molecular weight 396 g/mol
Melting point 135-136 ^{o}C

Ref: Ueno 1973

8.Moniliformin.

Moniliformin is the trivial name for 3-hydroxy-3-cyclobutene-1,2-dione, an anion usually occurring with potassium or sodium as a counter ion. (K/Na-salt of Semisquaric Acid), CAS [52591-22-7]. K or Na C4HO3, MW = 136.11 g/mol. It is mainly produced by *Fusarium* species (*F. moniliforme* , *F. fujikuroi, F. proliferatum, F. avenaceum, F. subglutinans*). MON is phytotoxic to *Lemna minor* (Vesonder et al., 1992). It causes 37% inhibition of seed germination of Orobanche at a concentration of 100 μM (Vurro et al., 2009). The Structural Formula and chemical characteristics are shown below:

$C_4H_2O_3$
Molecular weight 98 g/mol
MP 158 ^oC



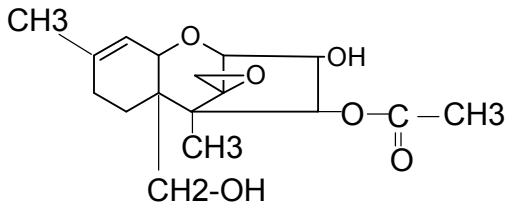
Ref: Cole et al 1973

9. Scirpenol derivatives.

This is produced *by Fusarium specie* e.g. *Fusarium scirpi, F. sambucinum.* It is toxic to animals, specifically birds causing loss of appetite, loss of body weight and oral lesions of broiler breeders (Brake et.al. 2000). It Inhibits cress root growth (Pathre et al 1976).

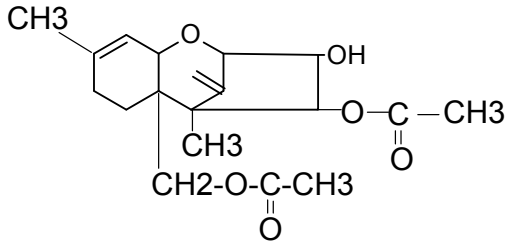
The structural Formula and chemical characteristics of its derivatives are shown below:

a)4-Acetoxyscirpendiol
$C_{17}H_{24}O_6$
Molecular weight 324 g/mol
Melting point 100-110 ^oC



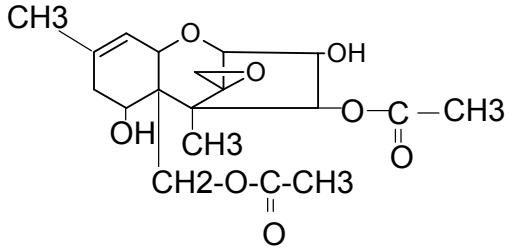
Ref: Ishii et al 1978

b) Deepoxy-Diacetoxyscirpenol
$C_{19}H_{26}O_6$
Molecular weight 350 g/mol



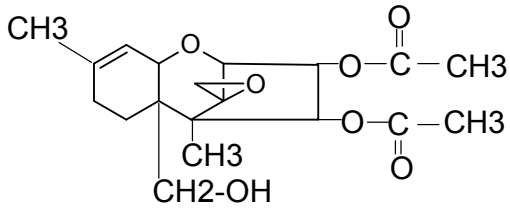
Ref : Chatterjee et al 1986

C) 4,15 – Diacetoxyscirpendiol
$C_{19}H_{26}O_8$
Molecular weight 382 g/mol
Melting point 201-203 ^oC



Ref : Ishii 1975

d) 3,4-Diacetoxyscirpenol
$C_{19}H_{26}O_7$
Molecular weight 366 g/mol

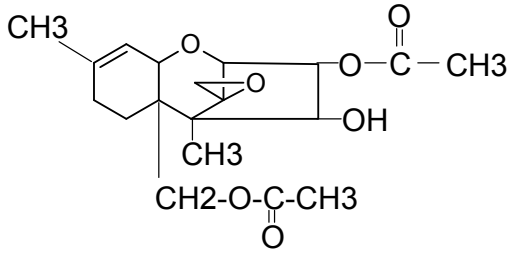


Ref : Chatterjee et al 1986

e) 3,15-Diacetoxyscirpenol

$C_{19}H_{26}O_7$

Molecular weight 366 g/mol

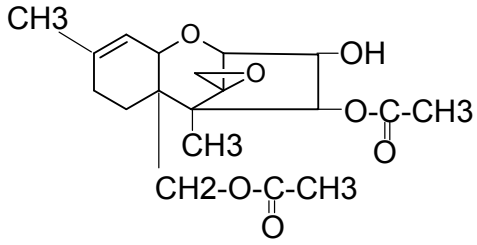


Chatterjee et al 1986

f) 4,15-Diacetoxyscirpenol

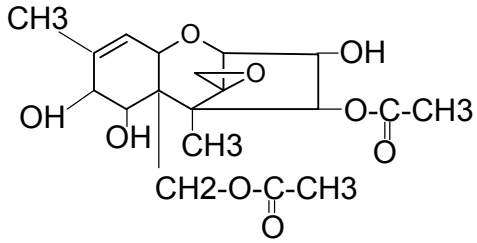
$C_{19}H_{26}O_7$

Molecular weight 366 g/mol



Ref : Ueno 1973

g) 4,15-Diacetoxyscirpentriol



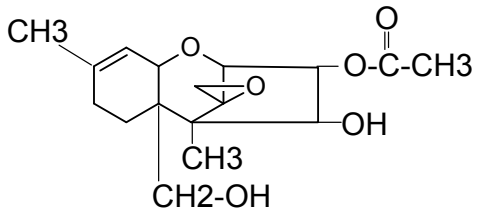
$C_{19}H_{26}O_9$

Molecular weight 398 g/mol

Melting point 167-169 ^{o}C

Ref : Ishii 1975

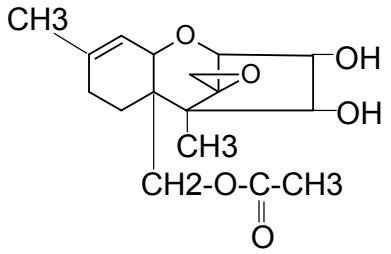
h) 3-Monoacetoxyscirpenol



$C_{17}H_{24}O_6$
Molecular weight 324g/mol

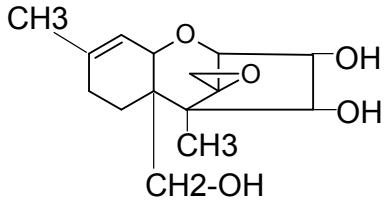
Mirocha et al 1986

i) 15- Monoacetoxyscirpenol
$C_{17}H_{24}O_6$
Molecular weight 324 g/mol
Melting point 172-173 ^{o}C



Ref : Pathre et al 1976

j) Scirpentriol



$C_{15}H_{22}O_5$
Molecular weight 282 g/mol
Melting point. 193 ^{o}C

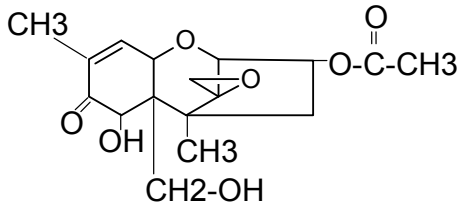
Ref : Pathre et al 1976

10.Deoxynivalenol derivatives.

These are produced by *Fusarium spp.* They are phytotoxic to some cereal species e.g. *Zea mays* (Michel McLean 1995). They inhibit root and shoot growth and also inhibit fresh mass accumulation. They cause 100% inhibition of seed germination of Orobanche at a concentration of 10 μM (Vurro et al., 2009).
The Structural Formula and chemical characteristics of its derivatives are shown below:

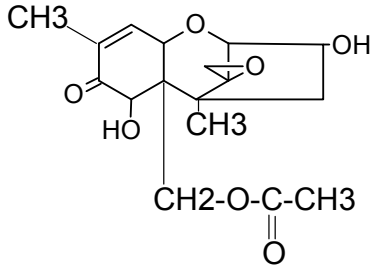
a*)* 3-Acetyldeoxynivalenol

$C_{17}H_{22}O_7$
Molecular weight 338 g/mol
Melting point 185.5-186 ^oC



Ref : Blight and Grove 1974

b) 15-Acetoxydeoxynivalenol

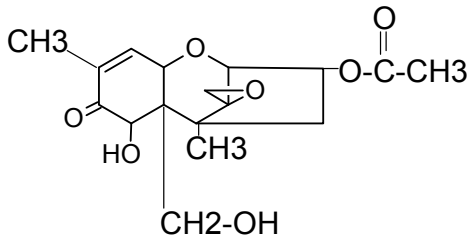


$C_{17}H_{22}O_7$
Molecular weight 338 g/mol
Melting point. 142-145 ^oC
Ref : Miller et al 1983

c) 3-Acetyl-7-deoxy-don

$C_{17}H_{22}O_6$

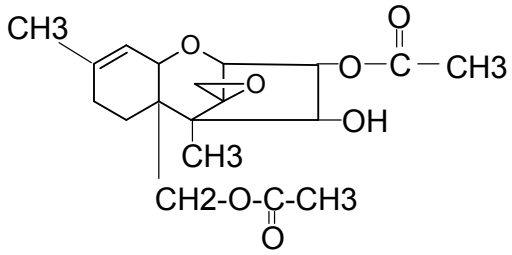
Molecular weight 322



Melting point 202-202.5 ^{o}C

Ref : Greenhalgh et al 1984

d) 3-15 –Diacetyldeoxynivalenol



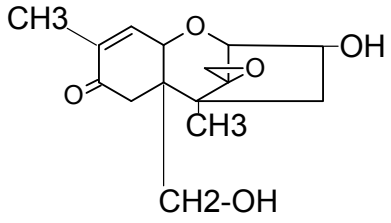
$C_{19}H_{14}O_8$

Molecular weight 380 g/mol

Melting point 117-118.5 ^{o}C

Ref : Yoshizawa and Morooka 1973

e) 4,7-Dideoxynivalenol

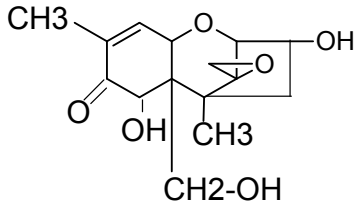


$C_{15}H_{20}O_5$

Molecular weight 280 g/mol

Ref : Bennett et al 1981

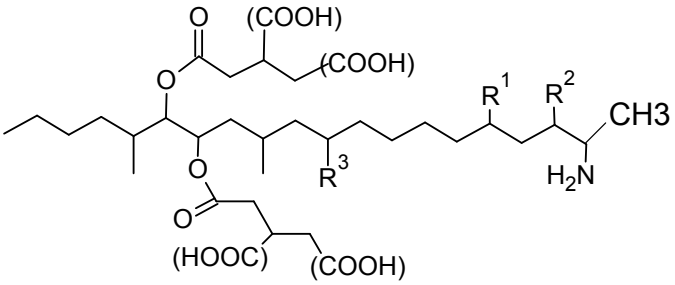
f)) Deoxynivalenol



$C_{15}H_{20}O_6$
Molecular weight 296 g/mol
Melting point 151-153 ^{o}C and 176-178 ^{o}C (bound water).
(Yoshizawa and Morooka, 1974., Vesonder et al 1976)

11.Fumonisin.

This is produced by *Fusarium species* e.g. *Fusarium verticillioides(syn. F. moniliforme), Fusarium proliferatum.*
Chemical structures of fumonisins B_1-B_4 (FB_1 – FB_4) are given in figure 1. Fumonisin B_1 is the diester of propane-1,2,3-tricarboxylicacid and 2S-amino-12S, 16R-dimethyl-3S,5R,10R,14S,15R-pentahydroxyeicosane in which the C-14 and C-15 hydroxyl groups are esterified with the terminal carboxyl group of propane-1,2,3-tricarboxylic acid. FB_2 – FB_4 show different hydroxylation patterns. They are phytotoxic to some cereal species e.g. *Zea mays* (Michel McLean 1995). They inhibit root and shoot growth and also inhibit fresh mass accumulation.
The Structural Formula and chemical characteristics of its isomers are shown below:



Fumonisin	B_1:	R1=OH;	R2=OH;	R3=OH;
Fumonisin	B_2:	R1=H;	R2=OH;	R3=OH;
Fumonisin	B_3:	R1=OH;	R2=OH;	R3=H;
Fumonisin	B_4:	R1 = H;	R2= OH;	R3=H;

1.12 TESTING CHEMICALS ON OROBANCHE

Because of the complex nature of Orobanche, several methods have been proposed for its control and consequently different ways for the testing of potential control chemicals have been suggested. Among the different methods for testing chemicals are:

1.12. a. Seed germination test.
Since *Orobanche spp* produce thousands of tiny seeds that remain in the soil for many years, some eventually exhibit dormancy or die during this period. The seeds of *Orobanche spp*. have special germination requirements, including preincubation (conditioning) in a warm wet environment for several days prior to the exposure to exogenous germination stimulants that are normally released by their hosts (Foy et al., 1989; Parker & Riches, 1993; Joel et al., 1995). Conditioning duration, temperature and water potential all affect the germination response and seed viability of *Orobanche crenata Forsk.* (Van Hezewijk et al., 1993; Kebreab & Murdoch, 1999a, 2000; Mauromicale et al., 2000; Song et al., 2005). To ascertain that *Orobanche spp* seeds are viable, a germination test is necessary. The germination test now makes way for testing chemicals on these seeds to determine if they have toxic or inhibitory effects on viable *Orobanche spp* seeds.

1.12.b. Leaf puncture test.

Leaf puncture bioassay has been reported by Hershenhorn et al 2004 as a test to evaluate the bioactivity of *Fusarium sp* on Orobanche spp. In essence, this test examines the toxicity effect of chemicals on the leaves of *Orobanche spp*. When the leaves of Orobanche spp are punctured and exposed to chemicals, it could be determined if the chemical has a systemic or contact effect on the Orobanche spp.

1.12.c. *In vitro* test.

In vitro test of chemicals on *Orobanche spp* is highly desirable by some researchers because it provides an opportunity to test the chemicals on tissues that have the same characteristics in terms of morphology and age. Calluses of Orobanche spp grown under laboratory conditions thus provides the necessary material needed for *in vitro* test of chemicals on Orobanche. For In vitro tests2,4 D at a concentration of 2.0mg/l has been reported to induce callus growth and cause vigorous growth of calluses (Syeda & Akbar 2000). Also Madhavi et al., 1992 reported that callus culturing with fruits before reaching climacteric with MS medium supplemented with 0.01mg/l of IAA and 2,4 D resulted in a high relative growth of 8.97 shoots for the callus. Since the localization and orientation of vascular tissues is determined by IAA flow (Berleth and Sachs 2001; Reinhardt et al., 2003; Scarpella et al., 2006) it could be supposed that IAA flow has a role in the infection process

of Orobanche. The possible importance of IAA in callus formation by *O. ramosa* was shown by Batchvarova et al (1999). Zhou et al (2004) have also reported that in calli of orobanche the formation of root like protrusions are induced by added IAA.

1.12.d. field test.

Because of the difficulties in producing large quantities of bioherbicides, most of these bioherbicides are tested through germination, leaf puncture, seedling and in vitro experiments. Synthetic herbicides such as imazapic, glyphosate, imazaquin, imazethapyr, and EPTC (ethyl dipropylthiocarbamate) have been mostly tested in field experiments because they are available in large quantities. However, they are also tested in germination test, leaf puncture test, seedling test and in vitro test to serve as standards or control tests.Kleifeld et al 1998 have also used sulfonylurea herbicides in controlling Orobanche in tomato fields. *O.cumana* has been controlled with pronamide in recent years (Diaz-Sanchez et al. 2003). In addition, pre-emergence treatment with herbicides belonging to the imidazolinone, sulfonylurea, and substituted amide families and post-emergence application of glyphosate or imazapyr at a very low rate, are effective treatments to control Orobanche in sunflower. However, the efficacy of those treatments depends on the crop growth stage, soil type, and environmental conditions (Garcias-Torres et al. 1994; Castejon-Munoz et al. 1990; and Garcia-Torres et al. 1995)

1.13 IDENTIFYING FUSARIUM METABOLITES

Isolation and identification of active components may include a multitude of different extractions, chromatographic fractionations, chemical modifications, and physical processes that often lead to re-identification of compounds that either are already known or have well-characterized mechanism of action (Ayer *et al.*, 1989). Dereplication processes involving the integration of bioassays, analytical instrumentation, and informatics are helpful in reducing duplication (Figure 5).
Thin layer chromatography (TLC) method has been used to detect secondary metabolites from *Fusarium* isolates (Thrane 1986, (Kačergius & Mačkinaitė, 2005; Kačergius et al. 2005)**.** Other methods for metabolite analysis include complicated extraction and/or analytical methods such as high performance liquid chromatography (e.g. Burmeister *et al.* 1985).
Metabolites could also be identified using the solid-phase extraction (SPE) and liquid chromatography technique of Gessner and Schmitt (1996).Fungal metabolites have been analyzed by reversed-phase HPLC, Gas chromatography/mass spectrometry(GC/MS) and NMR spectroscopy by Sutherland et al.1999. Miersch et al 1999 reported that Culture filtrate of *Fusarium oxysporum* f sp *matthiolae* was separated by chromatographic methods using DEAE-Sephadex A25, Lichrolut RP-18 and Eurospher 100-C18 followed by HPLC and GC-MS identification.

The development of new interfaces between high performance liquid chromatography (HPLC) and mass spectrometry (MS) (*i.e.*, electrospray ionization (ESI), thermospray (TSP), and atmospheric pressure chemical ionization (APCI), and between HPLC and nuclear magnetic resonance (NMR) have introduced new tools that simplify the identification of natural compounds. LC-MS can be used in conjunction with LC-NMR to

rapidly elucidate the structure of chemicals. Clearly, the use of the combined systems such as LC-MS-NMR is a more efficient method for complete structural elucidation of individual components in mixtures than more traditional methods. The direct identification of antibacterial sesquiterpene lactones from a partially purified extract of ironweed (*Vernonia fastigiata*), an Asteraceae, illustrates the efficiency of using LC-MS in conjunction with LC-NMR (Vogler *et al.*, 1998). Developments are also taking place in coupling NMR with supercritical fluid extraction, capillary electrophoresis, and centrifugal partition chromatography as alternate methods to the use of LC. Modern instrumentation reduces the need for and reliance on extensive databases of bioassay profiles since mixtures can be fractionated with fractions split for simultaneous bioassay and identification (*e.g.*, Cui *et al.*, 1998). Using such a method, fractions with known compounds and known activities are eliminated before bioassay.

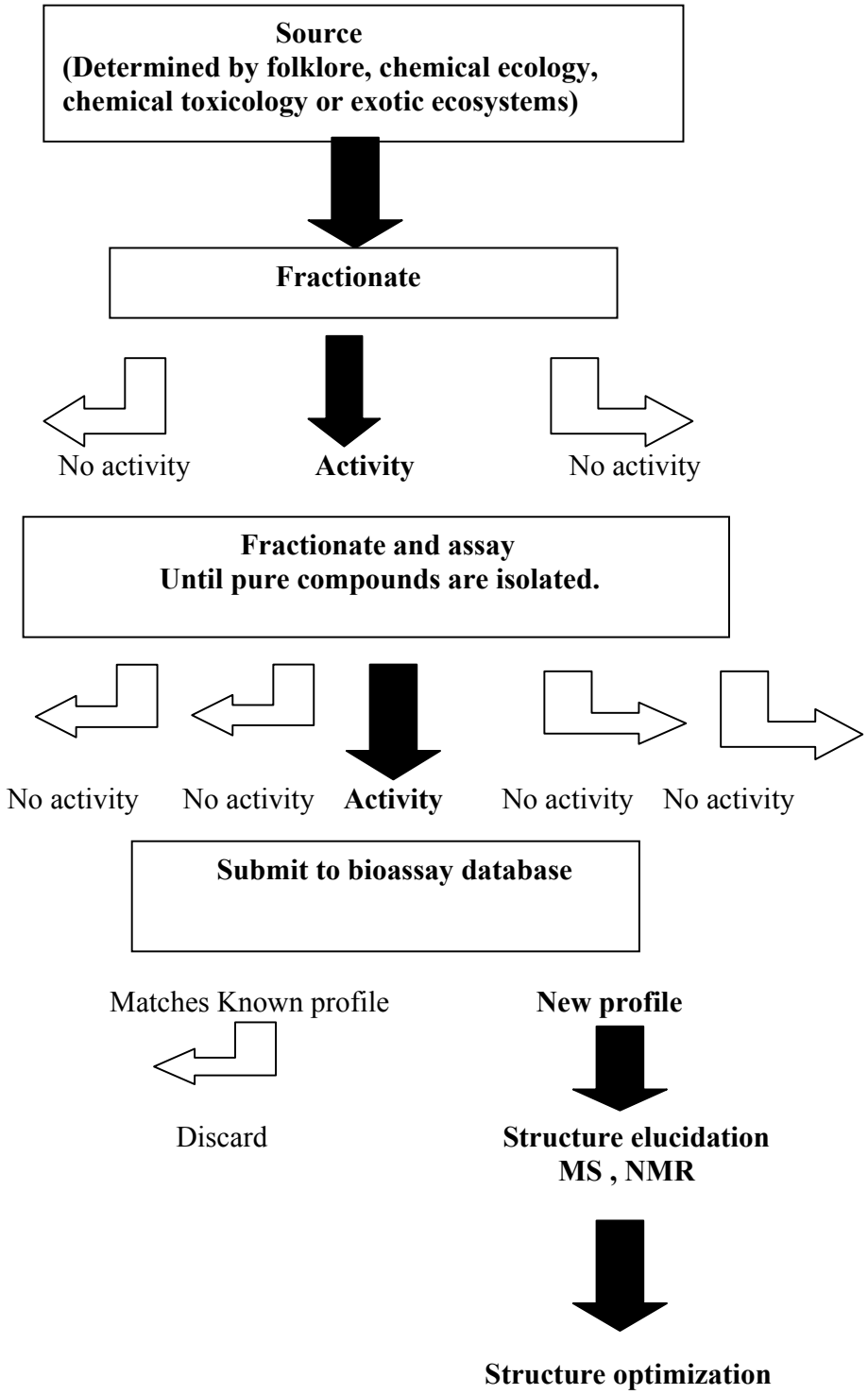


Figure 9. Schematic for bioassay-directed strategy for discovery of herbicides from natural sources.

Adapted from Dayan et al. 1999

1.14 HERBICIDES OF NATURAL ORIGIN

Microbial natural products, especially those produced by plant pathogens are good sources of potential natural herbicides. The use of natural products as herbicides or as lead structures for herbicide discovery programs is an alternative that has not been exploited as fully as it has been for insecticides and fungicides. Phytotoxic natural products are in general structurally more complex than synthetic herbicides and would not have been obtained by traditional synthetic approaches that tend to be limited by the cost of the synthesis of the final molecule. Henkel et al. (1999) demonstrated in a comparison between biologically active synthetic and natural molecules that natural products generally have higher molecular weights and more structural complexity than synthetic compounds, and "heavy" atoms such as halogens are seldom present. On the other hand, natural products have a greater proportion of oxygen and nitrogen, than most synthetic compounds. The diversity found in natural products is slowly being exploited to generate new classes of compounds in traditional synthetic programs. Dayan et al. (1999) reported that there is little overlap between the known molecular sites affected by synthetic and natural phytotoxins. Thus, plant-derived secondary compounds may provide a source of environmentally friendly herbicides with novel molecular sites of action. Johnson, 1994 studied *Xanthomonas* as a bioherbicide and screened many *Xanthomonas Campestris pv. poannua* for controlling annual bluegrass (*Poa annua L*) in burmudagrass (*Cynodon dactylon L. Pers*) . Imaizumi and Seiko Yokohama, 1996 , used mixture of *Xanthomonas campestris pv. poannua* (JT-P482) at 103-8 cfu/ml with sulfonylurea herbicides at 0.01–0.1% to control annual bluegrass and applied for a patent in 1996. They found that only this pathogen suspension of 108 cfu/ml would give excellent control. *Xanthomonas campestris pv. poannua* began to grow largely in inner plant (Imaizumi et al., 1998). When the field is 10 times larger, the concentration of suspension is increased to 1010cfu/g weekly, and this can provide 75% downy brome control after one month (Imaizumi and Fujimori, 1999). This bioherbicide, whose main component is *Xanthomonas campestris pv. poannua* is currently in the Japanese market and its effect is seen after 2 hours of spray. This bioherbicide from nature has not shown any harm to environment, human and animal, and would be suitable for use in soybean, golf course, roadside and lawns.

It is now possible to improve efficacy of plant pathogens by recombinant DNA methods. The use of adjuvant in bioherbicide and supplement some components in medium improves the performance. Charudattan and Dinoor (2000) has made an attempt to modify the host range and improve the virulence of *Xanthomonas campestris pv. campestris* by using genes encoding bialaphos production to control weed. Owen and Zdor (2001) reported *Pseudomonas putida* ATH-1RI/9 and *Acidovarax delafieldii* ATH2-2RS/1 with supplement can produce more HCN to control velvetleaf (*Abutilon theophrasti*). Gronwald et al (2002) have studied the effect of *Pseudomonas syringae pv. tagetis* as a biocontrol agent on Canada thistle in growth chamber, and found that foliar application of Pst109 cfu/ml plus Silwet L-77 (0.3%, v/v) on 4–5-week-old Canada thistle reduced shoot dry weight by 52%.

With the development of sustainable agriculture and consciousness of human environmental protection, government and enterprises will pay more attention to the study

and exploitation of microbial pesticide because of their potential benefits for the environment. With further study of weed control mechanism and establishment of many models of screening weed pathogen, microbial herbicide will have a high chance to develop successfully.

At present, the exploitation of chemical pesticide becomes more and more difficult. The chance of screening new pesticides is lower and lower, but their requirements become higher and higher. So screening new pesticides becomes more and more difficult, whilst cost becomes larger and larger. According to Weijing Chen, et al, 2002. Shangcheng Xu, 2002; Yibin Zhan, 1996, in the1950s the ratio of success was one in 800 compared to only 1 in 20,000 now. Moreover, the expense for exploiting one chemical pesticide is nearly USA$100 million over 8 years. On the contrary, microbial pesticides on exploitation cost USA$2 million, which is only 1 per 40 of chemical pesticide. However their development cycle is shortened by one–third of that of chemical herbicides. In 1990 global pesticide sales were USA$23 billion. Of this, herbicide sale was USA$10 billion and microbial pesticide sale was less than $100 million. In 1998 the sale was nearly USA$30 billion. Of this, herbicide was USA$15 billion and microbial pesticide was USA$300 million (Weijing Chen, et al, 2002. Shangcheng Xu, 2002; Yibin Zhan, 1996).

It can be expected that the growth rate of herbicide use will continue to increase, and microbial pesticide will also increase at the rate of 20% every year, because biology and biotechnology are developing quickly. In the meantime, they also give rise to a new way to explore new pesticide based on biosynthesis and molecular modification by gene technology. Therefore, the study and exploitation of microbial herbicide will become a focus area of new pesticide discovery.

Within the herbicide industry, most of the interest in sources of natural products as herbicides or herbicide leads has been in microbial sources (Duke et al., 1996). Hundreds of these compounds have been patented, but only two, bialaphos and phosphinothricin, have been successfully commercialized. Glufosinate (Basta®, Liberty®), the chemically synthesized form of phosphinothricin, acts directly on plants, whereas bialaphos must be metabolically converted into phosphinothricin by plants (Lydon and Duke, 1999). Bialaphos, from *Streptomyces viridochromogenes*, and *S. hydroscopicus* has been produced by industrial fermentation for a limited market in eastern Asia. The use of *Burholderia andropogonis* for controlling the growth of a weed belonging to the order Caryophyllales has been reported by Zhang et al (2000).There has been less interest in plants than in microbes as sources of lead compounds for herbicides, although many plant-derived compounds are highly phytotoxic. Some examples include cinmethylin (Cinch®), a herbicide with limited success, which was apparently derived from plant cineoles (Grayson *et al.*, 1987), and the triketones, a new class of herbicides from Zeneca, that were derived from the plant secondary metabolite leptospermone (Lee *et al.*, 1997).
Natural compounds tend to be rich in oxygen and nitrogen, have few sulfur or phosphate groups, and are structurally more complex, with more chiral centers, sp3–hybridized carbons, and rings than synthetic compounds. Such diversity may be useful to synthetic chemists in developing new classes of herbicides based on the carbon structure from phytotoxic‘lead structures’ found in nature.

The recent resurgence of interest in natural sources of bioactive compounds may, in part, be attributed to improved methods and instrumentation that has greatly reduced the time and effort required in natural products discovery programs. This renewed interest is also associated with several other factors, including the realization that nature has already selected for very specific biological activities, that many natural compounds have yet to be discovered, and that the biological activities of relatively few of the known natural products have been characterized. Moreover, the study of natural products may lead to the discovery of novel target sites, and/or new classes of chemistry that can be developed for weed management.

1.15 OBJECTIVE OF THE WORK

Toxins produced by *Fusarium* spp. are also phytotoxic to several plants (Duke, 1986) and therefore, their bioherbicidal effects have been tested against various weeds and crops (Hoagland, 1990; Abbas and Boyette, 1992). This provided an impetus to investigate firstly whether potential *Fusarium* isolates from infected *Orobanche crenata* plants are pathogenic to *Orobanche crenata* itself and other related species. Secondly, this study aimed at investigating if isolated *Fusarium oxysporum* is able to produce phytotoxic metabolites that have bioherbicidal effects against different developmental stages of *Orobanche sp* and selective effects on various *Orobanche spp* and other plants. To attain the objective of the work, green house and laboratory studies were conducted to determine the pathogenicity and phytotoxicity of *Fusarium oxysporum* on *Orobanche crenata* and related Orobanche species. These investigations are important in understanding how fungal pathogens and their mode of actions could be useful in the production of environmentally safe bioherbicides.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2.1 PLANT MATERIAL.

Orobanche crenata, Orobanche cumana and *Orobanche aegyptiaca* seeds were obtained from fields around Greece and Cyprus. *Orobanche crenata* was collected from mature *Orobanche crenata* plants parasitizing *Vicia faba* in a field at Marathonas village (approximately 40km from Athens). *Orobanche cumana* was collected from mature *Orobanche cumana* plants parasitizing *Helianthus annuus* in the University research farm in Orestiada, Northern Greece. *Orobanche aegyptiaca* was kindly donated by Dr Nikos Vouzounis of Cyprus Agricultural Research Institute after collecting them from fields in Cyprus. These seeds were stored dry in the dark in tightly closed bags under room temperature (25^{o}C) .

These seeds were used for germination tests, tissue culture and for the growing of Orobanche plants in situ on the respective host plants for pathogenicity experiments. The crop plants used for the experiments were *Vicia faba, Vicia sativa* and *Helianthus annuus.* Seeds of these plants were obtained from the Agronomy Laboratory of the Agricultural University of Athens.

2.2 FUNGAL STRAINS.

The *Fusarium oxysporum* strains OK7 and C11 used in the study were originally isolated from infected *Orobanche crenata* plants collected from Marathonas Village, Athens-Greece in 2003.These strains were coded based on the area of collection. In solid media culture of potato dextrose agar (PDA), the different strains had varying appearances. For both strains the aerial mycelium first appears white, and then changed to a variety of colors. C11 changed from purple to red while OK7 changed from rose to orange red.

Figure 10. One week old *Fusarium oxysporum* strains C11 and OK7 grown on Potato Dextrose agar (PDA) at 20^0C. Figure shows difference in morphology.

2.3. CHEMICALS AND REAGENTS.

Except Nijmegen 1 (synthetic analogue of strigol) that was obtained from Dr. B. Zwanenburg of The University of Nijmegen, The Netherlands. All other Chemicals and reagents used for the experiments were obtained from the following companies:

-Aldrich

2, 4-Dichlorophenoxyacetic acid (2, 4-D)
Indole-3-acetic acid (IAA)

-Mallinckrodt

Sulphuric acid (H_2SO_4)

-Merck

Acetic acid (CH_3COOH)
Agar
Boric acid (H_3BO_3)
Calcium chloride ($CaCl_2$)
Calcium nitrate tetra hydrate ($Ca(NO_3)_2.4H_2O$)
Copper sulphate ($CuSO_4$)
Detergent 7X
Diammonium sulphate ($(NH_4)_2SO_4$)
Disodium Molybdate Dihydrate ($Na_2MoO_4.2H_2O$)
Magnesium Chloride ($MgCL_2$)
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)
Manganese chloride hydrate ($MnCl_2.H_2O$)
Potassium chloride (KCl)
Potassium dihydrogen phosphate (KH_2PO_4)
Potassium nitrate (KNO3)

Sequestrene (Fe chelate)
Silica gel GF_{254}
Sodium chloride (NaCl)
Sodium hydroxide (NaOH)
Sodium sulphate (Na_2SO_4)
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)

-Sigma
Acetaldehyde (CH_3CHO)
Ethylenediaminetetraacetic acid, EDTA
Gamborg`s salt
Gamborg`s B5 vitamin
Magnesium chloride ($MgCl_2$)
Sucrose ($C_{12}H_{22}O_{11}$)
Phytagel
GR24 (3-[(2,5-Dihydro-3-methyl-2-oxo-5-furanyl)oxymethylene]-3, 3a,4,8b- tetrahydroindeno-[l,2-b] furan-2-one)
Ethylenediaminetetraacetic acid, EDTA

-Serva
2,3,5-Triphenyltetrazolium chloride (TTC)
Amicase (Casein hydrolysate)

-Agan Chemical Manufacturers Ltd
Glyphosate, N-(phosphonomethyl) glycine (99,5%)

-Reckitt Benckiser Healthcare
Detol (Chloroxylenol 4.8%)

Organic reagents of analytical grade were obtained from the following companies

-Lab Scan Ltd
Acetone
Acetonitrile
Benzene
Chloroform
Ethanol
Ethylacetate
Petroleum ether

-Merck
n-hexane

2.4 INSTRUMENTS.

In the course of the study, the following instruments were used:

-Autoclave (Tuttnauer 2340MK and Nuve OT 4060)
-UV light
-Optical microscope (Olympus CK 40)
-Fridges and deep freezers (-80^{0}C)
-Stereoscope (Olympus SZ 40)
-PH Metre (Metrolab Phm 240)
- Vacuum rotary evaporator (Buchi Rotavapor R-3000r.).

-High Performance Liquid Chromatography (HPLC)
-Microwave oven
-Water Distiller (Aquatron)
-Light, dark, temperature and humidity controlled Growth chambers
-Homogenizer (Virtis)
-Konica Minolta camera Z20
-Surgical knives for cutting tissues (Knife-maker, Reichert-taab)
-Centrifuge (Biofuge primo R and Heraeus Hermle Z200A)
-Laminar flow chamber
- Electronic balance (Weighing machines)
-Electric blender
-Spectrophotometer.

Various items

During the study various materials and items were used as listed below:
-Aluminium foil
-Microscope Slides
-Silver tape, 3M
-Parafilm (Pechinery plastic packaging)
-Nylon films
-Cooking gas
-Mortar and pistle
-Glass bottles with corks
-Glass pipettes (0.1-10mL)
-Glass funnels (10cm diameter)
-TLC glass plates for thin layer chromatography
-Glass column for column chromatography
-Glass graduated cylinders
-Whatman glass microfiber filter papers
-Conical flasks
-Surgical knife
-Cylindrical glass
-Plastic petri dishes (5 and 9 cm diameter)
-Plastic cylinders (50mL)
-Plastic eppendorf tubes (2mL)
-Various spatulas
-Dissecting needles
-Cotton
-Surgical gloves
-Scissors
-Plastic pots
-Plastic buckets
-Perlite
-Peat
-Chronometer
-Disposable syringe filter (pore size 0.2µm)

METHODS

2.5 ISOLATION OF FUNGAL PATHOGENS FROM INFECTED OROBANCHE PLANT TISSUES.

Sliced sections of the infected Orobanche plants were thoroughly rinsed in sodium hypochlorite solution and sterilized water before being placed on water agar medium containing 1% acetic acid in Petri dishes. After incubation for four to seven days at 20^0C, the fungi grown out from the medium were isolated for further propagation.

Single Fusarium spores were isolated by standard dilution plate methods in which the germinating spore is removed from the plate under a stereoscope with a needle. Single spore from the Fusarium isolate was placed on Potato-Dextrose Agar medium in petri dishes and incubated at 20^0C for 48 hours.

2.6 PREPARATION OF FUNGAL SUSPENSIONS FOR PATHOGENICITY TESTS.

For the preparation of Fusarium aqueous cell suspension, mycelia from 3 weeks old Fusarium cultures were scraped off from 10 Petri dishes and thoroughly smashed using a mortar. The smashed extract was filtered using a cheese cloth and the filtrate was made up to 35mL using sterilized deionized water. The filtrate was made up to 50mL .After shaking vigorously with an electric shaker; spore count was measured by haemocytometer. Concentrations of $3x10^4$spores/mL and $3x10^3$ spores/mL were made for future pathogenicity tests.

2.7. PREPARATION OF GROWTH MEDIA.

Fusarium oxysporum was cultured on potato dextrose agar medium or rice growth medium. The potato dextrose agar (PDA) medium was 30g of PDA (Sigma Aldrich) dissolved in 1litre of deionized water, sterilized and poured in 9cm diameter petri dishes. The rice culture medium was a medium composed of 100g of rice seeds soaked in 30mL of deionized water. This was then boiled for 15 minutes and later sterilized for 20 minutes using the autoclave.

2.7.a. Potatoes Dextrose Agar (PDA) medium.

This was prepared by measuring 30g of PDA (Sigma Aldrich) and dissolving in 1litre of deionized water. The pH was adjusted to 5.8 before sterilizing. After sterilizing at 121^0C for 20minutes, it was then poured into plastic petri dishes of diameter 9cm or test tubes and allowed to solidify for further use in inoculating the Fusarium strains.

2.7.b. Rice growth medium.

