Joint Workshop

INTERNATIONAL BIOHERBICIDE GROUP
and
EWRS – BIOCONTROL WORKING GROUP

Current status and future prospects in bioherbicide research and product development

Bari, Italy, Sunday 19 June 2005
(organizer: Maurizio Vurro)

09:00 – 11:00 **Discovery of weed biocontrol agents (chair: Raghavan Charudattan)**

Mohamed Abouzeid	Pattern and potentiality of fungal isolates from soil-rhizosphere and from young *Orobanche crenata* infesting *Vicia faba* fields in south of Egypt
Alexander Berestetskyi	Can mycelial inoculum be an alternative to conidia in the case of *Stagonospora cirsii* J.J. Davis, a potential biocontrol agent of *Cirsium arvense*?
Elena Gasich	Survival of *Stagonospora cirsii* J.J. Davis, a leaf pathogen of *Cirsium arvense*, in the soil
Geoff Hurrell	Do *Chondrostereum purpureum* and *Fusarium tumidum* have potential as mycoherbicides for gorse?
Linnea Wang	Culture stability of *Phomopsis cirsii*, a potential biocontrol agent of *Cirsium arvense*
Timothy Widmer	*Nigrospora oryzae* associated with shoot tip death of *Arundo donax*

11:00 – 11:30 **Coffee break**

11:30 – 13:10 **Understanding and managing weed biocontrol agents (chair: Raghavan Charudattan)**

Gavin Ash	Phylogeny, pathogenicity and diversity of biocontrol agents for Alismataceae weeds in Australia and Korea
Karen Bailey	Environmental fate of *Phoma macrostoma*, a fungus for broadleaf weed control in turfgrass
Joe Neal	Selective inhibition and promotion of ryegrass, wheat and canola by rhizobacteria from Australian soils
Gary Peng	Spray retention and its impact on bioherbicide efficacy
Emmanuel Yamoah	Does wounding of gorse plants enhance *Fusarium tumidum* infection?

13:10 – 15:00 **Lunch**

15:00 – 15:40 **Weed pathogens as sources of natural herbicides (Chair: Paul Hatcher)**

Anna Andolfi Fungal metabolites for management of *Orobanche ramosa*

Antonio Evidente Toxins produced by pathogenic fungi of grass weeds as potential natural herbicides

15:40 – 16:20 **Commercialisation of bioherbicides (Chair: Paul Hatcher)**

Raghavan Charudattan SolviNix: R&D of a bioherbicide for tropical soda apple

Graeme Bourdot Towards commercialisation of the fungus *Sclerotinia sclerotiorum* as a mycoherbicide for *Ranunculus acris* in dairy pasture

16:20 – 17.20 **General discussion – concluding remarks (chair: Graeme Bourdot)**

17:30 - **EWRS Welcome party**

List of expected participants

Abouzeid	Mohamed	Egypt
Andolfi	Anna	Italy
Ash	Gavin	Australia
Bagavathiannan	Muthukumar	India
Bailey	Karen	Canada
Berestetskyi	Alexander	Russia
Boari	Angela	Italy
Bourdot	Graeme	New Zealand
Chandramohan	S.	USA
Charudattan	Raghavan	USA
Evidente	Antonio	Italy
Falk	Stuart	New Zealand
Fracchiolla	Mariano	Italy
Gasich	Elena	Russia
Hatcher	Paul	UK
Hershenhorn	Joseph	Israel
Hurrell	Geoff	New Zealand
Montemurro	Pasquale	Italy
Mueller-schaerer	Heinz	Switzerland
Neal	Joe	USA
Netland	Jan	Norway
Peng	Gary	Canada
Pottinger	Brenda	New Zealand
Sheppard	Andy	France
Vurro	Maurizio	Italy
Waipara	Nick	New Zealand
Wang	Linnea	Norway
Widmer	Timothy	France
Yamoah	Emmanuel	New Zealand
Zonno	Maria Chiara	Italy

Pattern and potentiality of fungal isolates from soil-rhizosphere and from young *Orobanche crenata* infesting *Vicia faba* fields in South of Egypt

