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Assessment of the biocontrol agent *Fusarium oxysporum* for controlling *Phelipanche ramosa* in tobacco fields

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1 General introduction

Root-parasitic weeds of the family Orobanchaceae Vent. threaten the production of a wide range of economically important crops in warm-temperate as well as subtropical and tropical regions. The family contains the hemiparasitic *Striga* Lour. species (witchweeds), mainly parasitising cereals, and the holoparasitics *Orobanche* L. and *Phelipanche* Pomel species (broomrapes). The latters have lost their photosynthetic ability and entirely depend on their host regarding assimilates, water and nutrient supply. *Orobanche and Phelipanche* species infest numerous dicotyledonous crops (Sauerborn, 1991; Parker & Riches, 1993).

The genera *Orobanche* and *Phelipanche* comprise more than 100 species, but only seven are considered a threat to economically important crops, namely *Phelipanche aegyptiaca* (Pers.) Pomel (= *Orobanche aegyptiaca* (Pers.), *P. ramosa* L. Pomel (= *O. ramosa* (L.), *Orobanche minor* Sm., *O. cernua* Loefl., *O. crenata* Forssk., *O. cumana* Wallr. and *O. foetida* Poir. (Musselman, 1980; Rubiales *et al.*, 2005; Parker, 2009).

1.1 Biology of *Phelipanche ramosa* (branched broomrape)

The life cycle of broomrape plants consists of two stages; a hypogeal (underground) stage and an epigeal (aboveground) stage (Lolas, 1986).

Broomrape seeds only germinate in the presence of certain germination stimulants (sesquiterpene lactones, strigolactones) released by the host-plants (Bouwmeester *et al.*, 2003). After germination, they develop a specific attachment organ (haustorium) to directly connect to the vascular system of their hosts. Via the haustorium they are able to take up mineral nutrients and assimilates (Pate & Gunning, 1972; Dörr & Kollmann, 1974). Because of their limited root system, *Orobanche* and *Phelipanche* species are also dependent on water supply by their host plant. According to Harloff and Wegmann (1993) *Phelipanche* accumulates high amounts of mannitol in order to lower its osmotic potential to a much more negative value than the one of the host. The osmoregulation process is essential to *Phelipanche* for water and nutrient uptake (Delavault *et al.*, 2002).

In the *Phelipanche ramosa*/*Nicotiana tabacum* L. system, the parasitic plant emerges above ground after an underground shoot development for approximately 45 to 55 days, flowers 4 to 5 days later, and the tiny seeds (0.3 mm in diameter, approx. 100,000 per plant) ripen after another 20 to 25 days (Lolas, 1986; Wegmann, 1999). However, this can vary according to soil types and transplanting date (Gonzalez & Rodriguez, 1981).

1.2 Distribution and host range of *Phelipanche ramosa*

Among *Orobanche* and *Phelipanche* species, *P. aegyptiaca* and *P. ramosa* have the broadest host range, including plants of the families Asteraceae Bercht. & J. Presl, Solanaceae Juss., Cannabaceae Martinov and Brassicaceae Burnett (Musselman, 1980; Qasem & Foy, 2007).
P. ramosa mainly occurs in Europe, North Africa and the Middle East (Chater & Webb, 1972; Musselman, 1986). It has also be recorded in South Africa, Cuba and the United States (Parker & Riches, 1993; Musselman & Bolin, 2008) and recently, it has been accidentally introduced to South Australia and Chile (Diaz *et al.*, 2006; Panetta & Lawes, 2007).
Branched broomrape probably spread into Europe from Central Asia with increasing cultivation of hemp between the 16th and 17th century (Koch, 1887; Demuth, 1992). Under the changing agro-climatic conditions of Western Europe, *P. ramosa* infests today at a progressing rate host crops such as *Solanum lycopersicum* L. (tomato), *Nicotiana tabacum* (tobacco), *Cannabis sativa* L. (hemp), and *Brassica napus* L. (oilseed rape) (Gibot-Leclerc *et al.*, 2003; Buschmann, 2004; Benharrat *et al.*, 2005). As hemp production had lost importance in Germany, tobacco remained the main host for branched broomrape (Gonsior *et al.*, 2004). Since the middle of the 20th century the parasitic weed did not play an important role in German agriculture any more, but it recently started to become an increasing problem in tobacco production (Figure 1.1).

Figure 1.1: Tobacco field heavily infested by *Phelipanche ramosa,* Neupotz, Germany

This is inter alia due to the meanwhile almost exclusive cultivation of Virgin tobacco varieties which can be grown in monoculture up to 10 years and therefore promote the build-up of the *Phelipanche* seed-bank in soil (Buschmann, 2004; Buschmann *et al.*, 2005). According to Billenkamp (pers. comm.), a severe infestation can cause complete yield loss in tobacco fields.

In Germany *P. ramosa* mainly occurs in the southwestern region (Billenkamp, personal communication, Figure 1.2) appearing in approximately 7-11% of the tobacco-growing area. (Schwär, Wachowski, pers. comm.).

Figure 1.2: Distribution of *Phelipanche ramosa* parasitising tobacco in Germany
X = Tobacco cultivation sites infested with *P. ramosa*

1.3 Management of *Phelipanche ramosa* in tobacco

Application of conventional control methods against *Orobanche* and *Phelipanche* species is limited due to their complex biology, *i.e.* the plant reproduces by mean of tiny and long-living seeds, their very close affiliation with the host plant, and the fact that the plants can hardly be detected before they have irreversibly damaged the crop (Joel *et al.*, 2007). Management strategies should focus on reducing the soil seed-bank and interfering with the parasite's early development stages, since most of the damage to the host is inflicted before *Phelipanche* emerges above soil.

Management strategies can be based on physical methods (weeding, soil solarisation, soil tillage, flaming, flooding), chemical methods (soil fumigation, herbicides, germination stimulants) and biological methods (resistant varieties, biological control with insects and fungi, catch crops) (Dhanapal *et al.*, 1996).
Breeding for resistance against parasitic weeds is for most host plants difficult to achieve (Rubiales, 2003) because of the complex nature and low heritability of the resistance traits. Until now neither cultivars nor species that are naturally resistant have been found in the genus *Nicotiana* L. (Slavov *et al.*, 2005), although Covarelli (2002) reported one Virgin tobacco variety to be highly resistant to broomrape.
In Germany, the most common practice to control *P. ramosa* in tobacco is by foliar herbicides (at a very low dose) that have to be applied accurately timed (when the first tubercles are developed), and the application has to be repeated at least three to four times in the growing season. Drawbacks of this management approach are possible yield losses caused by phytotoxic effects of the active ingredients on tobacco (Lolas, 1986; Covarelli, 2002) and the delayed emergence of *P. ramosa* shoots after the tobacco harvest, which can lead to a further increase of the soil seed-bank.

1.4 Biological control possibilities of *Phelipanche ramosa*

Many pathogenic microorganisms, especially fungi of the genus *Fusarium*, have been described as a potential mean to control *Orobanche* and *Phelipanche* species (Bedi & Donchev, 1991; Amsellem *et al.*, 2001b; Boari & Vurro, 2004; Nanni *et al.*, 2005; Müller-Stöver & Kroschel, 2005; Alla *et al.*, 2008) and the mycoherbicidal approach seems to be a promising management tool. None of them has been developed into a commercially available mycoherbicide yet, because either virulence was considered too low for field use (Cohen *et al.*, 2002) or scarce data is available about the efficacy under field-grown conditions.
A recently found *Fusarium oxysporum* (Schlecht.) Snyd. & Hans. isolate (FOG), obtained from diseased tubercles of an *P. ramosa* population from Germany, showed an encouraging control ability of the parasite in preliminary experiments which justified further investigations under controlled and field conditions (Figure 1.3).

Advantages of the application of a soilborne fungus are its host specificity (Amsellem *et al.*, 2001b; Boari & Vurro, 2004) and the possible destruction of all underground stages of the parasitic plant, including seeds (Sauerborn *et al.*, 1996; Thomas *et al.*, 1999a) which can contribute to lower the seed-bank every year (Joel *et al.*, 2007). However, since the influence of potential mycoherbicides is often lower and not reliable under natural conditions compared to pot experiments (Sauerborn *et al.*, 2007; Zahran *et al.*, 2008), it is important to learn as much as possible about the ecology of any potential biocontrol organism. It is also essential to find appropriate formulations which can withstand adverse environmental conditions and to enhance and stabilise the efficacy of the biocontrol agents.

Figure 1.3 *Phelipanche ramosa* shoot infested with *Fusarium oxysporum*

Müller-Stöver *et al.* (2005) observed an increased reliability of biocontrol of *O. cumana* under controlled conditions when the application of the biocontrol fungus had been combined with a second control method, the use of a chemical resistance inducer (BION®, Syngenta, Basel, Switzerland, with its active component benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester [BTH]). BTH acts as a functional analogue of salicylic acid (SA) which activates induced resistance (Systemic Acquired Resistance, SAR).

After pathogen infection or treatment with chemicals, the induced SAR promotes cellular defence responses more rapidly and to a greater degree than in non-induced plants (Kohler *et al.*, 2002). The commercially available product BION® has shown its efficacy against bacterial, fungal and viral pathogens (Oostendorp *et al.*, 2001). It also controlled the parasitic weeds *O. cumana* and *P. ramosa* under greenhouse conditions (Sauerborn *et al.*, 2002; Gonsior *et al.*, 2004).

1.5 Identification of biocontrol agents applied to the environment

To optimise biocontrol efficacy under natural conditions, knowledge about the behaviour of the fungus *in situ* is essential. Furthermore, since the potential environmental risks associated with the use of biocontrol agents at high concentrations are of increasing public concern (Tebeest *et al.*, 1992; Hoagland *et al.*, 2007), it is important to have baseline information on the dispersal and survival of the biocontrol agent in the environment (Boss *et al.*, 2007b). This requires specific monitoring methods that allow a reliable tracking of the isolate in complex environments. The development of a fungal population in soil is often monitored using the dilution plate method, which is based on morphological identification of the fungal strain on a selective medium, but does not always permit a reliable distinction between the applied biocontrol agent and the autochthonous fungal population. Since the omnipresent *Fusarium* species consists of many pathogenic as well as non-pathogenic or beneficial strains (Lievens *et al.*, 2007), identification is required even below the species level, which is classically based on bioassays that are time-consuming and laborious (Recorbet *et al.*, 2003).

Currently, DNA-based methods offer a more precise tool to characterise fungal species and strains (Hermosa *et al.*, 2001). The RAPD (Random Amplification of Polymorphic DNA) technique, based on DNA-amplification with single or multiple arbitrary primers requiring no previous knowledge of the target DNA sequence, has been successfully used to differentiate *F. oxysporum* at *forma specialis* level (Wang *et al.*, 2001; Jana *et al.*, 2003; Bayraktar *et al.*, 2008).

1.6 Objectives

The objectives of this study were to investigate the impact of FOG on *P. ramosa* parasitising tobacco under controlled environmental conditions regarding its efficacy and host specificity, the susceptibility of the different development stages of *P. ramosa* to the fungus, as well as the influence of biotic and abiotic conditions on the performance of FOG. Consequently, the control efficacy of the fungus was tested under field-grown conditions in Germany, using different formulations and application techniques. Furthermore, the potential beneficial effect resulting from a combination of the biocontrol agent with the resistance inducer BTH was studied as well. To be able to distingiush the biocontrol agent from other *F. oxysporum* strains, a RAPD marker-based assay was applied, which allowed to verify the applied monitoring methods based on morphological criteria and to obtain information about the spread potential of the biocontrol agent into the environment.

1.7 Outline of the thesis

In Chapter 2, the newly found isolate FOG (*F. oxysporum*) attacking *P. ramosa* is described and several experiments, *e.g.* on host specificity, susceptibility of the different development stages of *P. ramosa*, or control efficacy under controlled environmental conditions are illustrated.
In Chapter 3, results of the combination of FOG with the resistance inducer BTH in greenhouse trials and their subsequent application to tobacco fields are presented.
Chapter 4 deals with measurement of the efficacy of two different fungal granular formulations (alginate, pesta) and their combination for controlling *P. ramosa* under field conditions. It also presents the results of monitoring of the fungus survival rate in the soil throughout all experiments.
Molecular detection of FOG after field release and its environmental impact is reported in Chapter 5.
The work is completed by a general discussion (Chapter 6).

2 A novel strain of *Fusarium oxysporum* from Germany and its potential for biocontrol of *Phelipanche ramosa* L.[1]

Summary

A newly isolated *Fusarium oxysporum* isolate was investigated for its biocontrol potential against the root parasitic weed *Phelipanche ramosa* (branched broomrape). The fungus was found to affect all developmental stages of the parasite. *Phelipanche* seed germination was reduced by 40% in the presence of fungal conidia *in vitro*. The number of underground developmental stages of the weed was reduced by the fungus by 55% compared to the control and 92% of tubercles were recorded as diseased in root chambers. In pot experiments, soil application of a granular formulation of the fungus resulted in a reduction of number and dry matter of *Phelipanche* shoots by more than 90%. Spraying of a conidial suspension on aboveground *Phelipanche* shoots caused the death of 75% of the population within 2 weeks. Data from initial host-range experiments indicate that the isolate is very host-specific, not even attacking shoots of other *Orobanche* species. Because of these promising results, we conclude that the fungal isolate should be investigated under field conditions and be compared with other *Fusarium* isolates proposed for biocontrol of *P. ramosa*.

Keywords: branched broomrape, parasitic weed, tubercle, biological control, fungus, plant pathogen, mycoherbicide

[1]This chapter is an amended version of MÜLLER-STÖVER D, KOHLSCHMID E & SAUERBORN J (2009) A novel strain of *Fusarium oxysporum* from Germany and its potential for biocontrol of *Orobanche ramosa*. *Weed Research* **49**, 175-182, with permission from Wiley-Blackwell.

2.1 Introduction

In the temperate zone of central Europe, *Phelipanche ramosa* L. (branched broomrape, hemp broomrape) (Figure 2.3) is the most common pest of the *Orobanchaceae* (Gonsior *et al.*, 2004) and causes severe damage to several crops (Benharrat *et al.*, 2005). The holoparasitic flowering plant is completely dependent on its host plant for the supply of carbohydrates, nutrients, and water resulting in both qualitative and quantitative damage to crops. In contrast to other *Phelipanche* species, *P. ramosa* can attack a broad range of plants. In central Europe, these are predominantly tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill. = *Solanum lycopersicum* L.) and hemp (*Cannabis sativa* L.). There is recent evidence for the increasing occurrence of branched broomrape in oilseed rape (*Brassica napus* L.) cultivars in Western France (Gibot-Leclerc *et al.*, 2003; Brault *et al.*, 2007).

The complex ecology of the parasite and its intimate physiological interaction with its host are the main difficulties that limit the development of successful control measures that can be accepted and used by farmers. Biological control may offer a promising solution. Many pathogenic organisms, especially fungi of the genus *Fusarium*, have been isolated to potentially control *Orobanche* and *Phelipanche* species, however, none of them has been developed into a commercially available mycoherbicide yet. Two *Fusarium* isolates, *F. arthrosporioides* Sherb. (strain: I-1621) and *F. oxysporum* (Schlecht.) Snyd. & Hans. (strain: I-1622), isolated in Israel have been described to be pathogenic to *P. ramosa* (Amsellem *et al.*, 2001b), but virulence was considered as too low for field use (Cohen *et al.*, 2002). More recently, Boari and Vurro (2004), isolated two fungal isolates in Italy. *F. oxysporum* (strain: FT2) and *F. solani* (Mart.) Sacc. (strain: ET4), that reduced the number and weight of emerging *P. ramosa* shoots by around 60%. However, it is reasonable to look for further more pathogenic isolates to be able to compare the strains regarding their efficacy and suitability for specific crop situations. Regulatory authorities are often hesitant to introduce bioherbicidal pathogens from foreign countries. In such situations, biocontrol strains have to be detected in each country and need to be developed independently into a bioherbicide .

Here we describe the potential of a further *F. oxysporum* strain, isolated out of diseased *P. ramosa* tubercles developed from seeds originating from Germany.

2.2 Materials and methods

2.2.1 Taxonomic identification and fungal culture

The *F. oxysporum* isolate (abbreviated FOG) used in this study was obtained from severely diseased *P. ramosa* tubercles (parasitising on tobacco roots) developing in root chambers. *P. ramosa* seeds had been collected in farmer's fields near the village Sandhausen (49°21'N, 8°39'E) and in experimental fields of the LTZ Augustenberg (Forchheim, 48°59'N, 8°19'E) in south-western Germany. The isolated strain caused diseased symptoms when applied to healthy tubercles in root chambers and could be reisolated out of the diseased plant parts. Taxonomic identification of the isolate was performed by the Federal Biological Research Centre for Agriculture and Forestry, Berlin, Germany. For experiments, stock cultures were stored up to 6 months on special nutrient-poor agar (SNA) (Nirenberg, 1976) plates at 5°C without subculturing. For long-term preservation, the isolate was stored on SNA amended with 5% (v/v) glycerol at –80°C at the Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Germany.

2.2.2 Inoculum production

Microconidia were produced in liquid culture using 250-mL Erlenmeyer flasks containing 100 mL autoclaved malt extract medium (20 mL malt extract [Biomalt, Kirn, Germany], 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 2 g yeast extract, 0.2 gL^{-1} chloramphenicol L^{-1} H_2O deionised). One agar plug (1 cm diameter) from a fungal culture on SNA medium was used to inoculate each flask. The flasks were closed with a cotton plug and aluminium foil and incubated on a rotary shaker (150 rpm) at ambient laboratory conditions (20 ± 3°C) for 5 days. The content of the flasks was homogenised for 5 s in a Waring blender at high speed and mycelial fragments were separated by filtration through four layers of cheesecloth.

Conidial density in the solution was determined with a haemacytometer and adjusted to the desired concentration by centrifugation or dilution with deionised water.

2.2.3 Influence of the substrate pH on growth and sporulation of FOG

Malt extract medium (see above) was used as standard medium. Appropriate amounts of citrate-phosphate buffer or phosphate buffer (Dhingra & Sinclair, 1985) were added to the medium to achieve pH ranges of 4, 5, 6, 7 and 8. The original pH of malt extract medium was 6. Medium was sterilised by autoclaving for 20 minutes at 121°C. Three replicate Erlenmeyer flasks (250 mL) containing 100 mL of each test medium were inoculated with one agar plug (1 cm diameter) taken from a fungal stock culture on SNA medium. After 5 days of incubation on a rotary shaker (150 rpm) at ambient laboratory conditions (20 ± 3°C), the content of each flask was blended for 5 s. Conidial density in the solution was determined with a haemacytometer. Fungal biomass was separated from the solution by centrifugation at 1700 g for 5 min. The supernatant was discarded and the pellet was placed on a filter paper and oven-dried for 24 h at 80°C. The experiment was repeated.