This was prepared by measuring 100g of commercial rice and soaking it in 30mL of deionized water in 500ml bottles. This was allowed to soak for 24hours under room

temperature before boiling for 15minutes. After boiling for 15 minutes, the content was then sterilized for 20 minutes at 121^0C.After sterilization, it was then allowed to cool for inoculation to be effected from *Fusarium oxysporum* earlier cultured on PDA.

2.7.c. Hoagland solution.

The Hoagland solution used for the Polyethylene bag experiment was a modified form of Hoagland and Arnon 1950. Stock solutions were made from which the final solution was made as indicated in the table below. The pH was adjusted to 5.8 and sterilized at 121^0C for 20minutes before filter sterilized sucrose was added by filter sterilizing it using disposable syringe filter.

Table 4 Hoagland solution composition

s/n	Component	Stock solution g/L	Hoagland solution ml/L
1.	$Ca(NO_3)_2.4H_2O$	72	10
2.	KCl	74	5
3.	$MgSO_4.7H_2O$	120	4
4.	KH_2PO_4	70	4
5.	KNO_3	50	5
6.	Fe.EDTA a) $FeCl_3.6H_2O$ 0.24g/500ml b) EDTA 0.75g/500ml		20
7.	Microelements		3
	H_3BO_3	2.86	
	$MnCl_2.H_2O$	1.8	
	$ZnSO_4.7H_2O$	0.22	
	$CuSO_4$	0.08	
	$Na_2MoO_4.2H_2O$	0.017	
	sucrose		10g

2.7.d Tissue culture medium.

The tissue culture medium was a modified form of Gamborg`s B5 medium (Gamborg et al 1968) containing 3M Indole-3-acetic acid (IAA) or 1M 2, 4-Dichlorophenoxyacetic acid (2, 4 D) to produce root like protruding calluses or amorphous calluses respectively.
The modified Gamborg B5 medium composed of:

Gamborg salt -3.1g
$MgCl_2.6H_2O$ - 0.204g
$CaCl_2.2H_2O$ - 0.147g
Amicase - 600 µg
Sucrose - 30g
Phytagel - 3g
All dissolved in 1litre of deionized water and adjusted to pH 5.8 before sterilization at 121^0C for 20 minutes.
After sterilization, the solution was half cooled in the laminar before 1ml of filter sterilized B5 vitamins was added. The 1 litre solution was then divided into two (500ml each) .In one of them, 4ml of 0.5 µm of 2,4D was added while in the other 1ml of 3 µm of IAA was added. Both IAA and 2,4 D were filter sterilized before addition. The solution was then poured into petri dishes and allowed to solidify while cooling. Sterilized preconditioned seeds of orobanche were then spread on the media for callus formation.
Callus formation began after about 2 weeks depending on the specie and every three weeks transfer of calluses to fresh medium was made so as to multiply the biomass.
The above were all dissolved in 1litre of deionized water, sterilized and poured into 9cm diameter petri dishes where the calluses were grown.

2.8. PREPARATION OF GROWTH STIMULANTS/HORMONES

Preparation of GR24 and Nijmegen 1 test solutions was done by dissolving 1 ml of 1M solution of the respective stimulants in 1L of water to give a concentration of 10^{-1} M. Continuous repetition of this process yielded the concentrations: 10^{-5} M, 10^{-6} M and 10^{-7} M which were used in the experiment. 1M and 3M of 2,4D and IAA respectively were used based on an earlier test conducted to determine the best concentration for callus production of *Orobanche crenata.* These plant stimulants and growth regulators were filter sterilized by passing through a 0.22-µm Millipore filter. IM solution of the various stimulants and growth hormones were made by dissolving quantities equal to their molecular weights in 1litre of deionized water.

2.9 PATHOGENICITY TESTS.

2.9. 1. Polyethylene bag tests.

The Polyethylene bags (PEB) were prepared according to Parker and Dixon (1983), Goldwasser et al. (1997) and Linke et al (2001) with modifications to suit this experiment (fig 11). This method permitted the observation of the host roots and the various stages of broomrape development in the parasitism process. Seed germination, tubercles formation and appearance of spiders characterized the various stages of development.
0.5mg of the sterilized broomrape seeds (about 100 seeds) were spread on 11.5 x 23 cm glass fiber filter papers (Whatman, GF/A, Whatman International Ltd., Maidstone, England) placed in transparent polyethylene bags 25x35 cm. One sunflower or vetch seedling at the cotyledon stage was mounted onto the glass fiber paper containing the broomrape seeds.

20ml of sterilized half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) was added into each bag to serve as nutrient for the plants. The polyethylene bags were placed upright in a dark box in the growth chamber with the following conditions: 20±2^{o}C temperature; 175 µmol m^{-2} s^{-1} light intensity and a 12 h photoperiod.
5ml of GR-24(10mg/L) was added in each bag two weeks after seed placement to ensure homogenous seed germination. The percentage of Orobanche seeds that germinated and attached to the host roots was determined three weeks after the addition of GR24 under a microscope at 30 x magnification by studying 50 Orobanche seeds per bag. Only seeds that were close to a host root (< 3mm) were considered. Two months later, the number of Orobanche tubercles per bag was counted. The PEB were nourished twice a week from the top of the bag with 20-30mL half-strength Hoagland nutrient solution depending on the level of uptake by the plants.
In each host plant the treatments were carried out in 5 replications. *Fusarium oxysporum* aqueous spore suspensions ($3X10^4$ & $3X10^3$ spores/mL) was sprayed on the filters after emergence of tubercles.
In all experiments, distilled sterile water, served as the control.
Observations were carried out with a stereoscope (Olympus TL2) and Microscope (Olympus BX40F-3), in order to detect and monitor the transition stages from seed attachment to tubercle and spider formation. The stereoscope microscope observations were centered on the host plant's roots where the broomrape attachments, tubercles and spiders were found.

Figure 11. *Helianthus annuus* in PEB showing roots of host in association with the parasite 3days after association.

Figure 12.*Vicia sativa* in PEB showing roots of host in association with the parasite 3days after association.

2.9. 2. Pot experiments.

Pot experiments were carried out in the in the greenhouse with daytime temperature maintained at 25^{o}C, whereas night temperature was kept at about 15^{o}C. These experiments were classified as Pre emergence and Post emergence.

2.9.2.1 Pre emergence Pot experiment.

The efficiency of various conidial density of *Fusarium oxysporum* to control *O. crenata* and *O .cumana* after soil incorporation was evaluated in pot experiments. Soil was mixed with peat. The soil was then autoclaved for one hour at 121^{o}C to kill pathogens. This was repeated after 24 hours to ensure complete disinfestation of the soil.

Three host plants were used: *Vicia faba* for *O. crenata*, *Vicia sativa* for *O. crenata* and *Helianthus annuus* for *O. cumana.*

For each host plant species, 12 pots were used viz: 3 for the negative control (Orobanche spp. without Fusarium strains), 3 for the positive control (neither Orobanche nor Fusarium),3 with Fusarium at a conidial density of $3x10^{4}$ spores ml^{-1} and 3 with Fusarium at a conidial density of $3x10^{3}$ spores mL^{-1} . Plastic pots (12x12x18cm) were filled with approximately 0.8kg soil/pot. Approximately 100 seeds of Orobanche spp. were sprinkled onto the surface in each pot. The seeds had been collected in Greece in 2003 and showed a viability of approximately 80% according to a triphenyl tetrazolium chloride (TTC) test (Aalders & Pieters, 1985) where after 72 hours at 37^{o}C in the dark, red or orange seeds were considered viable, while white seeds were considered dead. 5ml of each Fusarium aqueous cell suspension was sprayed on the soil surface of each pot. The pots were later filled with soil to the top. Soil irrigation was carried out immediately after inoculation and complete drying was avoided for the first 3 weeks in order to precondition the seeds. Subsequently, the soil was allowed to dry to a certain degree (i.e. irrigation was carried out every three days) not very harmful to the plants before irrigation. Host plants were sown alongside *Orobanche* species and later thinned to one plant per pot.

The experiment was evaluated when the host plants of the negative control as well as treatments with low inoculum doses of the fungus had stopped development due to Orobanche infestation. At this time, the number of emerged shoots was recorded. Shoots

were considered emerged as from 0.5 cm above soil level and the shoots emerged between 35 and 45 days after sowing. The shoots were also examined for symptoms of Fusarium infection.

2.9. 2.2.Post emergence Pot experiment.

The susceptibility of emerged shoots of Orobanche to the pathogen was evaluated in a separate experiment. The Orobanche species were cultivated on the respective host plants. Soil was mixed with peat to improve its fertility. The soil was then heated for one hour at 121^{o}c to kill pathogens. This was repeated after 24 hours to ensure complete disinfestation of the soil.

Three host plants were used: *Vicia faba* for *O. crenata*, *Vicia sativa* for *O. crenata* and *Helianthus annuus* for *O. cumana.*

For each host plant specie, 12 pots were used viz: 3 for the negative control (Orobanche to grow but no fusarium cell suspension to be applied), 3 for the positive control (neither Orobanche nor Fusarium cell suspension to be applied),3 for Fusarium cell suspension to be sprayed at concentration of $3x10^{4}$ spores ml^{-1} and 3 for Fusarium cell suspension to be sprayed at concentration of $3x10^{3}$ spores ml^{-1} .Plastic pots (12x12x18cm) were filled with approximately 0.8kg soil almost to the top. Approximately 100 seeds of Orobanche sp were sprinkled onto the surface of pots that required orobanche spp. The seeds had been evaluated for viability according to the triphenyl tetrazolium chloride (TTC) test (Aalders & Pieters, 1985) described above where after 72 hours at 37^{0}C in the dark, red or orange seeds were considered viable, while white seeds were considered dead. Host plants were sown alongside *Orobanche sp* and later thinned to one plant per pot.

5ml of aqueous cell suspensions at concentrations of $3X10^{4}$ & $3X10^{3}$ spores/mL were sprayed on the emerged shoots in each pot. The selected shoots for treatments were between 8 and 10 cm high. Deionized water was sprayed as a control treatment. After spraying, the plants were covered with polyethylene bags for 48hours to increase humidity. Observations were taken 7 and 14 days after inoculation.

2.10. INCUBATION AND GROWTH OF FUSARIUM STRAINS.

Single Fusarium spores were isolated and placed on Potato-Dextrose Agar (PDA) medium in petri dishes and incubated at 23^{o}C for 48 hours. For multiplication of biomass and production of enough metabolites, several Petri dishes of PDA(for green house experiments) or boiled and sterilized rice(for laboratory bioassay) were later infected with plugs of PDA containing fusarium. These cultures were incubated at 23^{o}C for one month before extraction.

2.11. EXTRACTION OF METABOLITES.

For toxin production, the *Fusarium oxysporum* strains were cultured on rice for one month at 20^{o} C in the dark. Afterwards the cultures were extracted with acetone. The extract was dried over anhydrous sodium sulphate (Na_2SO_4). It was later on passed through Whatman filter paper No.1. The filtrate was evaporated to dryness at 30^{o}C using a vacuum rotary evaporator. The extract was then collected with 3ml of acetone and stored for further analysis. It was

further separated using TLC plates to obtain purer fractions which were then subjected to further analysis using HPLC, MPLC, GCMS and NMR.

2.12 IDENTIFICATION OF METABOLITE.

2.12.1 HPLC Separation.

Separation by HPLC was performed using a Varian ProStar System equipped with a ProStar Solvent Delivery Module 230, Injector Rheodyne 7125, ProStar 330 UV–Vis photo diode array detector. Chromatographic separations were performed on a Pinnacle II C-18 column (250 × 4.6 mm i.d., 5 μm) including Pinnacle C-18 guard column (10 × 4 mm i.d., 5 μm) (Restek, Bellefonte, USA). For gradient elution mobile phase A contained methanol; solution B contained mixture of 72.5% acetonitrile and 15% HPLC grade water adjusted to pH 7.35 with concentrated phosphoric acid. The flow rate was 0.5 mL min^{-1}. Operating conditions were as follows: column temperature, 20 °C, injection volume, 20 μL, UV–Vis photo diode array detection at 254 nm. All the samples were filtered through 0.2 μm membrane (Millipore) using a filtration syringe system. Each run was repeated thrice and detector response was measured in terms of peak area.

2.12.2 MPLC Separation.

Medium pressure liquid chromatography (MPLC) was carried out with a preparative MPLC system of Buchi, on silica gel (Merck 0.015-0.04 mm) with an applied pressure of 200 mbar.

2.12.3. TLC Separation.

Precoated TLC silica 60 F_{254} plates (Merck) were used for thin-layer chromatography (0.25 and 2 mm layer thickness for analytical and preparative TLC, respectively). Spots were visualized using UV light, vanillin-sulphuric acid.

2.12.4. GC-MS analysis.

The GC-MS analyses have been carried out using a Hewlett Packard 5973-6890 GC-MS system operating on EI mode at 70 eV. Helium was used as carrier gas (flow 0.8 ml/min) and the capillary columns used were a HP 5MS (30m x 0.25mm; film thickness 0.25μm), and a HP Innowax (30m x 0.25mm; film thickness 0.50μm). The initial temperature of the column was 60°C and then it was heated to 280^{o}C with a 3°C/min rate. The temperatures of injector and transfer line were set at 220^{o} C and 280^{o}C respectively.

The identification of the compounds was based on comparison of their retention indices (RI), retention times (RT) and mass spectra with those from Wiley libraries spectra, the NIST/NBS and literature data.

2.12.5. NMR analysis.
Nuclear magnetic resonance (NMR) spectra were obtained on Bruker 600 MHz spectrometers using $CDCl_3$ (Aldrich) as solvent. The 2D-NMR experiments (COSY, LRCOSY, HMQC, HSQC and HMBC) were performed using standard Bruker microprograms.

2.12.6. MS.
Mass spectrometry APCI-HRMS were run on a Thermo Scientific LTQ Orbitrap Discovery mass spectrometer with the diffusion method.

2.13. PHYTOTOXICITY TESTS.

2.13.1: Polyethylene bag experiment.
Broomrape seeds were surface sterilized by dipping in 70%ethanol for 1.5 minutes. Later seeds were dipped in sodium hypochlorite solution for 10 minutes and later rinsed thoroughly with distilled sterile water. The viability of the seeds was determined by the 2,3,5-triphenyl tetrazolium chloride test (Linke & Saxena, 1991).
0.5mg of the sterilized broomrape seeds(about 100 seeds) were later spread on 11.5 x 23 cm glass fiber filter papers (Whatman, GF/A, Whatman International Ltd., Maidstone, England) placed in transparent polyethylene bags 25x35 cm .One sunflower or vetch seedling at the cotyledon stage was mounted onto the glass fiber paper containing the broomrape seeds .20ml of sterilized half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) was added into each bag to serve as nutrient for the plants. The polyethylene bags were placed upright in a dark box in the growth chamber with the following conditions: 20 ± 2^0C temperature; 175 µmol m^{-2} s^{-1} light intensity; 12 h photoperiod.
5ml of GR-24(10mg/L) was added in each bag two weeks after sowing to ensure homogenous seed germination. The percentage of Orobanche seeds that germinated and attached to the host roots was determined three weeks after the addition of the GR24 under a microscope at 30 x magnifications by studying 50 orobanche seeds per bag. Only seeds that were close to a host root (< 3mm) were considered. Two months later, number of Orobanche tubercles per bag was counted. The PEB were nourished twice a week from the top of the bag with 20-30mL half-strength Hoagland nutrient solution depending on the level of uptake by the plants.
In each host plant the following treatments were carried out in 5 replications:
1-*Fusarium oxysporum* extract (C11 and OK7) incorporated in PEB at the time of sowing the broomrape seeds.
2-*Fusarium oxysporum* extract (C11 and OK7) sprayed on broomrapes after emergence of tubercles.
In all the treatments, distilled sterile water, served as the control.
Observations were carried out with a stereoscope (Olympus TL2) and Microscope (Olympus BX40F-3), in order to detect and monitor the transition stages from seed attachment to tubercle and spider formation. The stereoscope observations were centered on the host plant's roots where the broomrape attachments, tubercles and spiders were found. Host roots of about 7mm long were harvested separately from each of the treatments at different developmental stages.

2.13.2 Bioassay on stems, flowers and tissue cultures for phytotoxicity evaluation.

The Fusarium extracts (OK7 & C11) were subjected to thin layer chromatography (TLC). Analytical silica gel plates were marked under ultra violet (UV) light and then each band was carefully scraped off. Compounds from each band were re-extracted in acetone after centrifuging it.
In order to detect biological activity, each compound was prepared and subjected to flower and stem bioassay by dissolving in 1mL of deionized water.
Flowers from the upper part of the stem were detached from the plant. Also the upper part of the stem i.e.1cm from the apex was cut off and sliced into pieces of 0.2 cm in length .These plant parts were immersed in solutions from the various bands and observations made after 12 hrs. Two sets of control were set up. One with pure deionized water and the other with glyphosate (at 2, 5 and 10 ppm concentrations).
The fractions used were those that migrated with Rf values of 0.06, 0.13, 0.23, 0.35, 0.42, 0.62, 0.77 and 0.87.
For tissue culture bioassay, calluses from the Orobanche spp were used. These calluses were placed in Eliza plates and the various fractions of the extracts were added. Similarly, glyphosate at concentrations of 2, 5, and 10 ppm and water were also tested as the negative and positive controls. The Eliza plates were sealed with parafilm and kept in a growth chamber for observation and evaluation after 3, 6, 12, 24 and 48 hours.
A second bioassay was conducted using porcelain plates with wells. Calluses were placed on whatman filter paper discs with diameter of 2cm .The weights were taken before placing the discs in wells of a porcelain plate. Extracts of the various fractions at concentrations of full strength, half strength and a quarter strength were tested on these calluses. They were then placed in a covered container and placed in the refrigerator (10^{o}C). Results were taken after 48hours on the weight change as influenced by the various fractions. Similarly, glyphosate at concentrations of 2, 5, and 10 ppm and water were also tested as the negative and positive controls, respectively.

2.13.3.Orobanche germination test.

Sterilization of the Orobanche seeds was adapted from the various methods previously reported by Ben-Hod et al., 1991, Losner-Goshen et al., 1996 and Batchvarova et al., 1999.In this study, Orobanche seeds were sterilized in 70% ethanol for 1.5minutes and later in 5% sodium hypochlorite solution containing 0.1% Tween for 10 minutes with constant agitation to ensure complete sterilization. The seeds were later rinsed with sterilized water three times to ensure complete removal of the chemicals.
A viability test was conducted according to van Hezewijk et al.(1993) using TTC(2,3,5-triphenyl tetrazolium chloride).TTC is a white salt which gives a colorless solution in water but gets hydrated to a red coloration in living cells. The seeds were placed in small Petri dishes and drenched with 1% solution of TTC. The dishes were then kept in the dark at 28^{0}C. After two weeks, the seeds were examined if they are viable. Red or pink seeds were

considered viable while no colored seeds were considered dead. A stereoscope was used in the assessment.
After the viability test, sterilized seeds, approximately 20, were spread on a double layer sterile filter paper discs and placed on PDA for 10 days to allow for preconditioning. The dishes were then closed tightly to prevent water evaporation and kept in a dark incubator at 20^{o}C. After 10 days, clean (uncontaminated) filter paper discs were dried on filter paper (by placing the wet filter papers on dry filter papers) to remove any excess water and then transferred unto new filter paper discs containing GR24 and Nijmegen-1 stimulants for conditioning. The Petri dishes were then enclosed in aluminium paper to prevent water losses and incubated in the dark at 20^{o}c. After one week, the seeds were evaluated if they germinated by inspecting under the stereoscope if the root tip has protruded through the seed coat for about 1mm.

2.13.4. Chlorophyll content in relation to chemicals.

Five hundred milligrams of leaf tissue in fractions from the various experiments with glyphosate, tyrosol and 1H Indole-3-carboxaldehyde with water and rice extracts as control were placed in test tubes containing 3.5mL Dimethylsulfoxide (DMSO) and incubated at 60^{0}C for various times to extract the chlorophyll into the liquid. The extract liquid was transferred into other test tubes and made up to 5ml with DMSO and used for spectrophotometry analysis.
3.0ml of the chlorophyll extracts were transferred o a cuvette and the optical density (absorbance) values at 665nm were read in a Beckman DBG spectrometer against a DMSO blank.
The equation used for calculating the chlorophyll a content was adopted from Arnon 1949.
Chla (mg/g) = 0.0127x A665 – 0.00269x A645

2.14. STATISTICS.

Formular for calculating % growth inhibition
Initial weight – new weight ÷ new weight of control x 100.

Data were analyzed using Duncan's test with the JMP software (version 4.0.2; SAS Institute Inc., USA) and ANOVA. Also Excel statistical software of Microsoft was used.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 PATHOGENICITY OF THE ISOLATES OF FUSARIUM STRAINS.

Pathogenicity tests conducted showed the potential of the Fusarium strains C11 and OK7 to infect Orobanche species at pre and post emergence stages. Since it is difficult to assess the pathogenicity effect for pre-emergence stages since they are underground, the PEB offered the opportunity to evaluate the broomrape attachments, tubercles and spiders for pathogenicity effect of Fusarium. These results confirm the work of Thomas *et al*. (1999) who reported that conidial suspensions of *F. oxysporum* colonized and infected the seeds of *Orobanche cumana*, specie specific to sunflowers. The fungus was effective in penetrating seed testa. The cell walls of the endosperm were dissolved, cytoplasm degraded, and lipid body membranes were damaged in the infected seeds. The lipid and protein rich endosperm was presumably used by the fungus as a nutrient source. As reported by Gressel (2001), the use of mycelia as mycoherbicides for the control of Orobanche is desirable due to the high infectivity and the long term storage and quick germination of the fungi in media.
Abouzeid et al., 2004 and Dor et al., 2007 also found that *Fusarium oxysporum* is pathogenic to broomrape underground structures. Extracts from 3-4 weeks old *Fusarium oxysporum* cultures caused mortality of germinating seeds of *O. cernua*, *O. aegyptiaca, O. ramosa* and *O. cumana*. The results of the pathogenicity tests conducted in the present study confirm these findings. However, it is worth noting that all these pathogenicity tests conducted by the aforementioned researchers and the present study were tested under aseptic conditions in PEBs, which could be considered semi *in vitro* condition. Under the semi *in vitro* conditions in the PEB, the tested fungi do not have competition with other microorganisms. Under natural soil conditions where competition between microorganisms prevails, the results might not be the same.
From results obtained by Thomas *et al.,* 1998; Dor *et al*., 2007 and the present study, it is interesting to know that *Fusarium oxysporum* had no pathogenic effect on the roots of *H. annuus* and *V. sativa* in PEB. With a conidial density of 10^7 conidia/ml of water used by Thomas et al.,1998 for field tests and a conidial density of 10^4 conidia/ml of water used in the recent study it can be concluded that *Fusarium oxysporum* could be used in *H .annuus* and *V. sativa* fields for control tests.

3.1.1. Polyethylene bag (PEB) experiment.

O. cumana and *O. crenata* grown in PEB showed three underground stages of parasitism on their hosts which are essential in any control program. These are: Attachments, tubercles and spiders. Stereoscopic and microscopic observations of the roots in the polyethylene bags revealed that the parasite seeds germinated, their radicles extended towards the root and got attached to it. Development continued normally, leading to the formation of Orobanche tubercles and spiders. Controls (pots without broomrape seeds) yielded plants with healthy roots, whilst plants in pots with broomrape seeds were heavily parasitized.

Pathogenicity tests conducted on these underground stages of parasitism showed that pathogenicity increased with increasing fungal inoculum pressure. Conidial suspension of density $3x10^4$ spores / mL caused greater diseased symptoms than conidial density of $3x10^3$ spores / mL. Results obtained from the influence of the *Fusarium oxysporum* strains on the number of broomrape attachments, tubercles and spiders formed when applied at planting (table1) indicates that there was no significant difference in effect between the two strains.However, there was a significant difference between the strains and the control (water).The number of attachments, tubercles and spiders of both *O. crenata* and *O. cumana* were significantly higher in the control than the treatments containing the fusarium cell suspensions .

The pre emergence application of *Fusarium oxysporum* extracts from both strains on the broomrape seeds, reduced the number of attached seeds, tubercles and spiders formed compared to the control (water).

Also, when extracts from both strains were applied post emergence on the attached seeds, tubercles and spiders, more than a half of these structures were found dead three days after application. Those that were not dead had disease symptoms while no disease symptom was observed in the control indicating that these *Fusarium oxysporum* strains are pathogenic to *Orobanche crenata* and *Orobanche cumana*. In the post emergence experiment, healthy Orobanche tubercle cells with intact structures were seen in the non infested control pots. The pathogenicity test conducted using the fusarium cell suspensions of the two strains at densities of $3X10^4$ spores/mL and $3X10^3$ spores/mL showed these two strains are pathogenic to the broomrape structures (i.e. attachments, tubercles and spiders). In the present study, *Fusarium oxysporum* extracts from 4weeks old cultures caused a significant reduction in the number of underground structures (attachments, tubercles and spiders) compared to the control.

Results obtained from pre-emergence experiments(when fusarium conidial suspension was applied in the soil or in PEB) and Post-emergence (when Fusarium conidial suspension was sprayed on the plants) showed that the tested strains of *Fusarium oxysporum* could be further evaluated in the field to control *Orobanche crenata* and *Orobanche Cumana* using fusarium conidia as pathogenic agents to the broomrape.

Table 5: Effect of application of spore suspensions of Fusarium oxysporum strains on the formation of broomrape parasitic structures. Inoculum was applied either at sowing or post emergence on Orobanche species in PEB bags in relation to their respective hosts (hosts in parenthesis*).*
All data are the means of twelve replications

Inoculum strain (Spores/mL)	Number of broomrape structures formed following pre-emergence application of inoculum.						Disease incidence on Orobanche tubercles and spiders when inoculated with *Fusarium oxysporum*.Number of tubercles were 13 and 16 for *V.sativa* and *H.annuus* respectively while number of spiders was 10 for both *orobanche spp*.			
	O.crenata (*V. sativa*)			*O.cumana* (*H .annuus*)			*O.crenata* (*V.sativa*)		*O.cumana* (*H.annuus*)	
	Attachments	Tubercles	Spiders	Attachments	Tubercles	Spiders	Tubercles	Spiders	Tubercles	Spiders
OK7 $3X10^4$	15^{c}	6^{c}	3^{c}	18^{d}	8^{d}	5^{d}	100^{c}	100^{c}	100^{b}	100^{d}
OK7 $3X10^3$	18^{b}	7^{bc}	5^{b}	22^{b}	10^{c}	8^{b}	100^{c}	90^{b}	100^{b}	80^{b}
C11 $3X10^4$	12^{d}	6^{c}	4^{bc}	17^{d}	9^{c}	5^{c}	100^{c}	100^{c}	100^{b}	100^{d}
C11 $3X10^3$	14^{c}	8^{b}	4^{bc}	20^{c}	12^{b}	7^{c}	90^{b}	90^{b}	100^{b}	90^{c}
WATER	33^{a}	13^{a}	10^{a}	35^{a}	16^{a}	10^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Data on same column not connected by same letter are significantly different.

The number of attachments, tubercles and spiders of both *O. crenata* and *O. cumana* were significantly higher in the control than the treatments containing the fusarium cell suspensions .

Figure 13.Attachment of broomrape to host root as indicated by arrows.

Figure 14. Spider- developmental stage of broomrape.

Figure.15. untreated (yellow) and treated (black) Tubercles.Treatment was done with *Fusarium oxysporum.* Black tubercles signify dead tubercles while yellow ones signify living tubercles.

3.1.2 Pot experiment

Pot experiments were carried out to test the pathogenicity effect of the two strains of *Fusarium oxysporum* on *O.crenata* and *O.cumana* and to clarify two aspects of pathogenicity of these fungi.
a) Which Orobanche specie is controlled by each of these fungi?
b) Whether the fungi are pathogenic to the host.

Fusarium cell suspensions with inoculum densities of $3X10^4$ spores / mL and $3X10^3$ spores /ml were used based on a previous work by Paternotte 1987 whereby inoculum densities of 10^2 -10^6 were used. In the first experiment, a preemergence test was conducted to evaluate the effect of Fusarium on the germination and growth of Orobanche as expressed in the number of emerged Orobanche plants per pot.
Results obtained showed that there was significant difference between the treatments exposed to various inoculum level (spore density/ml and the control-water) of application on the emergence of both specie of Orobanche in all the three host plants i.e. *V.sativa , V. faba* and *H. annuus.*
The greater the spore density i.e. $3X10^4$ spore/ml the lesser the number of Orobanche species per pot in all the species of Orobanche. With regards to the effects of the various strains (C11 and OK7) on the influence of the emergence of the parasite, there was no significant difference (Table 5.).
In the second pre-emergence experiment, concerning the percentage number of emerged diseased orobanche plant per pot, it was recorded that there was no significant difference on the effect of the two strains on *O. crenata* but there was significant difference on the effects of the two strains on *O. cumana*. Strain C11 had more pathogenic effect than OK7.With regards to the influence of inoculum levels on disease severity, the higher level of $3X10^4$ spore/mL had a significant effect compared to the lesser level of $3X10^3$ spore/ml and the control (water).
Post emergence experiments were also conducted to evaluate the effects of these two strains on *Orobanche spp*. The first of this experiment was to evaluate the percentage number of diseased Orobanche plants/pots 7 and 14 days after the application of the fusarium inoculums. The disease level was significantly higher when assessed 14 days after application compared to when assessed 7 days after application. However, in both assessments, the disease level was significantly higher than the control. This result was reflected in all the two species of Orobanche and the three host plants. Comparing the results obtained from the two strains (C11 and OK7) they were similar with regards to assessments after 7 and 14 days of application and dosage of applications ($3X10^4$ spore/ml and $3X10^3$ spore/mL).
In the second post emergence experiment regarding the percentage dead of Orobanche spp 7 and 14 days after the application of the inoculums, it was noted that there was significant difference in the results obtained 7 and 14 days after application of both strains of C11 and OK7. After 7 days of application, no dead Orobanche plant was recorded from *O. cumana* parasitizing *H. annuus* with both strains. Similar result was obtained from experiments with *O. crenata* parasitizing *V. sativa* with C11 strain.
Previous work done in PEB indicated that Fusarium strains are pathogenic to the developmental stages of *Orobanche cumana* and *Orobanche crenata*. Similar work

conducted by Amsellem et al 2001 confirmed that *Fusarium oxysporum* is pathogenic to the parasites: *Orobanche cumana, Orobanche aegyptiaca, Orobanche cernua* and Orobanche *ramosa* but not to their hosts.

When the inoculum C11 and OK7 were applied pre-emergence, they significantly reduced the number of emerged Orobanche spp/ pot compared to the control (table 6).

At a spore density of $3x10^4$ spores/ml, OK7 reduced *O.crenata* parasitizing *V.sativa*, *O.crenata V.faba* parasitizing and *O.cumana* parasitizing *H.annuus* by 61.85, 63.49 and 59%, respectively. Similarly C11 strain at a spore density of $3x10^4$ spores/ml reduced *O.crenata* parasitizing *V.sativa*, *O.crenata* parasitizing *V.faba* and *O.cumana* parasitizing *H.annuus* by 57.1, 57.6 and 68.1% respectively.

Also when the inoculum was applied pre-emergence, it had an adverse effect on the health of emerged Orobanche plants. OK7 at a spore density of $3x10^4$ spores/ml caused 100, 88.69 and 100% infection on *O.crenata* parasitizing *V.sativa*, *O.crenata* parasitizing *V.faba* and *O.cumana* parasitizing *H.annuus* respectively. While C11 strain at a spore density of $3x10^4$ spores/ml caused 100, 88.76 and 100% infection on *O.crenata* (*V.sativa*), *O.crenata* parasitizing *V.faba* and *O.cumana* parasitizing *H.annuus* respectively. At a lower spore density of $3x10^3$ spores/ml both strains also caused infections on the parasites (table 6).

These results confirm the work of Muller-Stover (2001) where *Fusarium oxysporum* applied at a dose of 0.5g/kg of soil (preemergence) cause a reduction efficacy of broomrape between 78-89%.

From the experiments conducted, no adverse effect of the inoculums was recorded on the host plants confirming the work of Bedi 1994; Heiko et al., 1998; that fusarium exclusively attacks *O. cumana*, *O. cernua* and *O.aegyptiaca.* Heiko et al 1998 reported that fusarium conidia at a dose of $3x10^4$ spores/mL caused a 28% reduction of shoot number. Also with this dose, 51% of the emerged shoots were diseased while 90% of the shoots were diseased at a dose of $3x10^8$ spores/ml and caused a 67% reduction of emerged shoots. Similarly, Bozoukov and Kouzmnova 1994 reported that pre-emergence application of fusarium mycelia caused broomrape (*O. ramosa and O. aegyptiaca*) reduction by 62-68% in the field.

However, it is likely that the combined attack on seeds, germ tubes and tubercles in soil by *Fusarium oxysporum* was responsible for the reduced number of parasite shoots in pre emergence pot experiments of the current study as well as those conducted by the above mentioned researchers.

Post emergence experiments were evaluated 7 and 14 days after the application of inoculum. The first incidence of fungal disease was observed on shoots one week after inoculation, starting with the development of lesions. The scales (rudimentary leaves) became brownish. The shoot browning gradually spread to the entire shoot.

Observations recorded after 7 days of application of OK7 at a dose of $3x10^4$ spores/mL caused a disease infection level of approximately 71.6% on *O.crenata* and 77.3% on *O.cumana*. At same inoculum level, observations after 14 days revealed the infection level was 88.6, 100, and 100% for *O.crenata* (V.faba as host), *O.crenata* (*V.sativa* as host) and *O.cumana* (*H. annuus* as host), respectively (table7).

At a lower inoculum level of $3x10^3$ spores/mL, observations after 7 days of application revealed the infection level was approximately 55.3% for *O.crenata* and 71.7% for *O.cumana.*

After 14 days, the infection level was 88.6% for *O.crenata* (*V.faba* as host), 77.1% for *O.crenata* (*V.sativa* as host), and 94.3% for *O.cumana* (*H.annuus* as host) (table 7).

Similarly, 7 days after the application of inoculum C11 at a dose of $3x10^4$ spores/mL, the percentage of diseased plants were 88.6% for *O.crenata* and 71.2% for *O.cumana*. At same dose after 2 weeks the results were 100% diseased plants for both broomrape species (Table 8).

At a lower inoculum level of $3x10^3$, the inoculum C11 caused an infection percentage of approximately 55% on both species of broomrape after 7days of inoculation and approximately 94.3% after 14days.

In this study, it is assumed that the reduction of emerged Orobanche when *Fusarium oxysporum* strains were applied in the soil at time of planting is because they produced some toxins that inhibited the germination of the parasitic seeds. Other potentially effective method of controlling parasitic weeds such as Orobanche is to stimulate germination of their seeds in the absence of the host crop. When the germinated seedling fails to find the roots of a susceptible host, thereby reducing the quantity of the parasite in the seed bank. To achieve this, a synthetic germination stimulant such as GR24 or ethylene is applied to the soil .An alternative method has been reported by other researchers such as El-Kassas et al., 2004 that some strains of fungi when applied to the soil produce ethylene which stimulate the germination of the parasite. Shifrin and Anderson 1999, like other researchers have another suggestion for the reduced emergence of parasites when fungi are incorporated in the soil. The fungi is said to produce some toxins that act as germination inhibitor by reducing protein synthesis through the inhibition of peptidyltransferase. Application of spore suspensions of the two *Fusarium oxysporum* strains on broom rape structures of *O.crenata* and *O.cumana* (table 5) revealed that both strains reduced the number of structures of broom rape. OK7 applied at inoculum density of $3x10^4$ spores/ml reduced attachments by 54.5 and 48.6% for *O.crenata* and *O.cumana* respectively.C11 applied at same spore density reduced attachments by 63.6 and 51.4% for *O.crenata* and *O.cumana* respectively. Other structures i.e. tubercles and spiders were also controlled by these Fusarium strains when applied pre-emergence in PEB even at a lower dose application of $3x10^3$ spores/ml . Amsellem et al. 2001 had earlier reported that *Fusarium oxysporum* reduced tubercle growth by 75.9 % in PEB. When same strains were applied post emergence on the Orobanche structures, they infected all the structures (100%) when applied at a spore density of $3x10^4$ spores/mL .Previous research by Abbas and Boyette (1992) on the pathogenicity of *Fusarium oxysporum*, provided an impetus to investigate whether the potential Fusarium isolates are able to produce pathogenic effects against different developmental stages of broomrape.

Table 6. Effect of *Fusarium oxysporum* strains on O*robanche crenata* and *Orobanche cumana* in pots on their respective hosts (hosts in parenthesis) when inoculum was applied pre-emergence at sowing. Observations on shoot emergence and symptoms development were made 45 and 60 days respectively after sowing. All data are the means of twelve replications

Inoculum Strain (Spores/mL)	No. of *Orobanche spp* shoots/pot when inoculum was applied pre-emergence			*No. of diseased *Orobanche spp* shoots /pot when inoculum was applied pre-emergence		
	O.crenata (V.sativa)	*O.crenata (V. faba)*	*O.cumana (H. annuus)*	*O.crenata (V. sativa)*	*O.crenata (V. faba)*	*O.cumana (H. annuus)*
OK7 ($3X10^4$)	2.67^{c}	2.3^{b}	9.00^{d}	2.67^{b}	2.08^{a}	9.0^{a}
OK7 ($3X10^3$)	3.67^{b}	2.67^{b}	14.00^{b}	3.08^{ab}	2.08^{a}	8.166^{a}
C11 ($3X10^4$)	3.00^{bc}	2.67^{b}	7.00^{e}	3.0^{b}	2.33^{a}	7.0^{b}
C11 ($3X10^3$)	3.67^{b}	3.00^{b}	12.00^{c}	3.66^{a}	2.66^{a}	8.004^{a}
WATER	7.00^{a}	6.3^{a}	22.00^{a}	0.0^{c}	0.0^{b}	0.166^{c}

* Shoots with lesions

Data on same column not connected by same letter are significantly different.