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The Mediterranean basin countries account for nearly 25% of both the total global area planted to faba bean (*Vicia faba* L.) and its production. The average yield from 1991 to 2001 in Egypt was remarkably high (9423-9565kg/ha) and was approximately double the average for developing countries (4610-4659 kg/ha). Abiotic factors such as drought, high temperature, inadequate supply of nutrients and biotic factors such as microorganisms, parasitic weeds and nematodes, play important roles in reducing *Vicia faba* yields. Infestation of the parasitic weed *O. crenata* Forsk. (broom rape) in food legumes especially in faba bean is of high significance. While not the major agricultural problem in this area, it nevertheless deserves attention. The parasite was present throughout the whole of the Beni-Suef governorate (South of Cairo) infesting 53 % of the faba bean fields surveyed and resulting in 23% reduction in yield. Fields of faba bean highly infested with *O. crenata* were investigated in the governorate of Beni-Suef. 188 fungal isolates, obtained from the rhizosphere area of *Vicia faba* parasitised by *O. crenata* were compared to 26 isolates obtained from the youngest possible infected shoots of the parasite for a preliminary evaluation of the isolated fungi as biocontrol agents. This article records their identification and their *in vitro* bioassay variation against germination of *O. crenata* seeds.

Fungal metabolites for management of *Orobanche ramosa*

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Extensive surveys carried out in Southern Italy allowed us to isolate many pathogenic fungi from diseased plants of *Orobanche ramosa*, a parasitic weed heavily infesting several important crops in the Mediterranean area. These fungi were evaluated both as biocontrol agents of this weed and as producers of natural herbicides. The ability of fifty-three strains to produce bioactive metabolites in solid and liquid cultures was ascertained, in order to identify metabolites inhibiting the germination of *O. ramosa* seeds. Among them, the organic extracts from liquid cultures of one strain of *Fusarium compactum* and one of *Myrothecium verrucaria* caused total inhibition of germination. In this communication, the isolation, identification and biological activity both of verrucarins, roridin A, isotrichoverrin B produced from *M. verrucaria*, and of neosolaniol monoacetate from *F. compactum*, will be described. Their potential use in biological and integrated control of *O. ramosa* well also be discussed.

Phylogeny, pathogenicity and diversity of biocontrol agents for Alismataceae weeds in Australia and Korea.

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Plants species in the Family Alismataceae are significant weeds of rice in Australia and south East Asia. In Korea and Japan, *Sagittaria trifolia* is considered the most important weed of rice while other members of the family including *Damasonium minus* (starfruit), *Alisma lanceolatum*, *S. montivedensis* and *A. plantago-aquatica* are weeds of concern in Australia. These weeds are difficult to control due to their herbicide tolerance/resistance and their season-long emergence. A related weed species, *S. graminae* is an important weed in irrigation supply channels in southern Australia and because of water quality issues, it has very limited chemical control options. In Australia and Korea, parallel research programs are investigating the use of inundative biological control of these weeds using the plant pathogenic fungi, *Rhynchosporium alismatis* and *Plectosporium tabacinum*. Recent studies in Australia involving the sequencing of the internal transcribed spacer from *R. alismatis* revealed a close relationship with the *Plectospheralla* genus and so the fungus has been transferred to the species *Plectosporium alismatis*. This finding demonstrated the close phylogenetic relationship between the organisms under investigation in the two countries.

During 2004, a survey of Alismataceae weeds in the southern regions of South Korea was undertaken to obtain isolates of fungi belonging to *Plectosporium* sp., which have potential as biocontrol agents for several weeds in NSW rice crops. From a total of 158 leaf samples of rice weeds collected over 10 days, forty five pure cultures were returned to Australia (under AQIS quarantine permits) and were cultured from single spores. Isolates that were morphologically similar to *Plectosporium* were tested for pathogenicity to *Alisma lanceolatum, Damasonium minus, Alisma lanceolatum* and *Sagittaria graminae* in glasshouse experiments. When compared to the Australian isolates, those from Korea where less virulent, only causing small lesions on emergent leaves. Preliminary data from the sequencing of the internal transcribed spacer of a number of the isolates confirm their identity as species of *Plectosporium*, with at least one isolate showing 100 % homology with the sequence of *Plectosporium alismatis*.