2.2.4 Pesta granulation procedure

To prepare standard ‘Pesta’ granules, 32 g durum wheat flour (Divella, Rutigliano, Italy), 2 g sucrose, 6 g Kaolin and 23 mL inoculum suspension were blended, modified after Connick *et al*. (1991). The mixture was thoroughly kneaded and extruded as a sheet through a hand-operated pasta maker set at the widest roller-gap setting. To homogenise the dough, the resulting sheet was folded and passed through the rollers at least four times. The dough sheet was then extruded at intermediate settings and finally at a setting that produced a sheet of 1-1.5 mm thickness. Sheets were air-dried for 24 h, ground using a laboratory mill and sieved to a particle size of 0.5-2 mm. Colony forming units (cfu) per g were assayed immediately after granulation by suspending 100 mg of the preparation in 10 mL sterile H_2O in a glass test tube. To dissolve the granules, the sample was vortexed from time to time together with 3 glass beads (0.6 cm diameter) until disintegrated.

Aliquots (100 µL) of appropriate dilutions were plated on half-strength PDA (Potato Dextrose Agar) amended with 100 ppm chloramphenicol (three plates per each of three samples) and cfu g^{-1} formulation were determined after incubation for 3 days at room temperature (20 ± 3°C). The formulated material used had *c.* 1-3 x 10^7 cfu g^{-1} and was stored in the refrigerator (4°C) in small sealed plastic bags.

2.2.5 Effect of *Fusarium oxysporum* on the germination of *Phelipanche* in vitro

For tests of *P. ramosa* germination, the methods as described by Linke (2001) were followed. *Phelipanche ramosa* seeds had been collected in Sandhausen, Germany. For preconditioning, surface-disinfected seeds were sprinkled onto 4 small segments of glass microfiber filter paper (Whatman GF/A; Whatman Int.; Maidstone, UK) in Petri dishes containing one layer of filter paper. Three milliliters of a conidial suspension of *F. oxysporum* at 1 × 10^6 conidia mL^{-1}, or 3 mL of water in the control treatments, were added to each petri dish. All petri dishes were sealed with parafilm and incubated in the dark at 25°C for 7 days. The small filter paper segments were put into a freshly prepared dish and 3 mL of the synthetic germination stimulant GR24 0.1 ppm were applied to each Petri dish. Seed germination was evaluated after 7 further days of incubation under a binocular microscope by counting the number of germinated seeds from 100 seeds of each filter-paper segment. Data were expressed as percentage of germination. To investigate if seeds have been irrevocably destroyed by the fungus or if changes in germinability were only temporary, 100 mg of fresh seeds were incubated in 10 mL of a conidial suspension of *F. oxysporum* (1 x 10^6 spores mL^{-1}) at 25°C. In the control treatment, seeds were incubated in water only. After 7 days, seeds were collected on a gauze and dried for 14 days at room temperature. Afterwards, seeds were surface-sterilised for 25 min. in a sodium hypochlorite solution (1% active chlorine), air-dried again for 24 h and subjected to a germination assay as described above. The experiments had four replicates and were repeated.

The influence of the fungus on the germinability of *O. cumana* seeds (originating from Romania) in the presence of FOG was also investigated.

One experiment was performed as described above and a second by incubating the seeds at 16°C and adding 2 mL of 1 ppm GR24 after preconditioning.

2.2.6 Root-chamber inoculation with *Fusarium oxysporum*

Root-chamber experiments as described by Linke *et al*. (2001) were set up to determine the effect of *F. oxysporum* on the underground development of *P. ramosa* and tomato (variety Hellfrucht). Surface-sterilised *P. ramosa* seeds were evenly sprinkled by hand to cover the whole surface of the filter paper. Ten milliliters of a conidial suspension containing 1×10^6 conidia mL^{-1} were dripped onto the filter paper strip of each chamber. The control chambers were treated with 10 mL water only. To be sure that the total number of *P. ramosa* seeds did not differ between treatment and control, seeds were counted in four samples of a defined surface area of each chamber. After 7 days of preconditioning at room temperature, the chambers were transferred to a growth chamber set to a temperature regime of 25/15°C day/night. Two pre-germinated tomato seeds were placed in each chamber. Two weeks after sowing, each chamber was fertilised with 10 mL of 0.2% Wuxal® (Bayer Crop Science, Langenfeld, Germany) N-P-K liquid fertiliser. Nine to 12 weeks after sowing, the Plexiglas cover was removed and the number of underground tubercles and shoots as well as the disease incidence was recorded. The experiment had four to six replicates and was repeated.

2.2.7 Pot experiments

P. ramosa (a mix of seeds collected in Germany) and tomato (variety Rentita) were grown in a greenhouse at a temperature regime of 25/15°C (day/night) with supplemental light provided by 400W Philips Son-t Agro lamps (Royal Philips Electronics N.V.; Amsterdam, the Netherlands) for 13h.

Plastic pots (13 x 13 x 13 cm) were filled with a compost growth substrate (10% organic matter, pH 6.6) up to two-thirds of the pot depth. The substrate had been steamed before at 80°C for four hours. Thirty mg *P. ramosa* seeds per pot were sprinkled onto the soil surface and mixed into the top 5 cm of the soil in the pot.

The granular formulation containing the biocontrol agent was incorporated into the soil along with the *P. ramosa* seeds. Finally all pots were entirely filled with substrate. Three tomato seeds were sown in each pot. The experiments were arranged in a completely randomised design with five replicates per treatment. Fourteen days after sowing, each pot was thinned to contain only one tomato plant and fertilised with 50 mL of a 2% (v/v) Wuxal® solution every 2 weeks. A series of pot experiments were performed.

2.2.7.1 Soil incorporation of Fusarium oxysporum *before planting*

The pots were prepared as described above. For the fungal treatments, 1 g or 2 g per pot of ‘Pesta’ formulation containing microconidia were incorporated pre-planting into the soil. Pots containing *P. ramosa* seeds without fungal formulation served as negative controls. Whereas those containing neither fungal formulation nor *P. ramosa* seeds were set as a positive control. The experiment concluded 4 weeks after the first emergence of *Phelipanche* shoots (which was observed *c.* 10 weeks after sowing). Soil was washed from tomato roots and the total number and dry weight (after drying for 48 h at 80°C) of parasite shoots and the dry weight of the tomato plants (without fruits) was determined. The experiment was repeated.

2.2.7.2 Soil incorporation of Fusarium oxysporum *before planting in two different substrates*

The effect of a different growth substrate (sandy loam with 0.2% organic matter and a pH of 7.9) on the control of *P. ramosa* with *F. oxysporum* in comparison to the compost growth substrate was investigated in further greenhouse experiments. Additionally, the number of emerged *Phelipanche* shoots was recorded. The experiment was repeated.

2.2.7.3 Post-emergence inoculation of Fusarium oxysporum *on* Phelipanche ramosa

To investigate the effect of *F. oxysporum* on emerged shoots, *P. ramosa* was raised in non-inoculated pots on tomato as described above. Emerged shoots were sprayed until run-off with an aqueous conidial suspension containing 10^7 conidia mL^{-1}. Control plants were sprayed with water only. The plants were kept under polyethylene bags for 48 h. The shoots were evaluated for disease symptoms 7 and 14 days after inoculation with a 0 to 4 rating scale (0 = no symptoms, 1 = 25% of the stem tissue necrotic; 2 = 50% of the stem tissue necrotic, 3 = 75% of the stem tissue necrotic; 4 = dead plant). The disease incidence was calculated by dividing the sum of all individual ratings by the number of inoculated plants. The experiment was repeated. In total, 53 plants were inoculated with the conidial suspension and 62 were treated as controls.

2.2.7.4 Host-range studies with Fusarium oxysporum

A preliminary study of the pathogenicity of *F. oxysporum* to *O. cumana* (seeds originating from Romania), a parasite of sunflower (*Helianthus annuus* L., cv. Albena) and *O. crenata* (seeds originating from Turkey), a parasite of faba bean (*Vicia faba* L., cv. ILB 1814) was carried out. Pot experiments with pre-planting application of 2 g ‘Pesta’ granules per pot were performed and repeated.
F. oxysporum was further evaluated regarding its potential pathogenicity towards important crop plants. The following plants were tested: carrot (*Daucus carota* L. ssp. *sativus*), faba bean, pea (*Pisum sativum* L.), sunflower, rape (*Brassica napus* L. var. *napus*), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and cucumber (*Cucumis sativus* L.). *F. oxysporum* was applied pre-planting to the soil at a rate of 2 g “Pesta” granules per pot as described above. Three to 10 seeds of the test plants (depending on the species) were sown per pot. Emerged plants were thinned to a single plant per pot after 14 days, with the exception of two plants per pot for wheat. Each treatment and a corresponding control treatment were set with three replicates. Plants were observed weekly for up to two months for the development of disease symptoms. At harvest, the plant shoot dry weight was determined after drying for 48 h at 80 °C.

2.2.8 Statistical analysis

Statistical analyses were performed using STATISTICA software (StatSoft, 1997). Analysis was done to the combined data of repeated experiments when they had homogenous variances. All tests of significance were conducted at $P < 0.05$. When data were not normally distributed or showed heterogeneity of variances, they were square-root or log-transformed before analysis. Percentage data were arcsine-transformed before analysis (Gomez & Gomez, 1984). Comparison of two means was performed using Fisher's t-test. Multiple mean comparisons consisted of analysis of variance (ANOVA) and Tukey's Honest Significant Difference test (HSD), except for the number of *Phelipanche* shoots. The latter has been analysed with the Mann-Whitney-U-test for two groups or a Kruskall-Wallis-Anova on ranks and a Bonferroni-corrected Mann-Whitney-U-test for three groups. The experiment with two different substrates was analysed as a two-way ANOVA for *Phelipanche* dry weight, followed by pairwise comparisons of treatment and respective control with the t-test. Disease ratings for the post-emergence spray application were compared using the Mann-Whitney-U-test. Mean ± SE are given in the text.

2.3 Results

2.3.1 Influence of the substrate pH on growth and sporulation of *Fusarium oxysporum*

The fungal isolate produced abundant microconidia in malt extract medium. After 5 days of incubation, *c.* 1 x 10^8 conidia mL^{-1} were determined. Dry matter production as well as sporulation were significantly decreased in a more alkaline (pH 7 and 8) medium compared with that at pH 6 (Figure 2.1). A pH of the medium of 4 or 5 did not affect fungal growth, only at pH 4 sporulation was significantly reduced compared with that of pH 6.

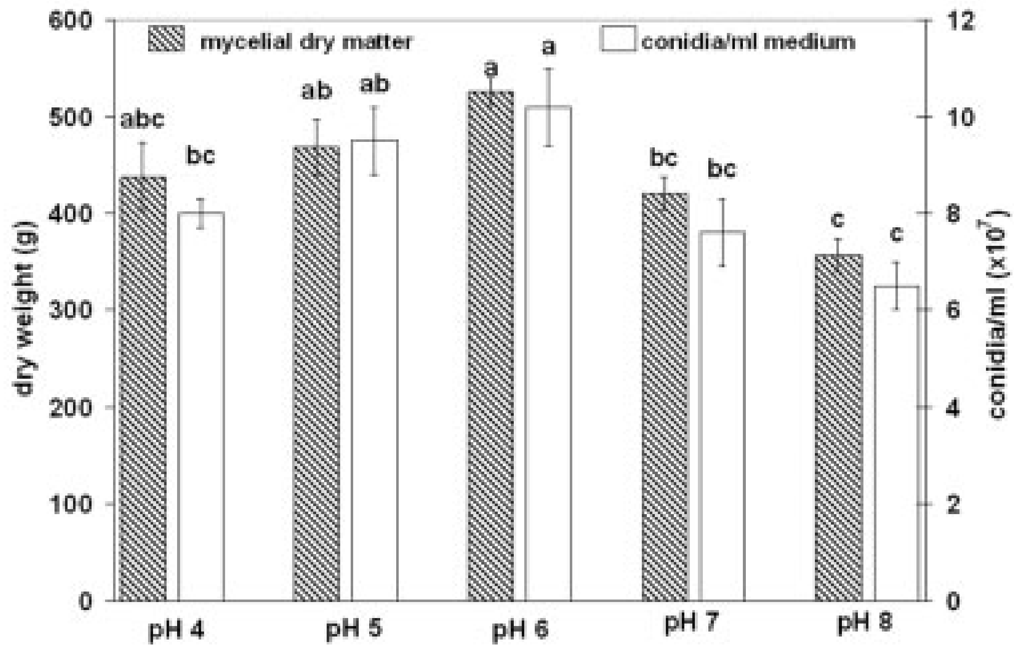

Figure 2.1: Influence of the substrate pH on dry matter production and sporulation of *Fusarium oxysporum* FOG in liquid malt extract medium. Multiple mean comparisons were performed using Tukey's HSD test. For each parameter, bars with the same letters are not significantly different (P < 0.05). Means ± SE given.

2.3.2 Effect of *Fusarium oxysporum* on early developmental stages of *Phelipanche ramosa* in vitro

The presence of fungal conidia beginning from the preconditioning phase significantly reduced the germination of *P. ramosa* from 78 ± 1.7% in the control to 46 ± 3.2% in the treated Petri dishes. However, when seeds had been exposed to a conidial suspension for 7 days and were surface-sterilised afterwards, no statistically significant difference regarding the germination rate occurred between treated and non-treated seeds. The number of underground developmental stages in root chambers was reduced by 55% after the application of fungal conidia. *Phelipanche* shoot development was much stronger in the non-treated root chambers. Furthermore, in the inoculated chambers, almost all tubercles and shoots were diseased or dead (92 ± 2.8%, compared to 5 ± 1.8% in the control treatments).

2.3.3 Pot experiments

The total number of parasite shoots was significantly decreased from 20 ± 5.7 in the control treatment to 1 ± 0.6 after the application of 1 g of 'Pesta' granules containing FOG.

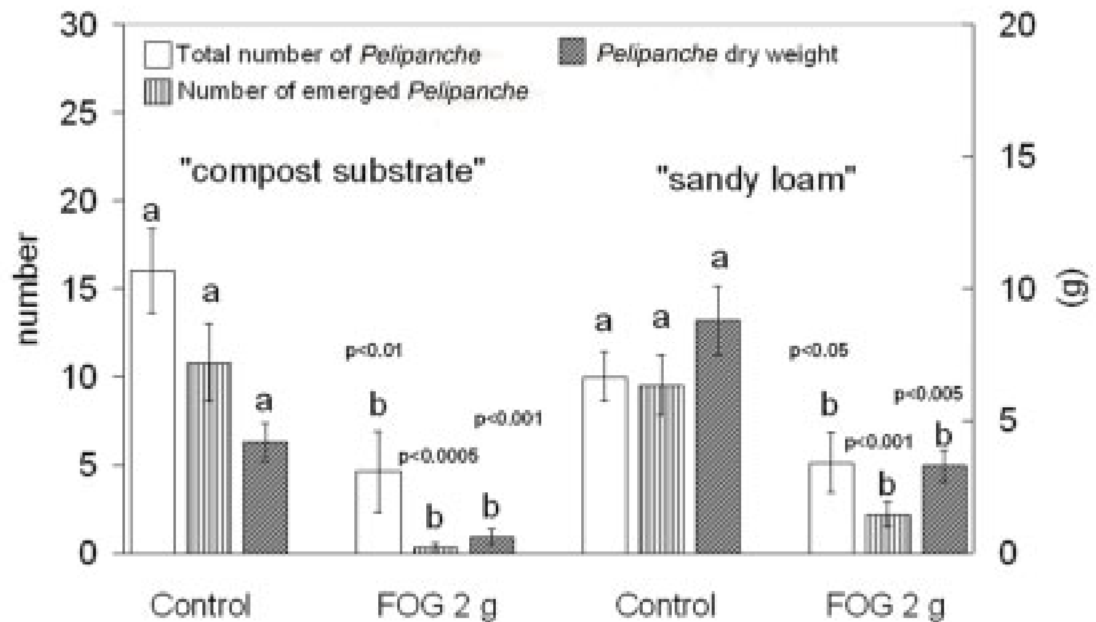

Figure 2.2: Influence of two different substrates on the control efficacy of *Fusarium oxysporum* (FOG) on the total number *Phelipanche ramosa* shoots, emerged shoots and the *Phelipanche* dry weight. Pairwise comparisons were performed using the Mann-Whitney-U-test for number of *Phelipanche* and Fisher's t-test for *Phelipanche* dry matter. For each parameter, bars with the same letters are not significantly different (P < 0.05). Means ± SE given.

Similarly, *Phelipanche* dry matter was decreased from 4.2 g ± 0.85 to 0.2 g ± 0.11. The application of 2 g Pesta granules did not bring any further control effect (number 1.3 ± 0.6, weight 0.3 ± 0.17). The dry weight of the tomato plants did not differ statistically between treatments (data not shown).

When comparing the influence of two different substrates on the biocontrol efficacy, significant effects could be recorded both from the fungus and the substrate on the *Phelipanche* dry matter, whereas the interaction *Fusarium* x substrate did not reveal any significant effect. However, the reductions in parasite number and weight compared to the respective untreated control were more significant in the compost substrate than in the sandy loam (Figure 2.2).

Data of the two experiments on post-emergence inoculation of *F. oxysporum* on *P. ramosa* were not combined because of heterogeneity of variances. The disease incidence of the water-inoculated control plants varied between the experiments from 0.1 ± 0.54 (no symptoms) in the first experiment to 2.2 ± 1.68 (50% necrotic tissue) in the second one. However, in both experiments disease severity was significantly higher (3.9 ± 0.36 and 3.6 ± 0.76, respectively) in the treated plants compared with control plants. Of the 53 inoculated plants, 39 (75%) were completely dead 2 weeks after inoculation. The fungal isolate did not cause any differences in number, emergence or dry matter of *O. crenata*. In the treated pots, the general level of disease was even lower than in the control pots. In contrast, the application of 2 g ‘Pesta’ granules containing FOG resulted in a significantly decreased number of *O. cumana* shoots per pot and a significantly reduced *Phelipanche* emergence compared to the untreated control (Table 2.1). However, the emerging shoots remained free of symptoms of fungal infection. The *in vitro* germination of *O. cumana* seeds was significantly reduced in the presence of fungal conidia compared to the control: 96% in the first and 98% in the second experiment.

However, the overall *in vitro* germination rate of *O. cumana* was very low and did not exceed 10% even in the untreated control (data not shown).

All inoculated crop plants neither differed in dry matter from the untreated control plants nor showed any visible signs of fungal infection (data not shown).