The inoculum C11 and OK7 significantly reduced the number of emerged Orobanche spp/ pot compared to the control .At a lower spore density of $3x10^3$ spores/mL both strains also caused infections on the parasites.

Table 7: Disease incidence of *Fusarium oxysporum* (OK7) on Orobanche spp grown in pots on their respective hosts (hosts in parenthesis*)* fungal inoculum was applied at $3X10^4$ and $3X10^3$ spore/mL and observations were made 7 and 14 days after inoculation. All data are the means of twelve replications.

treatments	*O.crenata (V.faba)*				*O.crenata (V.sativa)*				*O.cumana (H.annuus)*			
	*No. of diseased plants/pot		No. of dead plants/pot		*No. of diseased Plants/pot		No. of Dead plants/pot		*No. of diseased plants/pot		No. of dead plants/pot	
	7days	14days	7days	14days	7days	14days	7days	14days	7days	14days	7days	14days
$3X10^4$	7.16^b	8.91^a	0.583^c	6.08^a	7.33^c	10^a	0.583^c	4.41^a	7.75^c	10^a	0.0^c	6.08^a
$3X10^3$	5.58^c	8.91^a	0.0^d	3.83^b	5.5^d	7.83^b	0.0^d	1.16^b	7.17^d	9.5^b	0.0^c	3.91^b
Water	0.0^d	0.0^d	0.0^d	0.0^d	0.0^e	0.0^e	0.0^d	0.0^d	0.0^d	0.0^e	0.0^c	0.0^c

* Shoots with lesions
Data on same column not connected by same letter are significantly different.

After 7 days of application of OK7 at a dose of $3x10^4$ spores/mL caused a a very high disease infection level on *O.crenata* and *O.cumana*. At same inoculum level, observations after 14 days revealed the infection level was was also very high for *O.crenata* (V.faba as host), *O.crenata* (*V.sativa* as host) and *O.cumana* (*H. annuus* as host).

Table 8 : Pathogenicity (disease incidence) of *Fusarium oxysporium* (C11) on Orobanche spp grown in pots on their respective hosts (fungal inoculum was applied at $3X10^4$ and $3X10^3$ spore/mL and observation were made 7 and 14 days after inoculation). All data are the means of twelve replications.

treatments	*O.crenata (V.faba)*				*O.crenata (V.sativa)*				*O.cumana (H.annuus)*			
	*No. of diseased plants/pot		No. of dead plants/pot		*No. of diseased Plants/pot		No. of Dead plants/pot		*No. of diseased plants/pot		No. of dead plants/pot	
	7days	14days	7days	14days	7days	14days	7days	14days	7days	14days	7days	14days
$3X10^4$	8.83^a	10^a	1.166^b	4.91^a	8.91^b	10^a	0.0^c	5.0^a	7.16^b	10^a	0.0^c	4.91^a
$3X10^3$	5.58^c	9.416^b	0.58^{bc}	1.083^b	5.5^c	9.5^{ab}	0.0^c	1.16^b	5.515^c	9.5^a	0.0^c	1.08^b
Water	0.0^d	0.0^d	0.0^c	0.0^c	0.0^d	0.0^d	0.0^c	0.0^c	0.0^d	0.0^d	0.0^c	0.0^c

* Shoots with lesions
Data on same column not connected by same letter are significantly different.

After 7 days of application of C11 at a dose of $3x10^4$ spores/mL caused a a very high disease infection level on *O.crenata* and *O.cumana*. At same inoculum level, observations after 14 days revealed the infection level was was also very high for *O.crenata* (V.faba as host), *O.crenata* (*V.sativa* as host) and *O.cumana* (*H. annuus* as host).

Figure 16. Control experiment (without Fusarium extract) showing *Orobanche cumana* parasitizing *Helianthus annuus* in high numbers. Due to the absence of *Fusarium oxysporum* in the soil at time of planting, *Orobanche cumana* germinated and grew very well without hinderance.

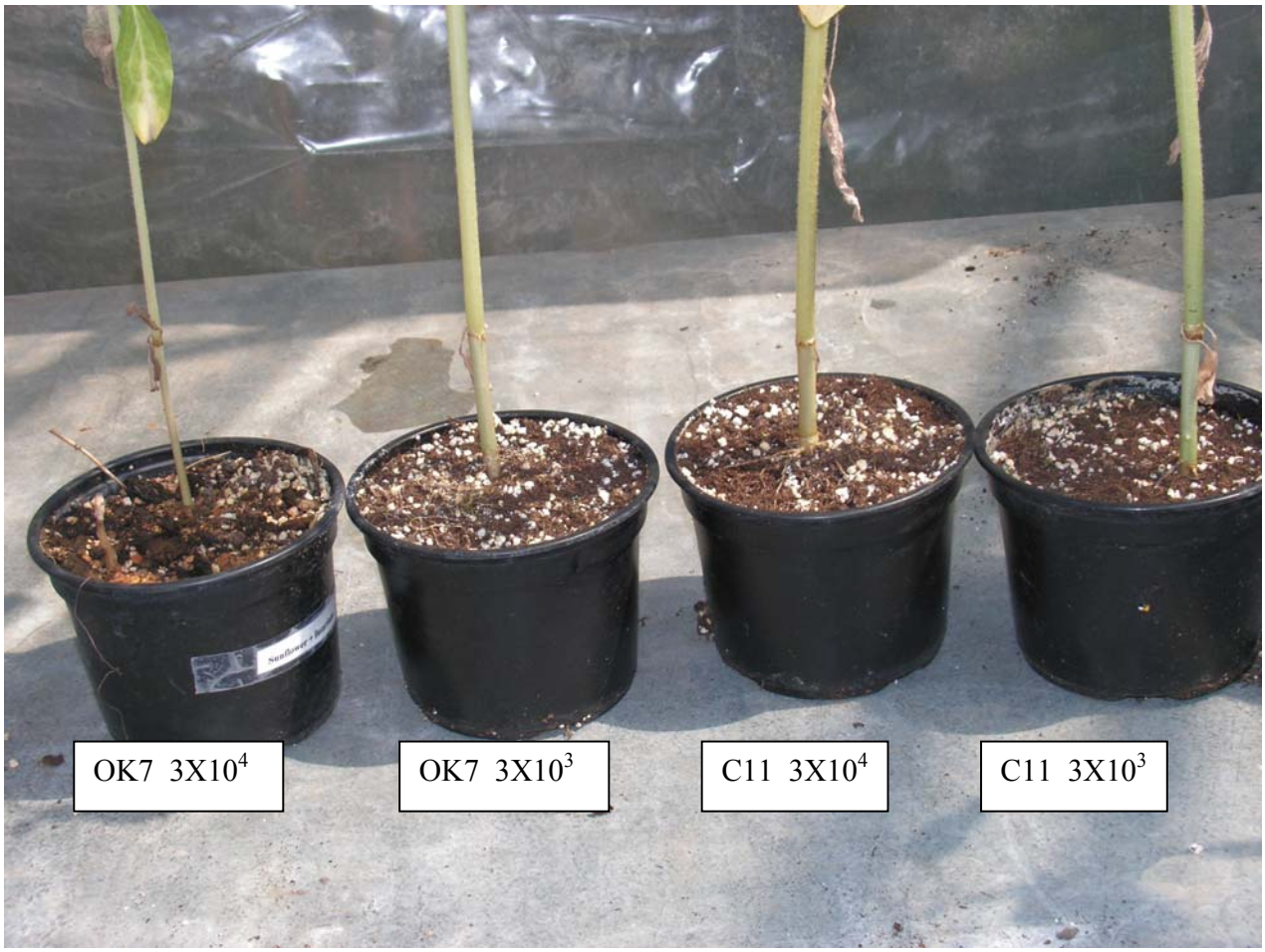


Figure 17. Preemergence treatment with Fusarium extract incorporated in soil at time of planting. Figure shows reduced or no emergence of *Orobanche cumana* because of the pathogenicity effect of *Fusarium oxysporum.*

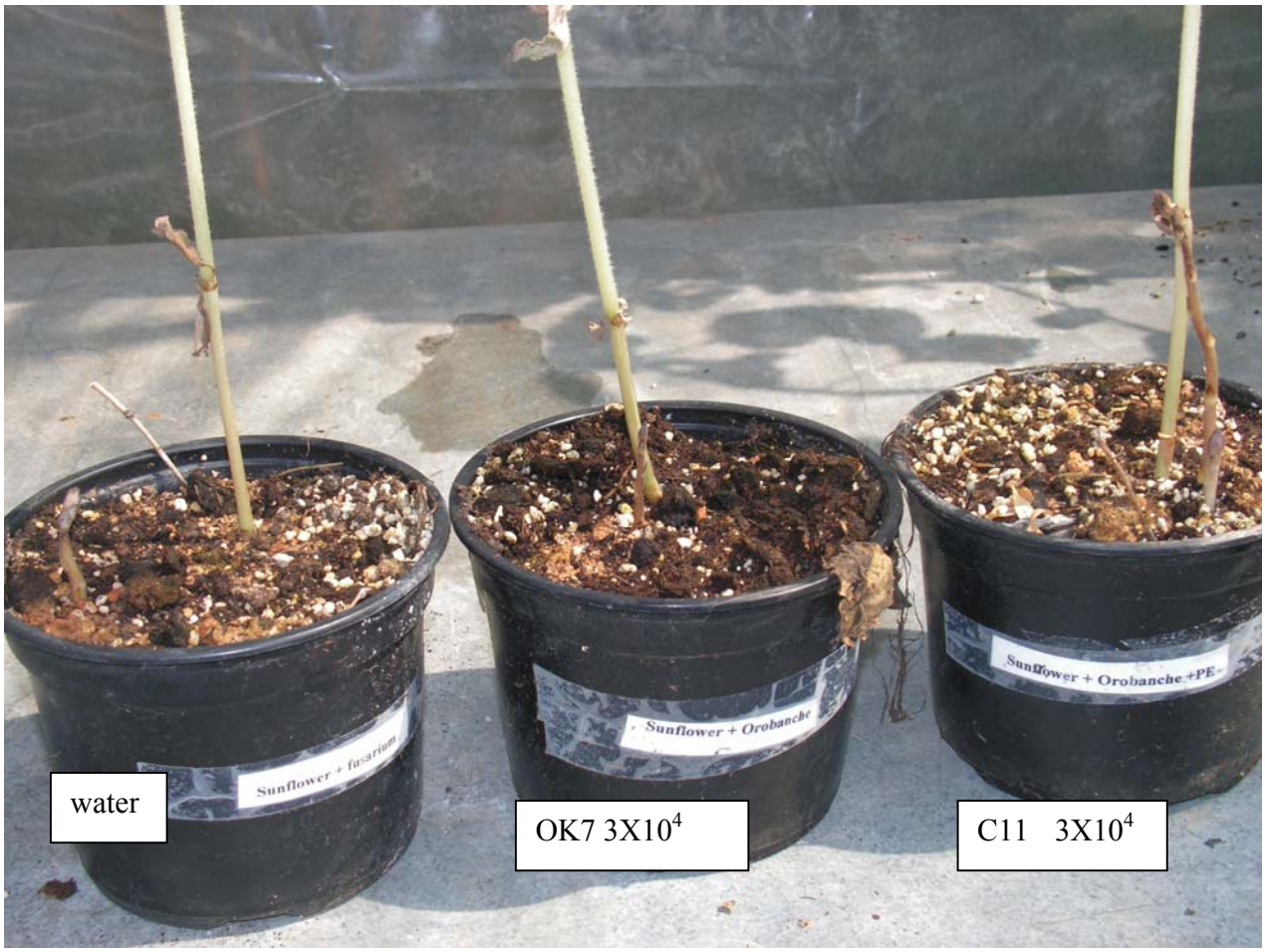


Figure 18. Post emergence -14 days after application of Fusarium extract. Figure shows the wilting of Orobanche caused by the fusarium extracts. This is field evidence of the pathogenicity and phytotoxicity of *Fusarium oxysporum* on Orobanche species.

Figure.19
Back row: Reduced emergence of Orobanche because of treatment with Fusarium extract before planting.
Front row: High emergence of Orobanche because of zero treatment before planting

Figure 20.Late appearance of *Orobanche crenata* after *Vicia faba* has matured and harvested.(Positive effect of *Fusarium oxysporum* applied at $3X10^4$ spore/mL before planting).

The polyethylene bag and pot experiments conducted in this study were successful in evaluating the effects of *Fusarium oxysporum* strains in controlling broomrape either in the pre-emergence or post emergence stages.

3.2 ISOLATION OF METABOLITES.

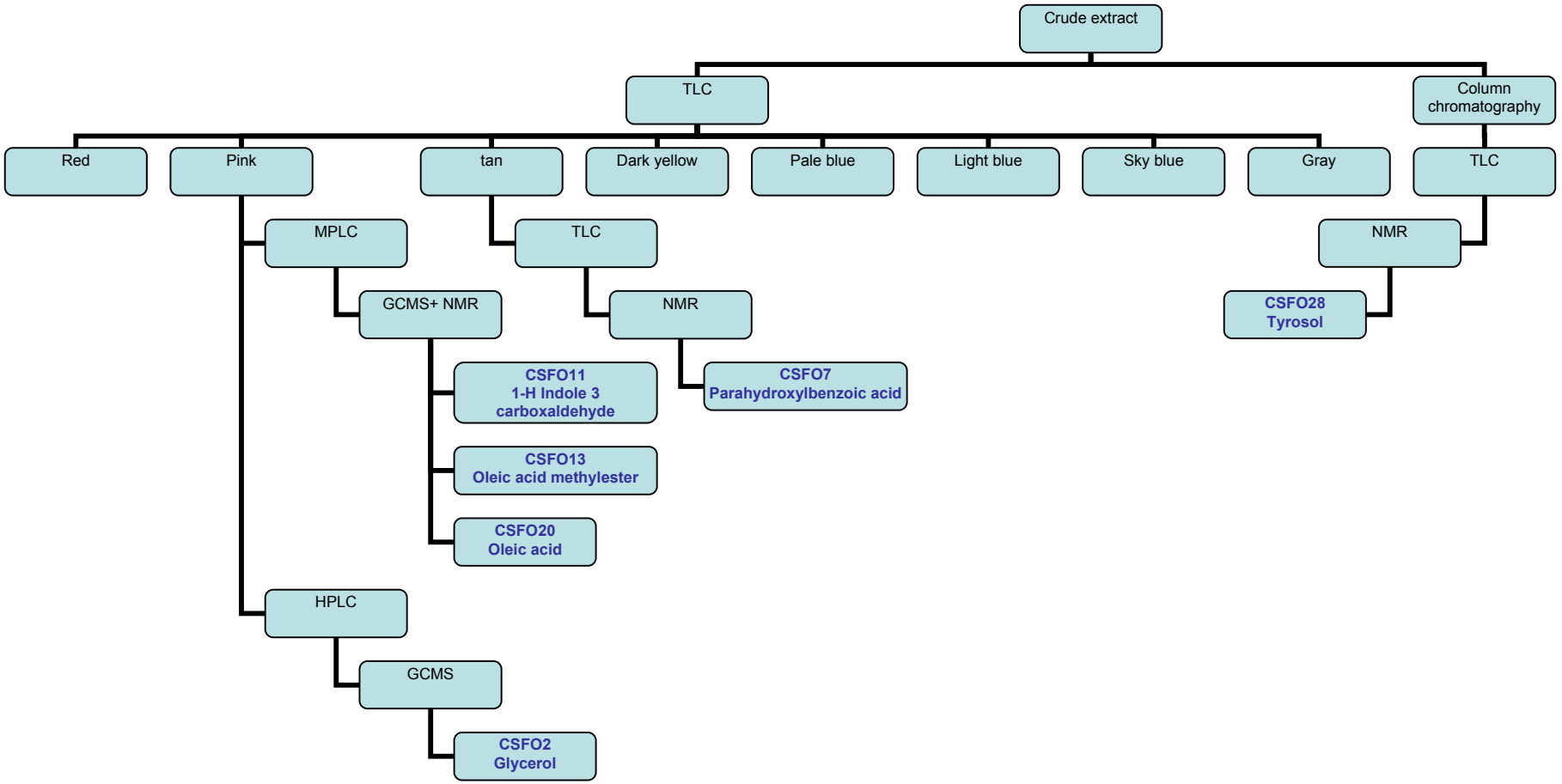
After removing the solvents used in the extraction process using a flash evaporator, a crude residue weighing 6g was collected which proved toxic to Orobanche species tested.
The crude residue on TLC using 6.5 petroleum ether: 3.5 ethylacetate contained 8 different bands as detected on TLC plate using short UV light (254nm). The various bands migrated with the R_f values indicated in Table 9.
Part of the crude extract (4.5 g) using the above system was separated by preparative TLC.
The various bands were collected in eppendorf tubes where they were later mixed with acetone. This was vigorously shaken with a shaker before centrifuging at 5000rpm/min at 4ºC for 15 minutes.The supernatant was collected and later evaporated using a vacuum evaporator to collect the residue. When these 8 bands were collected separately and rechromatographed, no other spots were detected. In different solvent systems, the compounds migrated with different R_f values. With acetone: ethyl acetate 7:3 solvent system, the R_f values of the pink and tan fractions were 0.81 and 0.75 respectively.
They are highly soluble in chloroform and sparingly soluble in water. They are fairly stable to heat.
In addition, the crude extract was also separated using column chromatography (6.5 petroleum ether: 3.5 ethylacetate) and medium pressure liquid chromatography (MPLC).
Dichloromethane (solvent A) and methanol (solvent B) were used in the MPLC extraction. The gradient consisted of 100% solvent A and 0% solvent B for the first 20minutes and later gradual reduction of solvent A and increasing solvent B for 120minutes before maintaining an isocratic system of 50% solvent A : 50% solvent B for the last 10 minutes. The flow rate was 10ml/minute and the column was catridge pp 12/150 and the maximum pressure was set at 10mbar while collection of fractions was by tube volumes of 15mL.

Table 9. Rf values and visual appearance of the various bands on TLC plates.

s/n	R_f values	Colour description	Code	Quantity
1	0.06	Red	CSFORD	25mg
2	0.13	Pink	CSFOPK	22mg
3	0.23	Tan	CSFOTN	28mg
4	0.35	Dark yellow	CSFODY	13mg
5	0.42	Pale blue	CSFOPB	27mg
6	0.62	Light blue	CSFOLB	8mg
7	0.77	Sky blue	CSFOSB	6mg
8	0.87	Gray	CSFOGR	43mg

The solvent system used to obtain these fractions (table 9) on TLC was petroleum ether: ethyl acetate 6.5:3.5.

Figure 21. Diagrammatic sketch of the Isolation and identification process of the metabolites



3.3 IDENTIFICATION OF METABOLITES.

The crude residue weighing 6g (obtained from 2kg of culture) when separated using 6.5 petroleum ether: 3.5 ethylacetate on TLC contained 8 different bands as detected on TLC plate using short UV light (254nm). The various bands migrated with the R_f values indicated in Table 9. In a preliminary experiment, 10mg of the pink band (CSFOPK) was ran on HPLC and the substance that eluded at a peak retention time of 4.475 minutes gave a yield of 2.2µg that was further analyzed by LCMS technique to give **glycerol** (**1**) .

6.25mg of the pink band (CSFOPK) with Rf value of 0.13 was further purified using MPLC with an isocratic solvent system of dichloromethane: methanol at a ratio of 98: 2.

This separation afforded three conspicuous fractions 1,2 and 3 with weights of 4.0µg, 19µg and 4.0µg respectively. Further investigations of the resulting conspicuous fractions were performed with GCMS and NMR 1D and 2D experiments. Fraction 1 afforded **1H-indole-3-carboxaldehyde (2),** fraction 2 afforded **Oleic acid methylester (3)** and fraction 3 **Oleic acid (4)** .

The crude extract that was separated by column chromatography (6.5 petroleum ether: 3.5 ethylacetate) gave three conspicuous fractions 1, 2 and 3 with dry weight yields of 40.7, 47.25 and 38.38mg respectively. While fraction 1 was stored for future use because it contains a lot of fatty substances, fractions 2 and 3 were combined and analysed using medium pressure liquid chromatography (MPLC) using dichloromethane: methanol at a ratio of 95:5. The results gave a conspicuous fraction (Rf 0.3) that was later purified on preparative TLC using ethylacetate: cyclohexane at a ratio of 50:50 to give a clean fraction with Rf 0.29 and weighing 5.2µg. This fraction when analyzed using NMR technique gave **tyrosol** (5).

The tan band obtained from TLC weighing 28mg was further developed on TLC using a system of dichloromethane: methanol at a ratio of 97:3. The resulting conspicuous fraction with Rf 0.12 weighing 17.3µg was analysed by NMR technique to give **parahydroxybenzoic acid (6).**

Preliminary phytotoxicity tests conducted with the identified metabolites proved that only 1H-indole-3-carboxaldehyde (2) and tyrosol (5) were selectively active against *Orobanche crenata* and thus were further investigated. Below are some characteristics of the identified metabolites with more emphasis on the two that were were active against *Orobanche crenata.*

3.3.1 Oleic acid methylester (((9Z)-Octadec-9-enoic acid methylester).
It has a chemical formular of $C_{19}H_{36}O_2$ and molecular weight of 296.49 g/mol and appears as a clear liquid. It is insoluble in water and has a density of 0.874 g/mL. The melting point is -20^{o}C and the boiling point is 218 ^{o}C.

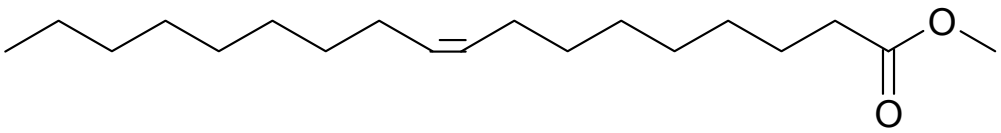


Figure 23 : The chemical structure of Oleic acid methylester

3.3.2. Oleic acid ((9Z)-Octadec-9-enoic acid).
It has a chemical formular of $C_{18}H_{34}O_2$ and molecular weight of 282.46 g/mol and appears as a pale yellow liquid. It is insoluble in water but soluble in methanol and has a density of 0.895 g/mL. The melting point is 13-14^{o}C and the boiling point is 360 ^{o}C.

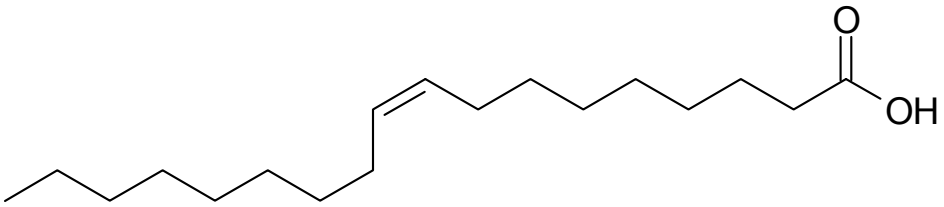


Figure 24.The chemical structure of Oleic acid

3.3.3 Glycerol (propan-1,2,3-triol).
It has a chemical formular of $C_3H_5(OH)_3$ and molecular weight of 92.09 g/mol and appears as a clear liquid. It is insoluble in water and has a density of 1.261 g/mL. The melting point is 17.8^{o}C and the boiling point is 290 ^{o}C.

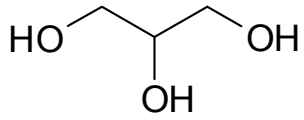


Figure 25.The chemical structure of glycerol

3.3.4.Parahydroxybenzoic acid (4-Hydroxybenzoic acid).
It has a chemical formular of $C_7H_6O_3$ and molecular weight of 138.12 g/mol and appears as a white crystalline solid. It is slightly soluble in water and has a density of 1.46 g/mL. The melting point is 214-217^{o}C and the boiling point is 133 ^{o}C.

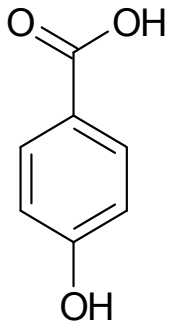


Figure 26.The chemical structure of parahydroxybenzoic acid

3.3.5 1H-indole-3-carboxaldehyde.
It has a chemical formular of C_9H_7NO and molecular weight of 145.16 g/mol. The melting point is 164^{o}C and the boiling point is 342.4 ^{o}C. It has a density of 1.385g/mL.
Indole-3-carboxaldehyde was isolated satisfactorily as a yellowish white amorphous. It has UV absorbances at λ_{max} 206, 243 and 298nm. The ESIMS spectrum showed the pseudo-molecular ion peak at *m/z* 146 $[M+H]^+$. The H-NMR spectrum data showed signals at δ_H 8.17 (1H, d, J=7.4Hz), 7.15 (1H, dt, J=7.4, 2.4 Hz), 7. 18(1H, t, J=7.4, 2.4 Hz), and 7.40 (1H, d, J=7.4 Hz), indicative for an ABCD aromatic spin system. This revealed the presence of an *ortho*-disubstituted benzene ring. The other signal at δ_H 8.00 (1H, s) taken together with the above mentioned signals suggested the presence of 3-substituded indole. The signal at δ_H 9.95 (1H, s) which was not exchangeable suggested the presence of an aldehydic group. This suggestion was confirmed from HMQC data which showed the direct correlation of H-8 with C-8 at δ_c 175.0 and from the HMBC spectrum which showed the correlations of H-2 with C-3, C-3a, C-7a, in addition to the correlations of H-5 with C-3 and C-7;H-4 with C-6 and C-7, and the correlations of H-8 with C-3 and C-3a. From the above data and through the comparison with the literature (Aldrich, 1992, Hiort, 2002) it was confirmed that the compound was Indole-3-carboxaldehyde.

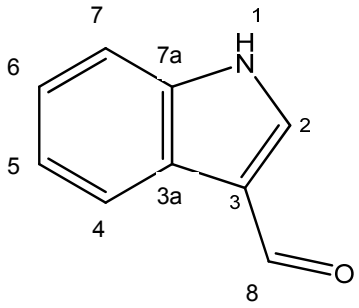


Figure 27.The chemical structure of 1H-indole-3-carboxaldehyde

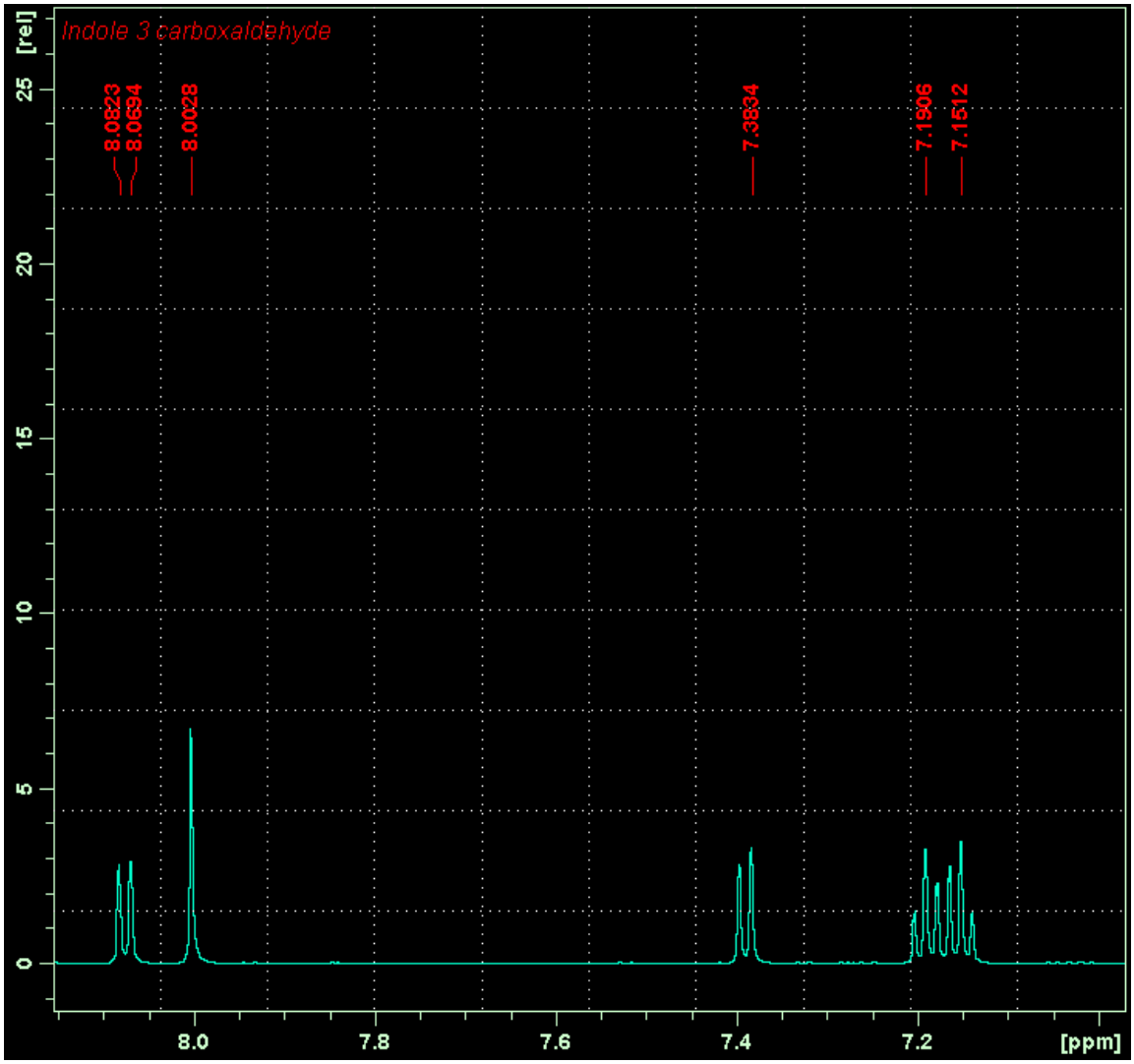


Figure 28. Proton NMR spectrum of 1H-indole-3-carboxaldehyde

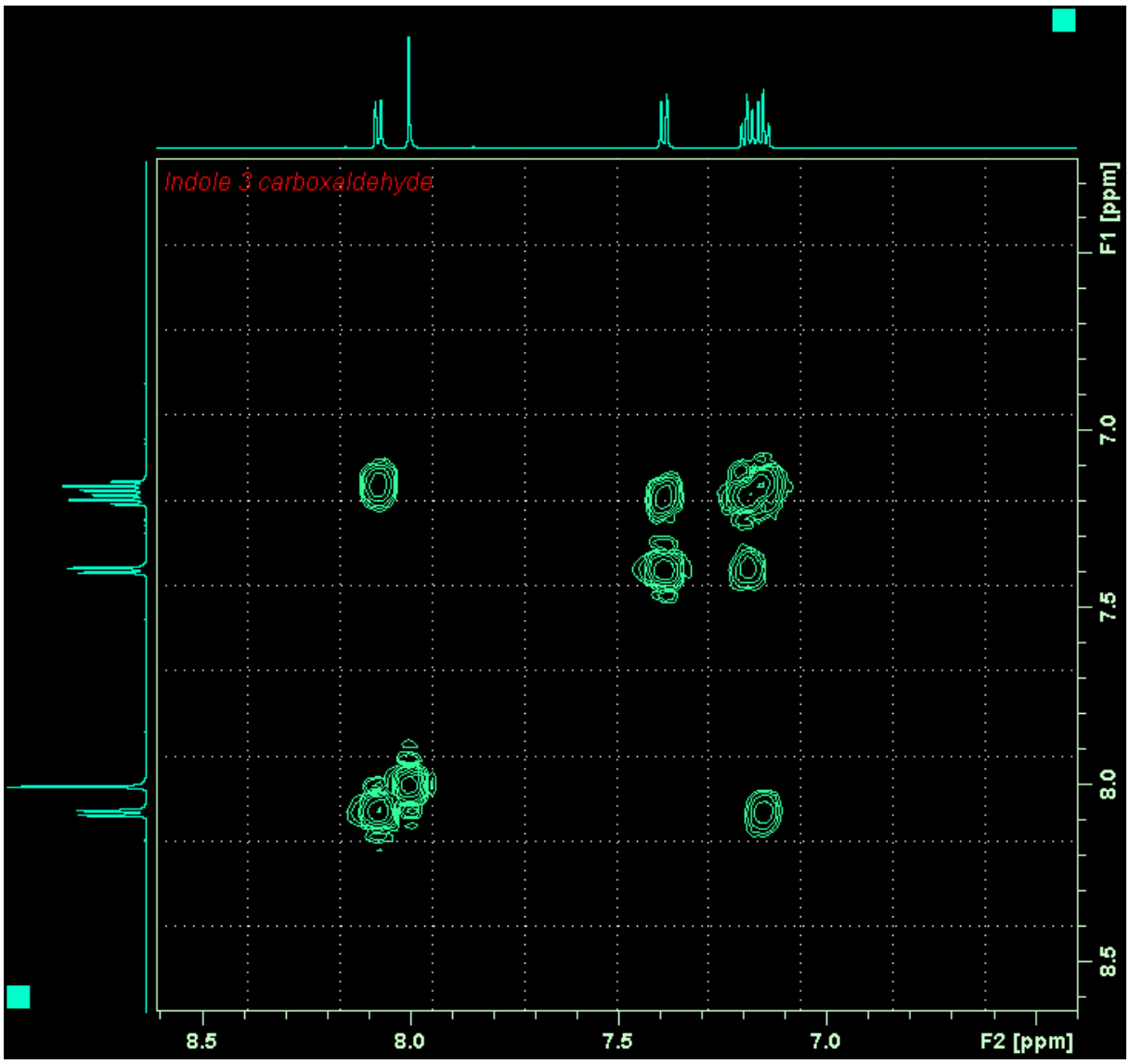


Figure 29.COSY image of 1H-indole-3-carboxaldehyde

In the COSY spectrum of 1H-indole-3-carboxaldehyde it is observed the corelation of the H- 4 at 8.17 ppm with H- 5 at 7.15 ppm, the correlation of H-5 at 7.15 ppm with H-6 at 7.18 ppm and the correlation of H-6 at 7.18 ppm with H-7 at 7.40 ppm.

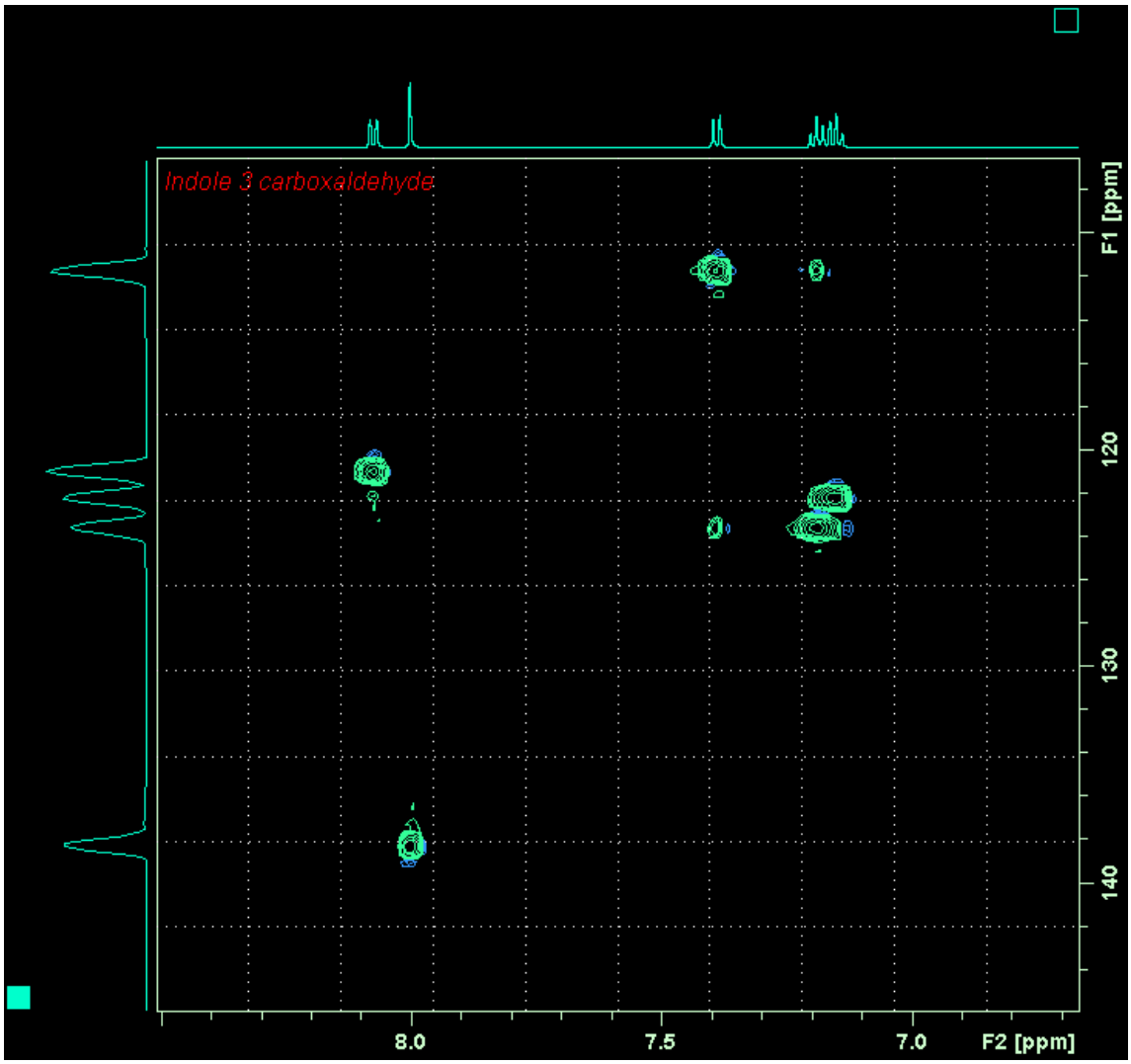


Figure 30.HMQC image for 1H-indole-3-carboxaldehyde

In the HMQC spectrum of 1H-indole-3-carboxaldehyde it is observed the direct proton - carbon shift correlations of H-4 at 8.17 ppm with the C-4 at 121.0 ppm, of H-2 at 8.00 ppm with the C-2 at 138.1 ppm, of H-7 at 7.40 ppm with C-7 at 112.1 ppm, of H-6 at 7.18 ppm with C-6 at 124.0 ppm and of H-5 at 7.15 ppm with the C-5 at 122.0 ppm.