Environmental fate of *Phoma macrostoma*, a fungus for broadleaf weed control in turfgrass

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Several isolates of *Phoma macrostoma* have demonstrated bioherbicidal activity against dandelions and other broadleaf weeds in turfgrass and this technology is being developed into a microbial weed control product by Agriculture & Agri-Food Canada and The Scotts Company. Because the fungus is applied at higher levels than those occurring in nature, we investigated aspects of environmental fate in order to assess the potential risk. A DNA probe highly specific to isolates of *P. macrostoma* with bioherbicidal activity was developed to detect the fungal DNA in plant and soil samples. Field experiments were conducted to monitor the presence or absence of *P. macrostoma* in space and time. The fungus had limited mobility in both the horizontal and vertical soil profiles and did not persist in plants or soil after 4 months. Studies under greenhouse conditions were conducted to determine the fate in susceptible and resistant plant species. The results showed that both resistant and susceptible plant species were colonized after application of the fungus to soil, but that the frequency of isolation from the rhizosphere declined after 10 days. These results suggest that *P. macrostoma* would have minimal environmental impact.

Can mycelial inoculum be an alternative to conidia in the case of *Stagonospora cirsii* J.J. Davis, a potential biocontrol agent of *Cirsium arvense*?

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The pycnidial fungus, *Stagonospora cirsii* is being evaluated as a potential bioherbicide for control of *Cirsium arvense*. One of the main features of this microorganism is dependence of pycnidial formation on near ultraviolet light. It is a constrain for large scale conidia production. In this study we compared temperature and pH requirements for germination of conidia and mycelium of *S. cirsii* C-163/6, and biocontrol efficacy of conidial and mycelial inoculum of the fungus. The mycelium was produced on autoclaved millet in the darkness, conidia were obtained on pearl barley under NUV. Optimum temperature for conidia germination was about 27°C, for mycelium the optimum was lower (24°C). Optimal pH for germination of conidia lied between 6 and 7. The mycelial inoculum propagated better at pH 5-6. It seems that mycelial type of inoculum will fit better to the environment than conidial inoculum of *S. cirsii*. In addition we studied efficacy of mycelial and conidial inoculum of the fungus applied on leaves of the weed or on soil surface. Millet colonized with the mycelium was dried, ground and applied at the rate 1 g/dm^2. For soil application, the concentration of conidial inoculum was $1 \cdot 10^6$ conidia per g of soil, and for foliar application the concentration was $5 \cdot 10^6$ conidia/ml (1 ml/plant). Plants of *C. arvense* at the rosette stage were inoculated and subjected to 24-h period of 100% relative humidity. By the 14-th day post inoculation the best results (more than 50% reduction of fresh biomass and dry weight of roots) were observed when mycelial inoculum of *S. cirsii* was deposited either on leaves of the weed or on the soil surface. Formulation of the mycelium in oil-based emulsion improved efficacy of *S. cirsii*.

Towards commercialisation of the fungus *Sclerotinia sclerotiorum* as a mycoherbicide for *Ranunculus acris* in dairy pasture

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Ranunculus acris (giant buttercup), a perennial species of European origin, has spread widely in dairy pastures in New Zealand. It caused a loss in milk solids revenue of $156 million in 2001-02 despite the use of the herbicides MCPA, MCPB, thifensulfuron-methyl and flumetsulam. MCPA and MCPB, once useful herbicides, now fail to control giant buttercup due to herbicide resistance. An additional problem with both MCPA and thifensulfuron-methyl is the damage they cause to clovers in treated pastures. These problems along with the high rainfall common in dairying regions (2 to 3 m/y), and the high-value of dairy production, have created an ideal environment for the development of *Sclerotinia sclerotiorum* as a mycoherbicide for use against *R acris*. This plant pathogenic pathogen does not attack clovers and there is no increase in disease risk in susceptible crops downwind of a treated pasture because the spore-trapping ability of dairy swards greatly limits the escape and aerial dispersal of the ascospores of the fungus that are formed in the treated pasture. Funded by the New Zealand government, the New Zealand dairy industry, and a joint-venture company, we have shown that control of *R. acris* to a level acceptable to dairy farmers (60% reduction) is attainable at an application rate of 50 kg/ha of a novel mycelium-based formulation applied with conventional farm spreader machinery. The effects remain evident for at least one year giving potential production benefits in two milking seasons. Registration and commercialisation of a mycoherbicide product are currently under consideration by a leading New Zealand fertilizer company.