Table 2.1: Effect of soil incorporation of FOG on *O. crenata* parasitising faba bean and *O. cumana* parasitising sunflower

	***O. crenata*/ faba bean**		***O. cumana*/sunflower**	
	Control	FOG	Control	FOG
Emerged *Phelipanche* shoots	4.5 (1.68)**a**	5.5 (1.70)**a**	7.5 (1.27)**a**	3.9 (0.95)**b**
Number of *Phelipanche* shoots	10.1 (2.47)**a**	12.5 (3.07)**a**	14.8 (2.85)**a**	6.2 (1.67)**b**
Phelipanche dry matter (g)	9.0 (2.95)**a**	8.9 (1.77)**a**	8.6 (1.26)**a**	5.2 (1.26)**a**
Diseased emerged *Phelipanche* shoots (%)	36 (14.6)**a**	12 (6.8)**a**	6 (3.0)**a**	8 (5.2)**a**

Pairwise comparisons were performed using the Mann-Whitney-U-test for number of *Phelipanche* and Fisher's t-test for *Phelipanche* dry matter and disease proportion (data presented as means with SE in parentheses). For each parameter and each host/parasite association, means with the same letters are not significantly different ($P < 0.05$).

2.4. Discussion

Among fungal parasites reported to attack parasitic weeds and thus being possible candidates for biological control, *Fusarium* spp. are predominant (Sauerborn *et al.*, 2007). Therefore it is not surprising that a further *Fusarium oxysporum* isolate has been found, this time attacking *P. ramosa* tubercles originating from Germany. *Fusarium* species have certain advantages regarding *Phelipanche* biocontrol. They are easy to culture on synthetic media and produce abundant propagules in liquid fermentation, a prerequisite for industrial production. Furthermore, they are soil-borne pathogens and might therefore be delivered via the soil and affect early developmental stages of the parasitic weed which are already very destructive to the host. However, the ability of many *Fusarium* strains to produce metabolites toxic to humans and animals could put their use as biocontrol agents into question (Dor *et al.*, 2007). As a preliminary assessment of the risk posed to the environment by the introduction of a mycoherbicide, it is of high importance to investigate each new strain regarding its toxin production potential.

However, before finally evaluating the risk, the evaluation of the real production *in vivo* has to be confirmed as well (Abouzeid *et al.*, 2004).

This newly found isolate of *F. oxysporum* affected all developmental stages of *P. ramosa*, from germination to underground tubercles and aboveground shoots. However, under the conditions used in this study, the reduction of germination occurred only when fungal propagules were present during the germination process. When removing them from the *Phelipanche* seed surfaces after 7 days of incubation, seed germination did not differ from untreated control seeds. This does not correspond to findings of Thomas *et al*. (1999a) who observed penetration of hyphae of *Fusarium oxysporum* f. sp. *orthoceras* into *O. cumana* seeds 26 h after inoculation which caused a complete destruction of the seed content. Similar processes were observed by Sauerborn *et al*. (1996) for *Fusarium nygamai* attacking *Striga hermonthica* (Delile) Benth. seeds. However, there are other reports of effective inhibition of *Phelipanche* germination by microorganisms without active destruction of the seed. E.g., purified toxins produced by *Fusarium* species significantly reduced *P. ramosa* germination (Zonno & Vurro, 2002; Andolfi *et al.*, 2005), which might also be a mechanism responsible for the observed germination inhibition in this study.

A comparison of efficacy with other *Fusarium* strains investigated as biocontrol agents against *P. ramosa* is difficult due to the varying test systems, inoculum application methods and pathogenicity evaluations applied. Amsellem *et al*. (2002) confirmed the pathogenicity of two *Fusarium* isolates to *P. ramosa* in polyethylene bag-assays without quantifying the effect. Boari and Vurro (2004) took mainly number and fresh weight of emerging parasite shoots into account when evaluating different fungi concerning their potential suitability as biocontrol agents. Their best isolate in this regard (*Fusarium oxysporum* FT2) caused a 70% reduction of emerged shoots after the application of at least 10^8 cfu per pot. In our study, the number of emerged shoots was only determined in the substrate experiment, where a 97% reduction in the compost substrate as well as a 77% reduction in the sandy loam was achieved after the application of at least 2×10^7 cfu, but using pots about one third the size of theirs. Boari and Vurro (2004) furthermore present one experiment where they counted the underground *Phelipanche* shoots as well.

Adding emerged and underground shoots, they achieved a 40% reduction in number with FT2 and a 60% reduction with *Fusarium solani* ET4. Fresh weight was reduced by 46% and 65%, respectively. In our study, the highest reduction in total number and dry weight of the parasite's shoots was 95% and 96%, respectively. Although this high control efficacy could not be consistently achieved in all experiments, the *F. oxysporum* strain described in our study seems to have a higher biocontrol potential than other strains pathogenic to *P. ramosa*. However, a direct comparison of the strains would certainly give a clearer picture.

In the pot experiments, the fungus was effective in reducing number and dry matter of *Phelipanche* shoots, irrespective of the substrate used. However, in the substrate with the high organic matter content, control of *Phelipanche* was more pronounced than in a sandy loam. The mechanisms lying behind can only be speculated about. Results from the laboratory experiments on the optimum pH for fungal growth can be a hint that soil pH is an important factor, since the fungus reacted quite sensitively to increasing alkalinity and the sandy loam had a pH of 7.9. However, further *in vivo* experiments are needed to confirm the influence of pH. A higher level of available nutrients in the compost substrate might also be a reason for the increased biocontrol efficacy. For example Yonli *et al*. (2006) observed an increased reduction of *Striga* seed germination by *Fusarium* isolates propagated on a compost substrate compared to chopped sorghum straw as growth substrate. Nutrient competition with other soil microorganisms (e.g. for carbon or iron as described in Lemanceau and Alabouvette (1993) and Duijff *et al.* (1994)), may also play a role in this regard. But also physical soil properties can be taken into account, as it has been shown by Otten *et al*. (2001) and Harris *et al*. (2003) that a phytopathogenic fungus showed a reduced spread with an increasing bulk density of the soil, probably because the fungus preferentially followed larger pores and avoided denser areas.The data from the host range experiment indicate that the fungal isolate is very host specific. None of the tested crop plants showed any sign of fungal infection or dry matter reduction when grown in FOG-amended soil. Even on the genus level, host specificity could be observed.

O. crenata parasitising faba bean was not affected by the application of this strain (FOG), whereas *O. cumana* did not show any symptom of fungal disease, but was reduced in numbers. This might be a result of a reduced germination of *O. cumana* seeds which could be confirmed in the presence of FOG conidia *in vitro*. Due to the promising results that have been achieved in the greenhouse, the impact of FOG should be investigated under field conditions as well. In order to bundle research activities in this field, it would be useful to gather all *Fusarium* strains that are under investigation as biocontrol agents against *P. ramosa* and compare their performance under identical conditions. Testing possible synergistic effects between the various strains (also under varying environmental conditions) in a “multiple-pathogen strategy” (Joel *et al.*, 2007) could be a tool to enhance the biocontrol efficacy.

Figure 2.3: *Phelipanche ramosa* parasitising tobacco

3 Mitigation of *Phelipanche ramosa* by *Fusarium oxysporum* and induced resistance[2]

Summary

Fusarium oxysporum (FOG), is a natural antagonist of *Phelipanche ramosa* and was isolated from diseased tubercles of *P. ramosa* seeds from Germany. Under controlled environmental conditions the antagonist was effectively reducing the incidence and reproduction of the parasitic weed on tomato and tobacco plants. Consequently, FOG was tested under field conditions using different application techniques. In-furrow application and broadcasting of the inoculum after tobacco planting as well as pre-planting subsoil application decreased the number of emerged *Phelipanche* shoots. In a second experiment in-furrow application of FOG markedly reduced *P. ramosa* shoots. In contrast to the results of the greenhouse experiments, no further reduction could be observed when the biocontrol agent was combined with the resistance inducer BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester). The results revealed the potential of the plant pathogen for *Phelipanche ramosa* control, however, future experiments should work on enhancing the observed effect under natural conditions.

Key words: *Fusarium oxysporum, Phelipanche ramosa,* benzothiadiazole, biological control, induced resistance, tobacco

[2]This chapter is an amended version of KOHLSCHMID E, MÜLLER-STÖVER D & SAUERBORN J (2008) Mitigation of *Orobanche ramosa* by *Fusarium oxysporum* and induced resistance. *Journal of Plant Diseases and Protection, Special Isssue* **XXI**, 473-478, with permission of Ulmer.

3.1 Introduction

Broomrapes (*Orobanche* and *Phelipanche* species) are obligate root parasites that cause serious damage to a wide range of economically important crops in warm, temperate as well as subtropical and tropical regions (Sauerborn, 1991). *Phelipanche ramosa* (branched broomrape) occurs in many parts of Middle, Eastern and Southern Europe, North Africa as well as in the Middle East (Chater & Webb, 1972). It was recently introduced to South Australia, South Africa and Chile. Branched broomrape has a broad host range that includes plants of the families Asteraceae, Solanaceae, Cannabaceae and Brassicaceae (Parker & Wilson, 1986). In Germany it infests at a progressing rate host crops such as hemp or tobacco and to an increasing degree oilseed rape in France (Gibot-Leclerc *et al.*, 2003; Buschmann, 2004). As a holo-parasite, it deprives its host plant of water, assimilates, and nutrients, which are transferred directly via a specific contact organ (haustorium) from the host's root. Depending on the infestation rate, this causes malnutrition, biomass reduction or death of the host plant. No control approach, which is effective and economically feasible, has been found for *P. ramosa* so far (Dhanapal *et al.*, 1996; Joel *et al.*, 2007). Research on biological control of parasitic weeds with phytopathogenic fungi has been carried out in the past 25 years (Hodosy, 1981; Amsellem *et al.*, 2001b; Boari & Vurro, 2004; Müller-Stöver & Kroschel, 2005) and the mycoherbicidal approach seems to be a promising control tool. A recently found *Fusarium oxysporum* (Schlecht.) Snyd. & Hans. isolate (FOG), gained from diseased tubercles of *P. ramosa* shoots originating from Germany, also showed encouraging results in controlling the parasite under greenhouse conditions (Chapter 2). However, in field experiments, the influence of the potential mycoherbicides is often lower and not reliable compared to pot experiments (Sauerborn *et al.*, 2007; Zahran *et al.*, 2008). Thus, it is essential to enhance and stabilise the efficacy of the biocontrol agents under natural conditions. Müller-Stöver *et al*. (2005) observed an increased reliability of biocontrol of *O. cumana* when it had been combined with a second control method, the use of a chemical resistance inducer (BION®, Syngenta, Basel, Switzerland, with its active component benzo (1,2,3) thiadiazole-7-carbothioic acid S-methylester [BTH]).

This commercially available product has shown its efficacy against bacterial, fungal and viral pathogens (Oostendorp *et al.*, 2001). It also demonstrated good control results in the greenhouse against *O. cumana* and *P. ramosa* (Sauerborn *et al.*, 2002; Gonsior *et al.*, 2004). The objectives of this study were to test the impact of FOG on *P. ramosa* under field conditions in Germany, using on the one hand different techniques of soil application and on the other hand a combination of the biocontrol agent with the resistance inducer BION®.

3.2 Materials and methods

3.2.1 Inoculum production and formulation of FOG

The fungal isolate FOG was maintained on Special Nutrient-poor Agar (SNA) (Nirenberg, 1976). High amounts of inoculum were produced in a bench-top fermenter (Figure 3.6) using liquid malt extract media (20 mL biomalt, 2 g yeast, 0,5 g $MgSO_4$, 0,5 g KH_2PO_4, 0,2 g Chloramphenicol, 1000 mL deonised water). Microconidia, needed as starter culture for the fermentation, were grown in 250 mL-Erlenmeyer flasks containing liquid malt extract medium for five days. The gained inoculum was incorporated into Pesta granules after the method of Connick *et al*. (1991). Wheat flour (32g), kaolin (5 g), sucrose (2 g) and iron (1 g) – commercial iron fertiliser Flory® 72 Fe – were mixed with 23 mL of liquid inoculum. The mixture was kneaded to a dough and passed several times through a hand-operated pasta maker. The thin dough sheets were air-dried, ground in a laboratory mill and sieved to a particle size of 250 µm to 2 mm. The number of viable colony forming units (cfu) was detected by dissolving 0.1 g of granules in 10 mL of water and plating 100 µL of appropriate dilutions on half strength Potato Dextrose Agar (PDA). Cfu per g formulated material were calculated after incubation for four days at room temperature.

3.2.2 Greenhouse experiment

The Tobacco cultivar HY Virgin 10 and its parasite *P. ramosa* were grown in a greenhouse at 25/15 °C (day/night) with supplemental light (1000 W) for 12 h. 1.7 L plastic pots were filled with a soil high in organic matter content, which had been steamed before at 80 °C for 12 hours.

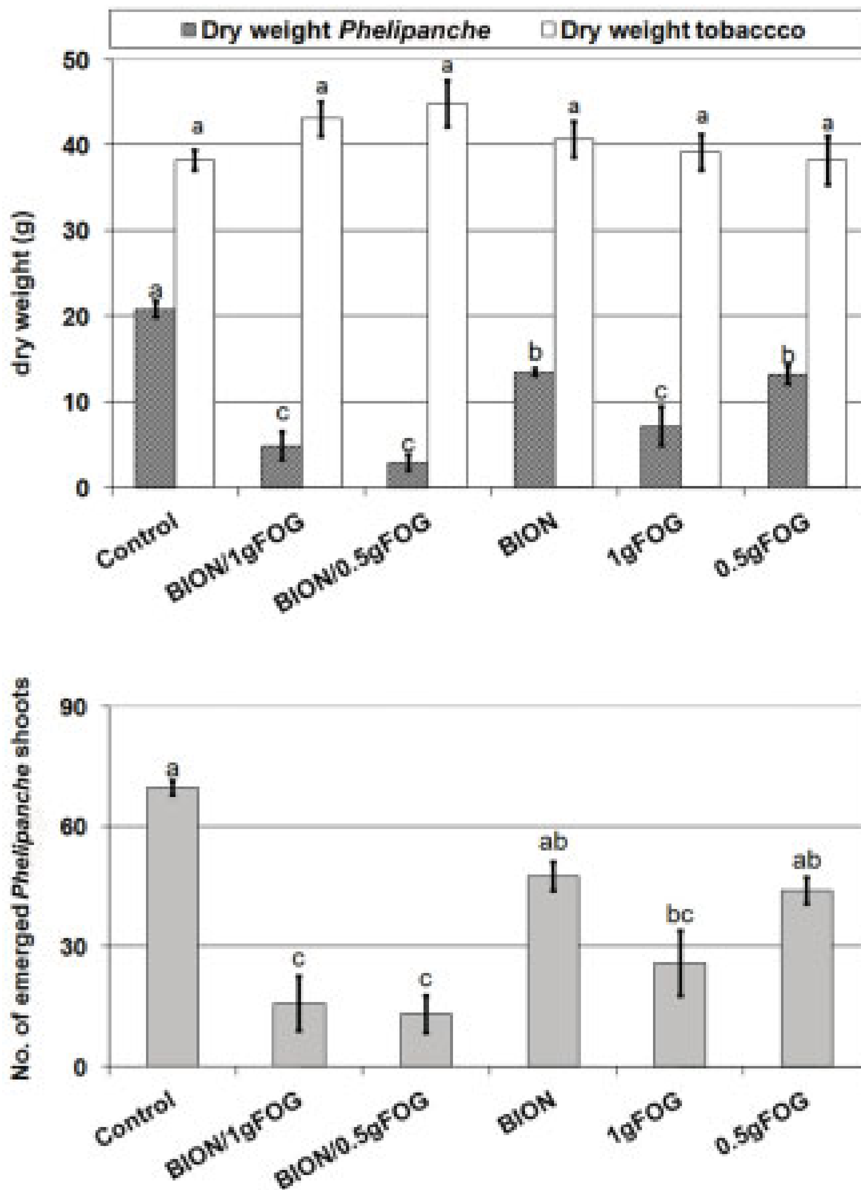

Figure 3.1:Experiment I: Influence of different inoculum dosages of *Fusarium oxysporum* and their combination with BION® on the number of emerged *Phelipanche ramosa* shoots and the *Phelipanche* and tobacco (HYV10) dry weight. Multiple mean comparisons were performed using Tukey's HSD test. Bars with diverse letters are significantly different ($P < 0.05$). **I** = ± standard error of means.

The formulated material (FOG) was incorporated into the soil at a rate of 1 g (10^7 cfu g^{-1} granules) per pot along with approximately 10,000 viable *P. ramosa* seeds. Pots containing only *P. ramosa* seeds served as a negative control.

Only in the repeated experiment a positive control was set up (pots without *P. ramosa* seeds and fungal population). The experiments were arranged in a completely randomised design.

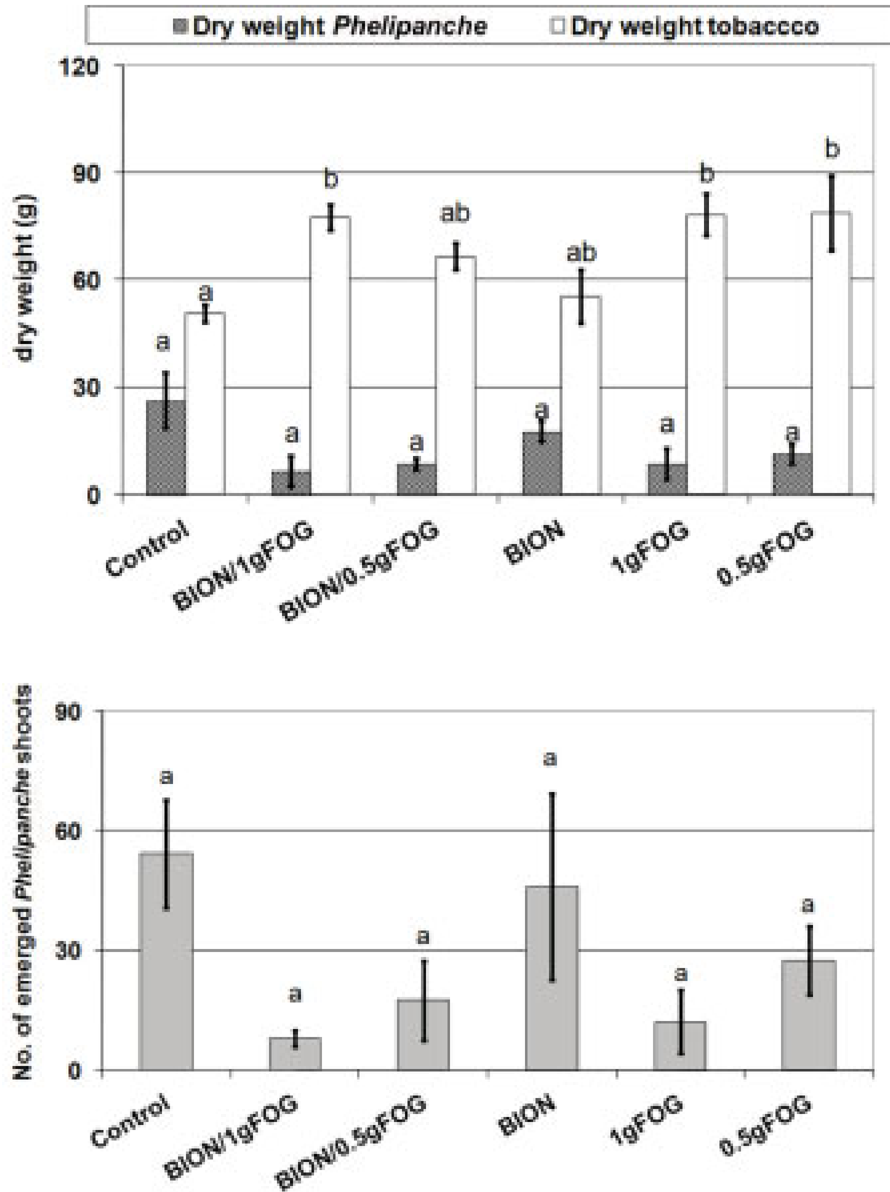

Figure 3.2: Experiment II: Influence of different inoculum dosages of *Fusarium oxysporum* and their combination with BION® on the number of emerged *Phelipanche ramosa* shoots and the *Phelipanche* and tobacco (HYV10) dry weight. Multiple mean comparisons were performed using Tukey's HSD test. Bars with diverse letters are significantly different ($P < 0.05$). **I** = ± standard error of means.