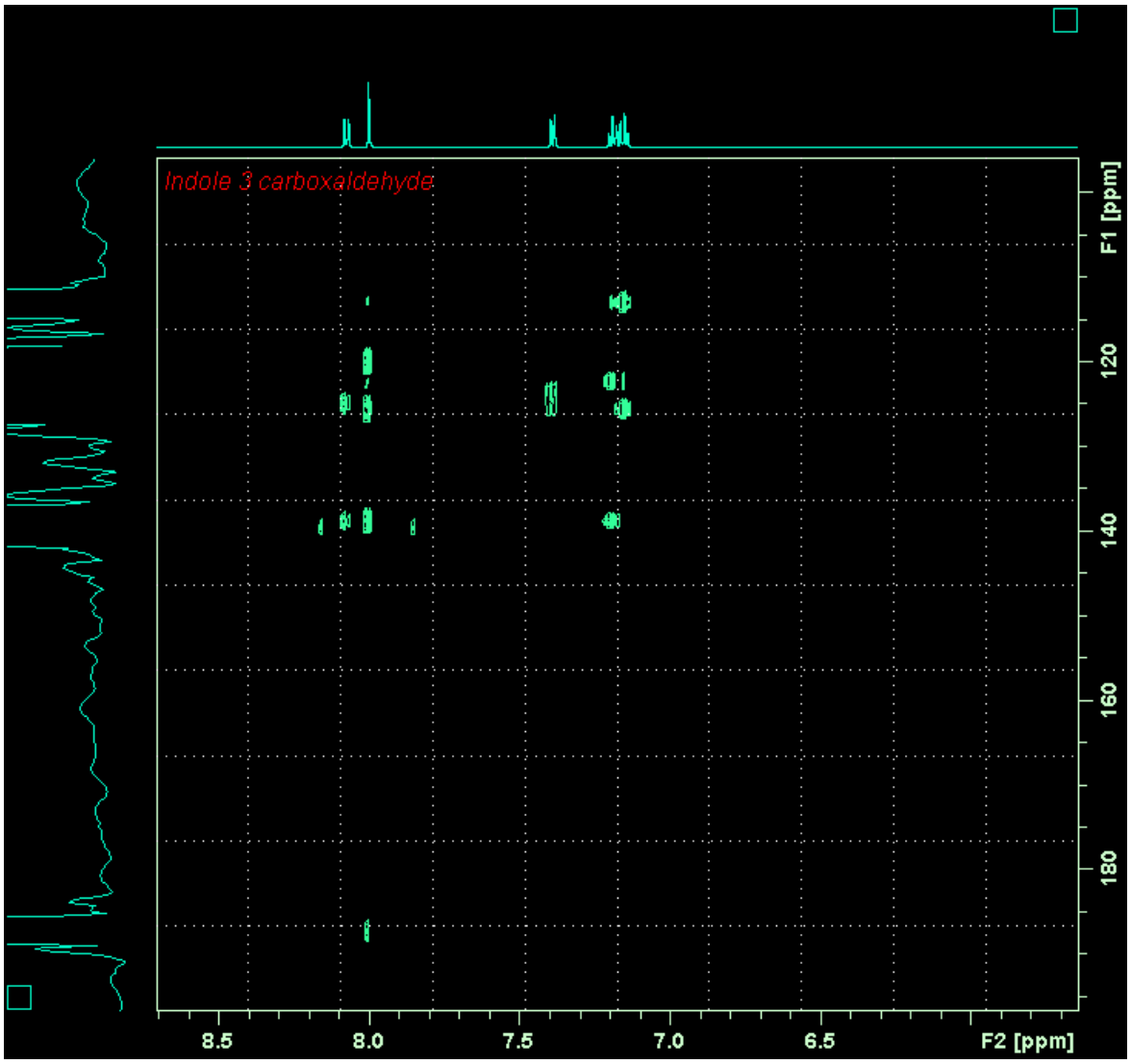


Figure 31.HMBC image for 1H-indole-3-carboxaldehyde

In the HMBC spectrum of 1H-indole-3-carboxaldehyde are observed mainly 2J correlations betwwen H and C of the molecules. More spesifically it is observed the corelation of the H-2 at 8.00 ppm with the carbonyl group of the aldehyde at 186.0 ppm. The same proton has also three other correlations with C-7a at 138.5 ppm, C-3a at 126.5 and the J2 correlation with C-3 at 119.1 ppm. On the second ring the correlation of H-4 at 8,17 ppm with the tertiary carbons C-7a at 138.5 ppm, C-3a at 126.5 is observed. Additionally there is a correlation of H-7 at 7.40 ppm with C-5 at 122.0 ppm, of H-6 at 7.18 ppm with C-7a at 138.5 ppm and C-4 at 121.0 ppm and of H-5 at 7.15 ppm with C-7 at 112.1 ppm and the tertiary C-3a at 126.5 ppm.

Table 10.1H and ^{13}C NMR data of 1H-indole-3-carboxaldehyde (600 MHz), δppm (j), CDCl3

Position	δH	δC
1	12.10(s)	-
2	8.00 (s)	138.1
3	-	119.1
3a	-	126.5
4	8.17 (*d*, 7.4 Hz)	121.0
5	7.15 (*dt*, 7.4, 2.4 Hz)	122.0
6	7.18 (*dt*, 7.4, 2.4 Hz)	124.0
7	7.40 (*d*, 7.4 Hz)	112.1
7a	–	138.5
8	9.95 (s)	186.0

3.3.6 Tyrosol (4-(2-Hydroxyethyl)phenol).

It has a chemical formular of $C_8H_{10}O_2$ and molecular weight of 138.16 g/mol. The melting point is 91-92^{o}C and the boiling point is 158 ^{o}C. It has a density of 1.168g/mL. The ^{13}C NMR spectra of the metabolite indicated the presence of 6 carbons in the molecule. The aromatic nature of this compound was verified by means of ^{1}H NMR spectrum that showed two doublets at δ 7.10 (2H, δ, J = 8.4 Hz) and δ 6.78 (2H, δ, J = 8.4 Hz), and to complete this spectrum, two methylenes were coupled according to COSY spectrum at δ 3.83 (1H, δ, J = 6.5Hz) and δ 2.80 (1H, δ, J = 6.5Hz). The spectra of ^{1}H NMR and HMQC confirmed the existence of a benzene ring with substitution 1,4. Spectrum HMBC allowed to establish the connectivities of quaternary carbons and to propose structure of the metabolite Tyrosol.

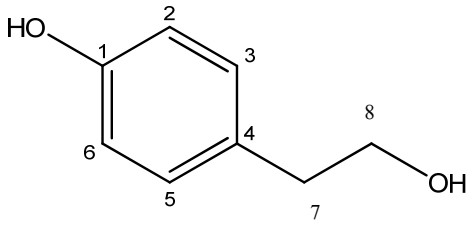


Figure 32.The chemical structure of Tyrosol

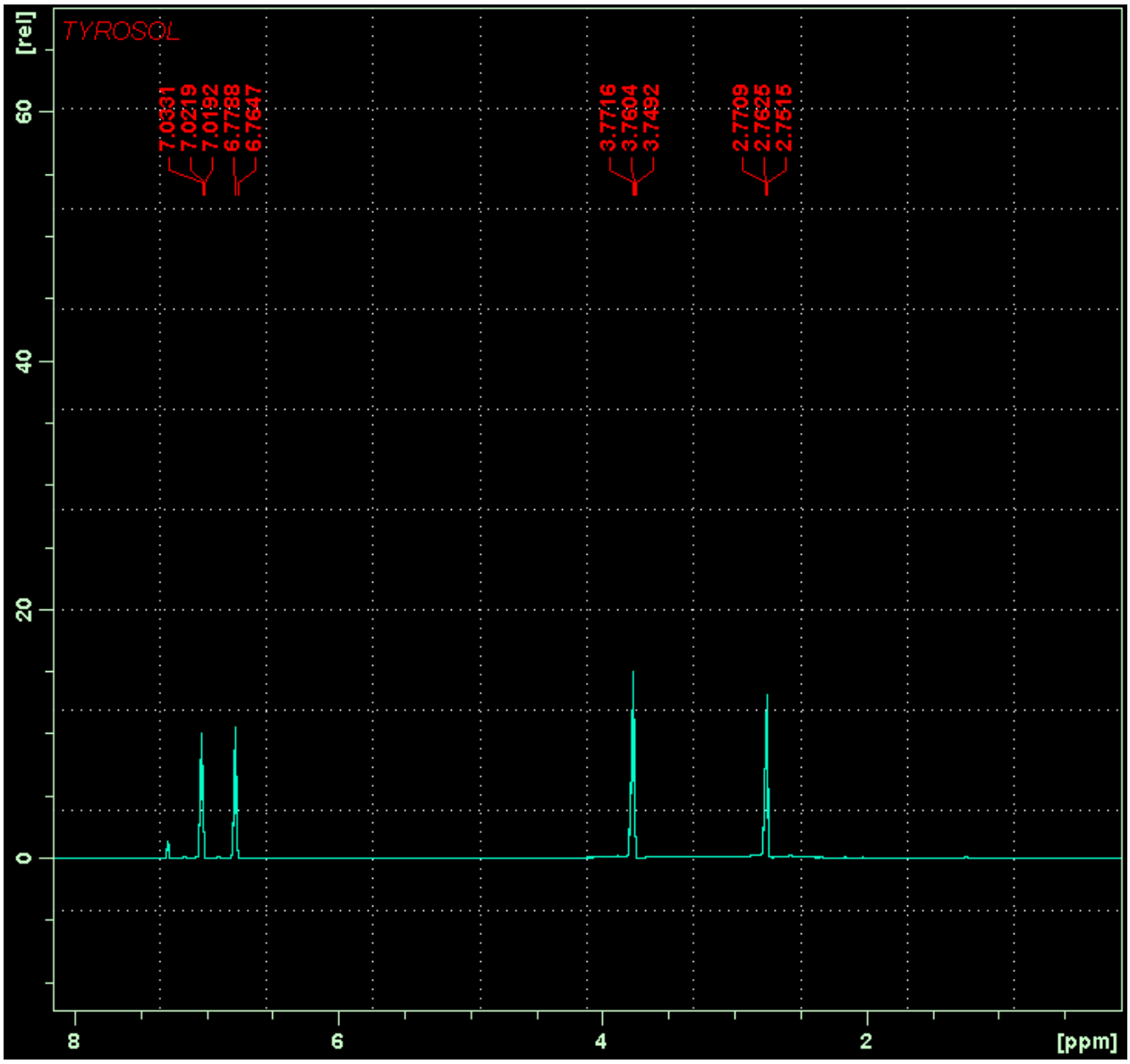


Figure 33.Proton NMR spectrum of Tyrosol

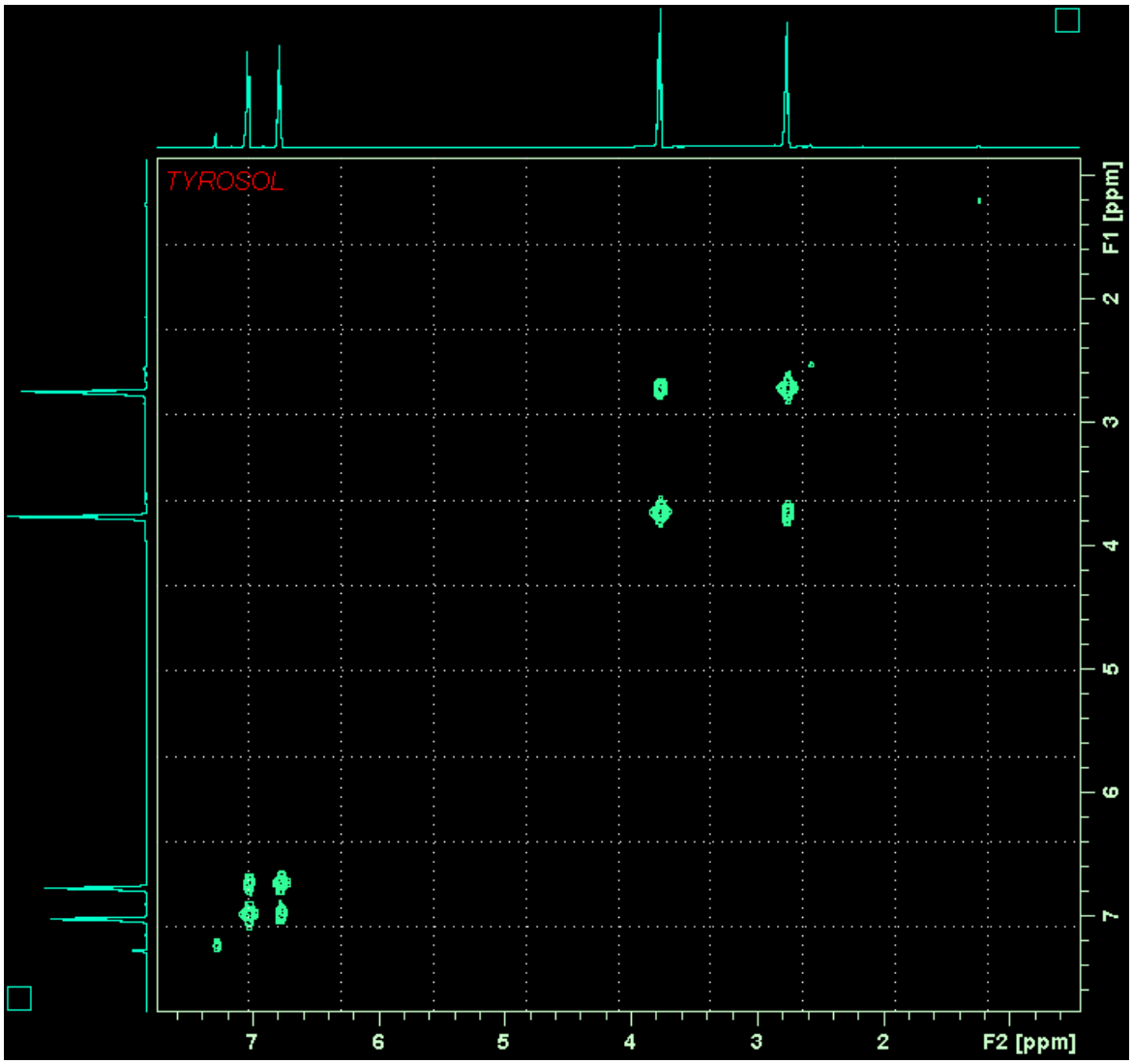


Figure 34.COSY image of Tyrosol

In the COSY spectrum of Tyrosol it is observed the correlation of the aromatic protons H-2 and H-6 with the aromatic protons H- 3 and H-5 respectively. Additionally it is observed the correlation of aliphatic proton H-7 at 2.80 ppm with the H-9 at 3.83 ppm.

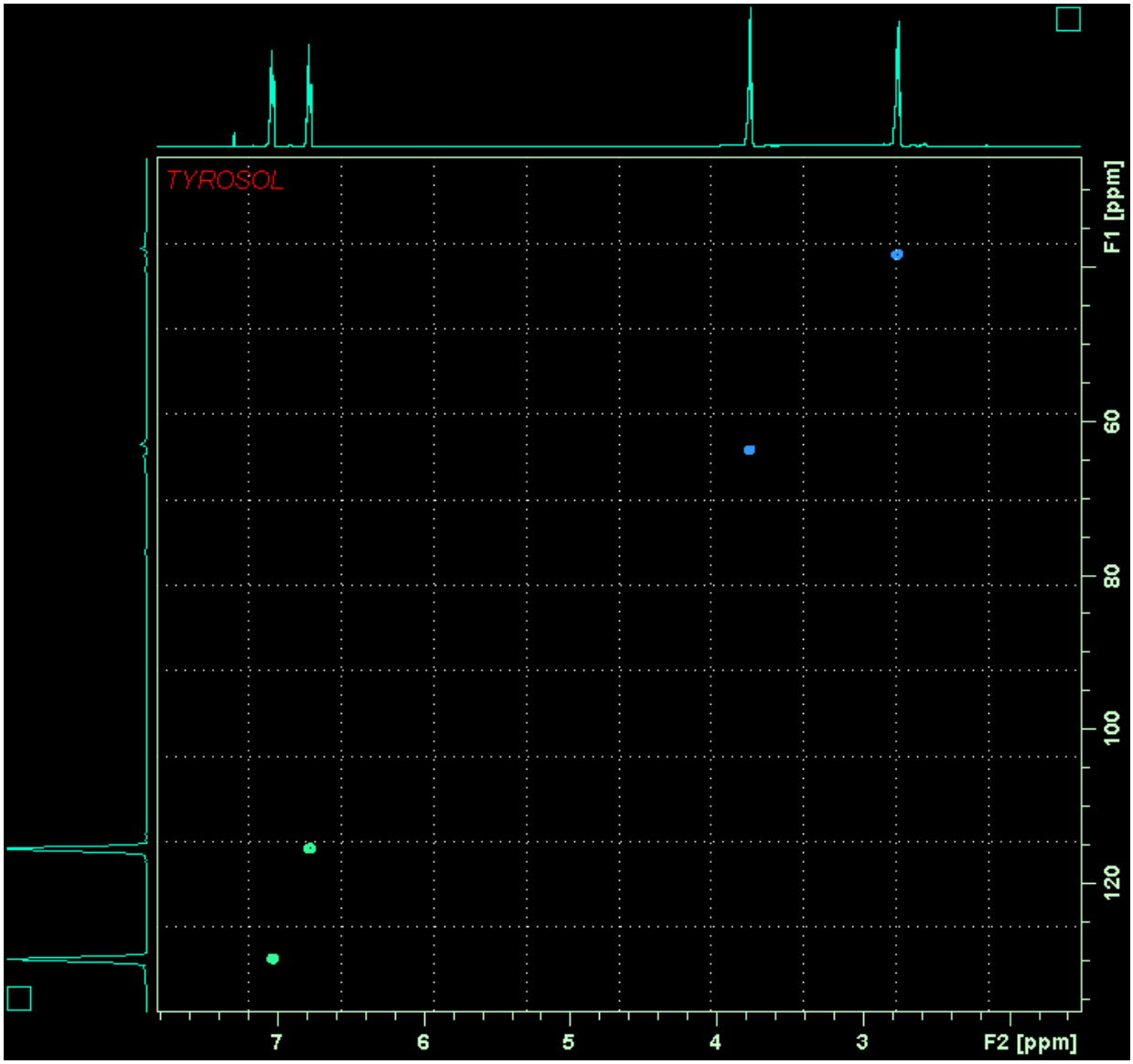


Figure 35.HMQC-DEPT image of Tyrosol

In the HMQC spectrum of Tyrosol it is observed the direct proton-carbon shift correlations of H-3 and H-5 at 7.10 ppm with the corresponding carbons C-3 and C-5 at 130.1 ppm as well as the corelation of H-2 and H-6 at 6.78 ppm with the coresponding carbons C-2 and C-6 at 115.4 ppm. Furthrmore the methylenes H-7 at 2.80 ppm and H-8 at 3.83 ppm are correlated with the coresponding carbons at C-7 at 38.2 and C-8 at 63.4 ppm.

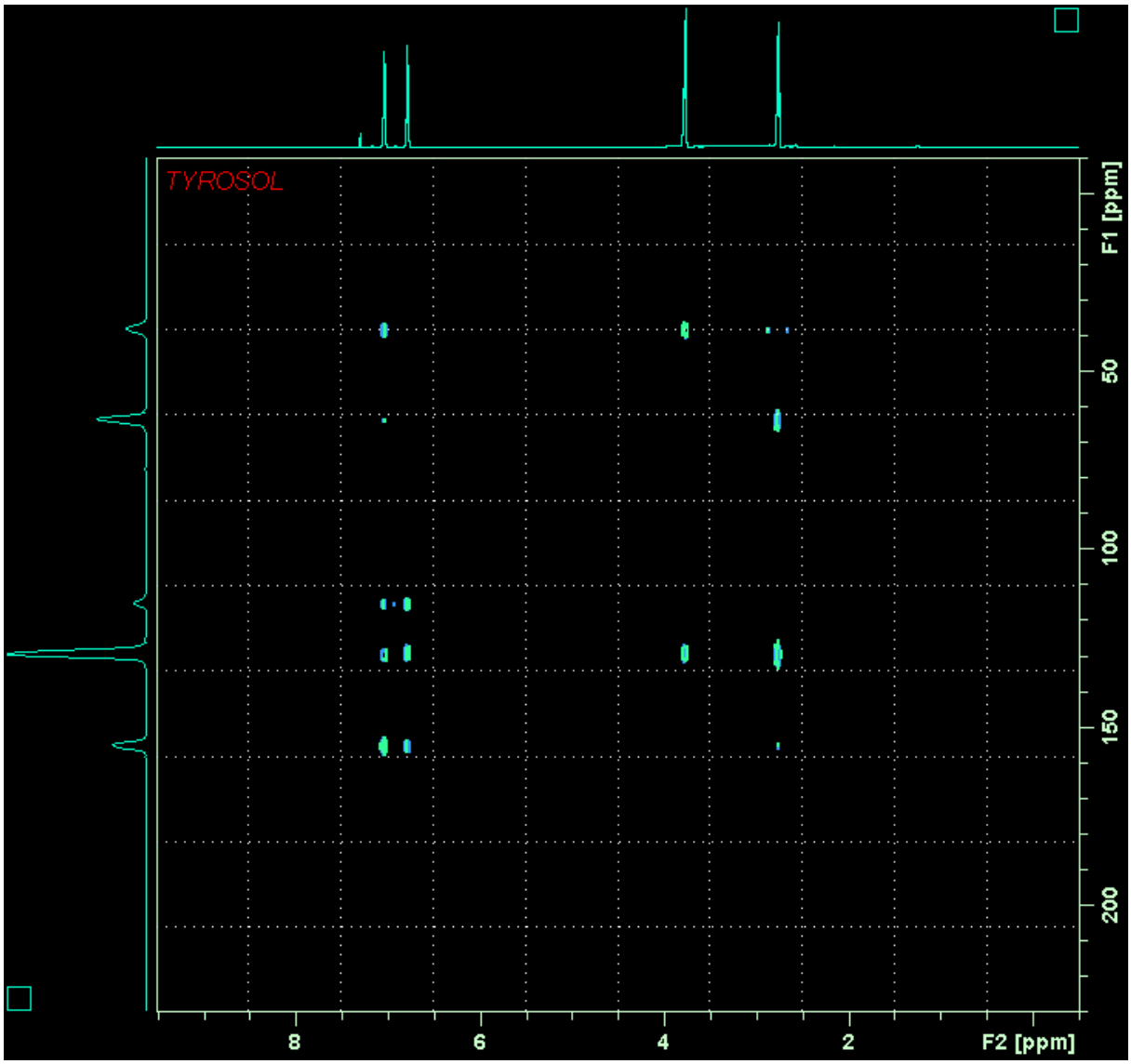


Figure 36.HMBC image of Tyrosol

In the HMBC spectrum of Tyrosol it is observed the J-2 and J-3 correlations of H-2, H-3, H-5 and H-6 with the carbons C-1 at 154.2 ppm, C-3 and C-5 at 130.1 ppm and with C-2 and C-6 at 115.4 ppm. Additionally protons H-3 and H-6 have a J-3 correlation with an aliphatic carbon at 38.2 ppm which corresponds to C-7. The same carbon has also correlation with the proton at 3.83 ppm which corresponds to H-8. This proton has also a J-2 correlation with the C-4 at 130.4 ppm. With the same carbon C-4 also a J-3 correlation of the proton H-7 at 2.80 ppm can be observed. This proton H-7 finally has a J-2 correlation with the carbon at 63.4 ppm that corresponds to C-8.

Table 11. ^{1}H and ^{13}C NMR data Tyrosol (600MHz), δ ppm (J), $CDCl_3$

Position	δH	δC
1		154.2
2	6.78d (8.4)	115.4
3	7.10d (8.4)	130.1
4		130.4
5	7.10d (8.4)	130.1
6	6.78d (8.4)	115.4
7	2.80t (6.5)	38.2
8.	3.83t (6.5)	63.4

3.4 INFLUENCE OF GROWTH STIMULANTS (GR24 AND NIJMEGEN) ON GERMINATION OF OROBANCHE SPP.

After conditioning and stimulation with GR24 and Nijmegen-1, the germination rates were far higher than what was obtained with non conditioned seeds. The most suitable concentration of these two stimulants was thus evaluated for three *Orobanche species* with results indicated in Table 15. GR24 at a concentration of 10^{-6}M gave the optimal germination response of *O .aegyptiaca, O. cumana* and *O. crenata* with corresponding germination percentages of 70, 55 and 65%, respectively. Increase in the concentration of the stimulant from 10^{-5} to 10^{-6} resulted in an increase in germination percentage in all the three species of Orobanche.On the contrary, an increase in concentration of the stimulant from 10^{-6} to 10^{-7} resulted in a decrease in germination percentage.

With Nijmegen as germination stimulant, a concentration of 10^{-5}M gave the optimum concentration for germination in all three Orobanche species. *O. aegyptiaca, O.cumana* and *O.crenata* recorded germination percentages of 40, 35 and 40% respectively. Further increase in concentrations from 10^{-5} to 10^{-6} and 10^{-7} M resulted in a decrease in germination percentages in all three species of Orobanche. Comparing the effectiveness of GR24 and Nijmegen 1 in stimulating Orobanche seeds, Nijmegen 1 is considerably less active than GR24 and probably that is the reason GR24 has been widely used as a germination stimulant by weed researchers. This is in agreement with the work done by Wigchert et al 1999.

This also confirms the research of Hess et al. (1992) which proved that Striga seeds require preconditioning before responding adequately to germination stimulants. Also, Nijmegen 1 has been reported by Nefkens et al (1997) as a germination stimulant for *Striga* and *Orobanche spp* seeds. As suggested by Rodriguez-Conde et al.(2004) for more studies to be

carried out to clarify the specific stimulant requirements for various *Orobanche* species, this research has made an effort to determine the required GR24 and Nijmegen 1 stimulant for *Orobanche crenata*, *Orobanche cumana* and *Orobanche aegyptiaca* for *in vitro* culture of Orobanche.

Table 12.
Effect of germination stimulants GR 24 and Nijmegen-1 on germination of broomrape seeds.

Growth stimulant	**Concentration** (M)	**Germination of *O.aegyptiaca*** % ± SEM	**Germination of *O.cumana*** % ± SEM	**Germination of *O.crenata*** % ± SEM
GR 24	10^{-5}	50 ±5.7ac	46.7 ± 6.6ab	55±2.8ab
	10^{-6}	70± 2.8b	55 ± 2.8a	65±5.0a
	10^{-7}	45 ± 2.8acd	40 ± 5.7ab	45 ± 2.8bcd
NIJMEGEN	10^{-5}	40± 5.7acd	35 ± 2.8ab	40±5.7cd
	10^{-6}	35±2.8d	35 ± 5.7ab	40±5.0cd
	10^{-7}	20±2.8e	15 ±5.0c	20±2.8e
Control(water)		5.3 ±0.33f	3.3 ± 0.33d	2 ± 0.33f

Data represent means of 3 replicates. Means for the same specie of Orobanche within a column followed by a different letter are significantly different at $P \leq 0.05$ according to Duncan student t test. (SEM = standard error mean).

3.5 EFFECT OF GROWTH HORMONES (IAA AND 2,4 D) ON THE MORPHOLOGY OF OROBANCHE CALLUSSES.

Between 21 and 60 days, germination of Orobanche seeds and formation of calli occurred when the seeds were transferred on the modified Gamborg's B5 medium depending on the Orobanche specie and the growth regulator (Table 15). *O. aegyptiaca, O. cumana* and *O. crenata* exposed to GR24 at a concentration of 10^{-6} M gave the highest germination percentage compared to Orobanche spp exposed to GR 24 at concentrations of 10^{-5} M and 10^{-7} M. Also *Orobanche spp* exposed to Nijmegen 1 at a concentration of 10^{-5} mol/L gave the highest germination percentage compared to Orobanche spp exposed to Nijmegen at concentrations of 10^{-6} M and 10^{-7} M. From results obtained, it is realised that *Orobanche aegyptiaca* produced more calluses than the other two species in both growth regulators (1AA and 2, 4 D).With 2, 4- D, 70% of the germinated seeds formed calluses as against 60% with IAA. It took *O. aegyptiaca* 21-30 days to germinate and form calli and the calluses survived for about eleven weeks on these media. With 2, 4- D,
O. aegyptiaca produced soft, white, large and massive calluses and with IAA as growth regulator, calluses with root-like protrusions were formed.
Germinated *Orobanche crenata* seeds formed 60% and 50% calluses on media containing 2,4 D and IAA respectively. It took *O. crenata* 30-60 days to germinate and form calluses and the calluses survived in these media for approximately 12 weeks. *O.crenata* survived longer than *O. aegyptiaca.* With 2,4 D, *O.crenata* produced soft, white, large and massive calluses while with IAA as growth regulator, calluses with root-like protrusions were formed from seeds of *O. crenata* .
Orobanche cumana seeds were poorly germinated resulting in fewer calluses production in both 1AA and 2, 4- D contained media. Just 40% of the germinated seeds formed calluses in both IAA and 2, 4- D containing media. It took *O.cumana* 30-45 days to germinate and form calluses and the calluses survived for a shorter period of 10 weeks. *O.cumana* produced a slow growing type which was yellow to brown in colour for both 2, 4-D and IAA supplemented media.
From results obtained, *O.cumana* responded least to the growth regulators compared to the other Orobanche species.
It is worth noting that the percentage of callus formation was not related to the percentage of germination since callus formation depended mostly on the growth regulator in the media. Also, at the early stage of development, the young calluses of all three *Orobanche species* were white and had a spherical shape.
Results also indicate that B5 medium without growth regulators could not support the development of calluses.
This research sheds some light into massive production of *Orobanche* spp calluses for anatomical, morphological, and physiological studies which could be useful in the search for a suitable herbicide in controlling this parasitic weed.
From results obtained, *O .cumana* responded least to the growth regulators compared to the other *Orobanche* species .This therefore is in agreement to Mangnus et.al., 1992 and Westwood, 2000 who reported that not all *Orobanche* sp. respond to the same type of stimulant. The above results agree with Wolf & Timko (1991) who reported that MS medium supplemented with 2, 4- D improved calluses yield of *Striga asiatica* L. Similarly, Ben Hod et al., 1991 and Batchvarova et al., 1999 had reported that growth regulators are necessary for the production of Orobanche callus.

While other researchers like Batchvarova et. al, have studied the influence of some growth regulators on callus production on *Orobanche ramosa*, little has been done to show the influence of these regulators and other regulators on other *Orobanche spp*. This research aimed at determining the influence of some growth regulators on three different *Orobanche species* .This is important because it gives a general overview of the type of calluses produced by different growth regulators and how long these calluses can survive on these media.

Zhou et al., 2004 had carried out a study on the influence of GA3 (Gibberellic acid), IAA (Indole-3- acetic acid) and NAA (1-naphthaleneacetic acid) on callus production of *O. ramosa*, *O. aegyptiaca* and *O. minor*.

In the absence of similar studies on *O. crenata* and *O. cumana* which are principal parasitic weeds in the Mediterranean region, this study therefore provides some information on production of *O. crenata* and *O. cumana* calluses using IAA and 2,4-D growth regulators.

Table 13
Callus production and morphology in relation to influence by 2, 4 Dichlorophenoxyacetic acid 2,4-D and Indole-3- acetic acid (IAA)

Chemical(M)	**Callus production**					
	O.crenata		***O.cumana***		***O.aegyptiaca***	
	% callus formed from germinated seeds.	**morphology**	**% callus formed from germinated seeds.**	**morphology**	**% callus formed from germinated seeds.**	**morphology**
2,4-D (1)	60±2.8a	Soft, white to yellow, large, massive and amorphous protrusions.	40±5.7 a	Soft, yellow to brown, slow growing	70 ±2.8a	Soft, white, large, massive and amorphous protrusions
IAA(3)	50 ±2.8a	Hard, yellow to brown, large root-like protrusions	40±5.0 a	Hard, yellow to brown, slow growing	60 ±4.4a	Hard, white to brown, large many root-like protrusions with attachment organs
Control B5(0)	0±0b		0±0b		0±0b	

Data represent means of 3 replicates. Means for the same specie of Orobanche within a column followed by a different letter are significantly different at $P \leq 0.05$ according to Duncan student t test.

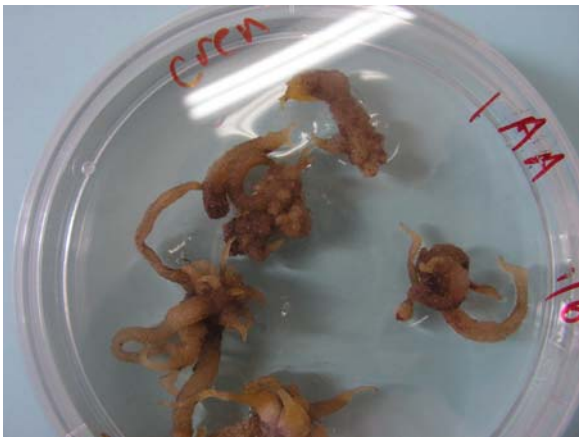


Figure 37 . Morphological appearance of *Orobanche crenata* callus on IAA Medium 14 days after first appearance. Hard, yellow to brown, large root-like protrusions as a result of influence of IAA.

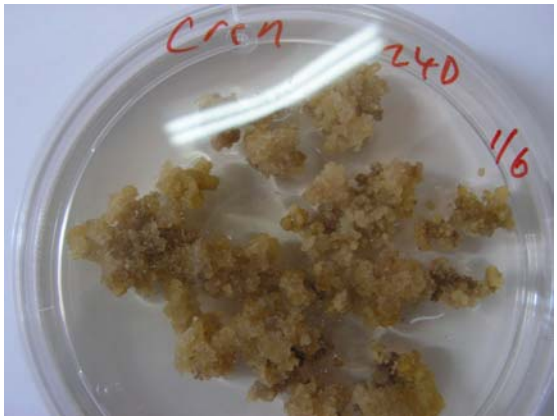


Figure 38. Morphological appearance of *Orobanche crenata* callus on 2, 4-D Medium 14 days after first appearance. Soft, white to yellow, large, massive and amorphous protrusions as a result of influence of 2, 4-D.

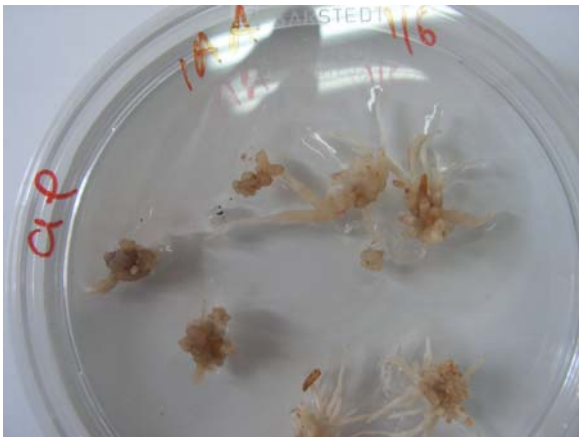


Figure 39. Morphological appearance of *Orobanche aegyptiaca* callus on IAA Medium 14 days after first appearance. Hard, white to brown, large many root-like protrusions with attachment organs as a result of influence of IAA.

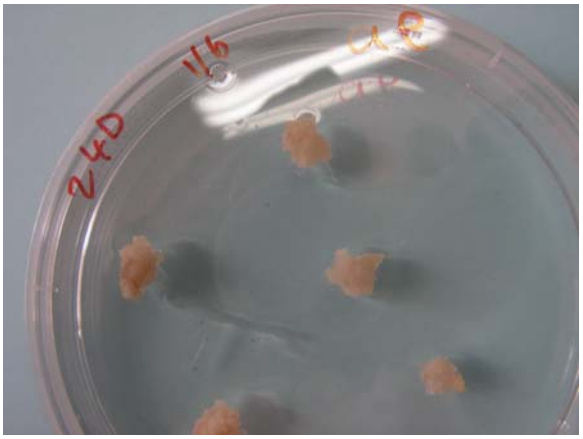


Figure 40. Morphological appearance of *Orobanche aegyptiaca* callus on 2, 4-D 14 days after first appearance. Soft, white, large, massive and amorphous protrusions as a result of influence of 2, 4-D.

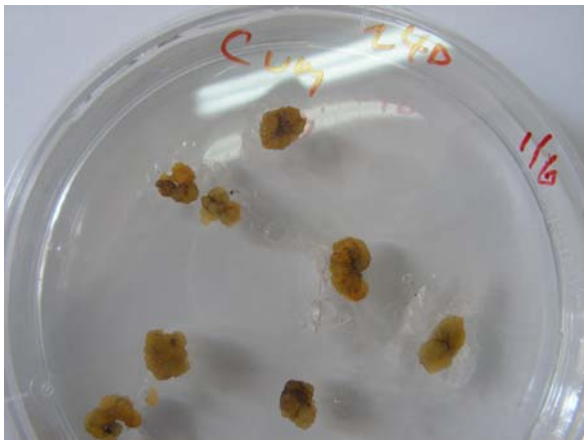


Figure 41. Morphological appearance of *Orobanche cumana* callus on 2, 4-D Medium 14 days after first appearance. Soft, yellow to brown, slow growing callus as a result of influence of 2, 4-D.