SolviNix: R&D of a Bioherbicide for Tropical Soda Apple

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Tropical soda apple (TSA; *Solanum viarum*) is a highly invasive noxious weed in Florida and the southeastern USA. We have shown that *Tobacco mild green mosaic tobamovirus* (common name: tobacco mild green mosaic virus; TMGMV), a plant virus that occurs worldwide on some *Nicotiana* spp., kills TSA upon infection by eliciting a lethal hypersensitive response. Typically, two to three weeks after virus inoculation, TSA plants of all ages wilt suddenly and die quickly and completely without regrowth. The TSA-killing ability of TMGMV is rather specific to this plant-virus interaction. We have successfully field tested TMGMV in several locations in Florida with repeatable, nearly 100% weed-kill. The virus could be applied over large areas, such as open ranch lands, with a tractor-mounted spray boom or a wiper applicator. TSA can be spot-treated with a backpack-sprayer or a pressure-jet spray. In wooded areas and over uneven terrain, an ATV-mounted spot-sprayer or a wiper could be used. Based on an extensive host-range study we have conducted, the virus is clearly adapted to plants in the Solanaceae. Of nearly 420 plants in 57 families tested, including 175 solanaceous plants, 68% were immune or resistant to TMGMV. Among plants outside the Solanaceae, 98% were immune or resistant. Among the susceptible plants (including asymptomatic and symptomatic plants), only peppers (*Capsicum* spp.) and tobaccos (*Nicotiana* spp.), are of any real concern. However, the risk to peppers and tobaccos and the other susceptible plants is negligible and manageable since the virus has no natural vectors and does not spread except through physical contact. TMGMV does not infect humans, animals, birds, fish, or insects and hence poses no risks to these organisms. We are currently following a small-business technology-transfer model to develop and register this bioherbicide. This R&D model, which may have broad applicability to other bioherbicide projects, will be discussed in detail. We intend to register TMGMV as the world's first commercial viral bioherbicide.

Toxins produced by pathogenic fungi of grass weeds as potential natural herbicides

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The ban of some dangerous herbicides, and the loss of efficacy of others due to the appearance of resistant weeds render the need for new active compounds particularly urgent, especially for the control of grass weeds which are among the worst weeds in many crops in the world. Among the possible sources of natural compounds, fungal pathogens of weeds appear to be particularly interesting. Toxins of plant pathogens could be used as new natural herbicides, both in their native forms, or as derivatives and analogues. For this purpose, many pathogens of grass weeds were collected, and their ability to produce toxic metabolites was ascertained. A strain of *Drechslera siccans* proved to be particularly interesting. This communication will describe the optimisation of the production *in vitro* of the phytotoxins produced by this fungus, its isolation from the fungal culture filtrates and its chemical and biological characterisation. The possible use of this toxin in integrated strategies for grass weed control will also be discussed.

Survival of *Stagonospora cirsii* J.J. Davis, a leaf pathogen of *Cirsium arvense*, in the soil

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The fungus, *Stagonospora cirsii* is a potential biocontrol agent of *Cirsium arvense*. In preliminary experiments, the pathogen infected the weed plants at soil surface application. The objective of this study was to evaluate survival and population dynamics of *S. cirsii* in the sterile soil. An artificial soil mixture (peat: sand 3: 1) and a field sod-podzol soil were used in the experiments. Survival of *S. cirsii* in the soils was studied with an adapted technique using membrane filters. In both soils the fungus survived on the filters for 4 weeks and more, but the survival rate of soil surface inoculum of *S. cirsii* was higher than survival rate of the inoculum incorporated into the soil at the depth of 3 cm. In the soil or on its surface conidia of *S. cirsii* germinated and formed mycelium. In 3-4 weeks the contents of the mycelial cells became granular, the most of the cells looked collapsed. The population dynamics of the fungus in the sterile soil was studied by a dilution technique. For two initial concentrations of *S. cirsii* (10^3 and 10^6 conidia per g of the soil), the CFU "decay" curves were similar: from decreasing CFUs (first 15 days) to a low constant level (15-90 days), and to the period of population growth (month 6). The fungus is not soil-borne, however, it is possible that selection of some individuals of *S. cirsii* capable of living in the sterile soil took place. [This work was funded by EU, project FOOD-CT-2003-001687, 2E-BCAs in Crops.]