Tobacco seeds had been sown into trays containing substrate sand mixture and were transplanted to the pots three weeks after emergence. BION® was applied as a soil drench with 5 g in 50 ml water three times every second week starting three weeks after potting the tobacco seedlings.

Three month after planting, above ground shoots of branched broomrape were counted. Tobacco as well as the emerged and subterranean *Phelipanche* shoots were harvested and dried in an oven at 80 °C for 48 hours.

3.2.3 Field trials

Field trials were carried out in loamy sand at the research station “Landwirtschaftliches Technologiezentrum Augustenberg”, Southern Germany in 2006. Tobacco (variety HYV10) seeds were sown in March in the greenhouse and the emerged plants were transplanted to the field at the beginning of May (Fig 3.7). The site was naturally infested with *P. ramosa.*

In the first experiment, different application techniques of Pesta granules (3×10^7 g^{-1}) were investigated: subsoil application (2 g planting per hole), in-furrow application after tobacco planting (20 cm width, 13 g per running m) and broadcasting application after tobacco planting (50 cm width, 29 g per running m). The experiment was set as a randomised complete block design with eight replications. Each plot (1.8 m x 3.0 m) contained four rows with 16 plants, but only four plants in the centre of the plot were investigated to avoid border effects. In the second experiment, the combination of the biocontrol agent (applied in-furrow) with a soil-drench of BION® (300g ha^{-1}, 10 mg in 100 mL per plant) applied twice, starting three weeks after transplanting the tobacco was compared to the sole application of the biocontrol agent. It was also set as a randomised complete block design with six replications. Each plot (2.3 m x 3.0 m) consisted of four rows and 20 tobacco plants, six of which were taken for investigation. The following parameters were recorded throughout the duration of the field experiment:

- number of *Phelipanche* shoots emerged;
- distance of the broomrape shoots to the tobacco plant;
- number of diseased *Phelipanche* shoots;
- dry matter of the *Phelipanche* shoots;
- fresh and dry matter of tobacco plant.

A data logger was incorporated into the field, which measured air and soil temperature. More climatic data was provided from the experimental station nearby. In regular intervals, soil samples were taken from the treated and untreated plots to follow the development of the fungal population. Dilutions of the soil samples were plated on PCNB (Pentachloronitrobenzene) plates (Fauzi & Paulitz, 1994) and cfu were counted after four days incubation at room temperature.

Statistical analysis was conducted using multiple comparison procedures. Multiple mean comparisons consisted of analysis of variance (ANOVA), Tukey's studentised range test (HSD) and protective LSD test. When data showed heterogeneity of variances they were square-root or log-transformed before analysis. All tests of significance were conducted at $P < 0.05$.

Table 3.1: Effect of the different application types of FOG on the dry weight of tobacco at harvest.

	Tobacco dry weight (g per plant)	Standard error of means
Untreated control	47a	+/-3
In-furrow application	48a	+/-2
Broadcasting application	51a	+/-2
Planting hole application	55a	+/-2

Multiple mean comparisons were performed using the protected LSD test. Bars with the same letters are not significantly different ($P < 0.05$).

3.3 Results

3.3.1 Greenhouse experiments

The repeated experiments were analysed separately due to heterogeneity of variances. In both experiments the *Fusarium* treatment using the higher dosage of inoculum (1 g) and the combined applications with BION® showed the lowest number of emerged *Phelipanche* shoots and the lowest amount of *Phelipanche* dry matter (Figures 3.1 and 3.2). In the first experiment the combined treatment with the lower dosage of FOG (0.5 g) performed best regarding the control of *P. ramosa* and differed significantly from the respective single treatments.

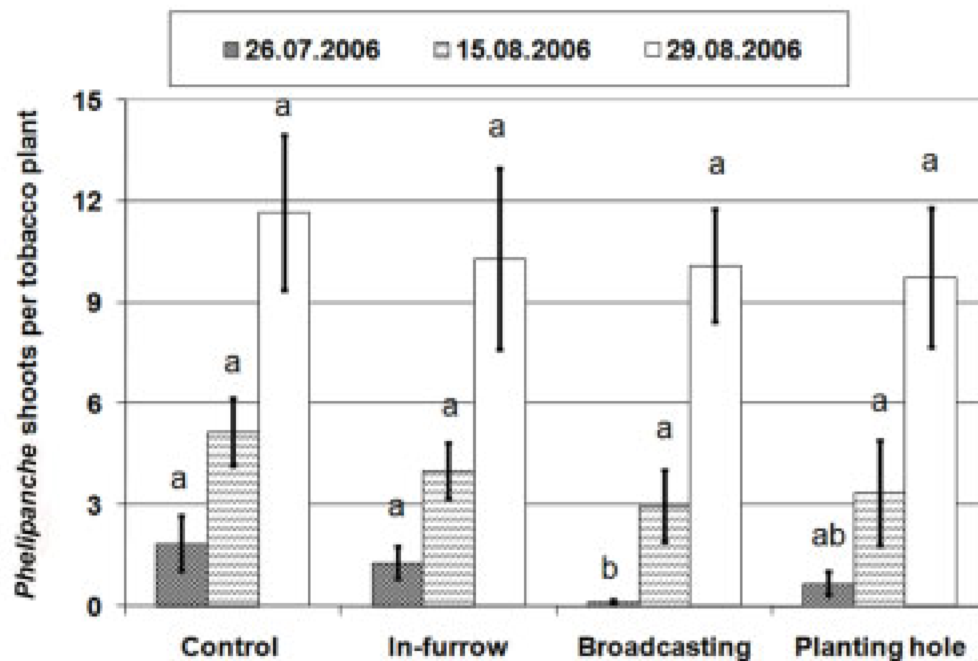

Figure 3.3: Field trial: The effect of different application techniques of *F. oxysporum* (FOG) on the number of *P. ramosa* shoots at different dates. Multiple mean comparisons were performed using the protected LSD test. Bars with the same letters are not significantly different ($P < 0.05$). **I** = ± standard error of means.

However, in the second experiment, the single and combined treatments did not significantly differ from each other. But again one of the combined treatments (FOG high dosage with BION®) provided the best *Phelipanche* control. The BTH treatment alone was only efficient in controlling *P. ramosa* in the first experiment but not in the second one.

The tobacco dry weight did not differ among the treatments in the first experiment but significant differences occurred in the second one. It was significantly higher in the FOG treatments alone and in combination (high dosage of FOG and BTH) when compared to the negative control. Only the dry matter of the sole BION® application and the negative control differed significantly from the positive control.

3.3.2 Field trials

The broadcasting application had a better effect on *Phelipanche* control than in-furrow or subsoil application early in the season (Figure 3.3).
The number of cfu detected per g soil was approximately twice as high for the broadcasting application compared to in-furrow application. However, the effect disappeared in the course of the experiment. Generally, biocontrol efficacy decreased during the season from 50% to 10%. The fungal population in the soil accordingly decreased strongly (up to 70% compared with the initial number of cfu) within two months (Figure 3.5). At harvest, dry weight of tobacco in the biocontrol treatments was not significantly increased compared to the control (Table 3.1). In the second experiment, the biocontrol treatments were generally more efficient (Figure 3.4). However, a better *Phelipanche* control by combining FOG with the resistance inducer compared with the single treatments was only observed at the beginning of the season, but had disappeared by the end of the season.

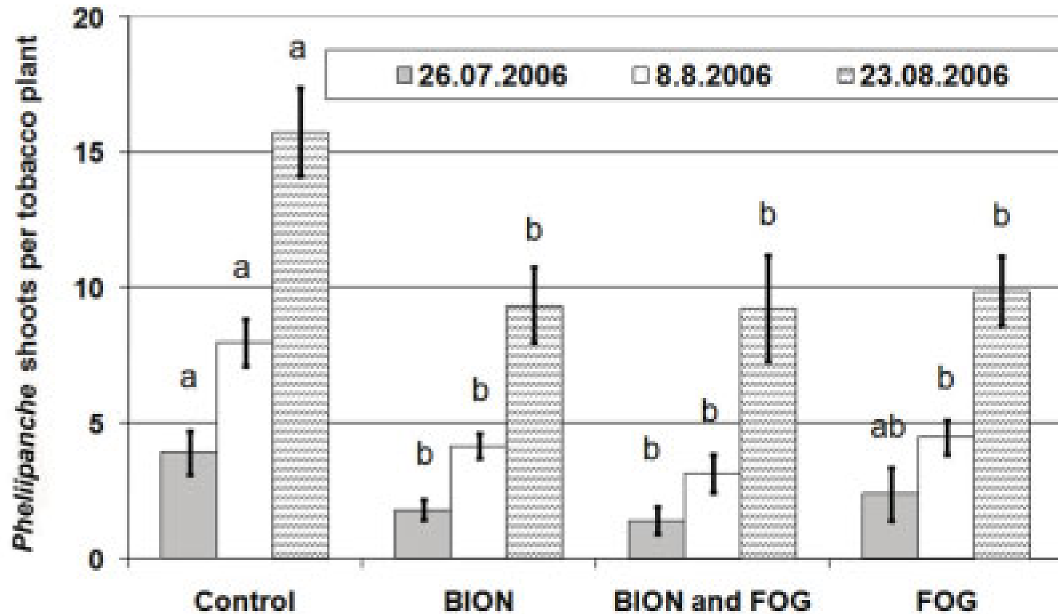

Figure 3.4: Field trial: The effect of *F. oxysporum* (FOG), BION® and their combination on the total number of *P. ramosa* shoots at different dates. Multiple mean comparisons were performed using the protected LSD test. Bars with diverse letters are significantly different ($P < 0.05$). **I** = ± standard error of means.

3.4 Discussion

In the greenhouse experiments, the combination of BION® and the biocontrol agent resulted in a more reliable *P. ramosa* control than it could be achieved by the use of a single control agent. Similar results were achieved by Müller-Stöver *et al*. (2005) with *O. cumana* and sunflower. The authors assumed that the combination of control strategies already affected the early stages of *Phelipanche,* but could not find any direct effects of BTH on the performance of the fungus. It is known that non-pathogenic *Fusarium* strains can induce an accumulation of PR (pathogenesis related) proteins as well as phytoalexins (Fuchs *et al.*, 1999; Cachinero *et al.*, 2002). Yu and Mühlbauer (2001) found that BTH and *Fusarium* induced different pathways of defence in wheat. Therefore it was hypothesised that BTH and the biological control agent triggered distinct defence mechanisms in sunflower and that their combination resulted in a lower broomrape infestation.

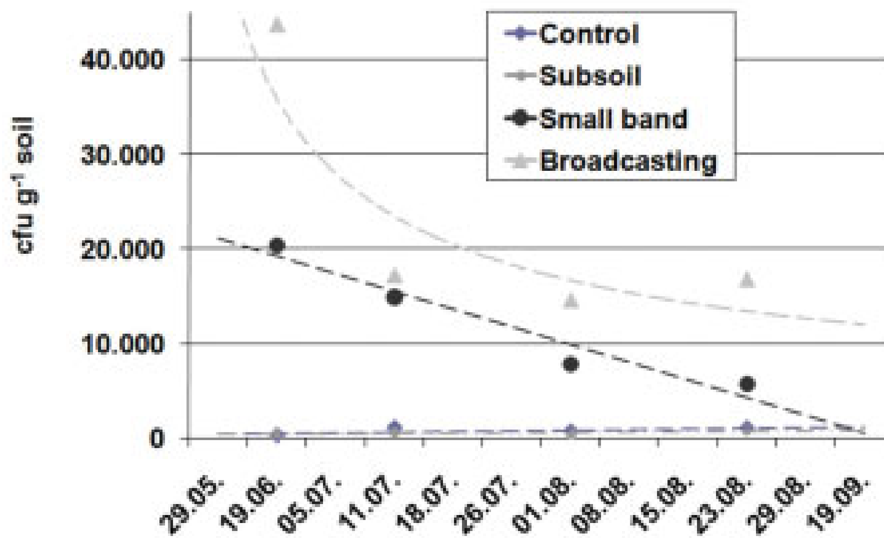

Figure 3.5: Development of the fungal population in the soil (15 cm depth).
Soil = ovendried.

As very variable control levels were achieved under natural conditions by working either with biocontrol agents (Sauerborn *et al.*, 2007) or BION® (Perez-de-Luque *et al.*, 2004), the combination of both control approaches seemed promising. The fluctuating success of *Phelipanche* control by systemic acquired resistance could even be observed in the performed greenhouse experiments where a better control level was achieved in the first experiment than in the second one. However, in the field the expected effect of combining two control agents was only observed at the beginning of the season. In the end (before harvest), no differences could be noticed to the FOG or BION treatments alone. The results are corroborated by those of Müller-Stöver (unpublished) investigating the combination of BION® and a different biocontrol agent against *O. cumana* in sunflower under field conditions. If the improved control effect of the combined treatment in the greenhouse is actually based on inducing distinct defence mechanisms in the host plant by both components, it seems that a defence-related response was not or only weakly induced by *F. oxysporum* under field conditions, since the single BTH treatment alone showed an effect against broomrape.

This could be due to the strongly decreasing concentration of the fungus in the soil. Fuchs *et al.* (1999) observed a more effective protection against *Fusarium* wilt when inoculating tomato roots with a higher-concentrated conidia suspension of a non-pathogenic *F. oxysporum* strain compared to a lower concentration. Furthermore, induced resistance requires an exposure of the plant to the inducing agent prior to the pathogen attack. As the inoculum was not directly applied to the roots of tobacco at transplanting, a possible induced resistance caused by FOG was probably not effective.

Broadcasting (50 cm width) of the inoculum gave the best results for *Phelipanche* control among the application techniques at the beginning of the season which was also reflected in the higher number of cfu in the soil. The better efficacy of this treatment could either be due to the better distribution of the fungal propagules in the soil or to the absolutely higher amount of inoculum (29 g per running m compared to 13 g per running m and 4 g per running m) being applied. However, the difference to the other application modes disappeared in the course of the experiment while generally a loss of biocontrol efficacy was observed. Most probably the climatic conditions enhanced *Phelipanche* growth but not the development of the FOG population in the soil. The very hot and dry July and the quite low soil temperatures in August (19.6 °C average) did probably not favour the fungal growth, as can be assumed by observing the cfu of the broadcasting treatment that dropped down from 40,000 cfu g^{-1} soil to 17,000 cfu g^{-1} soil within three weeks in July.

This study again shows the varying results achieved by the application of mycoherbicides even in neighbouring fields in the same season, influenced by various factors on a small scale. However, the combination of biocontrol agents with BTH does not seem to stabilise the control efficacy against *P. ramosa* under field conditions. Therefore, further studies should work on enhancing the performance of the fungus alone, e.g. by different formulation and application techniques that might result in a slower release of fungal propagules and therefore retard the decrease of the fungal population in the soil.

Figure 3.6: Bench-top fermenter, obtained inoculum after three days of fermentation

Figure 3.7: Tobacco planting, field trial in 2006

4 Impact of *Fusarium oxysporum* on the holoparasitic weed *Phelipanche ramosa*: biocontrol efficacy under field-grown conditions[3]

Summary

Under the changing agro-climatic conditions of western Europe, the parasitic weed *Phelipanche ramosa* infests at an increasing rate host crops such as tomato, hemp, tobacco and oilseed rape. A *Fusarium oxysporum* isolate (FOG), that had effectively reduced the parasite's incidence under controlled environmental conditions, was tested in different granular formulations (pesta granules, alginate pellets) regarding its impact on *P. ramosa* parasitising tobacco under field-grown conditions. To better understand field performance of this biocontrol agent, additional greenhouse and laboratory studies were conducted using soil from the experimental site.

FOG reduced number and biomass of *Phelipanche* shoots between 50% and 70% in three consecutive years (2006–2008). Sole pesta application did not show consistent results throughout the seasons: 50% reduction of *Phelipanche* biomass (DM) in the first year could not be repeated in the following years (20-30%). An alginate formulation applied solely performed better, however, combination of pesta granules with alginate pellets had the highest reliable control efficacy (60-70%) of all treatments in two seasons compared to the untreated control. Fungal population counts in soil samples did not show a close correlation to biocontrol efficacy. The greenhouse study revealed some fungistatic effects of the field soil which partly explain the reduced efficacy (-40%) in the field compared to results obtained under controlled conditions.

Results show the potential of FOG for *P. ramosa* control. Since formulation affected the bioncontrol agent's efficacy, it may be worthwhile to test how the delivery system can be changed in order to achieve increased disease development in the field.

Key words: biological control, fungi, mycoherbicide, weed control, *Fusarium oxysporum, Phelipanche ramosa, Nicotiana tabacum,* pesta granules, alginate pellets

[3]This chapter is an amended version of KOHLSCHMID E, SAUERBORN J & MÜLLER-STÖVER D (2009) Impact of *Fusarium oxysporum* on the holo-parasitic weed *Phelipanche ramosa*: biocontrol efficacy under field-grown conditions. *Weed Research* **49**, Supplement 1, 56-65, with permission of Wiley-Blackwell.

4.1 Introduction

The obligate root parasite *Phelipanche ramosa* L. (branched broomrape) has a wide host range that includes plants of the families Asteraceae, Solanaceae, Cannabaceae and Brassicaceae and causes serious damage to economically important crops (Parker & Riches, 1993). *Phelipanche* occurs in many parts of Europe, North Africa, the Middle East, and West Asia (Chater, 1972). In France, it has become an increasing problem in oilseed rape (*Brassica napus* L.) and tobacco (*Nicotiana tabacum* L) (Gibot-Leclerc *et al.*, 2003; Brault *et al.*, 2007), while in Germany it mainly infests tobacco and hemp (*Cannabis sativa* L.) at an increasing rate (Buschmann, 2004). Broomrape deprives its host plant of water, assimilates, and nutrients, which are transferred directly from the host root's vascular system. According to Lolas (1986) and Billenkamp (pers. comm.), the weed may cause complete yield losses in tobacco fields.