3.6 PHYTOTOXICITY OF METABOLITES.

Fungi of diverse taxa and ecological groups have previously been reported to produce phytotoxic metabolites (Vey et al., 2001). Strains (or even a single strain) of a certain fungus may synthesize several phytotoxins differing in the spectra of their biological activities. Also it has been reported by other researchers (Berestetskiy, 2008) that microorganism strains are genetically unstable and their storage or reinoculation may affect their ability to produce toxins. Before this research, no work has been carried out on *Fusarium oxysporum* strains obtained in Greece and their potential of producing toxins that are phytotoxic to Orobanche species. The two strains obtained in Greece from infected *Orobanche crenata* were tested for their ability to produce phytotoxic metabolites. They produced 1H-indole-3-carboxaldehyde and Tyrosol. This confirms the work of Dor et al 2007 which reported that *F.oxysporum* contains at least two main toxic metabolites (fusaric acid and 9, 10-dehydrofusaric acid) that caused necrosis and wilting of various plants and led to mortality of germinating seeds of *O. cernua, O. aegyptiaca, O. ramosa* and *O. cumana.*

3.6.1. Bioassay with fractions obtained from TLC.

From results obtained from bioassay conducted on *Orobanche crenata* calluses and the host plants, *Vicia faba* and *Vicia sativa*, it is of interest to learn that *Fusarium oxysporum* produces metabolites that are toxic to broomrape even at half strength concentrations but only toxic to host plants at full strength concentrations as is the case with Red (table 14 and 15). While so much work has been done on the influence of chemicals on the germination and growth of Orobanche tissue cultures, there is lack of literature on the effect of chemicals on orobanche tissue cultures when exposed to chemicals. Results obtained from the bioassay conducted with the various bands on *O. crenata* and *Vicia faba / Vicia sativa* using glyphosate as control, indicated that the chemicals contained in these bands could be successfully used in controlling *O.crenata* without harm on the host at various concentrations (table 14,15 and 16). When compared to glyphosate, a synthetic herbicide, red, pink and dark yellow had effect on Orobanche at full strength similar to glyphosate at 5 and 10 $\mu g.mL^{-1}$. Glyphosate at 10 $\mu g.ml^{-1}$ caused 31.59% growth inhibition of cultures of *O.crenata* after 36hours of exposure while pink, dark yellow , red and tan at full strength caused 27.64, 27.07, 16.1 and 15.46% growth inhibition respectively.At same concentrations glyphosate, dark yellow, pink, red and tan caused 32, 30, 25, 15 and 8% growth inhibition respectively on *V.faba* leaf tissues after 36hours of exposure.

From visual observations, glyphosate at 10 $\mu g.ml^{-1}$ effected 11-30, 5-10 and <5% discoloration of *O.crenata*, *V.faba*, and *V.sativa* tissues respectively. On the otherhand, dark yellow at full strength effected 11-30% discoloration on both *O. crenata* and *V. faba* tissues but <5% discoloration on *V.sativa*. Among all the chemicals tested on *V.sativa*, only pink at full strength caused a <5% discoloration of the tissues. On *V.faba*, pink at full strength and glyphosate at 10 $\mu g.ml^{-1}$ effected 5-10% discoloration of tissues while dark yellow at full strength caused 11-30% discoloration. On *O. crenata* , pink and dark yellow at full strength and glyphosate at 10 $\mu g.ml^{-1}$ had the highest effect 11-30% discoloration followed by glyphosate 5 $\mu g.mL^{-1}$, dark yellow half and a quarter strength, tan full strength, pink half strength and red full strength all effecting 5-10% discoloration of tissues.

Pink at full strength concentration, had a mild effect on *Orobanche crenata* but relatively no effect on *V. faba* and *V. sativa.*

Similarly, Pink and dark yellow at full strength had serious effect on *Orobanche crenata* same as glyphosate used at 10 $\mu g.ml^{-1}$ while they had mild effect on *V. faba* except BG that also had serious effect on *V. faba*.

Table 14: Effect of preparations of *Fusarium oxysporum* strain OK7 on weight of tissue culture of *O.crenata* after 36hours of exposure.

TREATMENTS	INITIAL WT (g)	WT AFTER 36HRS(g)	%growth inhibition
R full strength	1.0	0.8573	16.1
R ½ strength	1.0	0.937	8.27
R ¼ strength	1.0	0.9506	6.95
P full strength	1.0	0.7392	27.64
P ½ strength	1.0	0.8649	15.34
P ¼ strength	1.0	0.949	7.1
T full strength	1.0	0.8636	15.46
T ½ strength	1.0	0.9558	6.44
T ¼ strength	1.0	0.9620	5.83
DY full strength	1.0	0.7450	27.07
DY ½ strength	1.0	0.8651	15.32
DY ¼ strength	1.0	0.8670	15.12
Glyphosate 2 $\mu g.ml^{-1}$	1.0	0.9565	6.36
Glyphosate 5 $\mu g.ml^{-1}$	1.0	0.8503	16.76
Glyphosate 10 $\mu g.ml^{-1}$	1.0	0.6989	31.59
water	1.0	1.0215	

Key: R = Red band
P = Pink band
T = Tan band
DY = Dark yellow band

Full strength means 5mg of substance dissolved in 50mL of water.

½ strength means 5mg of substance dissolved in 100mL of water.

¼ strength means 5mg of substance dissolved in 200mL of water.

Table 15 : Effect of preparations of *Fusarium oxysporum* strain OK7 on weight of *Vicia faba* leaf tissues after 36hours of exposure

TREATMENTS	INITIAL WT (g)	WT AFTER 36HRS(g)	%growth inhibition
R full strength	0.05	0.0425	15
R ½ strength	0.05	0.0475	5
R ¼ strength	0.05	0.0475	5
P full strength	0.05	0.0375	25
P ½ strength	0.05	0.0465	7
P ¼ strength	0.05	0.0477	4.6
T full strength	0.05	0.0460	8
T ½ strength	0.05	0.0481	3.8
T ¼ strength	0.05	0.0481	3.8
DY full strength	0.05	0.0350	30
DY ½ strength	0.05	0.0420	16
DY ¼ strength	0.05	0.0477	4.6
Glyphosate 2 $\mu g.mL^{-1}$	0.05	0.0481	3.8
Glyphosate 5 $\mu g.mL^{-1}$	0.05	0.0375	25
Glyphosate 10 $\mu g.mL^{-1}$	0.05	0.0340	32
water	0.05	0.05	

Key: R = Red band
P = Pink band
T = Tan band
DY = Dark yellow band

Full strength means 5mg of substance dissolved in 50mL of water.

½ strength means 5mg of substance dissolved in 100mL of water.

¼ strength means 5mg of substance dissolved in 200mL of water.

Table 16: Visual observation of the effect of chemicals on *Orobanche crenata* and its hosts.

TREATMENTS	*Orobanche crenata*	*V.faba*	*V.sativa*
R full strength	+	-	-
R ½ strength	-	-	-
R ¼ strength	-	-	-
P full strength	++	+	+
P ½ strength	+	-	-
P ¼ strength	-	-	-
T full strength	+	-	-
T ½ strength	-	-	-
T ¼ strength	-	-	-
DY full strength	++	++	-
DY ½ strength	+	-	-
DY ¼ strength	+	-	-
Glyphosate 2 $\mu g.mL^{-1}$	-	-	-
Glyphosate 5 $\mu g.mL^{-1}$	+	-	-
Glyphosate 10 $\mu g.mL^{-1}$	++	+	-
water	-		

Key:

- < 5% discoloured tissue
+ 5-10% discoloured tissue
++ 11-30% discoloured tissue

Full strength means 5mg of substance dissolved in 50mL of water.

½ strength means 5mg of substance dissolved in 100mL of water.

¼ strength means 5mg of substance dissolved in 200mL of water.

Table 17: Bioassay on stem and flower of Orobanche crenata using Fusarium oxysporum extract.

Plant part	(crude)	Rf 0.06 red	Rf 0.13 pink	Rf 0.23 tan	H_2O	Glyphosate 1ppm	Glyphosate 2 $\mu g.mL^{-1}$	Glyphosate 5 $\mu g.mL^{-1}$	Glyphosate 10 $\mu g.mL^{-1}$
Flower	++	++	++	++	-	-	-	+	++
Stem	++	+	+	++	-	-	-	++	+++

Key :
+++ Severe
++ Moderate
+ Mild
- No symptom

Figure 42. Morphological effects of metabolite on the inflorescence of *Orobanche crenata* 12 hours after application.Clear colour as is the case with control (water) shows healthy tissues while dark colour as is the case with treated samples shows dead or sick tissues.

3.6.2 Bioassay with 1H- indole-3- carboxaldehyde and tyrosol.

Results obtained from the bioassay on *Orobanche crenata* proved that glyphosate is the most active chemical against *Orobanche crenata* compared to tyrosol and 1H-indole-3-carboxaldehyde at all concentrations i.e. 2,5,10,20 and 50 $\mu g.mL^{-1}$. Tyrosol and glyphosate at 2 $\mu g.mL^{-1}$ after 12 hours had a positive effect of -0.77% and -2.14% inhibition respectively on the growth of *Orobanche crenata* similar to the controls of -2.3% and -2.7% inhibition for rice extract and water respectively . After 36hours, it was noted that rice extract and water used as controls did not inhibit the growth of *Orobanche crenata* (fig. 40; table 18 and 22) as they showed an inhibition of -0.19% and -0.77% respectively.

Experiment conducted with 1H-indole-3-carboxaldehyde, showed that 1H-indole-3-carboxaldehyde at 10, 20 and 50 $\mu g.mL^{-1}$ had similar effects like glyphosate at same concentrations after 24 and 36hours. However glyphosate had a stronger effect than 1H-indole-3- carboxaldehyde after 12hours (fig.41;table 19 and 23).After 36hours, glyphosate at 50 $\mu g.mL^{-1}$ caused the highest inhibition of 35.3% compared to 34.99 % and 17.3% inhibition caused by 1H- indole-3- carboxaldehyde and tyrosol respectively at same concentrations.

A similar bioassay conducted on leaf tissues of *Vicia faba* showed that tyrosol had no effect on *Vicia faba* after 24hours (2.0% inhibition). However, tyrosol at higher concentrations of 20 $\mu g.mL^{-1}$ and 50 $\mu g.mL^{-1}$ had a mild effect on *Vicia faba* after 36hours causing 4.08% and 6.12% inhibition respectively .After 12hours only glyphosate at 2 and 5 $\mu g.mL^{-1}$ had a mild effect on *Vicia faba* (4.08% and 6.12% inhibitions respectively) while at concentrations higher than 5 $\mu g.mL^{-1}$ the effect was serious.(fig. 42; table 20 and 22).

Similarly, 1H indole carboxaldehyde had no effect on *Vicia faba* after 12 and 24hours. However 1H- indole-3-carboxaldehyde at concentrations higher than 10 $\mu g.mL^{-1}$ had mild effect on *Vicia faba* after 36hours with results of 4.08% and 6.12% inhibitions from 20 $\mu g.ml^{-1}$ and 50 $\mu g.mL^{-1}$ concentrations respectively (fig. 43 ; table 21 and 23).

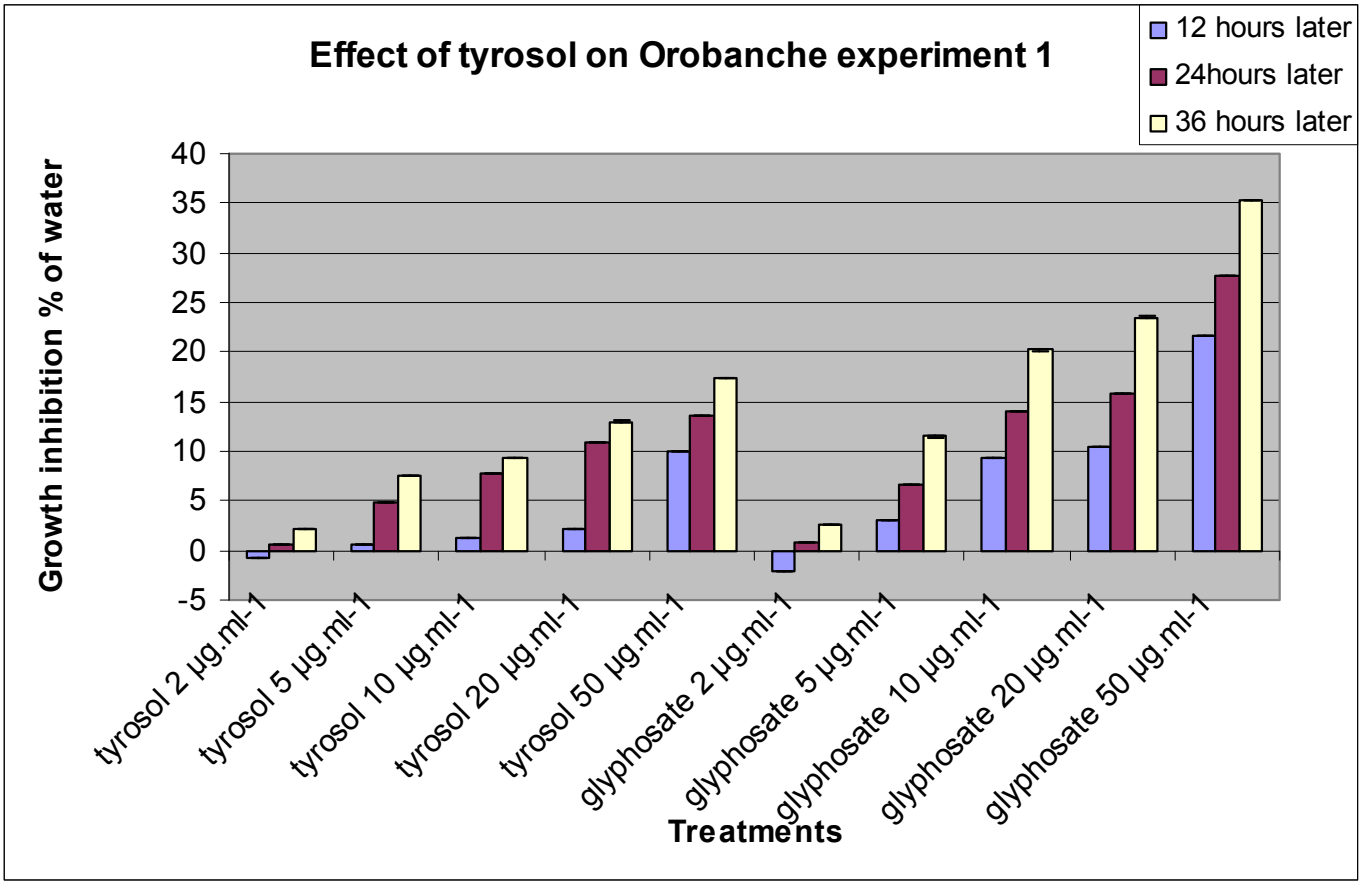


Figure 43(*see appendix table 18*).Effect of Tyrosol on weight (g) of tissue culture of *Orobanche crenata* 12, 24 and 36 hours after application. Results show increasing effect of tyrosol and glyphosate on the growth of *Orobanche crenata* relative to concentration of chemicals and time of exposure.

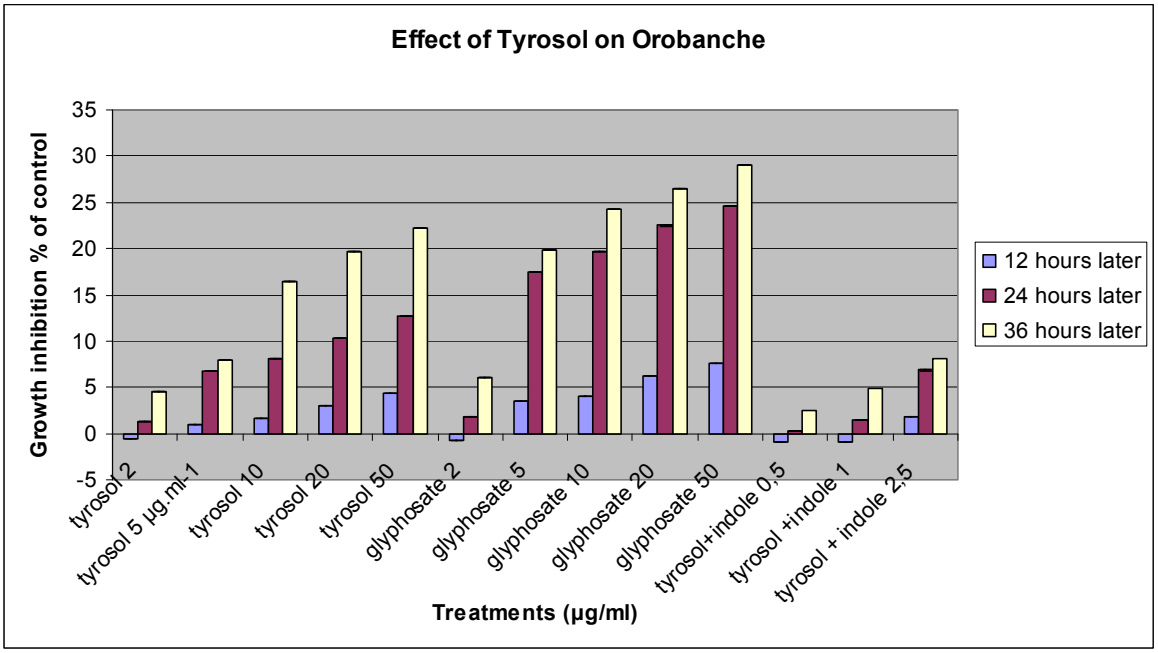


Figure 44(*see appendix table 18*).Effect of Tyrosol on weight (g) of tissue culture of *Orobanche crenata* 12, 24 and 36 hours after application. Results show increasing effect of tyrosol and glyphosate on the growth of *Orobanche crenata* relative to concentration and time of exposure.When tyrosol was applied in combination with 1H-Indole-3-carboxaldehyde, there was no significant diffirence from what is obtained when it is applied independently.

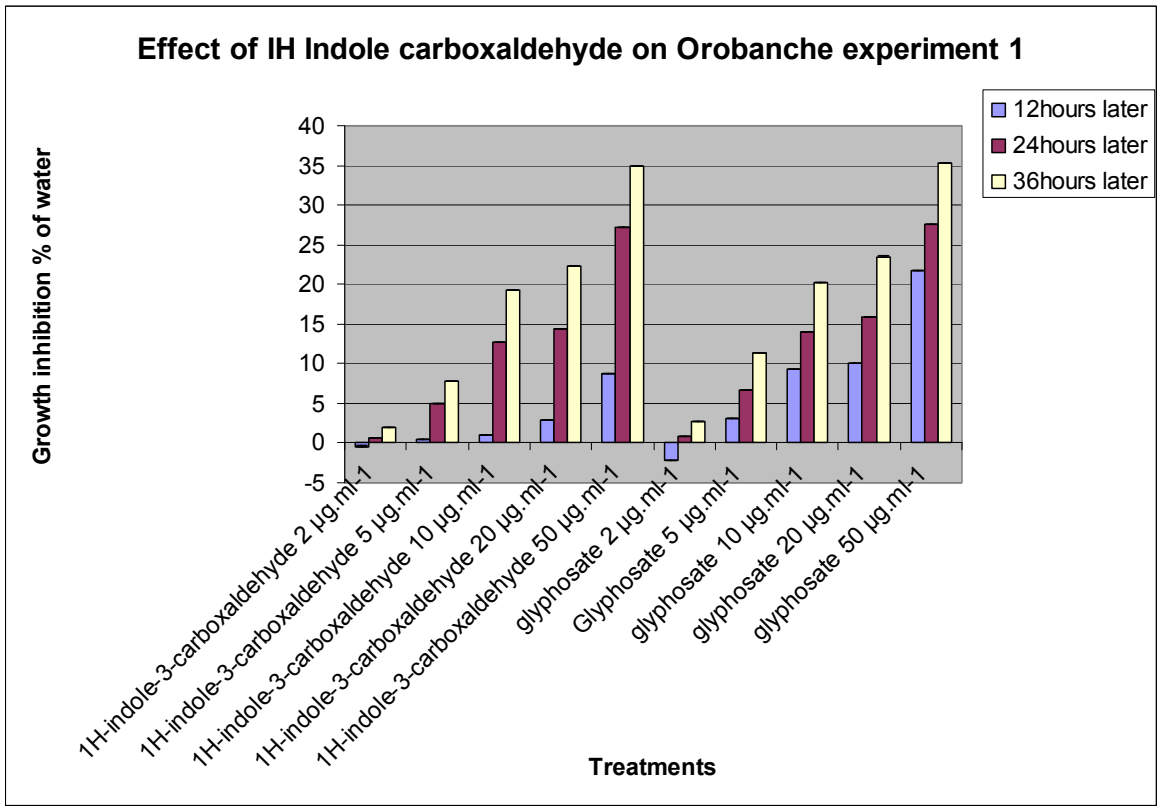


Figure 45(*see appendix table 19*).Effect of 1H-indole-3-carboxaldehyde on weight (g) of *Orobanche crenata* 12, 24 and 36 hours after application. Results show increasing effect of 1H-Indole-3-carboxaldehyde, and glyphosate on the growth of *Orobanche crenata* relative to concentration of chemicals and time of exposure.

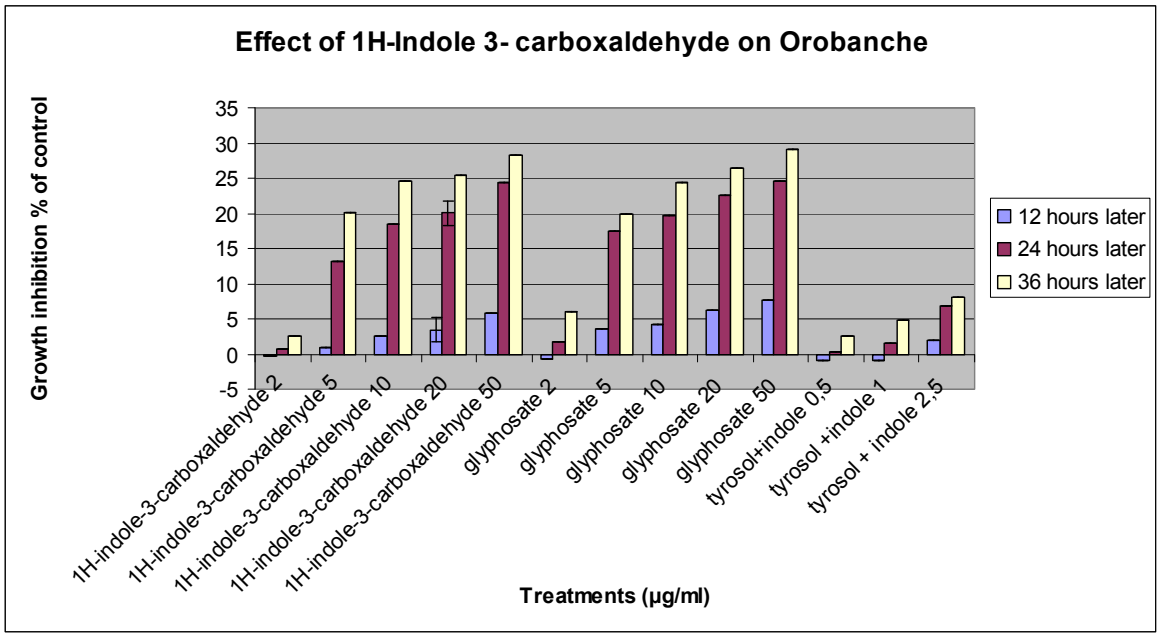


Figure 46(*see appendix table 19*).Effect of 1H-indole-3-carboxaldehyde on weight (g) of *Orobanche crenata* 12, 24 and 36 hours after application. Results show increasing effect of 1H-Indole-3-carboxaldehyde and glyphosate on the growth of *Orobanche crenata* relative to concentration and time of exposure.When 1H-Indole-3-carboxaldehyde was applied in combination with tyrosol, there was no significant diffirence from what is obtained when it is applied independently.

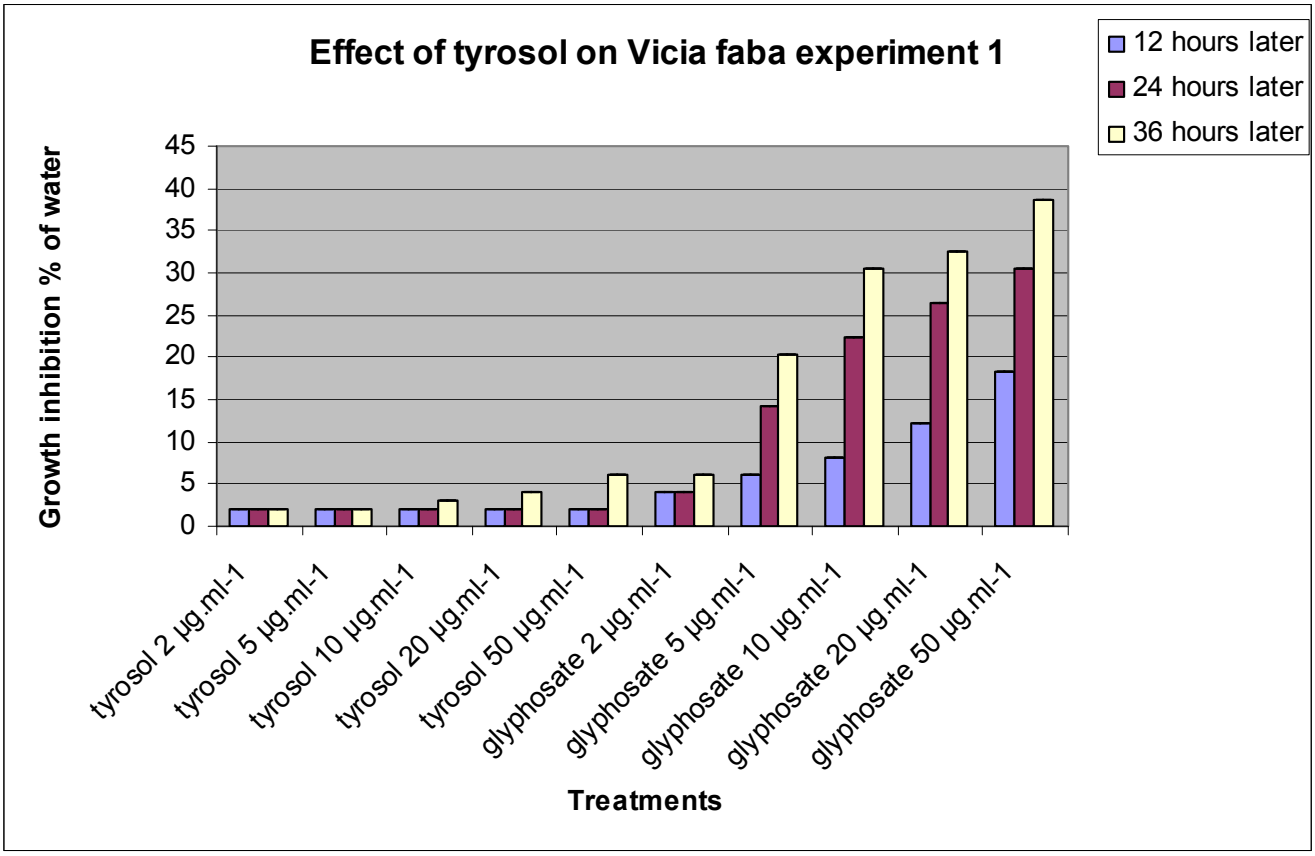


Figure 47(*see appendix table 20*).Effect of Tyrosol on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application. Results show that tyrosol has little or no phytotoxic effect on *Vicia faba* tissues compared to glyphosate that has a great phytotoxic effect on the tissues of *Vicia faba.*

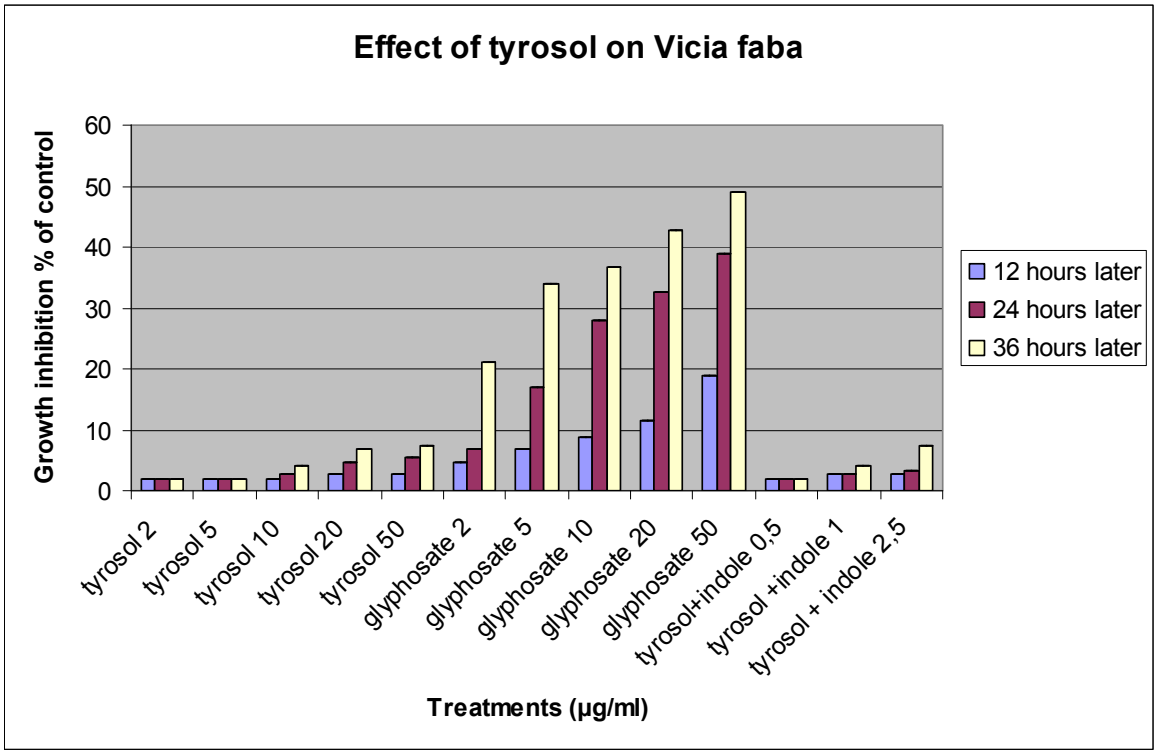


Figure 48 (*see appendix table 20*).Effect of Tyrosol on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application. Results show that tyrosol has little or no phytotoxic effect on *Vicia faba* tissues even in combination with 1H-Indole-3-carboxaldehyde compared to glyphosate that has a great phytotoxic effect on the tissues of *Vicia faba*.

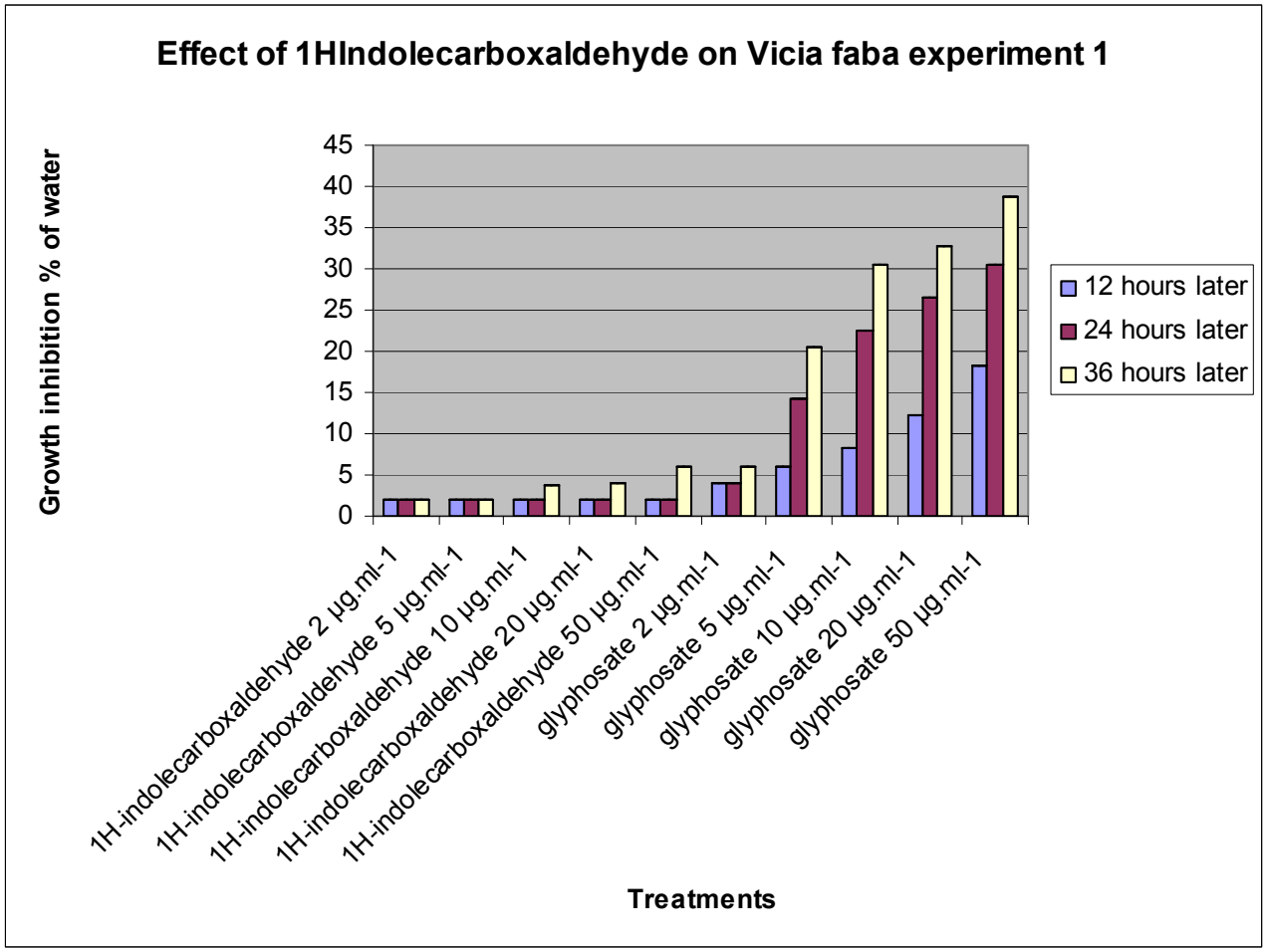


Figure 49 (*see appendix table 21*).Effect of 1H-indole-3-carboxaldehyde on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application. . Results show that 1H-indole-3-carboxaldehyde has little or no phytotoxic effect on *Vicia faba* tissues compared to glyphosate that has a great phytotoxic effect on the tissues of *Vicia faba.*

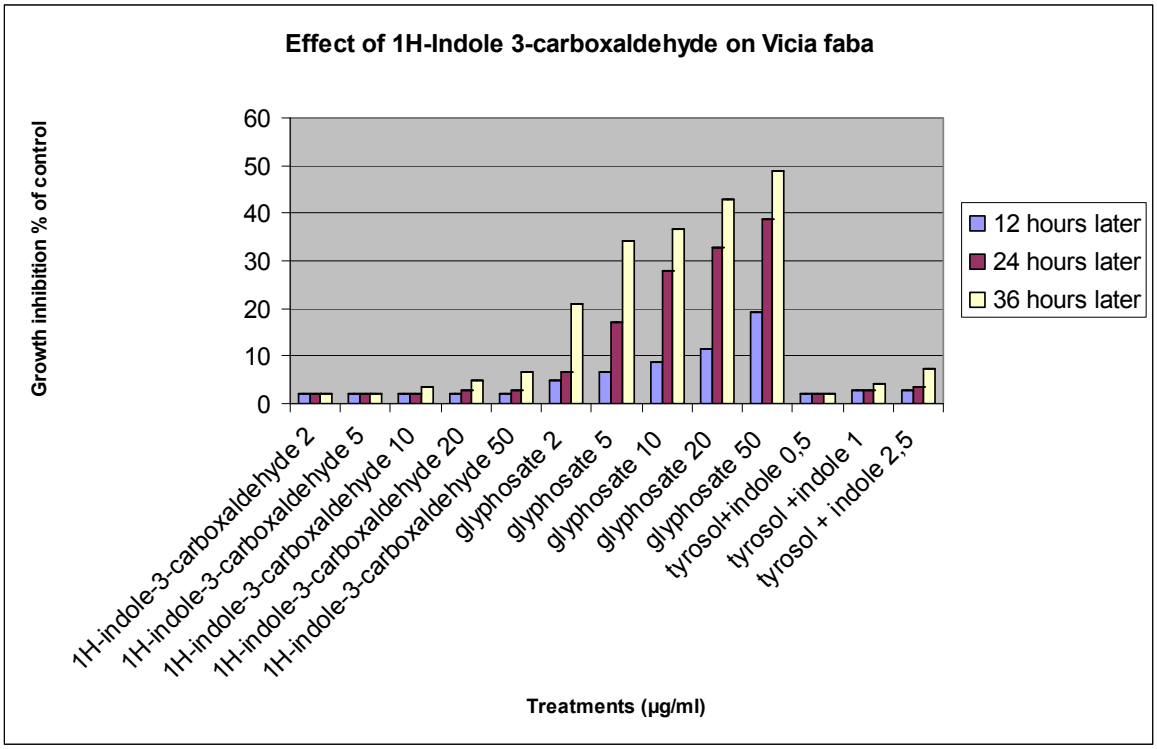


Figure 50 (*see appendix table 21*)**.**Effect of 1H-indole-3-carboxaldehyde on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application. Results show that 1H-indole-3-carboxaldehyde has little or no phytotoxic effect on *Vicia faba* tissues even in combination with tyrosol compared to glyphosate that has a great phytotoxic effect on the tissues of *Vicia faba.*

3.6.3 Effect of tyrosol and 1H-indole-3-carboxaldehyde on the chlorophyll content of *Vicia faba.*

Results obtained from the effect of tyrosol and 1H- indole carboxaldehyde on the chlorophyll a content of leaf tissues of *Vicia faba*, proved that glyphosate had a serious negative effect on the chlorophyll a content of *Vicia faba*. At a concentration of just 2 $\mu g.mL^{-1}$, glyphosate after 12hours had a serious effect on the chlorophyll a content of *Vicia faba* similar to effects caused by 1H-indole-3-carboxaldehyde at 50 $\mu g.mL^{-1}$ after 36hours (fig 44; table 24 and 25).While the control (water) had a chlorophyll a content of 0.0190mg/g after 12hours, glyphosate at 2 $\mu g.mL^{-1}$ had a chlorophyll a content of 0.0123mg/g after 12hours compared to 0.0116mg/g of 1H- indole-3- carboxaldehyde at 20 $\mu g.mL^{-1}$ after 36hours.