Do *Chondrostereum purpureum* and *Fusarium tumidum* have potential as mycoherbicides for gorse?

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Chondrostereum purpureum and *Fusarium tumidum* occur naturally on gorse (*Ulex europaeus* L) in New Zealand. Both pathogens have been associated with disease in this woody weed in the pastures and natural environments that it invades, and we have begun to explore their potential as mycoherbicides. Here we discuss the results of two experiments (Bourdôt et al. 2005) in which the response of gorse to these pathogens was evaluated. In the first experiment we found that summer-autumn (Feb-May) or late winter-early spring (Aug-Sept) applications of agar cultures of *C. purpureum* were effective on decapitated gorse stems, halving stem stump survival (from an average of 56% to 29%). In the second experiment, in which *F. tumidum* spores were applied in an invert emulsion to the shoots on gorse stems regenerating following decapitation, with and without wound treatment with *C. purpureum*, there was no evidence of synergism between the two fungi. Each independently reduced the density of regenerating shoots on the decapitated stems by 39-63% averaged over the 12 months following their respective applications. In neither experiment did the effects progress beyond the treated stems. The results confirm that both pathogens have potential as mycoherbicides for gorse. The contrasting modes of action of these two fungi (*C. purpureum* invading wounds on woody tissue and *F. tumidum* affecting young foliage) could be exploited by applying them to gorse plant stumps and regrowth foliage respectively. New research is planned to investigate this approach.

Bourdôt, G.W.; Barton, J.; Hurrell, G.A.; Gianotti, A.; Saville, D. 2005: *Chondrostereum purpureum* and *Fusarium tumidum* independently reduce regrowth in gorse (*Ulex europaeus*). *Biocontrol Science and Technology* Submitted March 2005

Selective inhibition and promotion of ryegrass, wheat and canola by rhizobacteria from Australian soils

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Deleterious rhizobacteria (DRB) have been demonstrated to selectively suppress the growth of several annual weeds in North America; however, limited research has been conducted on the use of DRBs for biological control of weeds in Australia. Thirty six rhizobacteria isolates, from several Australian soils with varying crop histories, were screened for impacts on shoot and root growth of rigid ryegrass (*Lolium rigidum*), wheat (*Triticum aesitivum* 'Janz') and canola (*Brassica rapa*). Rhizobacteria were grown in nutrient broth for 48 hours at 25C then diluted to approximately 1 x 10^7 cfu/ml. Ten seeds of each species were placed in Petri dishes with 5 ml of diluted rhizobacteria cultures. Root and shoot growth were measured when roots in water controls reached approximately 40 mm in length. Data were converted to percent of the controls and compared using analysis of variance; means were separated using a least significant difference procedure. Means were also subjected to cluster analysis. Root and shoot growth responses differed among species and isolates. Several isolates promoted root and shoot growth; fewer isolates inhibited both root and shoot growth. Isolates were also identified that selectively inhibited ryegrass root growth with little or no effect on wheat or canola. Although further research will be needed to identify isolates with adequate efficacy and safety for field use, these data demonstrated that it is possible to select rhizobacteria from native Australian soils that selectively promote or inhibit ryegrass, canola, and wheat root or shoot growth.