Due to close affiliation to the host plant, complex life cycle, and longevity of seeds (about 10 years), economically feasible control approaches are not available for all affected agro-ecosystems (Dhanapal *et al.*, 1996; Joel *et al.*, 2007). Management strategies should focus on reducing the soil seed-bank and interfere with the parasite's early development stages, since most of the damage to the host is inflicted before *Phelipanche* emerges above soil.

In this context, many pathogenic fungi, especially *Fusarium* spp., have been reported to potentially control *Orobanche* and *Phelipanche* species (Bedi & Donchev, 1991; Bozoukov & Kouzmanova, 1994; Amsellem *et al.*, 2001b; Boari & Vurro, 2004; Nanni *et al.*, 2005; Müller-Stöver & Kroschel, 2005) and seem to be a promising management tool. A recently found *F. oxysporum* (Schlecht.) Snyd. & Hans. isolate (FOG) obtained from diseased tubercles of an *P. ramosa* population from Germany, showed a high control efficacy in greenhouse trials (Müller-Stöver *et al.*, 2009). This isolate is very host specific and attacks all developmental stages of the parasite, such as germinating seeds, tubercles and aboveground shoots. According to Müller-Stöver *et al*. (2009), FOG seems to have a higher mycoherbicidal potential than other already found *F. oxysporum* isolates. However, efficacy of mycoherbicides is often lower and less reliable under field conditions compared to pot experiments (Sauerborn *et al.*, 2007; Kohlschmid *et al.*, 2008).

Environmental conditions such as temperature, moisture, sunlight, and abiotic and biotic soil characteristics can greatly influence biocontrol agents' efficacy (Handelsman & Stabb, 1996; Larkin & Fravel, 2002). Therefore, knowledge about biocontrol organism's ecology is important to find appropriate formulations that can withstand adverse environmental conditions.

The objectives of this study were to assess the impact of FOG on *P. ramosa* under field conditions in Germany and to find a suitable soil delivery system. Since first year pesta granule application showed varying biocontrol efficacy (Kohlschmid *et al.*, 2008), greenhouse and *in vitro* studies were conducted to investigate the influence of abiotic and biotic conditions of the soil from the experimental site. In the following seasons an alternative formulation (alginate pellets) was introduced. Additionally, the combination of a fast (pesta granules) and a slow release (alginate pellets) formulation – as observed by Gracia-Garza and Fravel (1998) – was investigated to assess whether this could produce a consistently high fungal soil-population and therefore result in more endurable *Phelipanche* control.

4.2. Materials and methods

4.2.1 Inoculum production and formulation of FOG

The fungal isolate FOG was maintained on special nutrient-poor agar (SNA), (Nirenberg, 1976) plus glycerol at –80°C. Stock cultures were stored up to 6 months on SNA plates at 5°C. Inoculum was produced in a bench-top fermenter (Labfors®, Infors AG, Switzerland) using liquid malt extract media (2% (v/v) biomalt (Biomalt, Kirn, Germany), 0.2% (w/v) yeast extract, 0.05% (w/v) $MgSO_4$, 0.05% (w/v) KH_2PO_4, 0.02% chloramphenicol, dissolved in deionised water). Starter cultures, grown for five days in a 250 mL-Erlenmeyer flask containing 100 mL liquid malt extract medium, were used to inoculate 4 L of growth medium (pH 5-6). The fermenter was run for 48-60 h at 28°C, with a constant air supply (1 L min^{-1}), and an agitation speed of 300-500 rpm. Every 10 min, 1 mL of Antifoam A (Sigma-Aldrich, Steinheim, Germany, 10 mL L^{-1}) was automatically added.

Inoculum was incorporated into pesta granules using the method of Connick *et al.* (1991) modified by Müller-Stöver and Sauerborn (2007), or into sodium alginate pellets (Walker & Connick, 1983).

To prepare pesta granules, 80% (w/w) durum wheat flour (Divella, Rutigliano, Italy), 12.5% (w/w) kaolin, 5% (w/w) sucrose and 2.5% (w/w) iron fertiliser (Flory® 72 Fe (PLANTA-Düngemittel GmbH, Germany)) were mixed with 57.5% (v/w) fungal inoculum. The mixture was kneaded and passed through a hand-operated pasta maker to produce 1 mm thick dough sheets, which were air-dried, ground in a laboratory mill, and sieved to a particle size of 250 µm to 2 mm. The number of colony forming units (cfu) was determined by completely dissolving 0.1 g of granules in 10 mL water and plating 100 µL of appropriate dilutions on half strength potato dextrose agar (PDA). Cfu per g formulated material were calculated after incubation for four days at room temperature.

For preparation of sodium alginate pellets, 1.2% (w/v) sodium alginate was dissolved in hot deionised water. The solution was then blended with kaolin (6.7%, (w/v)), durum wheat flour (8.9%, (w/v)), and 1.5% (w/v) of sucrose. After cooling the preparation to 30°C, 18.5% (v/v) of liquid inoculum was added and the pH was adjusted to 7. The mixture was stirred thoroughly to ensure complete dissolution of all ingredients and dripped into 0.1 M calcium chloride using a peristaltic pump. Obtained beads were left for one hour in calcium chloride for hardening, rinsed with tap water, and maintained an additional hour in a gelatine solution (5 g L^{-1}) (Bashan, 1986). Cfu g^{-1} alginate pellets were determined by dissolving 0.1 g of pellets in 1% sodium carbonate and plating appropriate dilutions on half-strength PDA (three replicates per preparation, three Petri dishes per dilution). Cfu for both formulations ranged from 3 to $5*10^7$ g^{-1} granules.

4.2.2 Laboratory experiments

To determine the development of the fungal population in soil under different moisture regimes, 0.2 g of pesta granules were incorporated into Petri dishes filled with 50 g dry topsoil from the experimental site (Forchheim soil: loamy sand, 1.6-2% organic matter, pH 5.6-6.0).

The moisture content was adjusted with tap water to 100% (27% (w/w)), 70% (19% (w/w)) and 20% (6% (w/w)) of the soil's maximum water holding capacity. For each treatment (three replicates), a corresponding control treatment without fungal inoculum was prepared. The experiment was carried out for 16 weeks in a dark incubator (Heraeus, Thermo Fisher Scientific, Ulm, Germany) at 25°C. After the first and third week, two samples of 0.5 g soil were taken from each Petri dish and dispersed in 10 mL water. 200 µL of 1:100 dilutions were streaked on PDA-PCNB agar (pentachloronitrobenzene, Sigma-Aldrich, Steinheim, Germany) (Fauzi & Paulitz, 1994). For each sample, two plates were inoculated and cfu showing the same morphological features as the FOG isolate (scarce pale violet mycelia) were counted after four days of incubation at room temperature (20 ± 2°C). Identicalness of counted colonies with FOG was randomly confirmed by RAPD-PCR (Chapter 5). The experiment was repeated.

4.2.3 Greenhouse experiment

The biocontrol agent's efficacy in soil from the experimental site (Forchheim soil) was compared to that in compost substrate (10% organic matter, pH 6.6) previously used in greenhouse experiments. The experimental design comprised three factors: (a) treatment (positive control without *Phelipanche* and fungus, negative control with *Phelipanche* only, and fungal treatment with *Phelipanche*), (b) substrate (compost, loamy sand), and (c) sterility (substrates non-autoclaved or autoclaved twice at 121°C for one hour).

Tobacco seedlings (var. HYV 10) were nurtured in a climate chamber (Percival Scientific, Inc., Perry, USA) at 25°C for three weeks, and then transplanted to plastic pots (1.7 L) which had been filled with the respective substrate and partially mixed with 30 mg *P. ramosa* seeds (collected in tobacco fields of south-western Germany) and 1 g pesta formulation. Pots were arranged in a completely randomised design with five replicates per treatment on the greenhouse bench. Plants were grown at a temperature regime of 25/15 ± 5°C (day/night) with supplemental light provided by 400 W Philips Son-T Agro lamps for 13 h, and fertilised with 2% (v/v) Wuxal® solution (Bayer Crop Science, Langenfeld, Germany) fortnightly. After three months, above ground shoots of *Phelipanche* were counted and harvested together with subterranean shoots.

The biomass (DM) of the parasites and their host plants (without roots) was determined after drying in an oven at 80°C for 48 hours. The experiment was repeated.

4.2.4 Field trials

Field trials were conducted at the research station "Landwirtschaftliches Technologiezentrum Augustenberg" (48°59'N, 08°19'E), Forchheim, Germany, in 2006, 2007, and 2008. The climate is temperate with an annual precipitation of 742 mm and an average annual temperature of 10°C. The experiments of 2006 and 2007 were performed at the same site, but due to a heavy weed establishment a different site was used in 2008. The soil of the experimental fields was naturally infested with *Phelipanche.* Basic fertilisations (N, P, K, Mg) were applied every year in April. Tobacco seedlings (HYV 10 in 2006 and 2008, HYV 17 in 2007) were nurtured in a greenhouse from March to May and afterwards transplanted to the field. In 2006 the field was supplementary irrigated and mechanically weeded. In 2007 weeds were removed manually and with the herbicides Fusilade Max (125 g Fluazifop-P L^{-1}, Syngenta, applied twice) and Flexidor (500 g Isoxaben L^{-1}, Spiess-Urania, applied once). The latter was also applied in 2008 combined with mechanical weeding. The fungicide Acrobat Plus WG (600 g Mancozeb kg^{-1} + 90 g Dimethomporph kg^{-1}, BASF) was used in all three years. In 2007 and 2008, Ranman (400 g Cyazofamid, Belchim L^{-1}) and Forum (150 g Dimethomorph L^{-1}, BASF) were applied to control blue mold (*Peronospora tabacina* Adam.) which is a severe problem in this region.
An automatic weather station was used to measure precipitation, air and soil temperature (10 cm depth). Experiments were set up as a randomised complete block design with six replications, however, in 2006 block 6 was not taken into account for statistical analysis, because *Phelipanche* did not emerge there.
In **2006** pesta formulation, applied in-furrows (20 cm width, 13 g per running m), and additional treatments (soil-drench of BION® applied twice and four times, Kohlschmid *et al.* 2008) were compared to an untreated control. Each plot (2.3 m x 3.0 m) consisted of four rows and 20 tobacco plants, six of which (in total 30 plants for each treatment) were taken for investigation to avoid border effects.

In **2007** different fungal formulations were tested using in-furrow application (20 cm width, 13 g per running m): (a) pesta granules, (b) alginate pellets (Figure 4.6), and (c) pesta plus alginates. Six plants (in total 36 plants for each treatment) out of 20 from each plot (2.3 m x 2.6 m) were investigated.
In **2008**, the field trial of 2007 was completely repeated with a plot size of 2.8 m x 5.4 m and 42 tobacco plants per plot, all of which were investigated due to a lower *Phelipanche* infestation.

The following parameters were recorded during the field experiments:

- number of emerged *Phelipanche* shoots above ground (weekly),
- number of diseased *Phelipanche* shoots (weekly),
- biomass (DM) of *Phelipanche* shoots, by digging out emerged shoots around the six plants in September. 2006: frame 1.5 m * 1.4 m; 2007 1.3 m * 1.4; 2008 whole plot. Shoots were dried at 80°C for 48 h.
- Yield and biomass (DM) of tobacco plants: 2006, ripe leaves of 10 plants of each plot were harvested twice in August, weighed and dried in an oven at 70°C for 4 days. In 2007, the six selected plants of the plot were cut, total fresh weight determined, chaffed, and 700-1000 g of fresh biomass was dried at 70°C for 3 days. Dry weight of one plant was calculated by applying the fresh weight ratio: dry weight to total fresh matter of the six plants devided by the number of plants.

At regular intervals, soil samples were taken with a N min auger (15 cm depth) from treated and untreated plots to monitor fungal survival rate. A mixed sample per plot was obtained from six sub-samples and cfu per g soil were determined as described in section 2.2.

4.2.5 Statistical analysis

Data which were not normally distributed or showed heterogeneity of variances were log or square root transformed before analysis. For the moisture experiment, a repeated-measures ANOVA was conducted with Statistica version 6.0 (StatSoft, 1984-2002). Greenhouse experiments were analysed together as ANOVA with three treatment factors and experiment as a block factor with the mixed procedure in SAS version 8.1 (SAS Institute, 1999-2000).

For field experiments, emergence rate of *Phelipanche* shoots in 2006 and 2007 and the survival rate of the fungal population in the soil (2007 and 2008) was $\log_{10}$ (number or cfu +1) transformed and a polynomial regression line which matched best according to the coefficient of determination was fit . Except for emergence of *Phelipanche* shoots in 2006 (linear regression $y=ax-b$), a quadratic curve ($y=ax^2+bx+c$) was adopted, with a being the curvature, b being the slope of the quadratic curve, and c being the axis intercept. Back transformed data are presented in Figure 4.4 and 4.5. ANOVA with two treatment factors and the block-factor as random factor with proc mix (SAS) was calculated for the parameters a, b and c (Mead *et al.*, 2003) and the dry weight of *Phelipanche* shoots. Since the whole plot (42 plants) was taken into account, no boundary effects could be eliminated in 2008. Therefore a covariate was introduced using the mean of the two bordering plots. Mean comparisons were performed using Tukey's studentised range test (HSD) or Fisher's t-test.

4.3. Results

4.3.1. Laboratory experiments

Results of one laboratory experiment are presented, because the repetition had similar results. In the experiment on fungal development in soil under different moisture regimes, cfu declined over time in all three treatments (Figure 4.1). Although initial number of fungal propagules was higher at 19% (w/w) moisture than at 6% (w/w), cfu were constantly higher at 6% (w/w) throughout the experiment. Significant differences were only observed between the 6% (w/w) and the 27% (w/w) moisture treatments. Interactions between time factor and treatment factor were not significant.

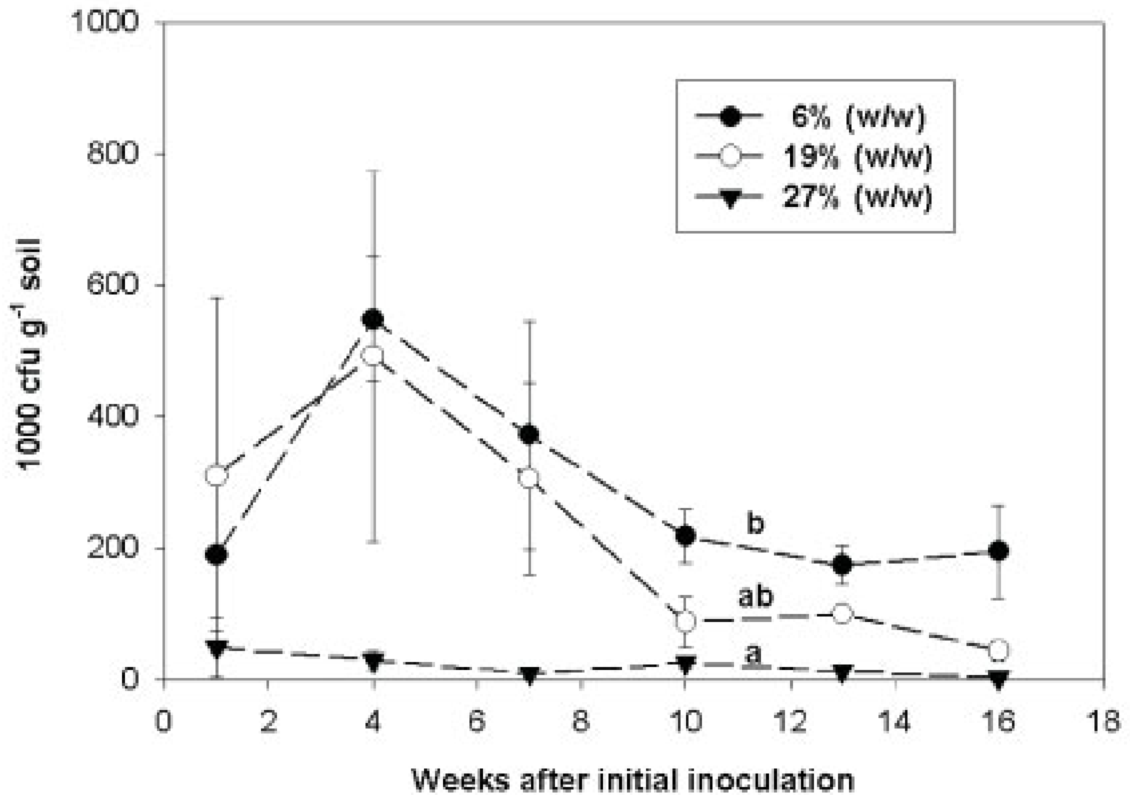

Figure 4.1: Influence of different water contents on FOG development in soil. Cfu observed in respective negative controls were subtracted from cfu counted in fungus-treated Petri dishes. Repeated measures ANOVA was performed with mean (n=3) comparison using Tukey's HSD test. Graphs with the same letters are not statistically different (P < 0.05). **I** = ± standard error of means, soil = oven-dried.

4.3.2. Greenhouse experiment

Statistical analysis of the two experiments revealed no interactions between treatment, soil type, and sterility, but between soil type and treatment (Figure 4.2). *Phelipanche* biomass (DM) was significantly higher in control pots containing compost compared to those with Forchheim soil, but DM in fungus-treated pots did not significantly differ among substrates which implicates a higher efficacy of the fungus in compost than in Forchheim soil. The biocontrol effect was even higher in sterilised soil.

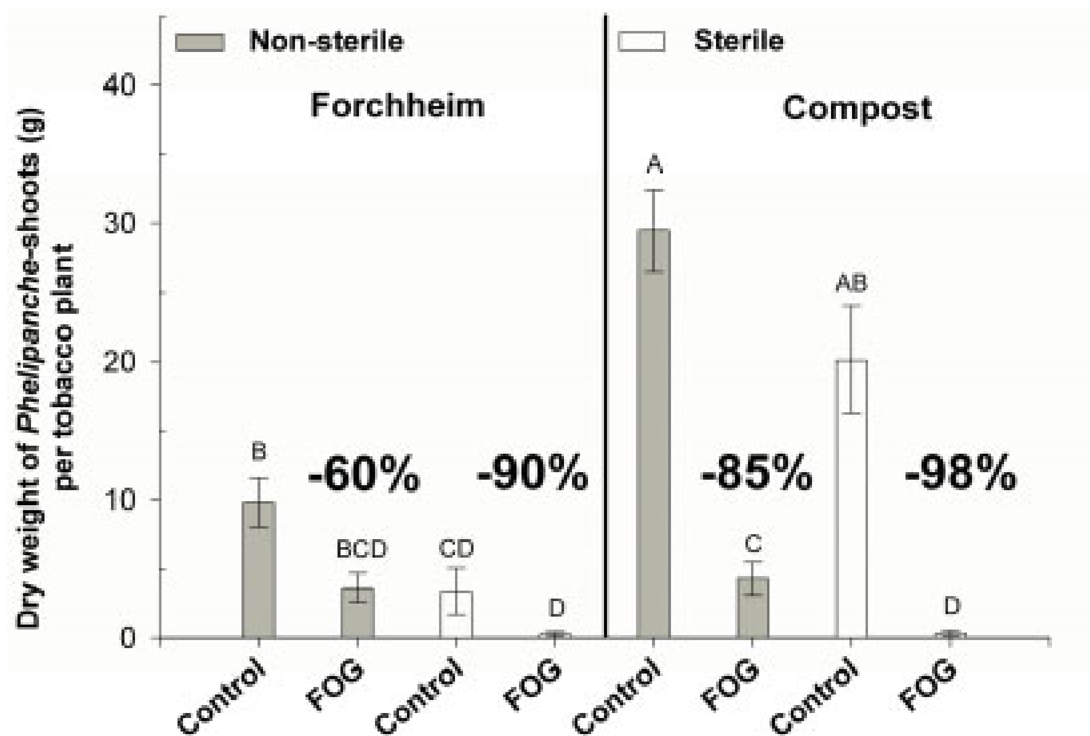

Figure 4.2: Influence of two different substrates and sterility on control efficacy of FOG. Bars (n=5) with the same letters are not significantly different (P<0.05) after Tukey's HSD test. **I** = ± standard error of means.