After 36hours, tyrosol at 50 $\mu g.mL^{-1}$ proved to have a mild effect on the chlorophyll a content of *Vicia faba* similar to 1H-indole-3-carboxaldehyde. However, 1H- indole-3-carboxaldehyde proved to have a stronger negative effect on the chlorophyll a content of *Vicia faba* than tyrosol (figures 44 and 45; tables 24 and 25).

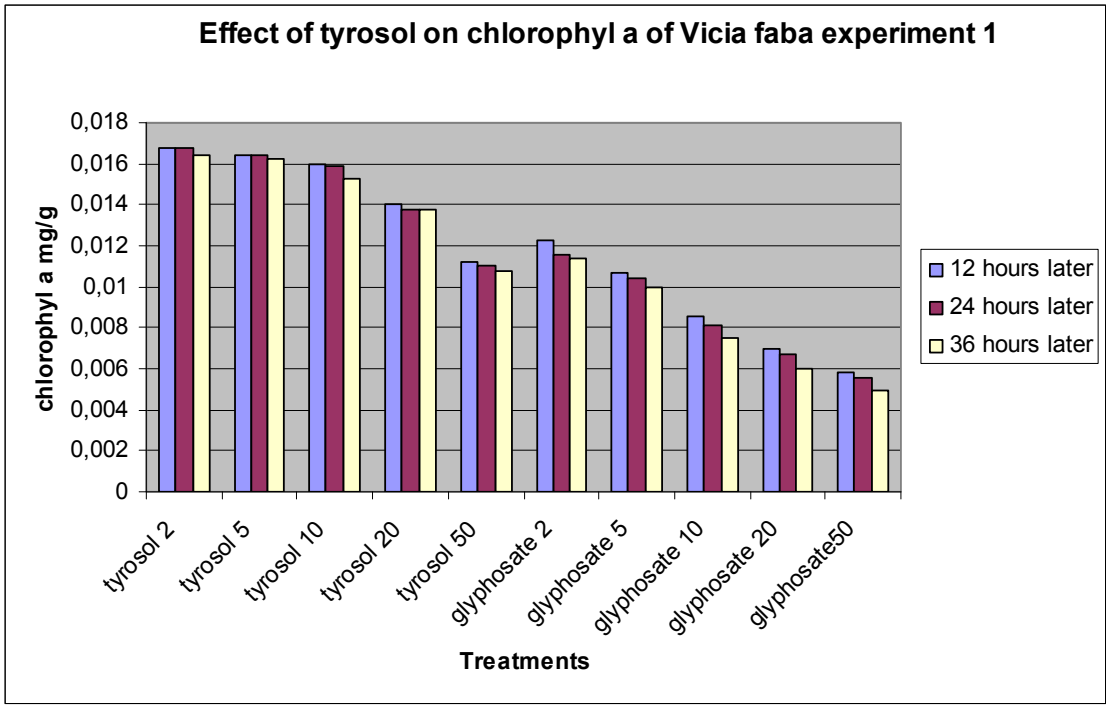


Figure 51(*see appendix table 24*).Comparative effect of tyrosol and glyphosate on the chlorophyll content of *Vicia faba.* Results show that tyrosol has little or no effect on the chlorophyll a content of *Vicia faba* up to a concentration of 20 $\mu g.mL^{-1}$ unlike glyphosate that has an adverse effect on the chlorophyll a content of *Vicia faba* even at a low concentration of 10 $\mu g.mL^{-1}$ similar to 50 $\mu g.mL^{-1}$ of tyrosol.

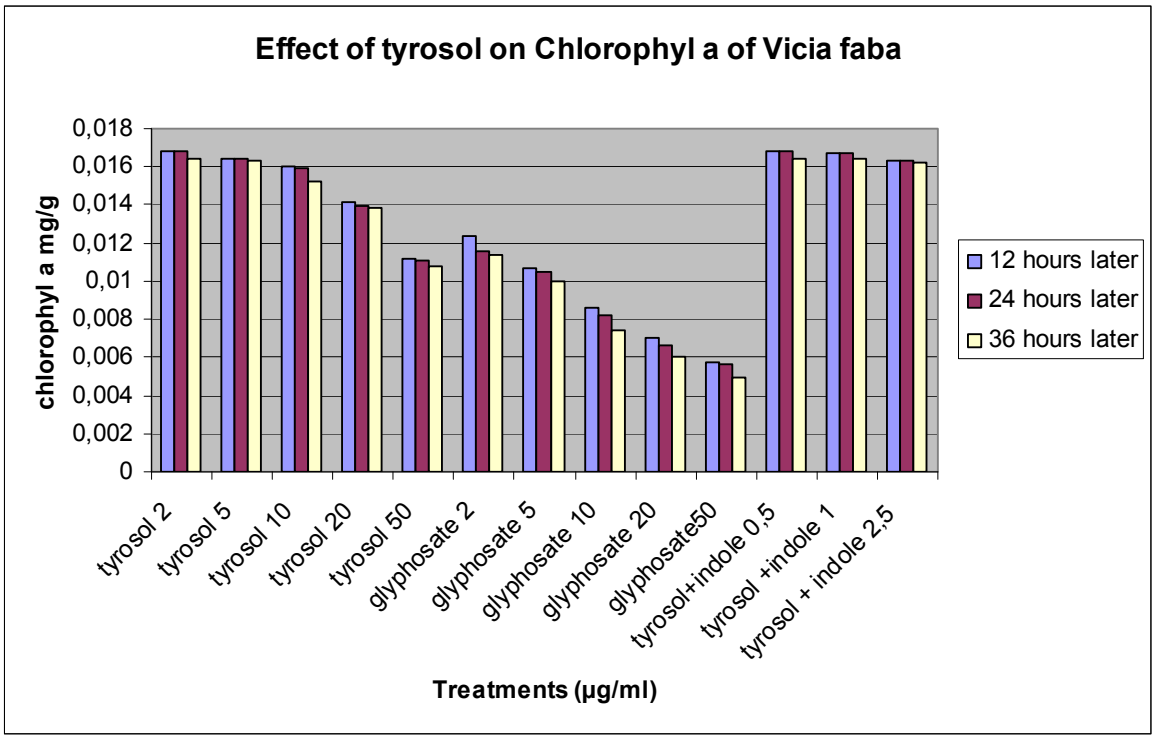


Figure 52 (*see appendix table 24*)**.**Comparative effect of tyrosol and glyphosate on the chlorophyll content of *Vicia faba.* Results show that tyrosol has little or no effect on the chlorophyll a content of Vicia faba unlike glyphosate that has an adverse effect on the chlorophyll a content of Vicia faba even at a low concentration of 10 $\mu g.mL^{-1}$ similar to 50 $\mu g.mL^{-1}$ of tyrosol. Even in combination with 1H-indole-3-carboxaldehyde there was still no adverse effect compared to glyphosate.

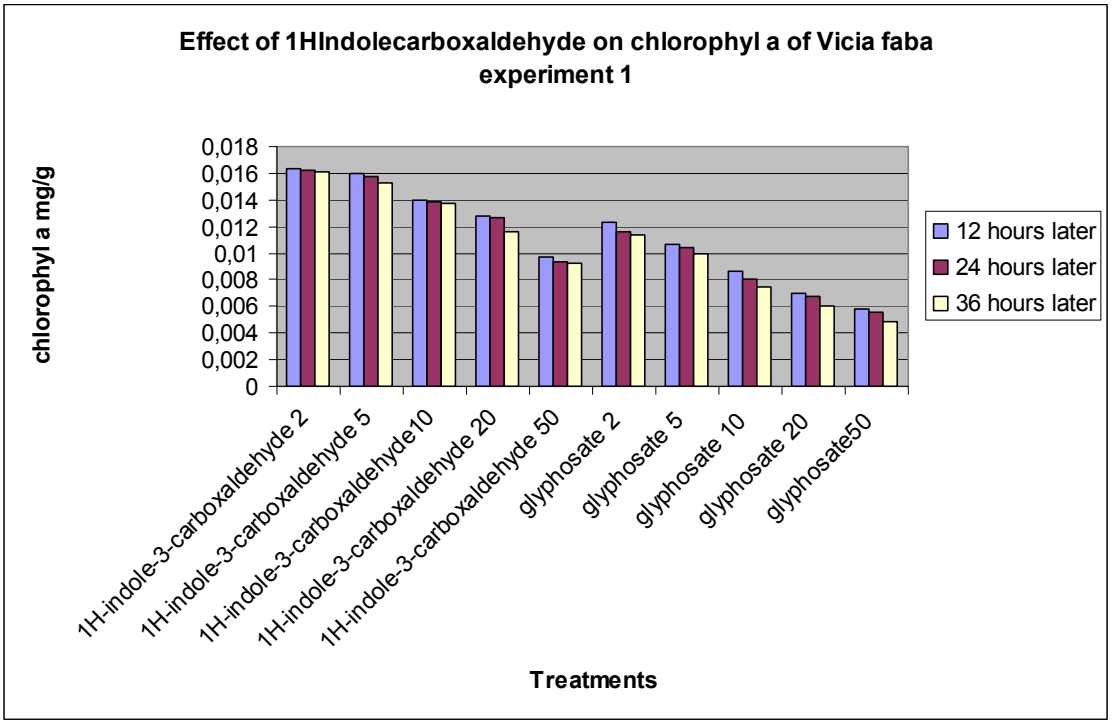


Figure 53(*see appendix table 25*). Comparative effect of 1H-indole-3-carboxaldehyde and glyphosate on the chlorophyll content of *Vicia faba.* Results show that 1H-indole-3-carboxaldehyde has little or no effect on the chlorophyll a content of *Vicia faba* up to a concentration of 20 µg.mL^{-1} unlike glyphosate that has an adverse effect on the chlorophyll a content of *Vicia faba* even at a low concentration of 10 µg.mL^{-1} similar to 50 µg.mL^{-1} of 1H-indole-3-carboxaldehyde.

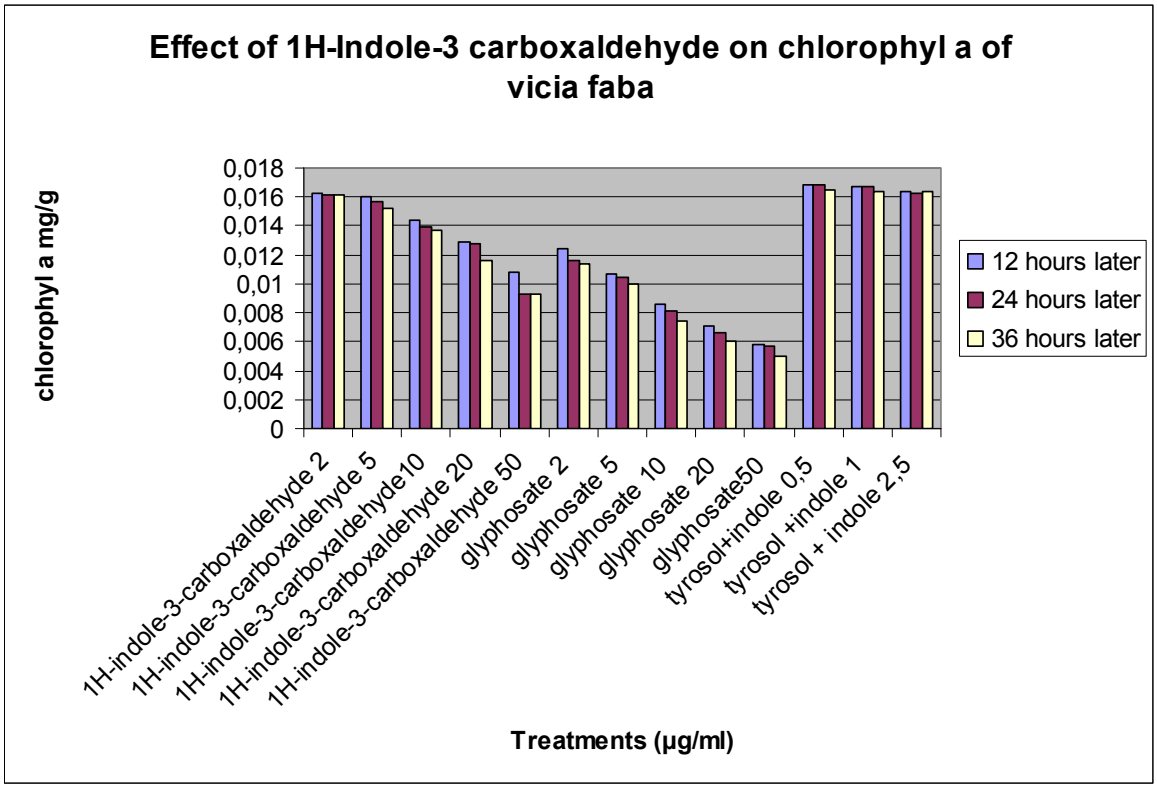


Figure 54(*see appendix table 25*). Comparative effect of 1H-indole-3-carboxaldehyde and glyphosate on the chlorophyll content of *Vicia faba.* Results show that 1H-indole-3-carboxaldehyde has little or no effect on the chlorophyll a content of *Vicia faba* up to a concentration of 20 µg.mL^{-1} unlike glyphosate that has an adverse effect on the chlorophyll a content of *Vicia faba* even at a low concentration of 10 µg.mL^{-1} similar to 50 µg.mL^{-1} of 1H-indole-3-carboxaldehyde. Even in combination with tyrosol there was still no adverse effect compared to glyphosate.

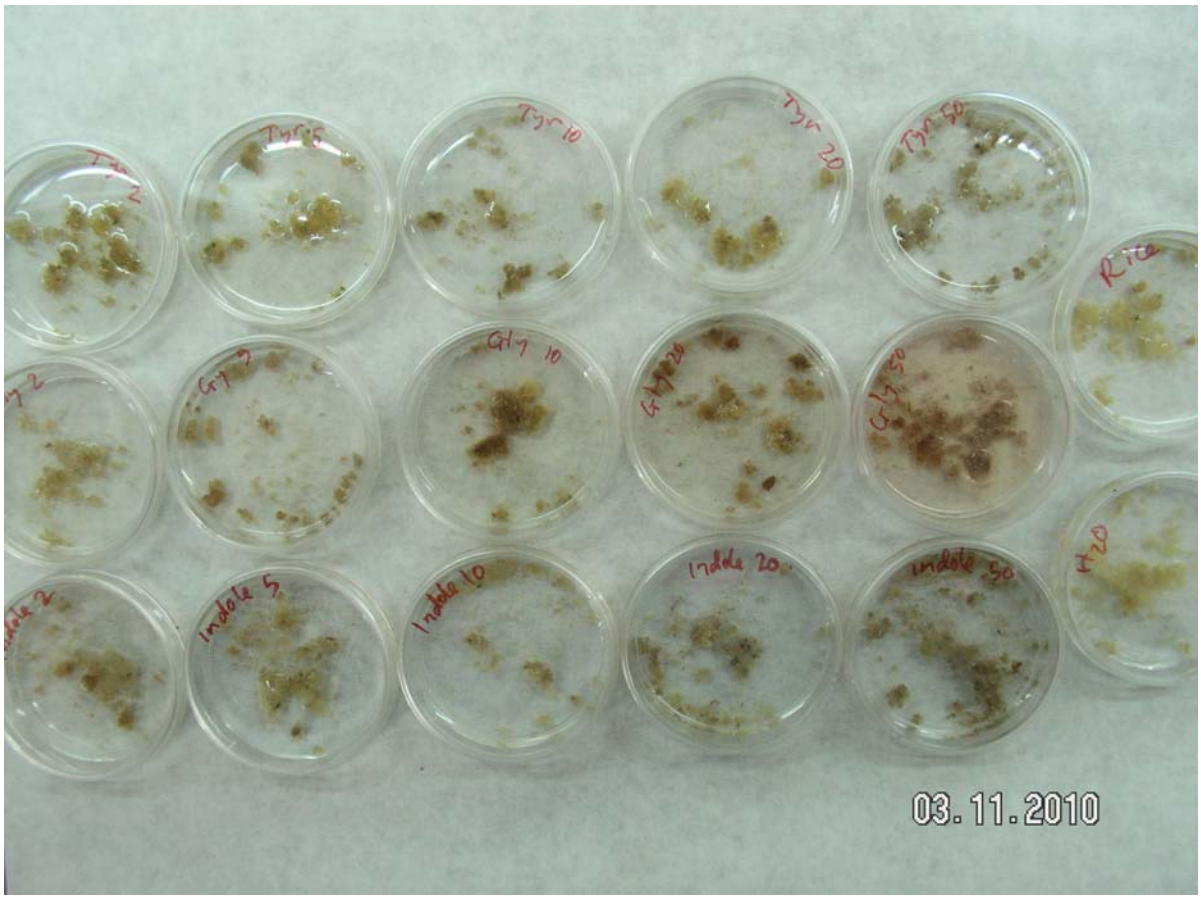


Figure 55. Visual effect of chemicals on the morphology of *Orobanche crenata* callus 12 hours after application. At 12 hours after application, adverse effects were seen on treatments with glyphosate ≥ 10 $\mu g.mL^{-1}$ and treatments with tyrosol and 1H-indole-3-carboxaldehyde ≥ 50 and 20 $\mu g.mL^{-1}$ respectively.
First row on top represents treatments with tyrosol at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Middle row represents treatments with glyphosate at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Bottom row represents treatments with 1H-Indole-3-carboxaldehyde at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
The two treatments at the extreme right in the middle represent the control.

Figure 56. Visual effect of chemicals on the morphology of *Vicia faba* 12 hours after application.
At 12 hours after application, adverse effects were seen on treatments with glyphosate ≥ 5 $\mu g.mL^{-1}$ and treatments with tyrosol and 1H-indole-3-carboxaldehyde ≥ 50 $\mu g.mL^{-1}$.

First row on top represents treatments with tyrosol at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Middle row represents treatments with glyphosate at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Bottom row represents treatments with 1H-Indole-3-carboxaldehyde at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
The two treatments at the extreme right in the middle represent the control.

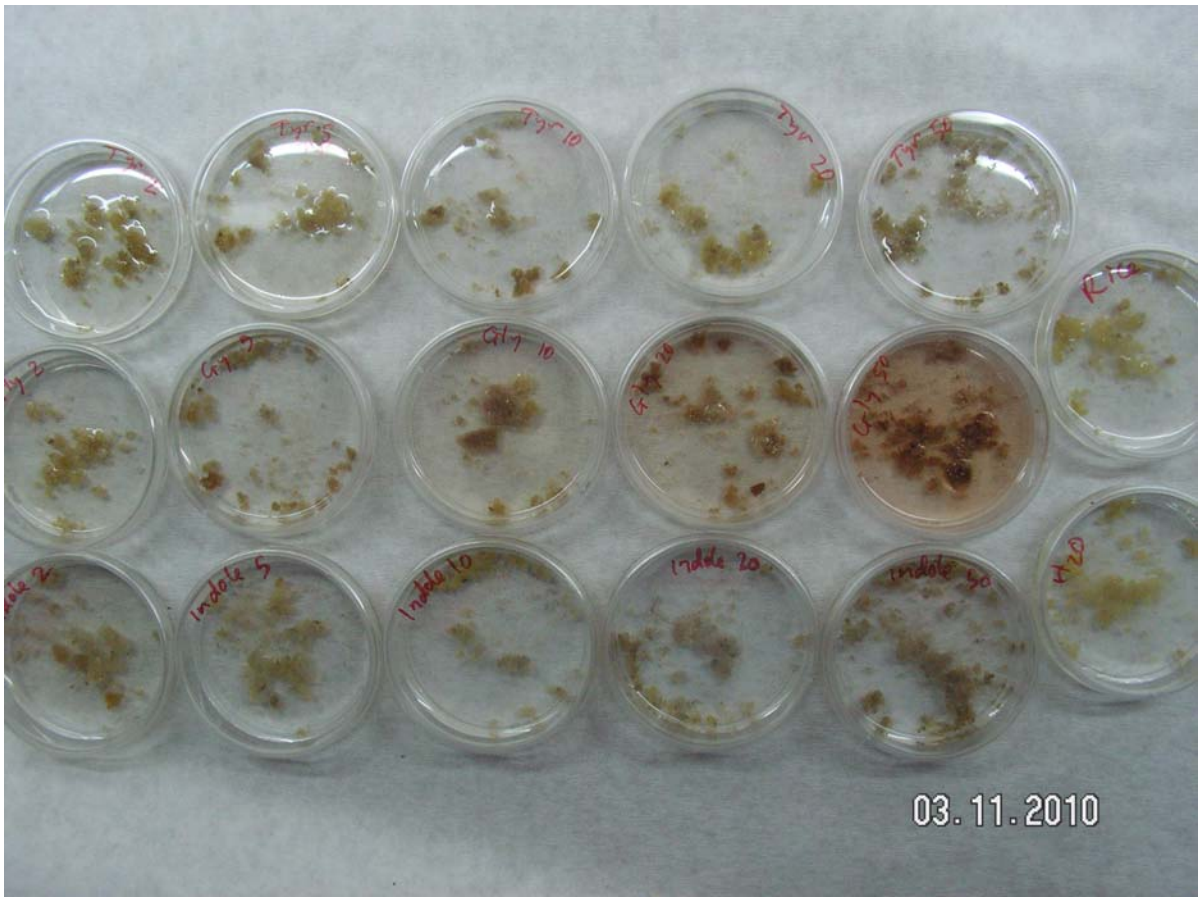


Figure 57. Visual effect of chemicals on the morphology of *Orobanche crenata* callus 24 hours after application. At 24 hours after application, adverse effects were seen on treatments with glyphosate ≥5 $\mu g.mL^{-1}$ and treatments with tyrosol and 1H-indole-3-carboxaldehyde ≥ 50 and 20 $\mu g.mL^{-1}$ respectively.

First row on top represents treatments with tyrosol at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Middle row represents treatments with glyphosate at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Bottom row represents treatments with 1H-Indole-3-carboxaldehyde at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
The two treatments at the extreme right in the middle represent the control.

Figure 58. Visual effect of chemicals on the morphology of *Vicia faba* 24 hours after application. At 24 hours after application, adverse effects were seen on treatments with glyphosate ≥ 5 $\mu g.mL^{-1}$ and treatments with tyrosol and 1H-indole-3-carboxaldehyde ≥ 50 $\mu g.mL^{-1}$.

First row on top represents treatments with tyrosol at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Middle row represents treatments with glyphosate at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Bottom row represents treatments with 1H-Indole-3-carboxaldehyde at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
The two treatments at the extreme right in the middle represent the control.

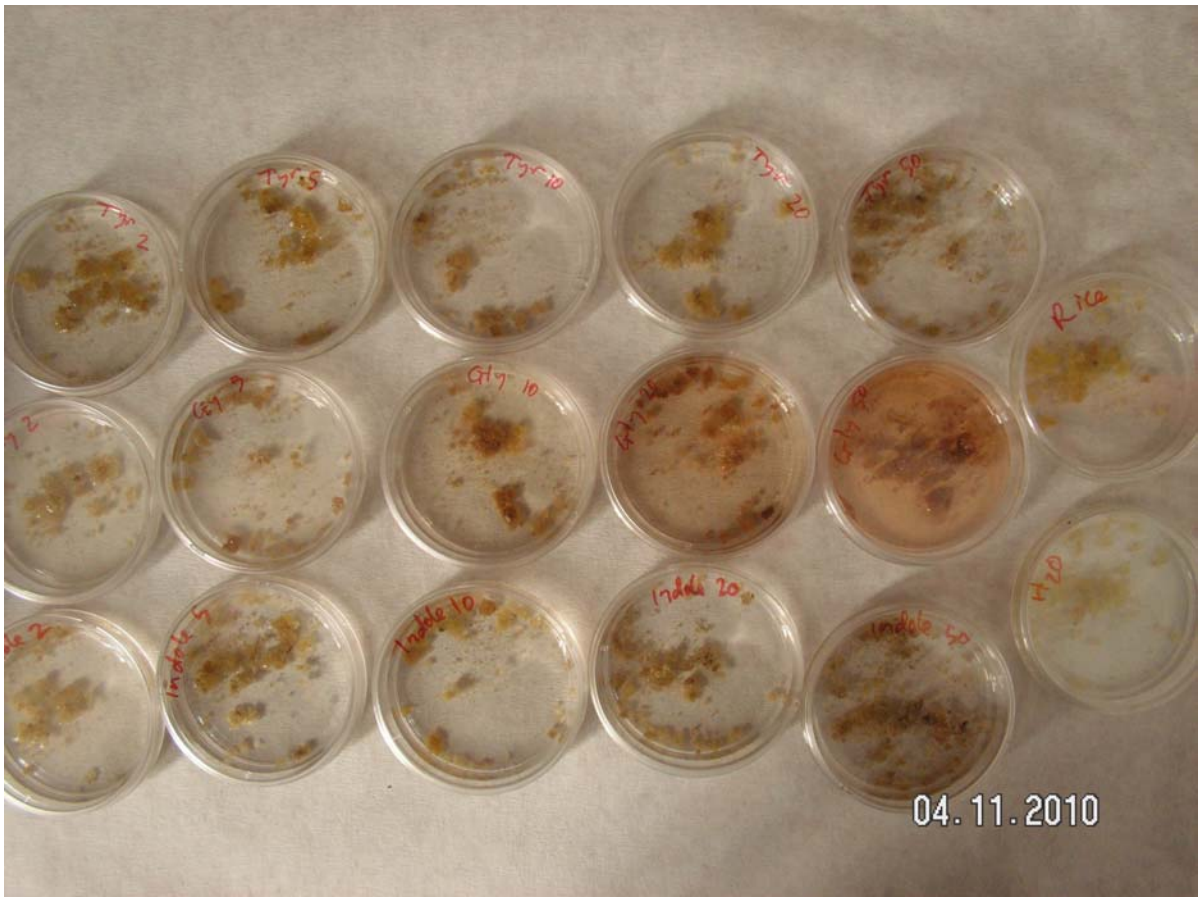


Figure 59. Visual effect of chemicals on the morphology of *Orobanche crenata* callus 36 hours after application. At 24 hours after application, adverse effects were seen on treatments with glyphosate ≥5 µg.mL^{-1} and treatments with tyrosol and 1H-indole-3-carboxaldehyde ≥ 2 and 5 µg.mL^{-1} respectively but with severity on treatments with glyphosate at higher concentrations followed by 1H-indole-3-carboxaldehyde.

First row on top represents treatments with tyrosol at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Middle row represents treatments with glyphosate at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Bottom row represents treatments with 1H-Indole-3-carboxaldehyde at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
The two treatments at the extreme right in the middle represent the control.

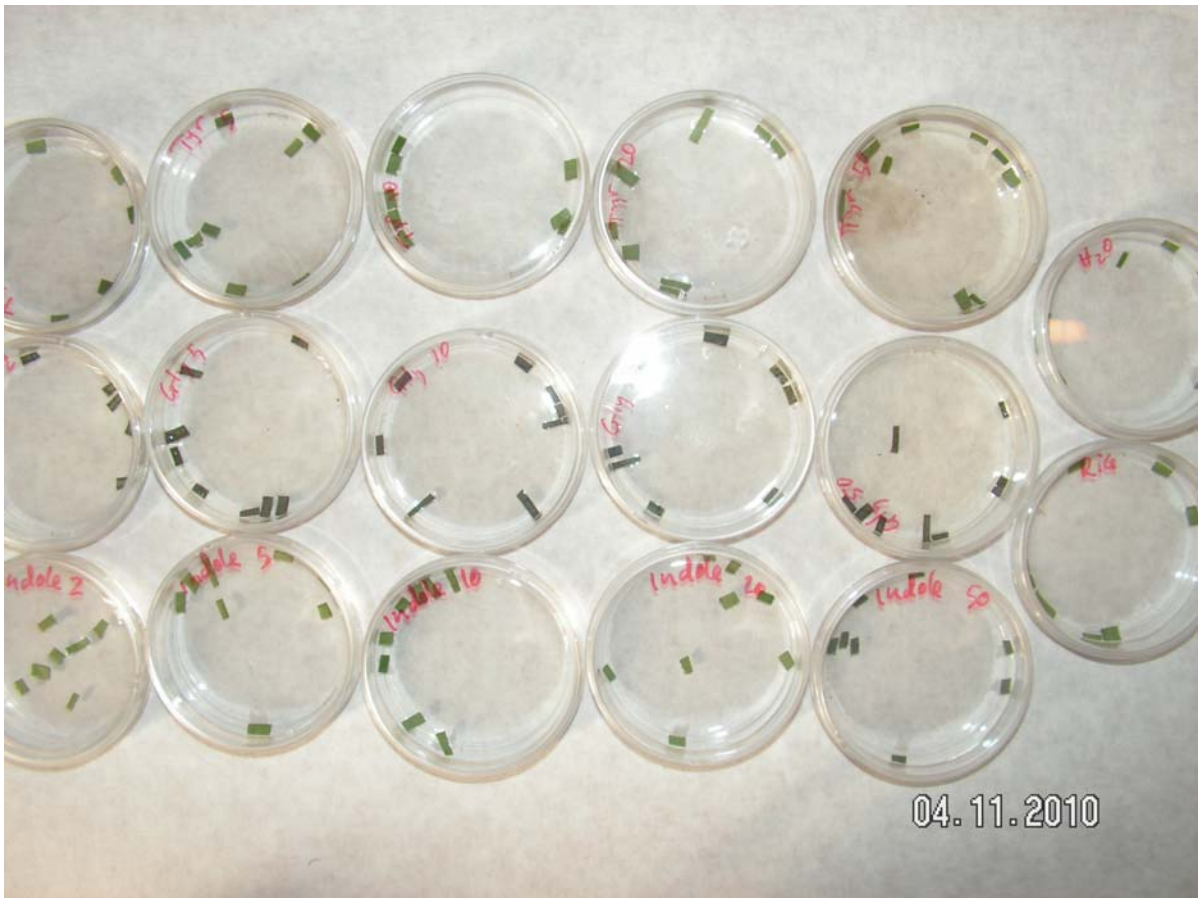


Figure 60. Visual effect of chemicals on the morphology of *Vicia faba* 36 hours after application. At 36 hours after application, adverse effects were seen on treatments with glyphosate ≥ 2 $\mu g.mL^{-1}$ and treatments with tyrosol and 1H-indole-3-carboxaldehyde ≥ 50 $\mu g.mL^{-1}$.

First row on top represents treatments with tyrosol at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Middle row represents treatments with glyphosate at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
Bottom row represents treatments with 1H-Indole-3-carboxaldehyde at concentrations of 2, 5, 10, 20 and 50 ppm from left to right respectively.
The two treatments at the extreme right in the middle represent the control.

3.7 DISCUSSIONS.

Today's agricultural sector is facing great economic losses due to the development of resistant plant-pathogens and crop destruction by various pests. Secondly, the use of chemicals in the agriculture sector, for controlling pests and plant pathogens is also causing environmental safety concerns. (Waller et al. 2005). Further, the development of resistance among weeds to herbicides is also a cause for concern (Ashrafi *et al*., 2007).
In this context, Fusarium oxysporum is been explored for the extraction of effective chemicals as agrochemicals.
Natural products are usually derived from microorganisms, plants or animals. Plants continue to remain a rich source of therapeutic substances since early civilizations. Moreover, even today, a major portion of new drugs (>60%) are obtained from natural products or their derivatives (Newman, et al., 2003).
Among the most fascinating and important properties of fungi is their ability to produce a tremendous variety of so-called secondary metabolites that display a broad range of biological activities (Demain et al. 2005).
Fungi are widely known for the production of compounds that have a deservedly negative reputation due to their activities as toxins. Such compounds include aflatoxins, ochratoxins, citreoviridin, trichothecenes, fumonisins, and various indole-derived tremorgenics (Miller and Trenholm 1994; Cole and Schweikert 2003).
This work was initiated on the basis that fungi produce bioactive. The rational of the work is that after its completion, at least one novel bioactive metabolite will be identified that could lead to the development of a herbicide.
Pathogenicity tests conducted showed the potential of the Fusarium strains C11 and OK7 to infect Orobanche species at pre and post emergence stages. Necrosis and chlorosis induced by the culture filtrates on Orobanche plants, besides the growth inhibition of germinating seeds, support this conclusion. These results confirm the work of Thomas *et al*. (1999) who reported that conidial suspensions of *F. oxysporum* colonized and infected the seeds of *Orobanche cumana*, specie specific to sunflowers. Factors contributing to pathogenesis of Fusarium spp have been identified as temperature (Cook and Baker 1983), PH (Peng et al 1999) and nutrition (Woltz and Jones 1981). These factors could be attributed to the results obtained where *Fusarium oxysporum* was found to be also pathogenic to broomrape underground structures. Extracts from 3-4 weeks old *Fusarium oxysporum* cultures caused mortality of germinating seeds of *O. cernua*, *O. aegyptiaca, O. ramosa* and *O. cumana*. However, it is worth noting that some of these pathogenicity tests conducted by previous researchers and the present study were tested under aseptic conditions as is the case of PEB, which could be considered semi *in vitro* condition. Under the semi *in vitro* conditions in the PEB, the tested fungi do not have competition with other microorganisms. Under natural soil conditions where competition between microorganisms prevails, the results might not be the same.
Pathogenicity tests conducted on the underground stages of parasitism showed that pathogenicity increased with increasing fungal inoculum pressure. Conidial suspension of density $3x10^4$ spores / mL caused greater diseased symptoms than conidial density of $3x10^3$ spores / mL. Results obtained from the influence of the *Fusarium oxysporum* strains on the number of broomrape attachments, tubercles and spiders formed when applied at planting (table1) indicates that there was no significant difference in effect between the two strains C11 and OK7. However, there was a significant difference between the strains and the

control (water).The number of attachments, tubercles and spiders of both *O. crenata* and *O. cumana* were significantly higher in the control than the treatments containing the fusarium cell suspensions indicating the pathogenic effect of *Fusarium oxysporum* on *Orobanche spp*.
The pre emergence application of *Fusarium oxysporum* extracts from both strains on the broomrape seeds, reduced the number of attached seeds, tubercles and spiders formed compared to the control.
Also, when extracts from both strains were applied post emergence on the attached seeds, tubercles and spiders, more than a half of these structures were found dead three days after application. Those that were not dead had disease symptoms while no disease symptom was observed in the control indicating that these *Fusarium oxysporum* strains are pathogenic to *Orobanche crenata* and *Orobanche cumana*. In the post emergence experiment, healthy Orobanche tubercle cells with intact structures were seen in the non infested control pots.
The pathogenicity test conducted using the fusarium cell suspensions of the two strains at densities of $3X10^4$ spores/mL and $3X10^3$ spores/mL showed these two strains are pathogenic to the broomrape structures (i.e. attachments, tubercles and spiders). In the present study, *Fusarium oxysporum* extracts from 4weeks old cultures caused a significant reduction in the number of underground structures (attachments, tubercles and spiders) compared to the control.
Results obtained from pre-emergence experiments(when fusarium conidial suspension was applied in the soil or in PEB) and Post-emergence (when Fusarium conidial suspension was sprayed on the plants) showed that the tested strains of *Fusarium oxysporum* could be further evaluated in the field to control *Orobanche crenata* and *Orobanche cumana* using fusarium conidia as pathogenic agents to the broomrape.
Pot experiments were also carried out to confirm the pathogenicity effect of the two strains of *Fusarium oxysporum* on *O.crenata* and *O.cumana* and to clarify two aspects of pathogenicity of these fungi strains viz: which Orobanche specie is controlled by each of these fungi strains and whether the fungi are pathogenic to the host.
Results obtained showed that there was significant difference between the treatments exposed to various inoculum level (spore density/mL and the control-water) of application on the emergence of both specie of Orobanche in all the three host plants i.e. *V.sativa , V. faba* and *H. annuus.* This also holds true for experiments in PEB.
The greater the spore density i.e. $3X10^4$ spore/mL the lesser the number of Orobanche species per pot in all the species of Orobanche. Concerning the effects of the various strains (C11 and OK7) on the influence of the emergence of the parasite, there was no significant difference (Table 5.).
In the second pre-emergence experiment, concerning the percentage number of emerged diseased orobanche plant per pot, it was recorded that there was no significant difference on the effect of the two strains on *O. crenata* but there was significant difference on the effects of the two strains on *O.cumana*. Strain C11 had more pathogenic effect than OK7.With regards to the influence of inoculum levels on disease severity, the higher level of $3X10^4$ spore/mL had a significant effect compared to the lesser level of $3X10^3$ spore/mL and the control (water).
Post emergence experiments were also conducted to evaluate the effects of these two strains on *Orobanche spp*. The first of this experiment was to evaluate the percentage number of diseased Orobanche plants/pots 7 and 14 days after the application of the fusarium inoculums. The disease level was significantly higher when assessed 14 days after application compared to when assessed 7 days after application. However, in both assessments, the disease level was significantly higher than the control. This result was

reflected in all the two species of Orobanche and the three host plants. Comparing the results obtained from the two strains (C11 and OK7) they were similar with regards to assessments after 7 and 14 days of application and dosage of applications ($3X10^4$ spore/ml and $3X10^3$ spore/mL).

In the second post emergence experiment regarding the percentage dead of Orobanche spp 7 and 14 days after the application of the inoculums, it was noted that there was significant difference in the results obtained 7 and 14 days after application of both strains of C11 and OK7. After 7 days of application, no dead Orobanche plant was recorded from *O. cumana* parasitizing *H. annuus* with both strains. Similar result was obtained from experiments with *O. crenata* parasitizing *V. sativa* with C11 strain.