Spray retention and its impact on bioherbicide efficacy

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Spray retention is often used as an indicator in herbicide delivery, but little is known about retention characteristics of fungal spores used for weed biocontrol. This study examined spore retention of three bioherbicide agents, *Pyricularia setariae*, *Colletotrichum* sp., and *C. gloeosporioides* f. sp. *malvae*, on their respective weed targets: green foxtail, scentless chamomile and round-leaved mallow. Spore suspensions, containing a sodium fluorescein tracer dye (2.5 ml/L), were applied at 500, 1000 and 2000 L/ha using a cabinet sprayer, and the liquid volumes as well as spores retained on the plants were quantified. On all three weed species, liquid and spore retention showed a high degree of correlation with increasing application volumes although differences existed depending on the weed and volume used. Liquid retention reflected spore retention more correctly on green foxtail and scentless chamomile but might overestimate the number slightly on round-leaved mallow, possibly due to different plant morphology and architecture as well as spray run-off patterns. There was a general trend for finer droplets to result in higher spray retention at the same application volume, but this retention difference was not consistently translated into efficacy enhancement for the three weed-bioherbicide systems tested. Possibly, higher retention increases are required for a more significant and consistent improvement in weed biocontrol.

Culture stability of *Phomopsis cirsii,* a potential biocontrol agent of *Cirsium arvense*

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A common problem of biocontrol agents is the loss of virulence during upscaling in submerged culture. In order to obtain a strain of *Phomopsis cirsii* with high stability, one-spore cultures of different Nordic strains of *P. cirsii* having different aggressiveness were prepared. Different culture traits, compatibility and DNA-profiles of the one-spore cultures were then compared. The aggressiveness of selected one-spore isolates was compared with that of the "mother" culture. Loss of aggressiveness was studied for correlation with changes in the DNA-profile.

Nigrospora oryzae associated with shoot tip death of *Arundo donax*

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Giant reed, *Arundo donax* L. [Poaceae], is a recent target for biological control. Plant stand density of giant reed in the Mediterranean basin is lower compared to California and Texas where it is considered an invasive weed. Observations of natural stands in France, Italy, Greece, Cyprus, and Morocco commonly show a shoot tip dieback. Dissection of the dead flag leaf reveals abundant large black spores at the point of necrosis on all samples. Based upon spore size these were identified as *Nigrospora oryzae*. This was confirmed by sequencing the ITS1 and ITS2 regions and comparing the sequences with a confirmed identifed isolate acquired from a repository in France. Abundant large black spores can also be observed in older tissue of *A. donax* sampled in New Mexico, but the conidial size was smaller than any previously recorded *Nigrospora* spp. In addition, sequences of the New Mexico isolate do not match with the *N. oryzae* samples collected from giant reed in the Mediterranean. This leads to the conclusion that this is a previously undescribed *Nigrospora* sp. not pathogenic to giant reed. Attempts to fulfill Koch's postulate and infect *A. donax* with the *N. oryzae* isolates have been unsuccessful using classical inoculation techniques. Frequently observed in both Mediterranean and U.S. locations in the giant reed tissue is a mite species that is capable of translocating *Nigrospora* spores on its back. In cotton, it was shown previously that a mite was needed in order for infection to occur with *N. oryzae*. Work has been started to demonstrate this synergism in *A. donax* as well.

Does wounding of gorse plants enhance *Fusarium tumidum* infection?

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Gorse (*Ulex europaeus* L.) is a serious weed in New Zealand. Wounding of gorse plants was assessed to determine if it enhances infection by *Fusarium tumidum* Sherb. This is an integral part of a long-term objective, using insects as deliberate vectors to disseminate spores of this pathogen to control gorse. Feeding and oviposition activities of these insects on the weed may provide wound sites for fungal entry, which could enhance infection. Plants used in this experiment were 1, 2, 4 and 8 months old. A fixed number of wounds per unit plant size were made in their stems, spines and leaves using needles. The plants were sprayed with a suspension of 10^6 *F. tumidum* conidia/mL immediately after wounding. The mean dry weights of wounded plants, which were not sprayed with the pathogen and that of the untreated control, were similar across all age groups at 5.6 ± 0.70 (SEM) g/plant. Wounding enhanced *F. tumidum* infection of all the gorse plants irrespective of their age at treatment. Wounded plants (1-4 months old) which were treated with the pathogen were shorter and had lower dry matter weight than the untreated control (P<0.001). Plant mortality by *F. tumidum* infection was higher in the 1 and 2-month old wounded plants compared with non-wounded plants. Wounding increased tip dieback infection in the 4 and 8-month old plants but not in the younger plants of which both wounded and non-wounded plants had 100% tip dieback. It is clear that wounding of older tissue will be required to facilitate *F. tumidum* infection of mature gorse plants.