4.3.3 Field experiments

In 2006 in-furrow application of FOG (pesta granules) significantly reduced *Phelipanche* biomass (DM) by about 50% (Figure 4.3 A). Statistical analysis of slopes and intercepts of the emergence rates of *Phelipanche* shoots (linear regression graphs) revealed no differences in relative shoot increase among treatments (Figure 4.4 A, Figure 4.7). The proportion of diseased shoots did not differ among treated plots and control plots (data not presented). Dry weight of harvestable tobacco leaves did not differ among control and FOG treatments (Table 4.1).

Ten weeks after transplanting, FOG population counts were significantly higher in the fungus-treated plots ($2.2*10^4$ cfu g^{-1} oven-dried soil) compared to control plots ($6.0 *10^2$ cfu g^{-1} oven-dried soil).

However, cfu declined by up to 80% (to $5.6*10^3$ cfu g^{-1}) in the treated plots within another 10 weeks.

Table 4.1 Effect of pesta formulation on tobacco-yield at two harvest dates in 2006.

Treatment	Leave dry weight (g $plant^{-1}$) 12 w.a.t.	SE of means	Leave dry weight (g $plant^{-1}$) 14 w.a.t.	SE of means
Control	23.3a	2.18	11.5a	1.07
Pesta (FOG)	20.4a	1.68	12.9a	1.35

Means (n=5) with the same letters are not statistically different (P<0.05) after the t-test.
w.a.t. weeks after transplanting.

Table 4.2: Effect of different formulations of FOG on tobacco biomass at harvest in 2007.

Treatment	Tobacco dry weight (g $plant^{-1}$)	SE of means
Control	76a	9.8
Pesta	95b	11.1
Alginate	80ab	13.8
Alginate/Pesta	94b	9.7

Means (n=6) with the same letters are not statistically different (P<0.05) after the t-test.

In 2007 a combination of pesta granules and alginate pellets was most effective in reducing number and dry matter of *Phelipanche* shoots by about 60% (Figure 4.3B, 4.4B). The control effect of the sole pesta treatment on *Phelipanche* set in later (Figure 4.4B), which was also observed in 2006 (Figure 4.4A). However, the fitted quadratic curves to the log transformed data of *Phelipanche* emergence did not statistically (P<0.05) differ in their curatives (coefficient *a*) and their slopes (coefficient *b*). Statistical analysis of the intercepts (coefficient *c*) revealed a significant influence of the factor alginate formulation but not of the factor pesta formulation on the level of *Phelipanche* emergence. At the end of the trial, the sole alginate treatment showed a similar efficacy as the sole pesta treatment (about 30%).

The proportion of diseased shoots did not differ among treatment and control plots (data not presented). A significantly higher tobacco biomass compared to the control was harvested from all fungus-treated plots with the exception of the sole alginate application (Table 4.2).

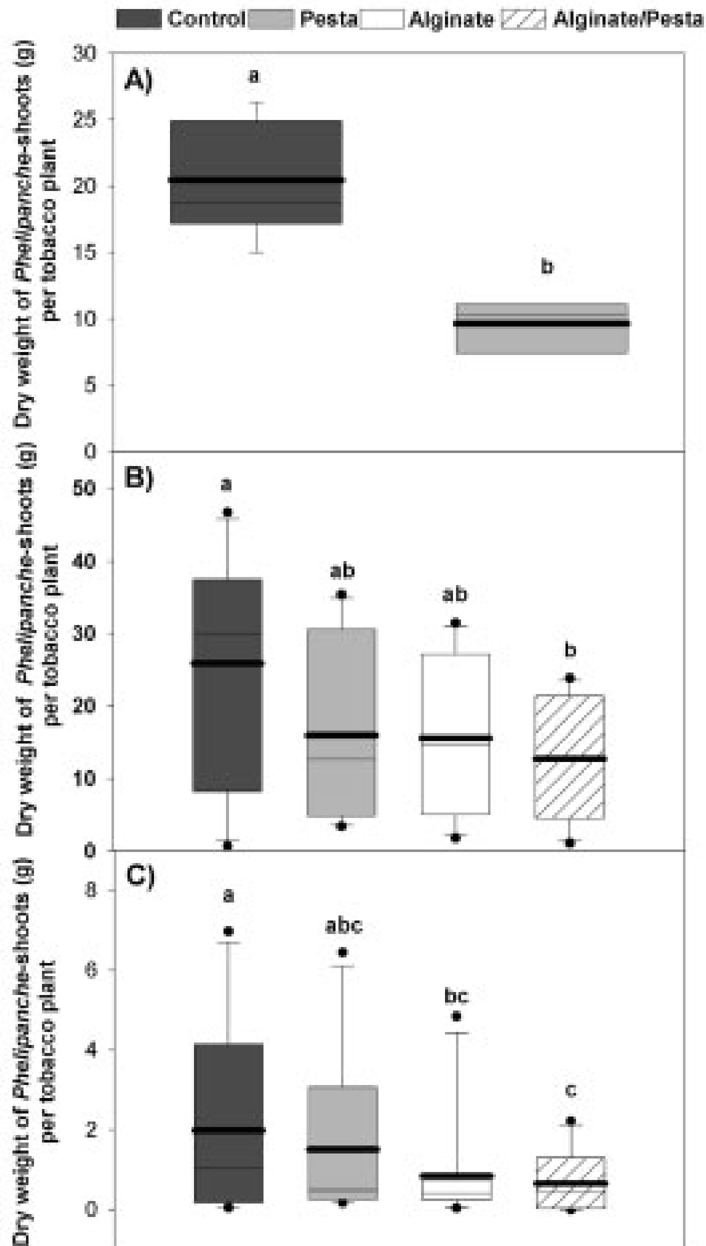

Figure 4.3: Influence of different formulations of FOG on biomass (DM) of *Phelipanche* shoots. Box and whisker plots illustrate the 25th, 50th (median), 75th percentiles and outliers (black dots). Thick black lines indicate the mean (n=5) in 2006, (n=6) in 2007 and 2008). Box plots marked with the same letters are not statistically different (P<0.05) after the t-test. A = 2006, B = 2007, C = 2008.

Fungal population in soil again decreased strongly (by about 90%) within three months (Figure 4.5B). Slopes (coefficient *b*) and curatives (coefficient *a*) of quadratic curves of the log transformed data did not significantly differ among treatments and control. Comparisons of intercepts (coefficient *c*) revealed significant higher levels of survival rates in all fungus-treated plots compared with control plots, but not among the different fungal formulations.

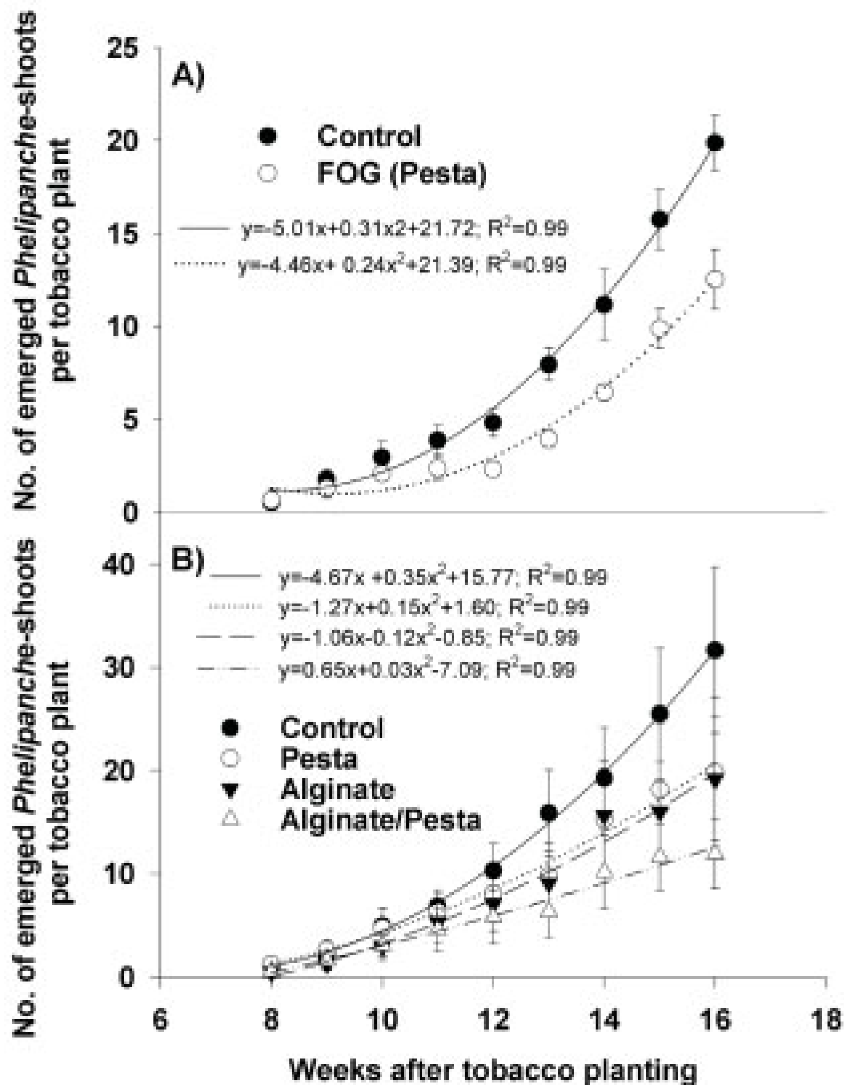

Figure 4.4: Influence of different formulations on number of emerged *Phelipanche* shoots. For statistical analysis data was log-transformed. A = 2006, means (n=5) ± SE given; B = 2007, means (n=6) ± SE given.

In 2008 the overall *Phelipanche* emergence was much lower than in previous years.

Combination of alginate pellets and pesta granules depleted the number and biomass of emerged *Phelipanche* shoots by about 70%. The sole alginate treatment had a better control effect than in 2007 (50% reduction compared to 38%), but efficacy of the sole pesta application was only around 20% (Figure 4.3C). Only the factor alginate formulation had a significant influence on *Phelipanche* emergence at the last counting date.

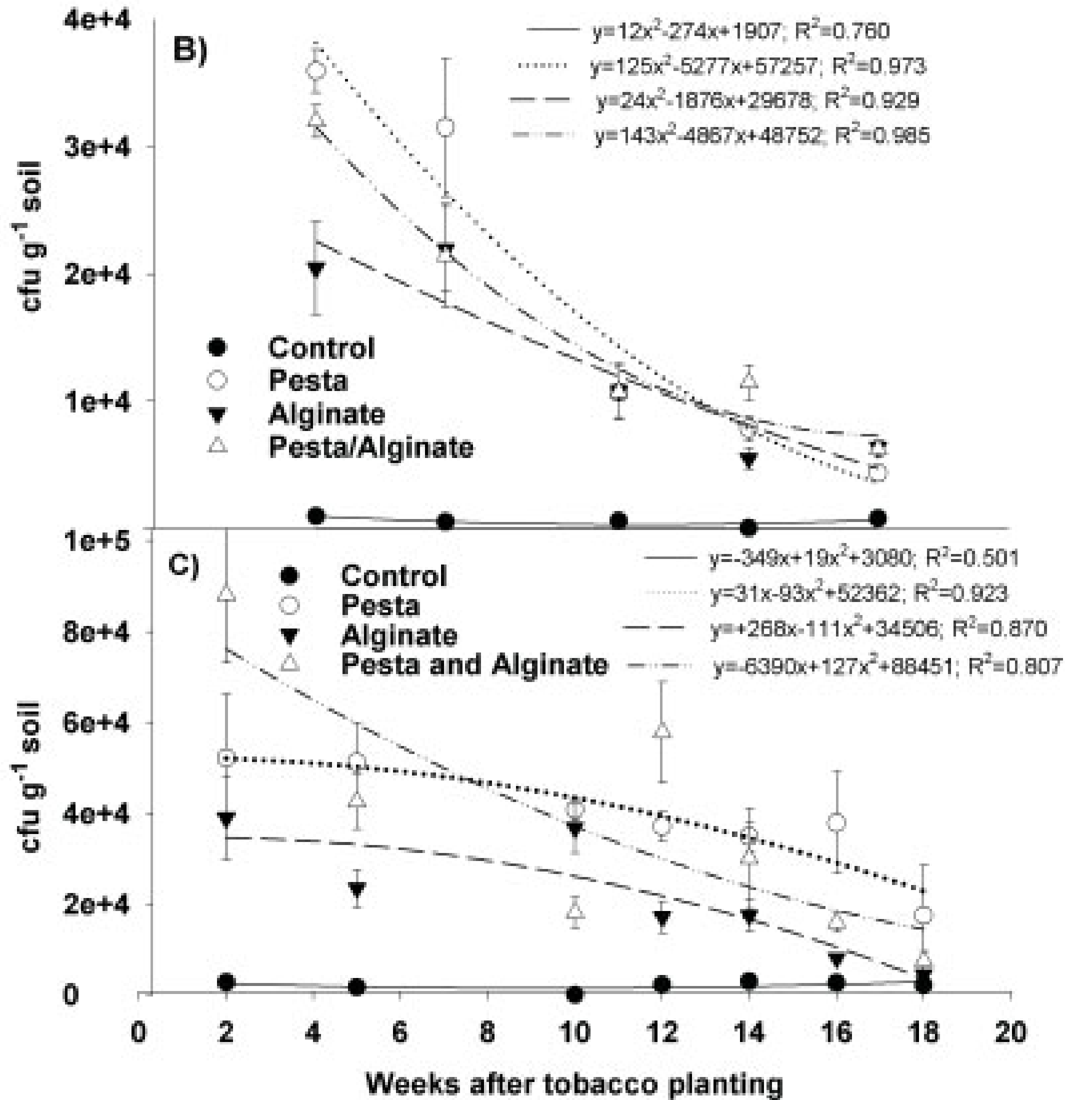

Figure 4.5: Survival rate of FOG in the soil. B = 2007, means (n=4) ± SE given; C = 2008 means (n=3) ± SE given. For statistical analysis data was log-transformed. Soil = oven-dried.

FOG soil-population decreased strongly by about 90% within three months. In the sole pesta treatment, decline was only 70% (Figure 4.5C).

Analysis of slopes and curatives did not show statistical differences of log transformed survival rates ($P<0.05$), except for the sole alginate treatment - the significant different curative compared to the control is caused by a strong cfu decline in this treatment at the end of the experiment. Comparison of intercepts (coefficient c) revealed a significant difference ($P<0.05$, t-test) in level of survival rate for the sole pesta treatment and combination alginate/pesta compared to the control, but not for the sole alginate treatment.

4.4. Discussion

The three field experiments revealed a biocontrol effect of FOG against *P. ramosa* under field conditions. The coefficient analysis of the polynomial regression lines showed that FOG had almost no influence on the relative increase of emergence of shoots. Slopes of the curves were the same in 2006 and 2007, but total shoot number was reduced by the fungus. This indicates that application of the biocontrol agent delays, but does not completely prevent appearance of *Phelipanche* shoots. The observed biocontrol effect in the field was lower compared to previously achieved results in the greenhouse (Müller-Stöver *et al.*, 2009). However, control efficacy of pesta granules in greenhouse experiments using soil from the experimental site was similar to that obtained in the field in 2006.

The control level obtained with pesta granules (the treatment that was applied consistently throughout the seasons) in 2006 could not be repeated in 2007 and 2008. Several reasons may be responsible for this reduction in efficacy, e.g. seasonal climatic conditions. Cook and Baker (1983) noted that the growth of *Fusarium* wilt pathogens is generally maximal at 28°C, inhibited above 33°C and not favoured below 17°C. This was also observed for FOG in laboratory studies, where no mycelial growth was visible at 35°, and growth rates differed greatly between temperature regimes of 15°C, 20°C and 25°C (Appendix 3). While in 2006 the average field soil temperature (top 10 cm) from May to September was 21°C, it was about 18°C in 2007 and 2008 which might have contributed to the reduced biocontrol efficacy.

The average soil moisture content may also be a reason for the differences in *Phelipanche* biocontrol among the years.

In the top 15 cm of soil, it was from May to September around 9% (w/w) in 2008, 11% (w/w) in 2007 and around 8% (w/w) in 2006 (Appendix 2). The Petri dish experiment revealed that FOG thrived better at a 6% (w/w, 20% saturation) soil moisture content, compared to 19% (w/w, 70% saturation) or 27% (w/w, 100% saturation). *Fusarium* spp. are strongly aerobic and greatly reduced in growth at soil moisture contents from 50 to 85% and almost nonexistent at 100% (Stover, 1953a; Oritsejafor, 1986). Stover (1953a) found for six *Fusarium* species an optimal growth and survival rate at 15% water saturation. Cook (1981) suggested for some *Fusarium* species that the higher the temperature in the range of 15°C to 36°C, the lower the optimal water potential for optimal growth. This may further explain the better control effect of the pesta treatment in 2006, where a comparatively high soil temperature was combined with a low moisture content.

Stover (1953a) obtained the highest bacterial populations at moisture levels above 50% with an optimum at 75%. A competitive bacterial flora at high water contents may therefore also be a factor in reducing *Fusarium* populations (Stover, 1953b). The possible influence of a competitive microflora could also be seen in the greenhouse study with different substrates. In non-sterilised soils the biocontrol agent's efficacy was relatively lower compared to sterilised soils, especially in the substrate from the experimental site, which could be due to reduction or elimination of antagonists of *F. oxysporum* by sterilisiation. According to Fravel *et al.* (1996), soils show suppressiveness to proliferation of a biocontrol agent if its population density is higher after soil-sterilisation. Forchheim soil seems to be more suppressive than compost substrate, which resulted in severe reduction of FOG propagules by up to 90% of the initial level within two or three months under natural conditions. However, it cannot be excluded that the application of fungicides (especially the broad-sprectrum component mancozeb) adversely affected the survival of FOG in the field soil.