Previous work done in PEB indicated that Fusarium strains are pathogenic to the developmental stages of *O.cumana* and *O.crenata*. Similar work conducted by Amsellem et al 2001 confirmed that *Fusarium oxysporum* is pathogenic to the parasites: *O. cumana, O. aegyptiaca, O. cernua* and O. *ramosa* but not to their hosts.

When the inoculum C11 and OK7 were applied pre-emergence, they significantly reduced the number of emerged Orobanche spp/ pot compared to the control (table 6).

Similarly C11 strain at a spore density of $3x10^4$ spores/mL reduced *O.crenata* parasitizing *V.sativa*, *O.crenata* parasitizing *V.faba* and *O.cumana* parasitizing *H.annuus* by 57.1, 57.6 and 68.1% respectively.

In addition, when the inoculum was applied pre-emergence, it had an adverse effect on the health of emerged Orobanche plants. OK7 at a spore density of $3x10^4$ spores/ml caused 100, 88.69 and 100% infection on *O.crenata* parasitizing *V.sativa*, *O.crenata* parasitizing *V.faba* and *O.cumana* parasitizing *H.annuus* respectively. While C11 strain at a spore density of $3x10^4$ spores/mL caused 100, 88.76 and 100% infection on *O.crenata* (*V.sativa*), *O.crenata* parasitizing *V.faba* and *O.cumana* parasitizing *H.annuus* respectively. At a lower spore density of $3x10^3$ spores/ml both strains also caused infections on the parasites (table 6).

These results confirm the work of Muller-Stover (2001) where *Fusarium oxysporum* applied at a dose of 0.5g/kg of soil (preemergence) cause a reduction efficacy of broomrape between 78-89%.

From the experiments conducted, no adverse effect of the inoculums was recorded on the host plants confirming the work of Bedi 1994; Thomas et al., 1998; that fusarium exclusively attacks *O. cumana*, *O. cernua* and *O.aegyptiaca.* Heiko et al 1998 reported that fusarium conidia at a dose of $3x10^4$ spores/ml caused a 28% reduction of shoot number. Also with this dose, 51% of the emerged shoots were diseased while 90% of the shoots were diseased at a dose of $3x10^8$ spores/mL and caused a 67% reduction of emerged shoots. Similarly, Bozoukov and Kouzmnova 1994 reported that pre-emergence application of fusarium mycelia caused broomrape (*O. ramosa and O. aegyptiaca*) reduction by 62-68% in the field.

However, it is likely that the combined attack on seeds, germ tubes and tubercles in soil by *Fusarium oxysporum* was responsible for the reduced number of parasite shoots in pre emergence pot experiments of the current study as well as those conducted by the above-mentioned researchers.

Post emergence experiments were evaluated 7 and 14 days after the application of inoculum. The first incidence of fungal disease was observed on shoots one week after inoculation, starting with the development of lesions. The scales (rudimentary leaves) became brownish. The shoot browning gradually spread to the entire shoot.

In this study, it is assumed that the reduction of emerged Orobanche when *Fusarium oxysporum* strains were applied in the soil at time of planting is because they produced some toxins that inhibited the germination of the parasitic seeds. Other potentially effective

method of controlling parasitic weeds such as Orobanche is to stimulate germination of their seeds in the absence of the host crop. When the germinated seedling fails to find the roots of a susceptible host, thereby reducing the quantity of the parasite in the seed bank. To achieve this, a synthetic germination stimulant such as GR24 or ethylene is applied to the soil .An alternative method has been reported by other researchers such as El-Kassas et al., 2004 that some strains of fungi when applied to the soil produce ethylene which stimulate the germination of the parasite. Shifrin and Anderson 1999, like other researchers have another suggestion for the reduced emergence of parasites when fungi are incorporated in the soil. The fungi is said to produce some toxins that act as germination inhibitor by reducing protein synthesis through the inhibition of peptidyltransferase. Application of spore suspensions of the two *Fusarium oxysporum* strains on broomrape structures of *O.crenata* and *O.cumana* (table 5) revealed that both strains reduced the number of structures of broom rape. OK7 applied at inoculum density of $3x10^4$ spores/ml reduced attachments by 54.5 and 48.6% for *O.crenata* and *O.cumana* respectively.C11 applied at same spore density reduced attachments by 63.6 and 51.4% for *O.crenata* and *O.cumana* respectively. Other structures i.e. tubercles and spiders were also controlled by these Fusarium strains when applied pre-emergence in PEB even at a lower dose application of $3x10^3$ spores/mL. Amsellem et al. 2001 had earlier reported that *Fusarium oxysporum* reduced tubercle growth by 75.9 % in PEB. When same strains were applied post emergence on the Orobanche structures, they infected all the structures (100%) when applied at a spore density of $3x10^4$ spores/mL .Previous research by Abbas and Boyette (1992) on the pathogenicity of *Fusarium oxysporum*, provided an impetus to investigate whether the potential Fusarium isolates are able to produce pathogenic effects against different developmental stages of broomrape.

The polyethylene bag and pot experiments conducted in this study were successful in evaluating the effects of *Fusarium oxysporum* strains in controlling broomrape either in the pre-emergence or post emergence stages.

Studies on the isolation of the phytotoxin responsible for the wilting observed in the pot experiments revealed that the pathogen produces several metabolites in culture filtrates as evident from TLC analysis. However, the principal phytotoxic compounds present in the culture filtrates were tyrosol and 1H-indole-3-carboxaldehyde. This is the first report on the isolation of tyrosol and 1H- indole-3-carboxaldehyde from broomrape specie in Greece. While tyrosol was isolated as a white substance with molecular weight of 138.164g/mol and melting point of 91-92^{o}C, 1H- indole-3-carboxaldehyde was isolated as a pale yellow substance with molecular weight of 145.16g/mol and melting point of 96-98^{o}C. These compounds were also found to be relatively stable at room temperatures.

These metabolites have already been reported as being produced by many other phytopathogenic fungi (Aldreidge et al., 1971; Evidente and Motta, 2001; Cabras et al., 2006; Rimando et al 2001). Tyrosol has been reported as a primary metabolite with phytotoxic and antifeeding activities (Marcela et al. 2001). The fungus *Epichloë festucae* employs indole-3-carboxaldehyde in its endophytic relationship with many different host plants, defending the host against fungal pathogens (Yue et al. 2000). This metabolite also has antibacterial characteristics against several human pathogens in vitro (Gutierrez-Lugo et al.2005). Violacein biosynthesis is a cell density-dependent factor, controlled via quorum sensing for the bacterial species *Chromobacterium violaceum*.

It is generally accepted that in a plant–pathogen interaction, the induction of key defense mechanisms in plants occurs as a response to phytotoxic metabolites produced by the fungus (Huang 2001).

One of the most notable modes of action of phytotoxic metabolites is the induction of programmed cell death. It is a genetically determined process that occurs in all multicellular organisms; it is characterized by a battery of morphological and biological changes such as nuclear condensation, cytoplasm shrinkage and cell death (Gilchrist 1998; Govrin and Levine 2000; Greenberg 1997). It has been reported by Gamboa-Angulo et al. 2000; Morales 2003 that some phytopathogenic fungi that cause early blight , synthesizes phytotoxic metabolites which, when tested using the leaf-drop assay on leaves mimic the symptoms of the disease.
As reported by Joaquin et al 2009, there is no known data about the phytotoxic activity at the cellular level, of tyrosol. It is also presently unknown whether the phytotoxic activity of tyrosol induces pathogenesis related responses, such as the hypersensitive response or toxic shock, and whether the activity is required for the successful infection by the pathogen. Joaquin et al 2009 also reported that almost 75% of all observed cells of marigold treated with tyrosol had reduced cell volume and a high rate of plasmolysis, which can explain the marked decrease in fresh mass and was found to be phytotoxic to marigold leaves. Similar phytotoxic results of tyrosol have been reported by Gamboa- Angulo et al. 2000.
Data from the present study confirm the report of Joaquin et al 2009 that tyrosol significantly reduced cell viability, fresh mass and the number of cells of marigold plant. It also induced lipid peroxidation.
Although, in some cases, a mode of action has been described for phytotoxic metabolites produced by phytopathogenic fungi, in most cases, this has not been enough to determine their role in pathogenesis.
Experiment conducted with 1H-indole-3-carboxaldehyde, showed that 1H-indole-3-carboxaldehyde at 10, 20 and 50 $\mu g.mL^{-1}$ had similar effects like glyphosate at same concentrations after 24 and 36hours on *Orobanche crenata*. However glyphosate had a stronger effect than 1H-indole-3-carboxaldehyde after 12hours (table 19 and 23). After 36hours, glyphosate at 50 $\mu g.ml^{-1}$ caused the highest growth inhibition of 35.3% compared to 34.99 % and 17.3% growth inhibition caused by 1H-indole-3-carboxaldehyde and tyrosol respectively at same concentrations. From literatures, the phytotoxic mode of action of 1H-Indole-3-carboxaldehyde has not been well proven unlike in animals that much has been studied. However, Bashan (1987) suggested that it has a similar action to all plant phytotoxins that it interferes with normal plant metabolism, increases or decreases plant-originated compounds, leading finally to visible chlorosis. Christoph et al 2009 attribute the phytotoxicity effect of Indole phytotoxins to the presence of the nitro group in the indole ring but also stated that the mode of action is unclear.
A similar bioassay conducted on leaf tissues of *Vicia faba* showed that tyrosol had no effect on *Vicia faba* after 24hours. However, tyrosol at higher concentrations had a mild effect on *Vicia faba* after 36hours. After 12hours only glyphosate even at a very low concentration had mild effect on *Vicia faba* while at a high concentration the effect was serious(table 20 and 22).This experiment confirms an earlier experiment by Evidente et al 2010 on the phytotoxic character of tyrosol. Capasso et al 1992 had also reported that tyrosol is selectively toxic during phytotoxicity tests carried out on tomato and vegetable marrow.

3.8. CONCLUSIONS.

In concluding, natural products play an important role in drug discovery and development. Plant and fungi in particular, have been recognized as key players in this aspect. It is well known that *Fusarium oxysporum* produces many metabolites with phytotoxic activities on plants. Interestingly, some of these metabolites have selective phytotoxic effects on plant species.
This study was initiated to prove the above hypothesis i.e. search for fusarium metabolites that are selectively phytotoxic to Orobanche spp.
In this study, it was observed that Fusarium oxysporum, isolated from infected broomrape plants, produce some metabolites that posses phytotoxic potentials against broomrape. These metabolites were identified using LCMS, GCMS and NMR techniques.
Tyrosol and 1H-indole-3-carboxaldehyde are known compounds with pharmaceutical and agrochemical interests. However, this is the first work to report their isolation and selective phytotoxicity on Orobanche spp in Greece.
The following major conclusions were drawn from this study:

1. From literatures, this is the first work conducted in Greece to determine the pathogenicity of *Fusarium oxysporum* to *Orobanche spp*. According to Rice 1983, plants differ in the production of allelochemicals depending upon the environment in which they are grown. This work therefore contributes to scientific information that *Fusarium oxysporum* infecting *Orobanche spp* in Greece have pathogenic and phytotoxic characteristics useful for the control of *Orobanche spp*.

2. While other researchers like Batchvarova et al., have studied the influence of some growth regulators on callus production on *Orobanche ramosa*, little has been done to show the influence of these regulators and other regulators on other *Orobanche spp*.This research attempted to determine the influence of some growth regulators on three Orobanche species. This is important because it gives a general overview of the type of calluses produced by different growth regulators and how long these calluses can survive on these media. Zhou et al., 2004 had also carried out a study on the influence of GA3 (Gibberelic acid), IAA (Indole-3-acetic acid) and NAA (1-naphthaleneaceticacid) on callus production of *O.ramosa*, *O. aegyptiaca* and *O.minor*.
In the absence of similar studies on *O.crenata* and *O.cumana* which are principal parasitic weeds in the Mediterranean region, this study therefore provides some information on production of *O.crenata* and *O.cumana* callusses using IAA and 2,4 D growth regulators with the aim of using them for bioassay with Fusarium phytotoxins.

3. From literatures there is lack of information on the exposure of Orobanche tissue cultures on chemicals for phytotoxic evaluation. This work therefore contributes to knowledge on the effect of phytotoxic chemicals to tissue cultures of Orobanche after exposure.

4. Two metabolites, Tyrosol and 1H-indole-3-carboxaldehyde with phytotoxic activity were identified for the first time from *Fusarium oxysporum* strains infecting *Orobanche crenata*.

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APPENDIX

APPENDIX 1

Table 18 A . Effect of Tyrosol on weight (g) of tissue culture of *O.crenata* 12, 24 and 36 hours after application.

	12HOURS				24HOURS			36HOURS		
TREATMENTS	Initial weight	mean	%Growth inhibition	STDV	mean	%Growth inhibition	STDV	mean	%Growth	STDV
tyrosol 2 µg.ml^{-1}	0,5	0,504	-0,77	0,004359	0,497	0,58	0,01513	0,489	2,13	0,01735
tyrosol 5 µg.ml^{-1}	0,5	0,4967	0,64	0,009923	0,4751	4,84	0,00518	0,4611	7,56	0,00267
tyrosol 10 µg.ml^{-1}	0,5	0,4935	1,26	0,005522	0,46	7,77	0,00557	0,452	9,33	0,00872
tyrosol 20 µg.ml^{-1}	0,5	0,4891	2,11	0,007252	0,444	10,88	0,00755	0,433	13,02	0,01058
tyrosol 50 µg.ml^{-1}	0,5	0,449	9,91	0,014933	0,43	13,6	0,01418	0,411	17,3	0,00529
glyphosate 2 µg.ml^{-1}	0,5	0,511	-2,14	0,005292	0,496	0,77	0,00794	0,469	2,7	0,02343
glyphosate 5 µg.ml^{-1}	0,5	0,484	3,11	0,060556	0,466	6,6	0,00361	0,441	11,46	0,0197
glyphosate 10 µg.ml^{-1}	0,5	0,452	9,33	0,004	0,428	13,99	0,00361	0,396	20,2	0,01418
glyphosate 20 µg.ml^{-1}	0,5	0,446	10,5	0,004359	0,418	15,9	0,00872	0,379	23,52	0,03716
glyphosate 50 µg.ml^{-1}	0,5	0,3885	21,67	0,003905	0,358	27,6	0,00964	0,318	35,3	0,007

APPENDIX 2

Table 18 B. Effect of Tyrosol on weight (g) of tissue culture of *O.crenata* 12, 24 and 36 hours after application.

	12HOURS				24HOURS			36HOURS		
TREATMENTS	Initial weight	mean	%Growth inhibition	STDV	mean	%Growth inhibition	STDV	mean	%Growth	STDV
tyrosol 2 µg.ml^{-1}	0,5	0,503	-0,58	0,002	0,49357	1,25	0,00031	0,47613	4,6	0,00035
tyrosol 5 µg.ml^{-1}	0,5	0,4953	0,91	0,000265	0,46553	6,71	0,00032	0,45943	7,9	0,00031
tyrosol 10 µg.ml^{-1}	0,5	0,49183	1,59	0,000252	0,4586	8,06	0,00036	0,41543	16,47	0,00031
tyrosol 20 µg.ml^{-1}	0,5	0,48543	2,92	0,000252	0,44727	10,32	0,00021	0,3987	19,7	0,00036
tyrosol 50 µg.ml^{-1}	0,5	0,4776	4,3	0,000557	0,43513	12,63	0,00045	0,38557	22,28	0,00042
glyphosate 2 µg.ml^{-1}	0,5	0,50433	-0,77	0,003512	0,4909	1,77	0,0004	0,4685	6,1	0,00036
glyphosate 5 µg.ml^{-1}	0,5	0,4821	3,48	0,0004	0,41073	17,39	0,0005	0,3945	19,8	0,0004
glyphosate 10 µg.ml^{-1}	0,5	0,47917	4,09	0,000306	0,39907	19,67	0,00045	0,3752	24,3	0,00053
glyphosate 20 µg.ml^{-1}	0,5	0,46833	6,17	0,000473	0,38457	22,49	0,00085	0,36433	26,42	0,0004
glyphosate 50 µg.ml^{-1}	0,5	0,46113	7,59	0,000252	0,3734	24,6	0,00046	0,35113	28,99	0,0006
tyrosol+indole 1µg.ml-1	0,5	0,504333	-0,84	0,002082	0,4984	0,31	0,000265	0,486867	2,55	0,000751
tyrosol +indole 2µg.ml-1	0,5	0,504733	-0,91	0,000208	0,4925	1,46	0,0004	0,4752	4,8	0,000458
tyrosol + indole 5µg.ml-1	0,5	0,490533	1,85	0,000153	0,464967	6,83	0,000416	0,4582	8,14	0,0007

APPENDIX 3

Table 19 A . Effect of 1H-indole-3-carboxaldehyde on weight (g) of *O.crenata* 12, 24 and 36 hours after application.

	12hours				24 hours			36hours		
TREATMENTS	Initial weight	mean	%growth	stdv	mean	%growth	stdv	mean	%growth	stdv
1H-indole-3-carboxaldehyde 2 µg.ml^{-1}	0,5	0,502	-0,388	0,003	0,497	0,58	0,015133	0,49	1,94	0,009074
1H-indole-3-carboxaldehyde 5 µg.ml^{-1}	0,5	0,498	0,39	0,007	0,474	5,05	0,007	0,46	7,77	0,005568
1H-indole-3-carboxaldehyde 10 µg.ml^{-1}	0,5	0,495	0,97	0,002646	0,435	12,64	0,012767	0,401	19,24	0,005568
1H-indole-3-carboxaldehyde 20 µg.ml^{-1}	0,5	0,485	2,92	0,006083	0,426	14,38	0,005568	0,385	22,35	0,002
1H-indole-3-carboxaldehyde 50 µg.ml^{-1}	0,5	0,455	8,74	0,004	0,36	27,2	0,005	0,32	34,99	0,014933
glyphosate 2 µg.ml^{-1}	0,5	0,511	-2,14	0,030643	0,496	0,77	0,014177	0,469	2,7	0,015716
Glyphosate 5 µg.ml^{-1}	0,5	0,484	3,11	0,006083	0,466	6,6	0,003606	0,441	11,46	0,007
glyphosate 10 µg.ml^{-1}	0,5	0,452	9,33	0,007	0,428	13,99	0,003	0,396	20,2	0,01044
glyphosate 20 µg.ml^{-1}	0,5	0,446	10,05	0,010583	0,418	15,9	0,003606	0,379	23,52	0,01044
glyphosate 50 µg.ml^{-1}	0,5	0,3885	21,67	0,008789	0,358	27,6	0,009644	0,318	35,3	0,007

APPENDIX 4

Table 19 B. Effect of 1H-indole-3-carboxaldehyde on weight (g) of *O.crenata* 12, 24 and 36 hours after application.

	12 hours				24 hours			36hours		
TREATMENTS	Initial weight	mean	%growth	stdv	mean	%growth	stdv	mean	%growth	stdv
1H-indole-3-carboxaldehyde 2 µg.ml^{-1}	0,5	0,50167	-0,32	0,001155	0,49647	0,687	0,000252	0,34363	2,59	0,000451
1H-indole-3-carboxaldehyde 5 µg.ml^{-1}	0,5	0,49533	0,909	0,004041	0,4324	13,167	0,010201	6,5886	20,1	0,000208
1H-indole-3-carboxaldehyde 10 µg.ml^{-1}	0,5	0,487	2,53	0,001732	0,40503	18,49	0,000416	9,24521	24,65	0,000416
1H-indole-3-carboxaldehyde 20 µg.ml^{-1}	0,5	0,48247	3,41	0,000351	0,39717	20,02	1,738228	10,8791	25,41	0,000153
1H-indole-3-carboxaldehyde 50 µg.ml^{-1}	0,5	0,46947	5,9	0,000351	0,37517	24,31	0,000306	12,1552	28,2	0,004967
glyphosate 2 µg.ml^{-1}	0,5	0,50433	-0,77	0,003512	0,4909	1,77	0,0004	0,8852	6,1	0,000361
glyphosate 5 µg.ml^{-1}	0,5	0,4821	3,48	0,0004	0,41073	17,39	0,000503	8,69525	19,8	0,0004
glyphosate 10 µg.ml^{-1}	0,5	0,47917	4,09	0,000306	0,39907	19,67	0,000451	9,83523	24,3	0,000529
glyphosate 20 µg.ml^{-1}	0,5	0,46833	6,17	0,000473	0,38457	22,49	0,00085	11,2454	26,42	0,000404
glyphosate 50 µg.ml^{-1}	0,5	0,46113	7,59	0,000252	0,3734	24,6	0,000458	12,3002	28,99	0,000603
tyrosol+indole 1µg.ml-1	0,5	0,504333	-0,84	0,002082	0,4984	0,31	0,000265	0,15513	2,55	0,000751
tyrosol +indole 2µg.ml-1	0,5	0,504733	-0,91	0,000208	0,4925	1,46	0,0004	0,7302	4,8	0,000458
tyrosol + indole 5µg.ml-1	0,5	0,490533	1,85	0,000153	0,464967	6,83	0,000416	3,41521	8,14	0,0007

APPENDIX 5

Table 20 A. Effect of Tyrosol on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application.

		12hours			24hours			36hours		
Treatments	initial wt	mean	%growth	stdv	mean	%growth	stdv	mean	%growth	stdv
tyrosol 2 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
tyrosol 5 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
tyrosol 10 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,0485	3,06	0,0005
tyrosol 20 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,048	4,08	0,001
tyrosol 50 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,047	6,12	0,001
glyphosate 2 µg.ml^{-1}	0,05	0,048	4,08	0,001	0,048	4,08	0,001	0,047	6,12	0,001
glyphosate 5 µg.ml^{-1}	0,05	0,047	6,12	0,001	0,043	14,2	0,004359	0,04	20,4	0,002
glyphosate 10 µg.ml^{-1}	0,05	0,046	8,16	0,001	0,039	22,45	0,00781	0,035	30,61	0,002
glyphosate 20 µg.ml^{-1}	0,05	0,044	12,24	0,001	0,037	26,5	0,002	0,034	32,65	0,001
glyphosate 50 µg.ml^{-1}	0,05	0,041	18,3	0,002646	0,035	30,6	0,002646	0,031	38,77	0,001

APPENDIX 6

Table 20 B. Effect of Tyrosol on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application.

		12hours			24hours			36hours		
Treatments	initial wt	mean	%growth	stdv	mean	%growth	stdv	mean	%growth	stdv
tyrosol 2 µg.ml^{-1}	0,5	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
tyrosol 5 µg.ml^{-1}	0,5	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
tyrosol 10 µg.ml^{-1}	0,5	0,049	2	8,5E-18	0,048667	2,7	0,000577	0,048	4,08	8,5E-18
tyrosol 20 µg.ml^{-1}	0,5	0,048667	2,7	0,000577	0,047667	4,75	0,000577	0,04667	6,79	0,000577
tyrosol 50 µg.ml^{-1}	0,5	0,048667	2,7	0,000577	0,047333	5,44	0,001155	0,04633	7,48	0,000577
glyphosate 2 µg.ml^{-1}	0,5	0,047667	4,75	0,000577	0,046667	6,79	0,001155	0,03967	21,08	0,000577
glyphosate 5 µg.ml^{-1}	0,5	0,046667	6,79	0,000577	0,041667	17	0,001528	0,03333	34,08	0,000577
glyphosate 10 µg.ml^{-1}	0,5	0,045667	8,83	0,000577	0,036333	27,89	0,001528	0,032	36,73	0,001732
glyphosate 20 µg.ml^{-1}	0,5	0,044333	11,57	0,000577	0,034	32,65	0,002646	0,029	42,85	0,002
glyphosate 50 µg.ml^{-1}	0,5	0,040667	19,04	0,000577	0,031	38,77	0,002	0,026	48,97	0,001
tyrosol+indole 1µg.ml-1	0,5	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
tyrosol +indole 2µg.ml-1	0,5	0,048667	2,7	0,000577	0,048667	2,7	0,000577	0,048	4,08	8,5E-18
tyrosol + indole 5µg.ml-1	0,5	0,048667	2,7	0,000577	0,048333	3,4	0,000577	0,046333	7,48	0,000577

APPENDIX 7

Table 21 A. Effect of 1H-indole-3-carboxaldehyde on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application										
	12hrs				24hours			36hours		
treatments	initial wt	mean	%growth	stdv	mean	%growth	stdv	mean	%growth	stdv
1H-indolecarboxaldehyde 2 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
1H-indolecarboxaldehyde 5 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
1H-indolecarboxaldehyde 10 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,0485	3,67	0,0005
1H-indolecarboxaldehyde 20 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,048	4,08	0,001
1H-indolecarboxaldehyde 50 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,047	6,12	0,001
glyphosate 2 µg.ml^{-1}	0,05	0,048	4,08	0,001	0,048	4,08	0,001	0,047	6,12	0,001
glyphosate 5 µg.ml^{-1}	0,05	0,047	6,12	0,001	0,043	14,2	0,004359	0,04	20,4	0,002
glyphosate 10 µg.ml^{-1}	0,05	0,046	8,16	0,001	0,039	22,45	0,00781	0,035	30,61	0,002
glyphosate 20 µg.ml^{-1}	0,05	0,044	12,24	0,001	0,037	26,5	0,002	0,034	32,65	0,001
glyphosate 50 µg.ml^{-1}	0,05	0,041	18,3	0,002646	0,035	30,6	0,002646	0,031	38,77	0,001

APPENDIX 8

Table 21 B . Effect of 1H-indole-3-carboxaldehyde on weight (g) of leaf tissues of *Vicia faba 12*, 24 and 36 hours after application

		12 hours			24hours			36hours		
treatments	initial wt	mean	%growth	stdv	mean	%growth	stdv	mean	%growth	stdv
1H-indole-3-carboxaldehyde 2 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
1H-indole-3-carboxaldehyde 5 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
1H-indole-3-carboxaldehyde 10 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,049	2	8,5E-18	0,04833	3,4	0,000577
1H-indole-3-carboxaldehyde 20 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,04867	2,7	0,000577	0,04767	4,75	0,000577
1H-indole-3-carboxaldehyde 50 µg.ml^{-1}	0,05	0,049	2	8,5E-18	0,04867	2,7	0,000577	0,04667	6,79	0,000577
glyphosate 2 µg.ml^{-1}	0,05	0,04767	4,75	0,000577	0,04667	6,79	0,001155	0,03967	21,08	0,000577
glyphosate 5 µg.ml^{-1}	0,05	0,04667	6,79	0,000577	0,04167	17	0,001528	0,03333	34,08	0,000577
glyphosate 10 µg.ml^{-1}	0,05	0,04567	8,83	0,000577	0,03633	27,89	0,001528	0,032	36,73	0,001732
glyphosate 20 µg.ml^{-1}	0,05	0,04433	11,57	0,000577	0,034	32,65	0,002646	0,029	42,85	0,002
glyphosate 50 µg.ml^{-1}	0,05	0,04067	19,04	0,000577	0,031	38,77	0,002	0,026	48,97	0,001
tyrosol+indole 1µg.ml-1	0,5	0,049	2	8,5E-18	0,049	2	8,5E-18	0,049	2	8,5E-18
tyrosol +indole 2µg.ml-1	0,5	0,048667	2,7	0,000577	0,048667	2,7	0,000577	0,048	4,08	8,5E-18
tyrosol + indole 5µg.ml-1	0,5	0,048667	2,7	0,000577	0,048333	3,4	0,000577	0,046333	7,48	0,000577

APPENDIX 9

Table 24 A Comparative effect of tyrosol and glyphosate on the chlorophyll content of *Vicia faba*										
	12hours			24hours			36hours			
treatments	665mean	645mean	chla	665mean	645mean	chla	665mean	645mean	chla	
tyrosol 2	1,453	0,6266	0,0168	1,4565	0,6263	0,0168	1,4221	0,6115	0,0164	
tyrosol 5	1,4227	0,6117	0,0164	1,4211	0,6105	0,0164	1,4116	0,6069	0,0162	
tyrosol 10	1,3875	0,5827	0,016	1,3776	0,5731	0,0159	1,3221	0,5685	0,0153	
tyrosol 20	1,2163	0,523	0,014	1,203	0,517	0,0138	1,2004	0,504	0,0138	
tyrosol 50	0,9687	0,387	0,0112	0,9534	0,381	0,011	0,9332	0,3719	0,0108	
glyphosate 2	1,068	0,4592	0,0123	1,0057	0,4324	0,0116	0,9874	0,4245	0,0114	
glyphosate 5	0,9276	0,3988	0,0107	0,9015	0,3876	0,0104	0,8712	0,3741	0,01	
glyphosate 10	0,7528	0,324	0,0086	0,7114	0,3233	0,0081	0,6514	0,2795	0,0075	
glyphosate 20	0,6116	0,264	0,007	0,5848	0,2632	0,0067	0,5261	0,2236	0,006	
glyphosate50	0,5115	0,223	0,0058	0,4896	0,211	0,0056	0,431	0,1849	0,0049	

APPENDIX 10

Table 24 B . Comparative effect of tyrosol and glyphosate on the chlorophyll content of *Vicia faba*										
	12hours			24hours			36hours			
treatments	665mean	645mean	chla	665mean	645mean	chla	665mean	645mean	chla	
tyrosol 2	1,4549	0,6244	0,0168	1,45305	0,61985	0,01679	1,42015	0,61035	0,016394	
tyrosol 5	1,4224	0,6103	0,01642	1,4211	0,60665	0,01642	1,41	0,60555	0,016278	
tyrosol 10	1,38435	0,5812	0,01602	1,3759	0,57065	0,01594	1,318	0,56205	0,015227	
tyrosol 20	1,22105	0,52365	0,0141	1,2095	0,519	0,01396	1,1985	0,498	0,013881	
tyrosol 50	0,9632	0,385	0,0112	0,94995	0,373	0,01106	0,93	0,37	0,010816	
glyphosate 2	1,0727	0,45675	0,01239	1,0011	0,4306	0,01156	0,9825	0,42205	0,011342	
glyphosate 5	0,92805	0,39685	0,01072	0,907	0,38335	0,01049	0,86835	0,37185	0,010028	
glyphosate 10	0,74215	0,32	0,00856	0,71095	0,3185	0,00817	0,64185	0,2751	0,007411	
glyphosate 20	0,6085	0,2495	0,00706	0,57305	0,24465	0,00662	0,5185	0,221	0,00599	
glyphosate50	0,50055	0,225	0,00575	0,4881	0,2103	0,00563	0,43	0,18285	0,004969	
tyrosol+indole 1µg.ml-1	1,47265	0,7103	0,016792	1,45505	0,6305	0,016783	1,42695	0,62355	0,016445	
tyrosol +indole 2µg.ml-1	1,4438	0,61285	0,016688	1,44285	0,61095	0,016681	1,4162	0,597	0,01638	
tyrosol + indole 5µg.ml-1	1,4126	0,60035	0,016325	1,40705	0,58905	0,016285	1,404435	0,59415	0,016238	

APPENDIX 11

Table 25 A. Comparative effect of 1H-indole-3-carboxaldehyde and glyphosate on the chlorophyll content of *Vicia faba*										
	12HOURS			24HOURS			36HOURS			
treatments	665mean	645mean	chla	665mean	645mean	chla	665mean	645mean	chla	
1H-indole-3-carboxaldehyde 2	1,4164	0,6088	0,0163	1,4048	0,6041	0,0162	1,3955	0,6	0,0161	
1H-indole-3-carboxaldehyde 5	1,3919	0,5985	0,016	1,316	0,5852	0,0157	1,3223	0,5685	0,01526	
1H-indole-3-carboxaldehyde10	1,2214	0,5252	0,014	1,2113	0,5209	0,0139	1,1896	0,5115	0,0137	
1H-indole-3-carboxaldehyde 20	1,1011	0,4347	0,0128	1,0987	0,4724	0,0127	1,0058	0,4324	0,0116	
1H-indole-3-carboxaldehyde 50	0,8396	0,361	0,0097	0,8114	0,3489	0,0094	0,8002	0,344	0,0092	
glyphosate 2	1,068	0,4592	0,0123	1,0057	0,4324	0,0116	0,9874	0,4245	0,0114	
glyphosate 5	0,9276	0,3988	0,0107	0,9015	0,3876	0,0104	0,8712	0,3741	0,01	
glyphosate 10	0,7528	0,324	0,0086	0,7114	0,3233	0,0081	0,6514	0,2795	0,0075	
glyphosate 20	0,6116	0,264	0,007	0,5848	0,2632	0,0067	0,5261	0,2236	0,006	
glyphosate50	0,5115	0,223	0,0058	0,4896	0,211	0,0056	0,431	0,1849	0,0049	

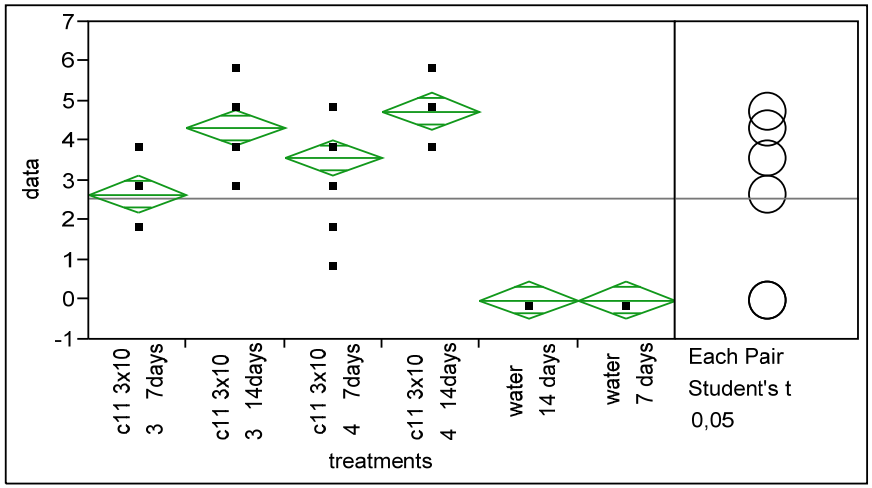
APPENDIX 12

Table 25 B. Comparative effect of 1H-indole-3-carboxaldehyde and glyphosate on the chlorophyll content of *Vicia faba*

	12HOURS			24HOURS			36HOURS			
treatments	665mean	645mean	chla	665mean	645mean	chla	665mean	645mean	chla	
1H-indole-3-carboxaldehyde 2	1,411	0,60365	0,016296	1,40315	0,60315	0,0162	1,39305	0,595	0,01609	
1H-indole-3-carboxaldehyde 5	1,3918	0,59255	0,016082	1,36035	0,5827	0,01571	1,3209	0,5669	0,01525	
1H-indole-3-carboxaldehyde10	1,2415	0,52285	0,014361	1,2107	0,51935	0,01398	1,18505	0,5099	0,01368	
1H-indole-3-carboxaldehyde 20	1,1045	0,4333	0,012862	1,09495	0,43115	0,01275	1,00155	0,42585	0,01157	
1H-indole-3-carboxaldehyde 50	0,9305	0,38475	0,010782	0,80085	0,34485	0,00924	0,80105	0,3215	0,00931	
glyphosate 2	1,0727	0,45675	0,012395	1,0011	0,4306	0,01156	0,9825	0,42205	0,01134	
glyphosate 5	0,92805	0,39685	0,010719	0,907	0,38335	0,01049	0,86835	0,37185	0,01003	
glyphosate 10	0,74215	0,32	0,008565	0,71095	0,3185	0,00817	0,64185	0,2751	0,00741	
glyphosate 20	0,6085	0,2495	0,007057	0,57305	0,24465	0,00662	0,5185	0,221	0,00599	
glyphosate50	0,50055	0,225	0,005752	0,4881	0,2103	0,00563	0,43	0,18285	0,00497	
tyrosol+indole 1µg.ml-1	1,47265	0,7103	0,016792	1,45505	0,6305	0,016783	1,42695	0,62355	0,016445	
tyrosol +indole 2µg.ml-1	1,4438	0,61285	0,016688	1,44285	0,61095	0,016681	1,4162	0,597	0,01638	
tyrosol + indole 5µg.ml-1	1,4126	0,60035	0,016325	1,40705	0,58905	0,016285	1,40525	0,55915	0,016343	

APPENDIX 13.
Attachments of *O.cumana* as in table 5

Oneway Analysis of data by treatments



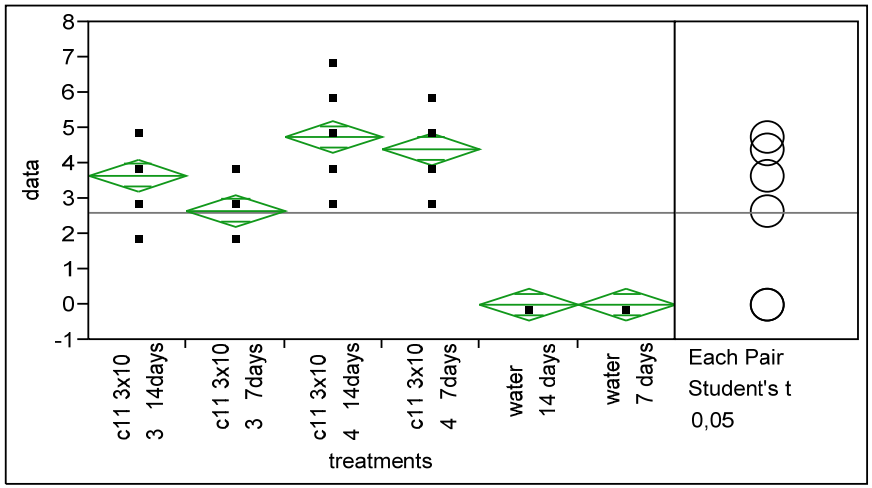
Means Comparisons

Level					Mean
c11 3x10 4 14days	A				4,7500000
c11 3x10 3 14days	A				4,3333333
c11 3x10 4 7days		B			3,5833333
c11 3x10 3 7days			C		2,6666667
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 14
Attachment of *O.crenata* as in table 5