The higher control efficacy in compost soil observed in the greenhouse may further be related to the higher organic matter (OM) content (10%) compared to Forchheim soil (1-2%). Müller-Stöver *et al.* (2009) also achieved a higher efficacy in compost substrate (OM 10%) compared to sandy loam substrate (OM 0.2%).

In many studies (Tilston *et al.*, 2002; Termorshuizen *et al.*, 2006; Perez-Piqueres *et al.*, 2006), compost had a significant negative influence on soilborne pathogens, however, Bonanomi *et al.* (2007) suggested that an occasional positive affect could be associated with their saprophytic growth promoted by organic matter.

In 2007 and 2008, alginate pellets proved to be superior to pesta formulation regarding reduction of *Phelipanche* infestation. Zahran *et al.* (2008) found that an alginate formulation of *F. nygamai* was slightly more effective against the parasitic weed *Striga hermonthica* (Delile) Benth. compared to a pesta formulation. In this study, a combination of alginate pellets and pesta granules resulted in a more reliable *Phelipanche* control, which seems to be related to a higher control effect of the alginate pellets at the beginning of the experiment combined with a delayed control effect of the pesta granules. Originally, this combination was meant to combine a fast outgrowth of fungal propagules from pesta granules with a slower release from alginate pellets – as reported by Gracia-Garza and Fravel (1998) and Bailey *et al.* (1998) - to achieve a consistently high fungal population level in soil. However, the observation of the development of FOG soil-population does not corroborate this hypothesis. In 2007 cfu in the soil were indeed higher in plots that had received the combined treatment compared to those where only alginate pellets had been applied, and did not decrease to the extent of the sole pesta treatment. In 2008 results were different: cfu in pesta-treated plots decreased only by about 70%, compared to around 90% in the other treatments. In contrast to the high and more persistent soil-population of this formulation, efficacy was generally quite low in 2008. In 2007 and 2008 the lowest numbers of cfu were detected in plots where alginate formulation had been applied, which neither corresponded to its biocontrol effect. Results indicate that the effectiveness of the formulation mix is not based on a constantly high level of fungal propagules in soil. Furthermore, the number of cfu in soil is obviously generally not very closely correlated to biocontrol efficacy. This confirms findings of Fravel *et al.* (1995), postulating that the potential of different carriers in alginate pellets to stimulate the growth of a biocontrol agent did not match with enhanced efficacy. Also Lewis and Papavizas (1985) did not find a correlation between population density of antagonists and their biocontrol impact.

However, since the underground infection mechanism of FOG is not known and estimating soil populations of sporulating organisms by dilution plate techniques does not distinguish between active hyphal growth and spore production (Thornton, 2008), obtained cfu from soil samples may not correctly reflect the infectious potential occurring in the different treatments.

The proportion of diseased *Phelipanche* shoots was not taken into account when evaluating biocontrol efficacy in the field due to occurrence of diseased *Phelipanche* shoots in control plots as well. Most likely, FOG had spread into control plots since isolates obtained from diseased *Phelipanche* shoots – but not from soil samples - from there were genetically identical to the original FOG isolate (Chapter 5). Although *F. oxysporum* was isolated from air (Rowe *et al.*, 1977; Rekah *et al.*, 2000), it is not well adapted to air dispersal (Burgess, 1981). It can be assumed that its aboveground spread is attributed to wind-blown soil organic debris, rain-splash (Ooka & Kommedahl, 1977), or the distribution of inoculum via hoeing the experimental plots.

Although a 50% reduction of *Phelipanche* shoots was achieved in 2006 in fungus-treated plots, tobacco yield was not significantly higher than in control treatments. This could be due to the relatively late onset of the control effect of the pesta treatment, which could not prevent that ripe tobacco leaves were reduced in biomass at harvest time. In 2007, the total tobacco biomass was significantly higher in the plots with the sole pesta treatment and the combination of alginate/pesta, although pesta formulation had not performed as good as in 2006. However, even in cases where no yield increase can be measured, a strong positive impact of the biocontrol agent is the reduction of emerging *Phelipanche* shoots and therefore the future soil seed-bank (Lolas, 1994).

The results indicate that FOG has a potential for field application, but future research should investigate in detail the effect of different formulation and application techniques on fungal growth and virulence under various environmental circumstances.

Figure 4.6: Application of alginate pellets (left) and pesta granules (right)

Figure 4.7: 2006: Control plot (left), FOG-treated plot (right)

5 Molecular detection of the biocontrol agent *Fusarium oxysporum* against *Phelipanche ramosa* after field release

Abstract

Aims: The main objectives of the present study were to assess the dispersal and survival potential of a *Fusarium oxysporum isolate* (FOG), a potential biocontrol agent for the parasitic weed *Phelipanche ramosa*, and to evaluate whether the RAPD marker technique can be used to identify the strain.

Methods and Results: Isolates were collected from soil, air, and diseased *Phelipanche* shoots in field experiments (2006 and 2007) and from a neighboring area (up to 10 m distance). The biocontrol agent FOG revealed fragment patterns clearly differentiable from another *F. oxysporum* strain (isolated from *Orobanche cumana*) when tested with 20 RAPD primers. In fungus-treated plots, most of the *Fusarium* isolates gained from *P. ramosa* shoots as well as all isolates from soil were 100% identical to FOG. In soil samples of control plots, FOG was only detected to a very limited extent. All isolates originating from plants or soil of the neighbor field in 10 m distance were clearly distinguishable from the biocontrol agent. However, FOG was discovered in diseased *Phelipanche* shoots in the control plots.

Conclusions: Reliable detection of FOG with the RAPD procedure was possible. The results indicate that the biocontrol agent has only a limited mobility and persistence in soil/air, but an aboveground spread over short distances seems to occur, possibly by rain-splash or human/animal activity.

Significance and Impact of Study: The ability to clearly re-detect a biocontrol agent released to the environment and studies about its proliferation and survival potential are very important for the overall acceptance by officials and farmers.

Key words: biocontrol, detection, fungi, granules, plant pathology, PCR, RAPD, parasitic weeds

5.1 Introduction

Broomrapes (*Orobanche* and *Phelipanche* species) are obligate root parasites that cause serious damage to a wide range of economically important crops in temperate as well as subtropical regions. As holoparasites, they deprive their host plants of water, assimilates and nutrients, which are transferred directly via a specific attachment organ, the haustorium (Sauerborn, 1991).
Phelipanche ramosa L. (branched broomrape) occurs worldwide (Parker & Riches, 1993; Musselman, 1994) and has a very broad host range that includes members of the families Asteraceae, Solanaceae, Cannabaceae, and Brassicaceae (Parker & Riches, 1993). Since there is no single method providing both effective and economically feasible control (Dhanapal *et al.*, 1996; Joel *et al.*, 2007), integrated management strategies should focus on reducing the soil seed-bank and interfering with the parasite's early development stages. In this context, biological control of *Orobanche* and *Phelipanche* species with soil-applied mycoherbicides based on *Fusarium* spp. isolates has been reported to have several advantages, as the fungi are able to attack the parasite already before the damage is inflicted upon the crop (Bozoukov & Kouzmanova, 1994; Thomas *et al.*, 1999b; Amsellem *et al.*, 2001b; Boari & Vurro, 2004). A *Fusarium oxysporum* (Schlecht.) Snyd. & Hans. isolate (FOG) was obtained from diseased tubercles of an *P. ramosa* population from Germany. This isolate effectively reduced the incidence of the parasitic weed on tomato (*Solanum lycopersicum* L.) and tobacco (*Nicotiana tabacum* L.) under controlled environmental conditions (Müller-Stöver *et al.*, 2009). It also showed its bioherbicidal potential in field experiments when inundatively applied in pesta or alginate pellets (Kohlschmid *et al.*, 2008). The development of the fungal population in soil during the experiments was monitored using the dilution plate method. This method is based on morphological identification of the fungal strain on a *Fusarium*-selective medium and does not always permit a reliable distinction between the applied biocontrol agent and the background *Fusarium* population. A similar problem occurred when investigating diseased *Phelipanche* shoots from different plots for the presence of FOG.

Because the species *F. oxysporum* have many pathogenic as well as non-pathogenic or beneficial strains (Lievens *et al.*, 2007), identification is required even below the species level, which is classically based on bioassays that are time-consuming and laborious (Recorbet *et al.*, 2003). However, obtaining more knowledge about the environmental fate of the organism is not only important regarding the optimisation of biocontrol efficacy, but also regarding safety matters, since potential environmental risks associated with the use of biocontrol agents at high concentrations are of increasing public concern (Tebeest *et al.*, 1992; Hoagland *et al.*, 2007). It was therefore necessary to find an inexpensive molecular method to be able to reliably identify the *F. oxysporum* isolate after its application to the environment. The RAPD (Random Amplification of Polymorphic DNA) technique, based on DNA-amplification with single or multiple arbitrary primers requiring no previous knowledge of the target DNA sequence, has been successfully used to differentiate *F. oxysporum* at *forma specialis* level (Wang *et al.*, 2001; Jana *et al.*, 2003; Bayraktar *et al.*, 2008). The aim of the present study was to use a RAPD marker-based assay to reliably recognise the field-applied biocontrol agent in order to i) verify the soil population counts on the dilution plates, ii) determine the spread potential of the biocontrol agent especially into non-treated control plots or into neighboring fields and iii) to corroborate whether the fungus can be disperse through the air, as it has been reported by Rekah *et al.* (2000).

5.2 Materials and methods

5.2.1 Fungal strains

The fungal isolates *F. oxysporum* (FOG) and *F. oxysporum* f.sp. *orthoceras* (Appel & Wollenw.) Bilay (Foo) (Figure 5.4), a pathogen of *O. cumana,* were preserved on special nutrient-poor agar (SNA), (Nirenberg, 1976) plus glycerol at –80°C at the Institute for Plant Production and Agroecology, University of Hohenheim, Germany. For experiments, stock cultures were stored up to 6 months on SNA plates at 5°C.

5.2.2 Fermentation, formulation, and field experiments

High amounts of inoculum were produced in a bench-top fermenter (Labfors®, Infors AG, Switzerland) using liquid malt extract media (2% (v/v) biomalt (Biomalt, Kirn, Germany)), 0.2% (w/v) yeast extract, 0.05% (w/v) $MgSO_4$, 0.05% (w/v) KH_2PO_4, 0.02% chloramphenicol, dissolved in deionised water) The obtained inoculum was incorporated into pesta granules, using the method described by Connick *et al.* (1991) and modified by Müller-Stöver and Sauerborn (2007), or into sodium alginate pellets (Walker & Connick, 1983). Field trials were carried out in 2006 and 2007 in tobacco fields at the research station "Landwirtschaftliches Technologiezentrum Augustenberg" (48°59'N, 08°19'E) in south-western Germany, where the soil was naturally infested with *P. ramosa*. In **2006**, different application methods of the biocontrol agent in pesta granules (3 x 10^7 cfu g^{-1}) were tested in comparison to an untreated control (Kohlschmid *et al.*, 2008), whereas in **2007** alginate pellets (5 x 10^7 cfu g^{-1}) were investigated as an alternate formulation (Chapter 4). The plot size was 6-7 m^2 and experiments were set up as a randomised complete block design with six replications.

5.2.3 Collection and isolation of the fungus

a) *from diseased* Phelipanche *shoots:* In 2006, diseased *Phelipanche* shoots with browning or wilting symptoms were randomly sampled weekly from the end of July until the end of August in fungus-treated plots as well as in non-treated plots. Shoots were also taken from a neighboring tobacco field in a distance of 10 m. In 2007, diseased shoots were sampled in the same way twice in August. Three to five shoots from each plot were selected after every sampling, and a small piece of necrotic tissue was cut out. The plant pieces were surface sterilised for 5 minutes in 1% sodium hypochlorite, then left three times in distilled water for 5 minutes, dried on filter paper and placed on Potato Dextrose Agar (PDA) plates amended with 100 ppm chloramphenicol. The plates were incubated at room temperature (20 ± 2°C) for one week.

Outgrowing *Fusarium* isolates which appeared morphologically identical to FOG (scarce pale violet mycelia and oval shaped, 0-septate microconidia) were sub-cultured on PDA and stored at 5°C. For long-term preservation, isolates were kept on SNA plus glycerol at –80°C.

b) from air: Detection of a possible air dispersal of fungal propagules was carried out in 2007 by trapping airborne conidia in Petri dishes (Rekah *et al.*, 2000) containing PDA-PCNB (pentachloronitrobenzene, Sigma-Aldrich, Steinheim, Germany), which is a *Fusarium*-selective medium (Fauzi & Paulitz, 1994). Every week from mid of July until the end of August, 15 to 20 plates were placed on paper sheets in a distance of 1 m to each other in the middle row (90 cm) of the field for three hours. The plates were then incubated at room temperature (20 ± 2°C) for 7 days. At each sampling date, 4 to 6 colonies exhibiting the FOG morphology could be obtained and were sub-cultured for molecular investigation.

c) *from soil:* at regular intervals, samples were taken in both years with a N min auger up to 15 cm soil depth from the treated and untreated plots to follow the development of the fungal population. One mixed sample per plot was obtained from six sub-samples. Additional four samples were taken once from the neighboring field (distance 10 m). In the laboratory, two sub-samples of 0.5 g soil were taken from each sample and dispersed in 10 mL water. 200 µL of 1:100 dilutions were streaked out on PCNB agar plates. For each sample, two plates were inoculated and cfu (colony forming units) were counted after four days of incubation at room temperature (20 ± 2°C). Colonies showing morphological features like FOG were randomly cut out and sub-cultured on PDA plates for further molecular investigation in 2007.

5.2.4 Genomic DNA extraction

The obtained *Fusarium* isolates from the field as well as the standard FOG and Foo strains were grown in liquid malt extract media for seven days on a rotary shaker (150 rpm) at room temperature (20 ± 2°C) in the dark. Fungal biomass was harvested by suction filtration on gauze frabric. The biomass was either used immediately or stored at -80°C for later use.

Genomic DNA was isolated using the CTAB method as described by Doyle and Doyle (1987) modified by Berndl (unpublished data).

5.2.5 RAPD analysis

After an initial screening of 100 decamer primers (Carl Roth GmbH, Karlsruhe, Germany) using the two standard *F. oxysporum* strains FOG and Foo, 20 primers were chosen for their ability to produce clear polymorphic and reproducible amplification patterns (Table 5.1).

Table 5.1: List of RAPD primers used in this study

Primer code	Sequence 5'->3'
A10	GTG ATC GCA G
A15	TTC CGA ACC C
A17	GAC CGC TTG T
A18	AGG TGA CCG T
A19	CAA ACG TCG G
L16	AGG TTG CAG G
L20	TGG TGG ACC A
M10	TCT GGC GCA C
M14	AGG GTC GTT C
M15	GAC CTA CCA C
M17	TCA GTC CGG G
M18	CAC CAT CCG T
N12	CAC AGA CAC C
N14	TCG TGC GGG T
N15	CAG CGA CTG T
N17	CAT TGG GGA G
U05	TTG GCG GCC T
U17	ACC TGG GGA G
U19	GTC AGT GCG G
U20	ACA GCC CCC A

These primers were used to genetically characterise the *Fusarium* isolates collected from the field trials in 2006 and 2007 and compare them to the standard isolates of FOG and Foo.

PCR reactions had a final volume of 10 µL. RAPD-PCR contained 10 ng template DNA, 200 µM of deoxynucleoside triphosphates (dNTP) mix (ABgene, Hamburg, Germany), 15 µM $MgCl_2$, reaction buffer (750 µM Tris-HCl pH 8.8, 200 µM $(NH_4)_2SO_4$ and 0.1% (v/v) Tween 20), 20 pmol of each primer (Carl Roth, Karlsruhe Germany) and 1 U of Taq DNA polymerase (Bioline, Luckenwalde, Germany). All PCR reactions were performed in a Biometra T-gradient Thermocycler (Göttingen, Germany). The PCR program initiated at 95°C for 3 min, followed by 34 cycles at 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min, continued with a final extension step at 72°C for 5 min. Ten µL of the PCR products plus 2 µL loading buffer were electrophoretically resolved in 2% agarose gels containing 1x Tris-borate-EDTA (TBE) buffer for 2.5 h at 4 V cm^{-1}, stained with 1% ethidium bromide, and visualised with an UV transilluminator (Intas GmbH, Göttingen, Germany) at 254 nm. Standard DNA samples (Gene Ruler 100bp Ladder, Roche Diagnostics GmbH, Mannheim, Germany) were used as molecular size markers for comparison.

5.2.6 Data analysis

Only amplicons that reliably amplified in repeated PCR reactions were included in the final analyses. RAPD fingerprints for all isolates were scored for the presence or absence of diagnostic amplicons (1 = presence and 0 = absence; Appendix 5). This binary matrice was analysed performed with the software package NTSYSpc version 1.80 (Exeter-Software, 1999). Jaccard´s similarity coefficients (JSCs) were calculated with the aid of SIMQUAL (Similarity for Qualitative Data) routine. Based on this data, a dendogram was constructed by UPGMA (unweighted pair group method with arithmetic averages) employing SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering).

Table 5.2: Source of *Fusarium* spp. isolates used for RAPD fingerprinting

Accession	Isolate	Genus	Location	Host/Place	Year	Geographic origin
1	Foo*	*F.oxysporum* f.sp. *othoceras*	Laboratory	*O. cumana* Wallr.	1991	Bulgaria
2	FOG+	*F. oxysporum*	Laboratory	*P. ramosa* L.	2003	Germany‡
3	3013	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2006	Germany§
4	3015	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2006	Germany§
5	3061-1	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2006	Germany§
6	3041-1	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2006	Germany§
7	3029-1	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2006	Germany§
8	3019-2	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2006	Germany§
9	949a	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2007	Germany§
10	14.08.-10	*Fusarium* spp.	Field Trial	Air	2007	Germany§
11	923-1b	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
12	952-2b	*Fusarium* spp.	Control Plot	Soil	2007	Germany§
13	948a	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
14	FF	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
15	944c	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2007	Germany§
16	942c	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
17	FGF	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
18	945b	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
19	3073-2	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2006	Germany§
20	924-2b	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
21	941-1b	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
22	954-1b	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
23	24.07.-7	*Fusarium* spp.	Field trial	Air	2007	Germany§
24	952-14	*Fusarium* spp.	Control Plot	Soil	2007	Germany§
25	948-1b	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
26	949-1b	*Fusarium* spp.	Control Plot	Soil	2007	Germany§
27	17.07.	*Fusarium* spp.	Field Trial	Air	2007	Germany§
28	930-2a	*Fusarium* spp.	Control Plot	Soil	2007	Germany§
29	3004-3	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2006	Germany§
30	N22	*Fusarium* spp.	Next Field (10m distance)	Soil	2007	Germany§
31	N42	*Fusarium* spp.	Next Field (10m distance)	Soil	2007	Germany§
32	N12	*Fusarium* spp.	Next Field (10m distance)	Soil	2007	Germany§
33	28.08.-1	*Fusarium* spp.	Field Trial	Air	2007	Germany§
34	922c	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
35	N31	*Fusarium* spp.	Next Field (10m distance)	Soil	2007	Germany§
36	31.07.-5	*Fusarium* spp.	Field Trial	Air	2007	Germany§
37	28.08.-4	*Fusarium* spp.	Field Trial	Air	2007	Germany§
38	N32	*Fusarium* spp.	Next Field (10m distance)	Soil	2007	Germany§
39	3001	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2006	Germany§
40	3024-5	*Fusarium* spp.	Control Plot	*P. ramosa* shoot	2006	Germany§
41	941d	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
42	923b	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
43	946d	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
44	922a	*Fusarium* spp.	Fungal Plot	*P. ramosa* shoot	2007	Germany§
45	927-1b	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
46	942-2b	*Fusarium* spp.	Fungal Plot	Soil	2007	Germany§
47	24.07.-5-1	*Fusarium* spp.	Field Trial	Air	2007	Germany§

* (Bedi & Donchev, 1991), +(Müller-Stöver *et al.*, 2009),

‡ unknown, § Ltz Augustenberg, Forchheim, Germany

5.3 Results

5.3.1 Sampling

a) from diseased Phelipanche *shoots*: In 2006, 212 necrotic plant pieces of *P. ramosa* shoots were investigated for the presence of *Fusarium* spp..