Oneway Analysis of data by treatments



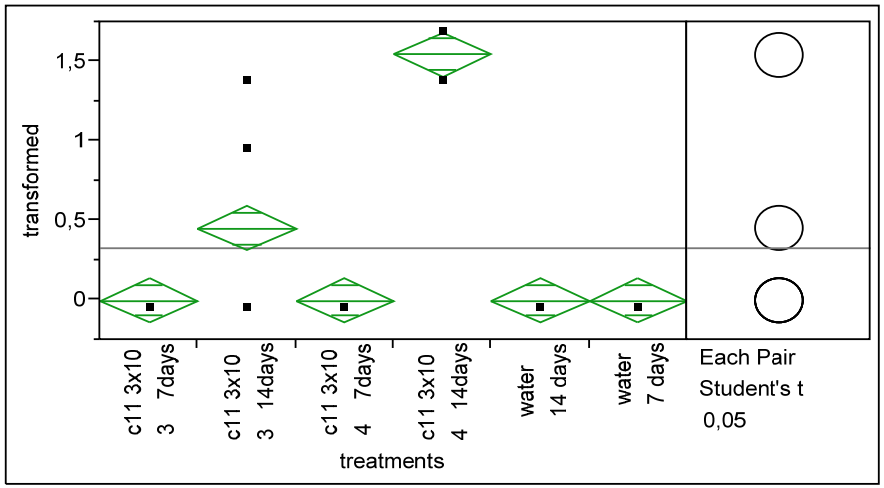
Means Comparisons

Level					Mean
c11 3x10 4 14days	A				4,7500000
c11 3x10 4 7days	A				4,4166667
c11 3x10 3 14days		B			3,6666667
c11 3x10 3 7days			C		2,6666667
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 15
Dead plants of *O. cumana* as in table 7

Oneway Analysis of transformed by treatments



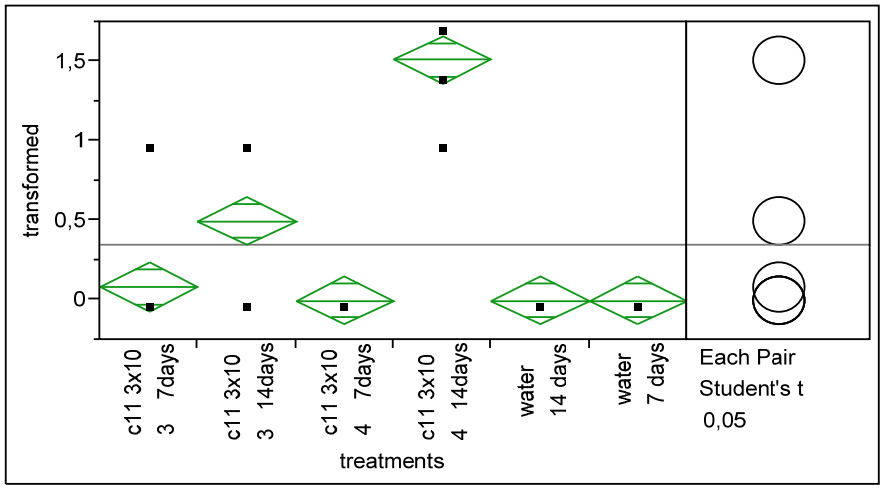
Means Comparisons

Level				Mean
c11 3x10 4 14days	A			1,5466457
c11 3x10 3 14days		B		0,4511845
c11 3x10 4 7days			C	0,0000000
c11 3x10 3 7days			C	0,0000000
water 14 days			C	0,0000000
water 7 days			C	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 16
Dead plants of *O. cumana* as in table 8

Oneway Analysis of transformed by treatments



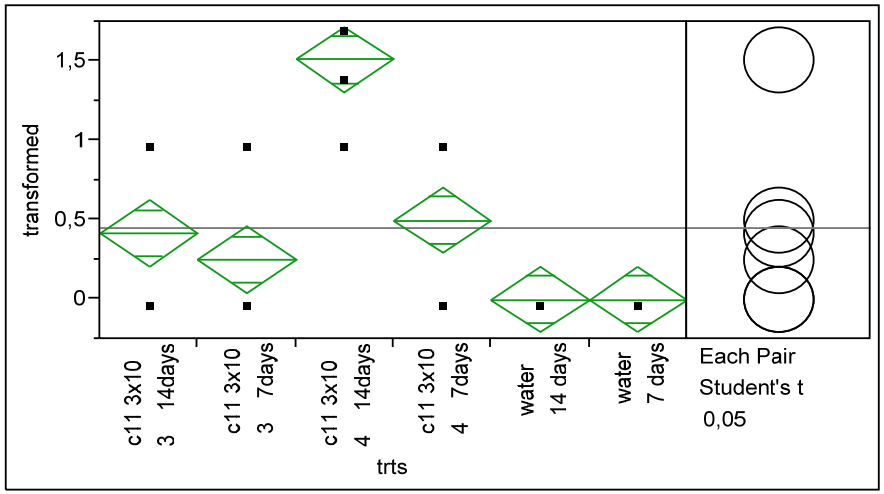
Means Comparisons

Level				Mean
c11 3x10 4 14days	A			1,5121280
c11 3x10 3 14days		B		0,5000000
c11 3x10 3 7days			C	0,0833333
c11 3x10 4 7days			C	0,0000000
water 14 days			C	0,0000000
water 7 days			C	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 17
Dead plants of *O.crenata* on *V.faba* as in table 8

Oneway Analysis of transformed by trts



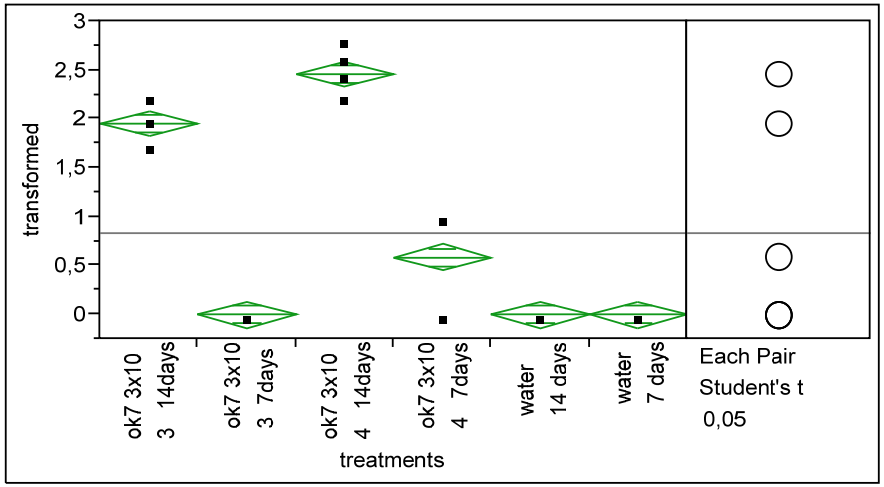
Means Comparisons

Level				Mean
c11 3x10 4 14days	A			1,5121280
c11 3x10 4 7days		B		0,5000000
c11 3x10 3 14days		B		0,4166667
c11 3x10 3 7days		B	C	0,2500000
water 14 days			C	0,0000000
water 7 days			C	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 18
Dead plants of *O.crenata* on *V. faba* as in table 7

Oneway Analysis of transformed by treatments



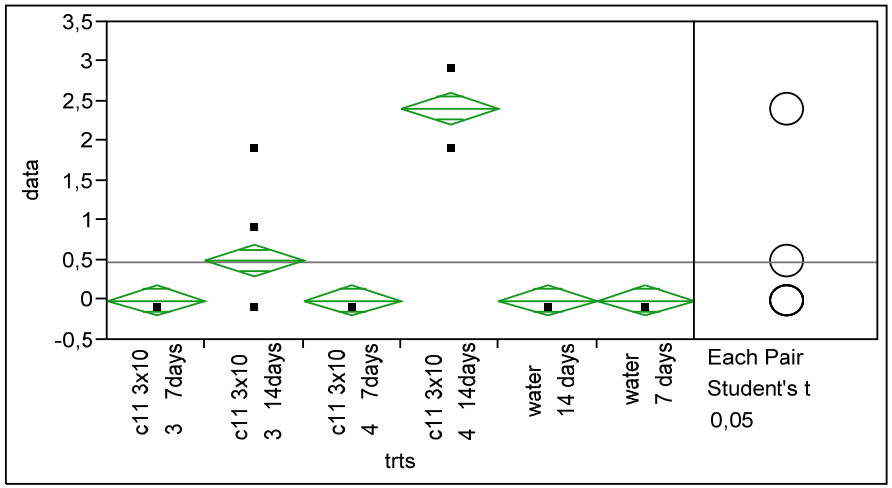
Means Comparisons

Level				Mean
ok7 3x10 4 14days	A			2,4589927
ok7 3x10 3 14days		B		1,9526850
ok7 3x10 4 7days			C	0,5833333
ok7 3x10 3 7days			D	0,0000000
water 14 days			D	0,0000000
water 7 days			D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 19
Dead plants of *O.crenata* on *V. sativa* as in table 8

Oneway Analysis of data by treatments



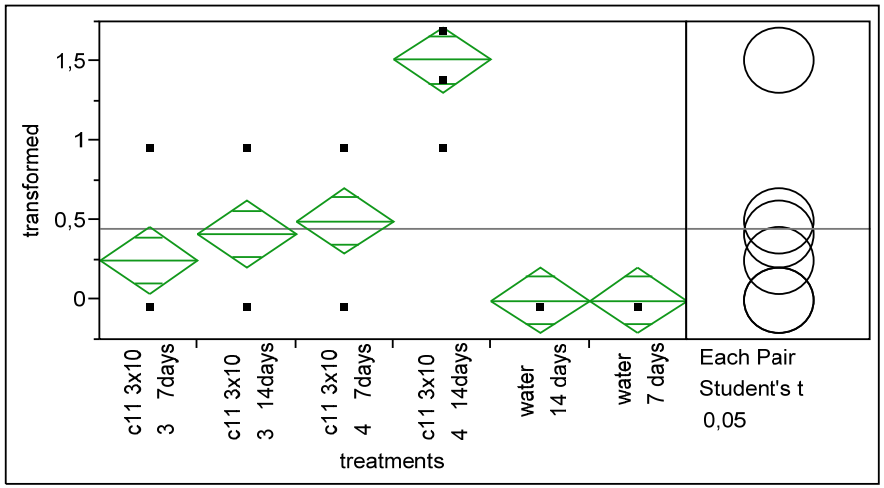
Means Comparisons

Level				Mean
c11 3x10 4 14days	A			2,4166667
c11 3x10 3 14days		B		0,5000000
c11 3x10 4 7days			C	0,0000000
c11 3x10 3 7days			C	0,0000000
water 14 days			C	0,0000000
water 7 days			C	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 20
Dead plants of *O.crenata* on *V. sativa* as in table 7

Oneway Analysis of transformed by treatments



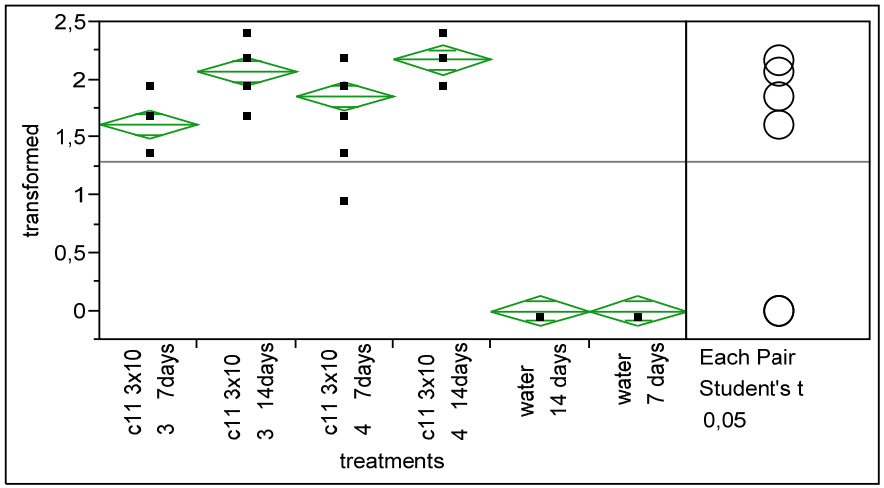
Means Comparisons

Level				Mean
c11 3x10 4 14days	A			1,5121280
c11 3x10 4 7days		B		0,5000000
c11 3x10 3 14days		B		0,4166667
c11 3x10 3 7days		B	C	0,2500000
water 14 days			C	0,0000000
water 7 days			C	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 21
Diseased *O.cumana* as in table 8

Oneway Analysis of transformed by treatments



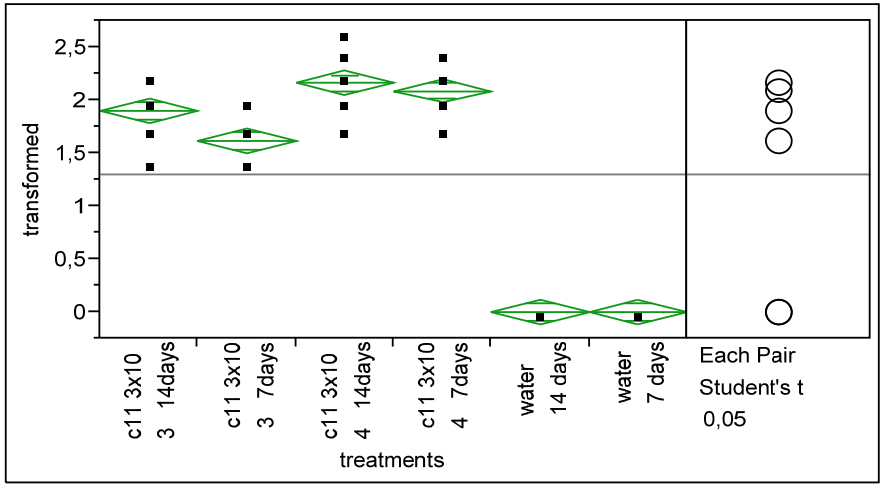
Means Comparisons

Level					Mean
c11 3x10 4 14days	A				2,1732766
c11 3x10 3 14days	A				2,0714886
c11 3x10 4 7days		B			1,8557235
c11 3x10 3 7days			C		1,6177904
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 22
Diseased *O. crenata* on *V.faba* as in table 8

Oneway Analysis of transformed by treatments



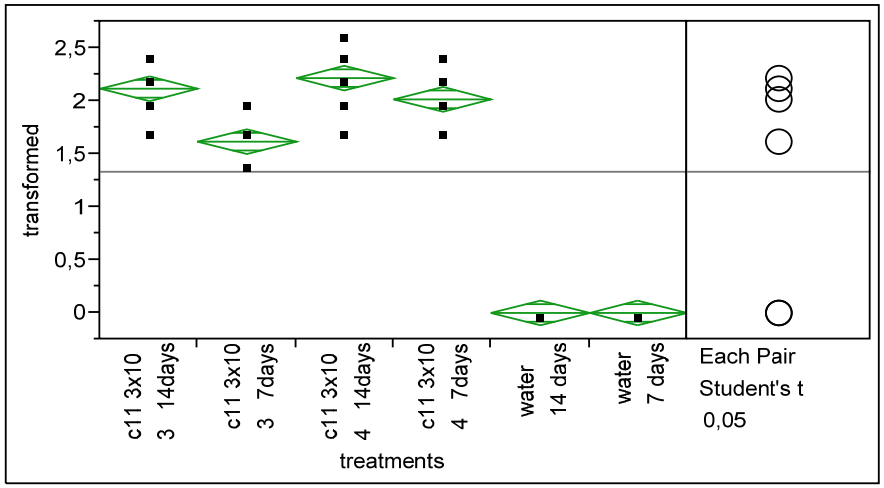
Means Comparisons

Level					Mean
c11 3x10 4 14days	A				2,1608715
c11 3x10 4 7days	A				2,0911609
c11 3x10 3 14days		B			1,9038695
c11 3x10 3 7days			C		1,6177904
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 23
Diseased *O.cumana* as in table7

Oneway Analysis of transformed data by treatments



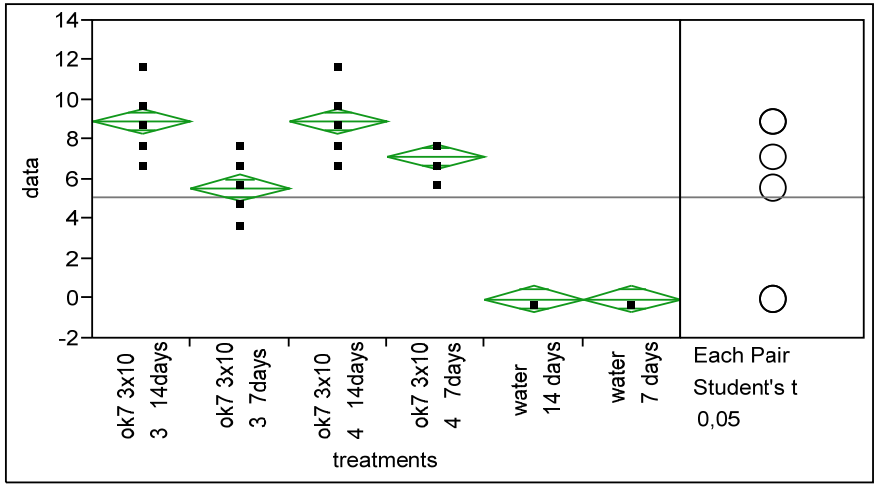
Means Comparisons

Level					Mean
c11 3x10 4 14days	A				2,2146841
c11 3x10 3 14days	A	B			2,1116029
c11 3x10 4 7days		B			2,0098148
c11 3x10 3 7days			C		1,6177904
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 24
Diseased *O. crenata* on *V.faba* as in table 7

Oneway Analysis of data by treatments



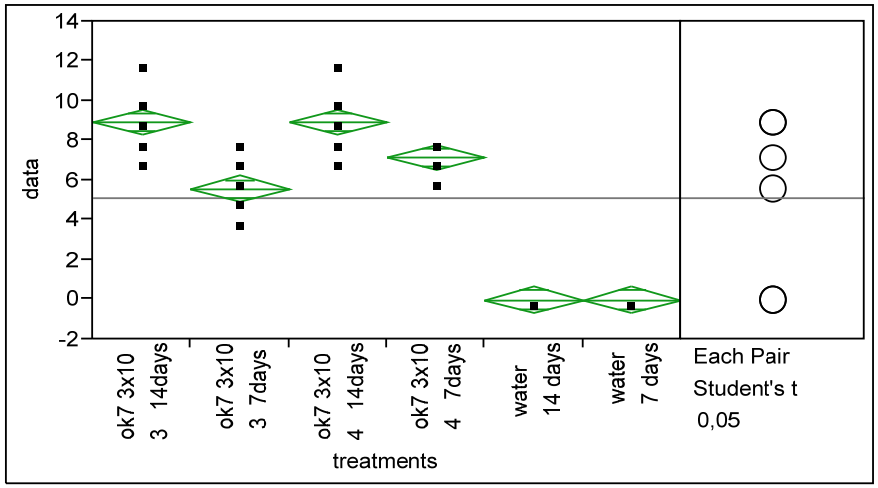
Means Comparisons

Level					Mean
ok7 3x10 3 14days	A				8,9166667
ok7 3x10 4 14days	A				8,9166667
ok7 3x10 4 7days		B			7,1666667
ok7 3x10 3 7days			C		5,5833333
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 25
Diseased *V.sativa* as in table 8

Oneway Analysis of data by treatments



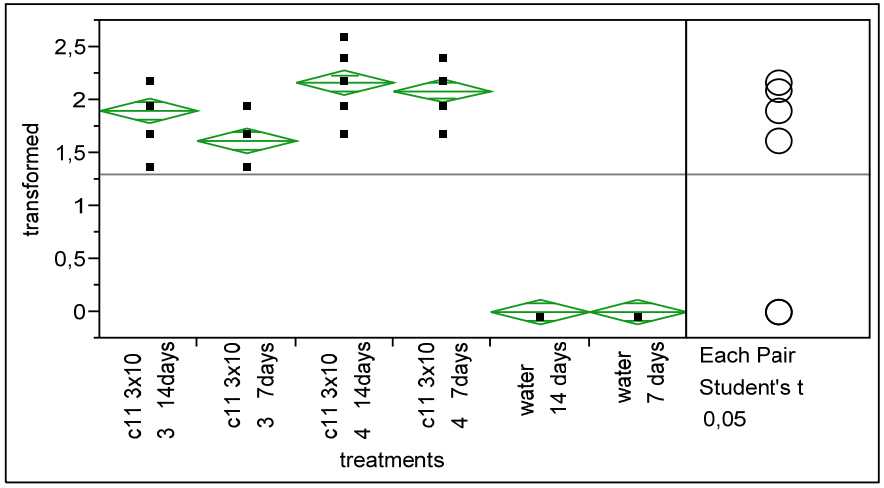
Means Comparisons

Level					Mean
ok7 3x10 3 14days	A				8,9166667
ok7 3x10 4 14days	A				8,9166667
ok7 3x10 4 7days		B			7,1666667
ok7 3x10 3 7days			C		5,5833333
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 26
Diseased *V.sativa* as in table 7

Oneway Analysis of transformed by treatments



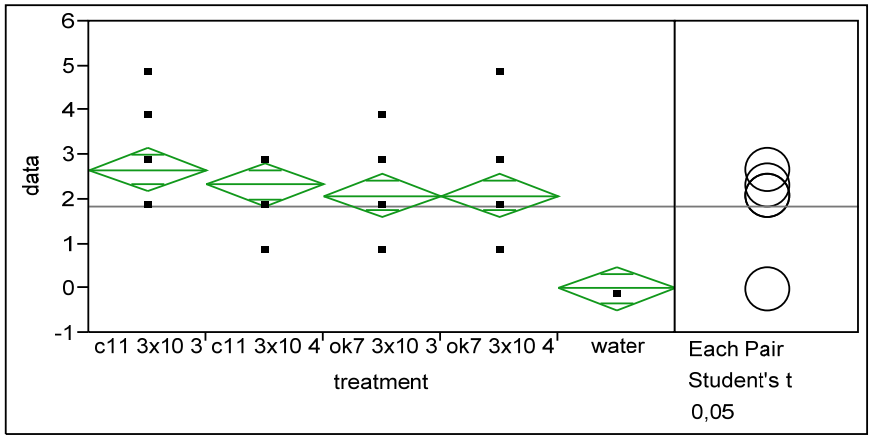
Means Comparisons

Level					Mean
c11 3x10 4 14days	A				2,1608715
c11 3x10 4 7days	A				2,0911609
c11 3x10 3 14days		B			1,9038695
c11 3x10 3 7days			C		1,6177904
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 27
No of *V.faba* as in table 6

Oneway Analysis of data by treatment



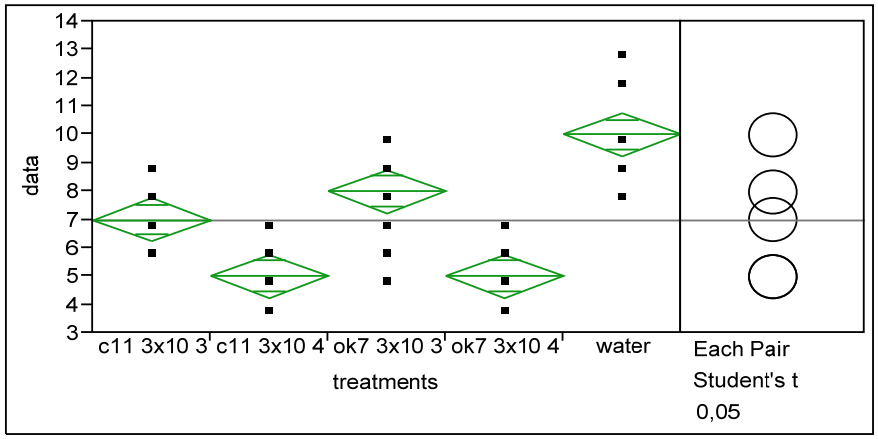
Means Comparisons

Level		Mean
c11 3x10 3	A	2,6666667
c11 3x10 4	A	2,3333333
ok7 3x10 3	A	2,0833333
ok7 3x10 4	A	2,0833333
water	B	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 28
Spider on *O.cumana* as in table 5

Oneway Analysis of data by treatments



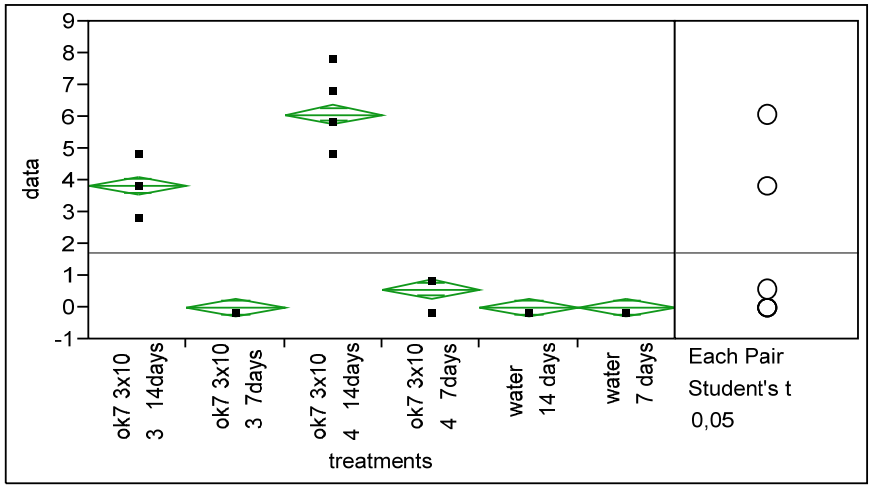
Means Comparisons

Level				Mean
water	A			10,000000
ok7 3x10 3		B		8,000000
c11 3x10 3		B		7,000000
c11 3x10 4			C	5,000000
ok7 3x10 4			C	5,000000

Levels not connected by same letter are significantly different.

APPENDIX 29
Spiders on *V.sativa* as in table 5

Oneway Analysis of data by treatments



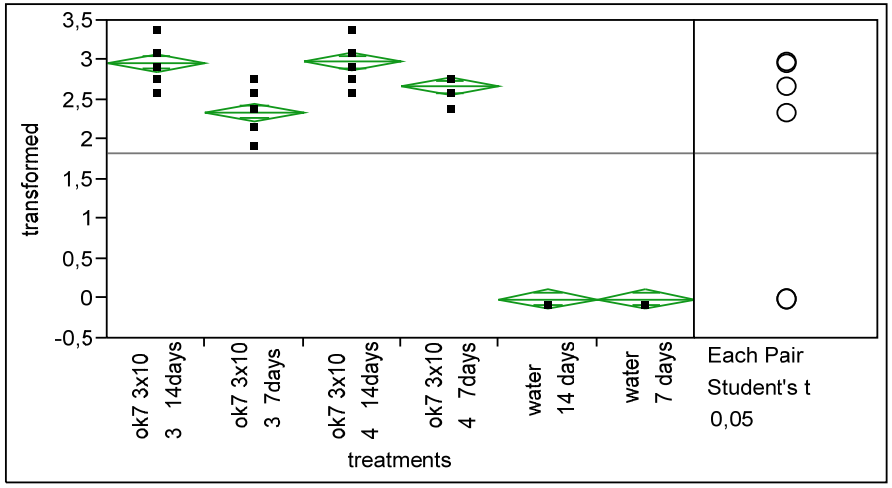
Means Comparisons

Level				Mean
ok7 3x10 4 14days	A			6,0833333
ok7 3x10 3 14days		B		3,8333333
ok7 3x10 4 7days			C	0,5833333
ok7 3x10 3 7days				0,0000000
water 14 days				0,0000000
water 7 days				0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 30
Tubercles on *V.sativa* as in table 5

Oneway Analysis of transformed by treatments



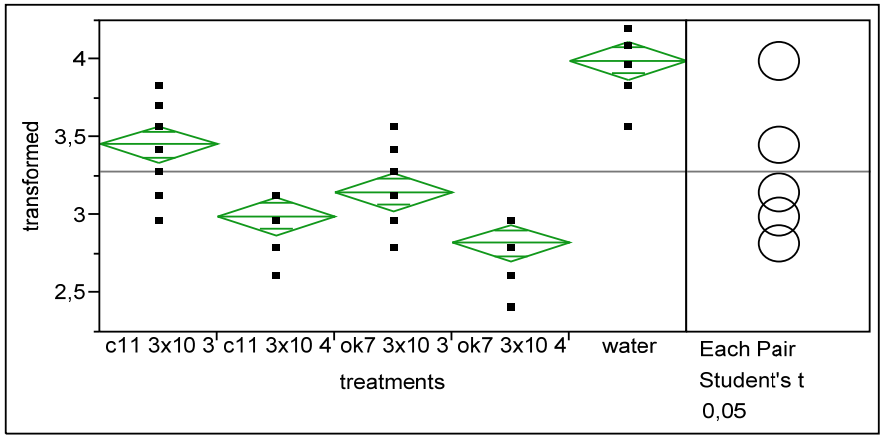
Means Comparisons

Level					Mean
ok7 3x10 4 14days	A				2,9797843
ok7 3x10 3 14days	A				2,9685724
ok7 3x10 4 7days		B			2,6750651
ok7 3x10 3 7days			C		2,3518246
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 31
Tubercles of *O.cumana* as in table 5

Oneway Analysis of transformed by treatments



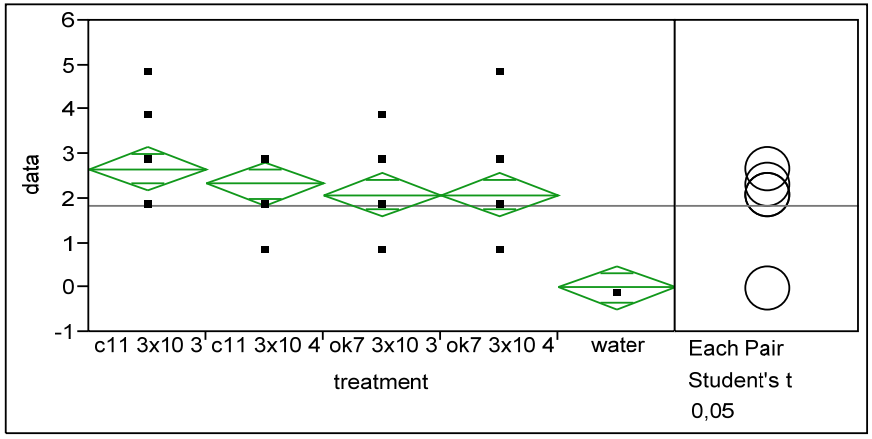
Means Comparisons

Level					Mean
water	A				3,9960069
c11 3x10 3		B			3,4564731
ok7 3x10 3			C		3,1526491
c11 3x10 4			C		2,9959764
ok7 3x10 4				D	2,8235940

Levels not connected by same letter are significantly different.

APPENDIX 32
Diseased *V. faba* as in table 6

Oneway Analysis of data by treatment



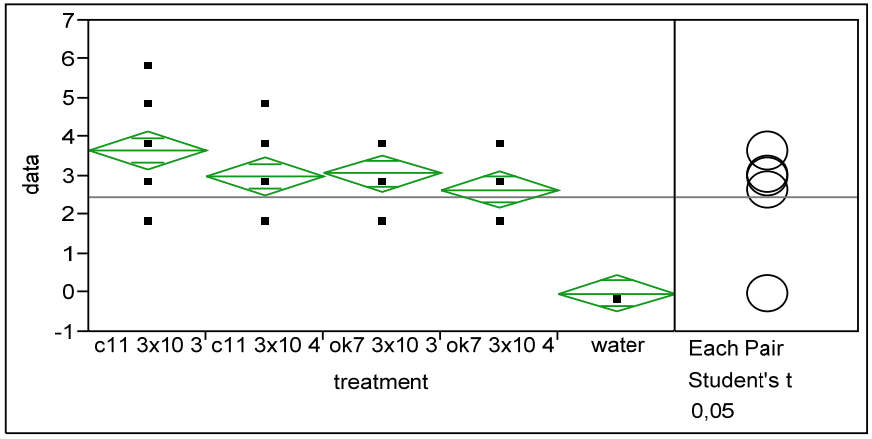
Means Comparisons

Level		Mean
c11 3x10 3	A	2,6666667
c11 3x10 4	A	2,3333333
ok7 3x10 3	A	2,0833333
ok7 3x10 4	A	2,0833333
water	B	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 33
Diseased *O. crenata* on *V.sativa* as in table 6

Oneway Analysis of data by treatment



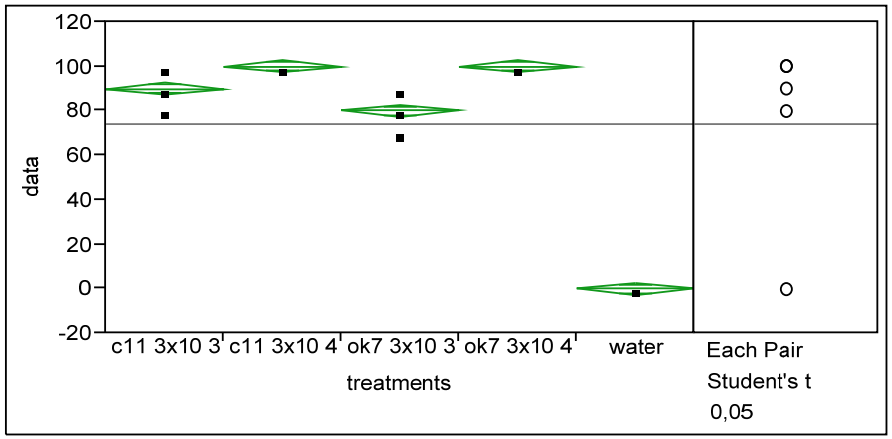
Means Comparisons

Level				Mean
c11 3x10 3	A			3,6666667
ok7 3x10 3	A	B		3,0833333
c11 3x10 4		B		3,0000000
ok7 3x10 4		B		2,6666667
water			C	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 34
Diseased *O. cumana* spiders as in table 5

Oneway Analysis of data by treatments



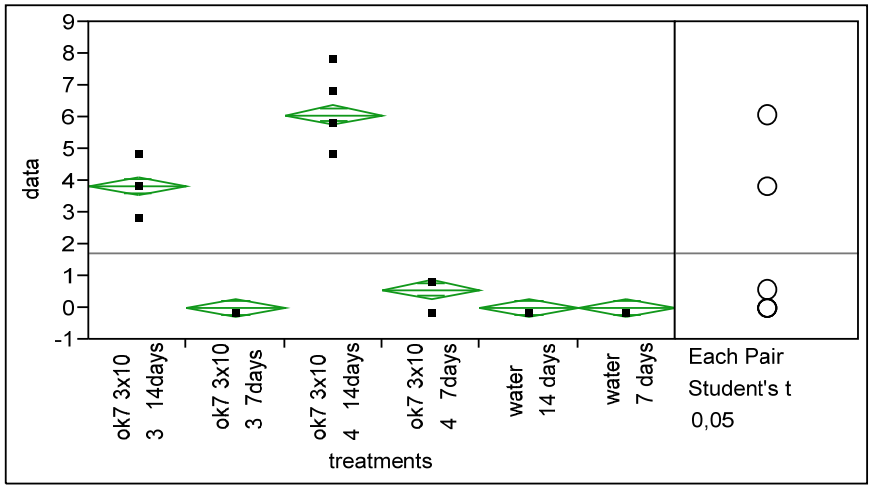
Means Comparisons

Level					Mean
c11 3x10 4	A				100,00000
ok7 3x10 4	A				100,00000
c11 3x10 3		B			90,00000
ok7 3x10 3			C		80,00000
water				D	0,00000

Levels not connected by same letter are significantly different.

APPENDIX 35
Diseased spiders of *O. crenata* as in table 5

Oneway Analysis of data by treatments



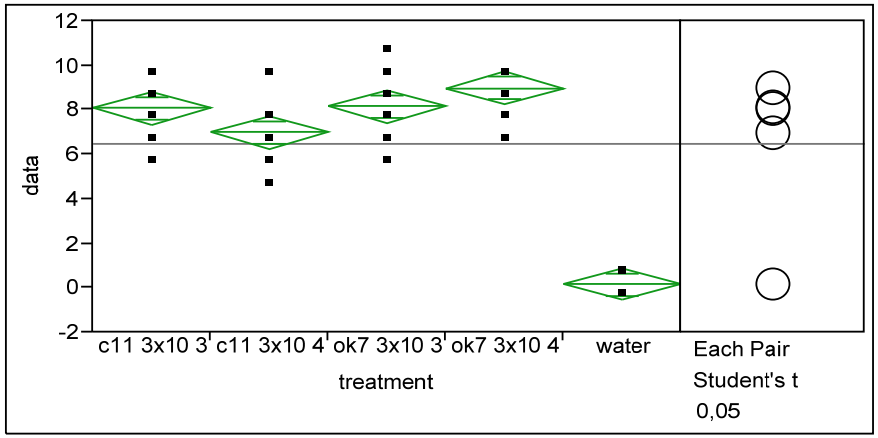
Means Comparisons

Level					Mean
ok7 3x10 4 14days	A				6,0833333
ok7 3x10 3 14days		B			3,8333333
ok7 3x10 4 7days			C		0,5833333
ok7 3x10 3 7days				D	0,0000000
water 14 days				D	0,0000000
water 7 days				D	0,0000000

Levels not connected by same letter are significantly different.

APPENDIX 36
***O.cumana* as in table 6**

Oneway Analysis of data by treatment



Means Comparisons

Level				Mean
ok7 3x10 4	A			9,0000000
ok7 3x10 3	A			8,1666667
c11 3x10 3	A			8,0833333
c11 3x10 4		B		7,0000000
water			C	0,1666667

Levels not connected by same letter are significantly different.