In total, 46 isolates showing similar features as the FOG isolate could be obtained.

Thirtyeight were gathered from fungus-treated plots and 8 from control plots. No *Fusarium* isolates could be gained from shoots collected in 10 m of distance from the field trial (36 necrotic pieces had been investigated). Six isolates from control plots and four from fungus-treated plots were used for molecular characterisation. In 2007, 21 isolates showing the morphological characteristics of FOG were obtained from 81 necrotic pieces of *P. ramosa* shoots on PDA plates. Four were gained from control plots and 17 from fungus-treated plots. Ten (2 from control plots, 8 from fungus-treated plots) of them were investigated further.

b) from air: Fourteen *Fusarium* isolates which showed similar morphology to FOG could be obtained from 105 agar plates laid out in the field, seven of which were taken for molecular fingerprinting.

c) from soil: 59 isolates which showed morphological similarities to the standard FOG isolate were randomly sampled from the PCNB plates used for monitoring the fungal soil population in the field trial. Thirteen (4 of control plots and 9 of fungus-treated plots) of them were selected for further molecular characterisation. Additional five isolates were obtained from the soil of the field in 10 m distance (Table 5.2).

5.3.2 Molecular characterisation with RAPD primers

Primers selected showed clearly repeatable fragment patterns. Moreover, it was possible to reliably distinguish FOG from Foo (Figure 5.1A). PCR amplification of the 45 *Fusarium* isolates obtained from the field experiments with the 20 different RAPD markers resulted in 99 amplicons used for scoring for presence versus absence (Figure 5.1 and Figure 5.2). Out of the 20 screened primers, one (A15) created a specific DNA fragment for FOG at 300bp (Figure 5.2). Eighteen different fingerprints could be distinguished. Thirty of the 46 tested isolates were genetically identical to FOG.

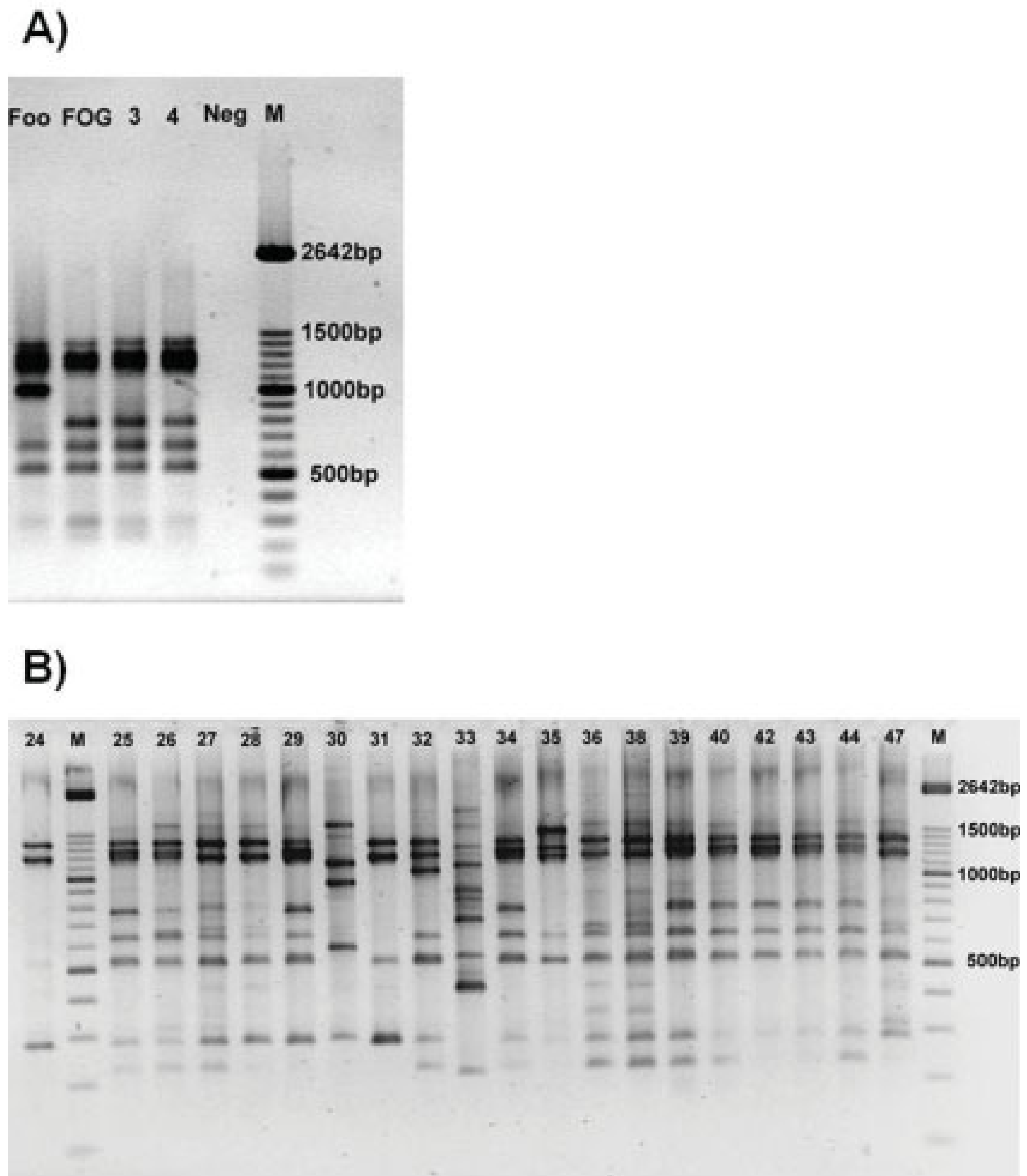

Figure 5.1: RAPD profiles of different isolates of *Fusarium* spp. obtained with primer A19 (Table 5.1).

M = Molecular weight marker (100bp). Neg = negative control. Accession numbers are listed in Table 5.2.

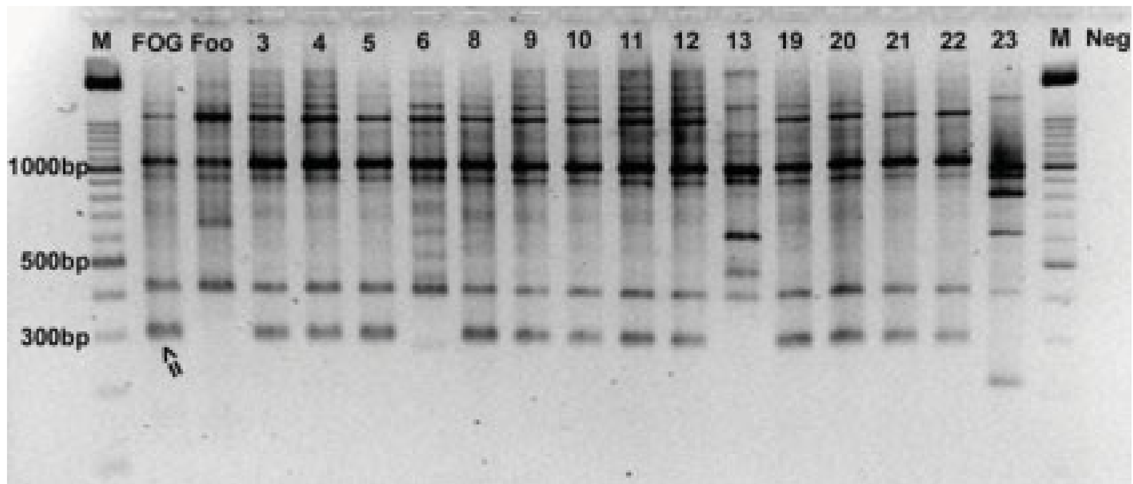

Figure 5.2: RAPD profiles of different isolates of *Fusarium* spp. obtained with primer A15 (Table 5.1).

M = Molecular weight marker (100bp). Neg = negative control. The arrow indicates a unique band only found for FOG isolates. Accession numbers can be found in Table 5.2.

5.3.3 Cluster analysis

Analyses of the RAPD fingerprints grouped the 47 isolates from our collection into 8 major branches showing similarity in the range of 33-100% to FOG (Figure 5.3; Appendix 6).

The composition of major branches varied from a minimum of one accession in branches C, D and E to a maximum of 33 accessions in branch H. Branch H holds the original FOG isolate (accession 2) and all isolates gathered from diseased *P. ramosa* shoots from fungus-treated and untreated plots as well as from the soil of fungus-treated plots with a maximum similarity index of 100%. Three accessions 1 (Foo), 6 (shoot, control plot), and 13 (shoot, fungal plot), showed a similarity between 65% and 69% to FOG.

Most of the air-borne isolates (accession 23, 27, 33, 36, 37, 47) were found in branch A, B, E, F, G and one was placed in branch H, 100% genetically identical to FOG. Three isolates from soil samples of the control plots (accession 24, 26, 28) were found in branch F exhibiting 63% genetic similarity to each other and between 58% and 62% to FOG. One of them (accession 12) was located in branch H. The isolates gained from the soil of the neighbor field (accession 30, 31, 32, 35, 38) are distributed over the branches A, C, D and G.

5.4 Discussion

Among the screened RAPD primers, A15 proved to be a reliable primer to distinguish FOG from Foo (pathogenic to *O. cumana),* as well as from two other *Fusarium* spp. isolates found on *P. ramosa* shoots and from other soil-inhabiting *Fusarium* strains. Jana *et al.* (2003) were also able to distinguish between different *Fusarium* species of different *formae speciales* by a single primer RAPD procedure. In a next step, the unique 300bps fragment created by primer A15 can be used to develop a specific primer for the biocontrol agent, as it has been done with a variety of biocontrol fungi, e.g. *Gliocladium catenulatum* Gilman & Abbott (Paavanen-Huhtala *et al.*, 2000), *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & Gams (Bulat *et al.*, 2000), *Aureobasidium pullulans* (de Bary & Löwenthal) Arnaud (Schena *et al.*, 2002) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Takatsuka, 2007). Specific primers can then be used in real-time PCR assays applied to quantitatively determine biocontrol fungi with DNA directly extracted from plant and soil samples (Cordier *et al.*, 2007). The ability to clearly re-detect a biocontrol agent released to the environment is very important for the overall acceptance by officials and farmers (Amsellem *et al.*, 2001b; Elzein *et al.*, 2008). The cluster analysis clustered FOG and Foo in the same branch. This aspect also indicates the close relatedness of *F. oxysporum* isolates attacking parasitic weeds, as it was already mentioned by Amsellem *et al.* (2001b), who found the *Phelipanche* pathogen E1D only distantly related to the crop-pathogenic strains of *F. oxysporum*. Most of the other isolates from the air, soil from the field in 10 m distance and the control plots were placed in distinct branches. In our study, monitoring the fungal population in soil via the agar plate method revealed a decrease of the fungal population at a rate of 80-90% over a three months period (Kohlschmid *et al.*, 2008).

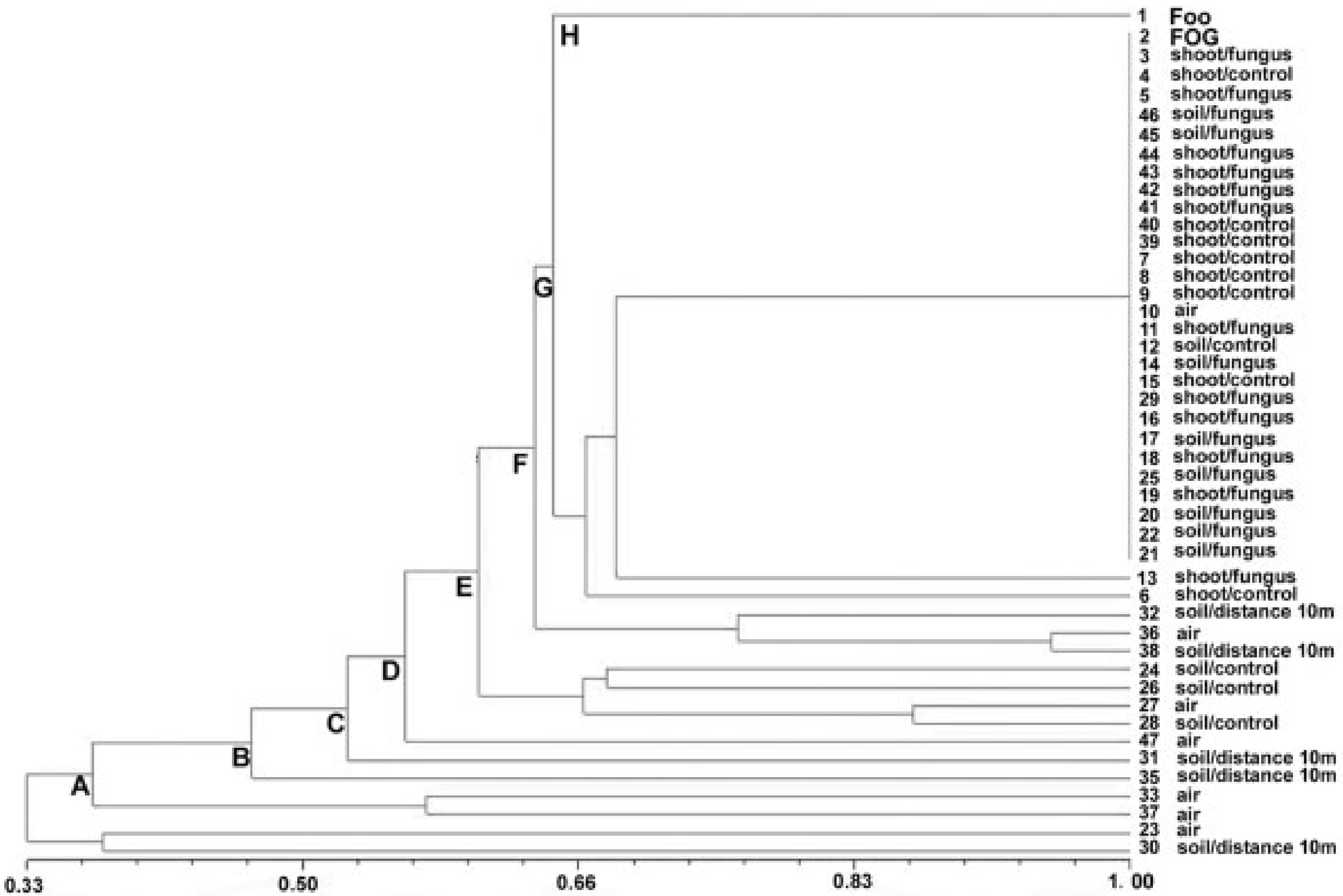

Figure 5.3: Dendogram obtained from 47 isolates of *Fusarium* spp. with UPGMA based on Jaccard's coefficient. Branches are labeled by accession numbers (Table 2). The line below the dendogram represents the similarity index. Fungus = treatment with FOG, control = no treatment.

Counting of colony forming units on selective or semi selective-media (Fauzi & Paulitz, 1994) have the advantage to only detect viable microorganisms compared to nucleid acid-based methods, but also several disadvantages: the counts may not be proportional to the amount of sample (Ridout & Harris, 1997), the results are not immediately available (Savazzini *et al.*, 2008), the method is very time-consuming, and it may lack specificity as non-targeted microorganisms with the same morphological characteristics are able to grow on the media as well (Hermosa *et al.*, 2001). This holds especially true for the omnipresent soil-inhabiting species *F. oxysporum*, of which exist many different pathogenic but also non-pathogenic strains. Therefore, a combination of two approaches in counting cfu on selective or semi-selective media and screening them randomly with PCR-based methods (Nunes *et al.*, 2008) will give a more accurate estimation of the fungal population in soil. All nine colonies randomly isolated from the soil of fungus-treated plots proved to be genetically identical to the original FOG isolate. On the plates prepared from soil of the control plots, generally very few colonies expressing similar features as FOG (maximum 3 per plate, compared to up to 100 from soil of fungus-treated plots) could be detected. One out of the four isolates obtained from the soil of the control plots, showed the same fingerprints as FOG. These results confirm that the dilution plate method is a valuable tool to monitor our biocontrol agent in the soil, with only a very small over-estimation of the population in the control plots. Risk assessment of microorganisms released to the environment is becoming a public concern in terms of allergenicity to humans or animals, competitive displacement, toxicity of metabolites and pathogenicity to non-target organisms (Cook *et al.*, 1996; Boss *et al.*, 2007a). The results of this study indicate that the capacity of dispersal and persistence of FOG in the environment is rather low. Only a very small number of colonies showing similar morphological features as FOG were found in soil of the control plots and on the PCNB plates placed in the field. Just one of the air-borne isolates and one isolate gained from the soil of the control plots were genetically identical to FOG. All five isolates originating from the soil of the neighbor field in 10 m distance (2007) could clearly be distinguished from our biocontrol agent and no *Fusarium* isolates could be obtained from shoots of the field in 10 m distance (2006).

As a result, it can be concluded that FOG has a limited mobility in soil and is not well adopted to air-dispersal, as it is reported for other *Fusarium* species (Burgess, 1981). However, 7 out of 8 isolates from diseased *P. ramosa* shoots from control plots showed the same RAPD fingerprints as FOG. This indicates a spread of the disease over short distances is possible, although it is not yet clear how the plants could be infected, as FOG was generally not detectable in the soil samples of control plots. It seems that the infection was transmitted via rain-splash, animal or human activity. The high proportion of diseased *Phelipanche* shoots that had been detected in the control plots in both years could have led to an underestimation of the real biocontrol efficacy of the fungus as discussed by Zahran *et al.* (2008).

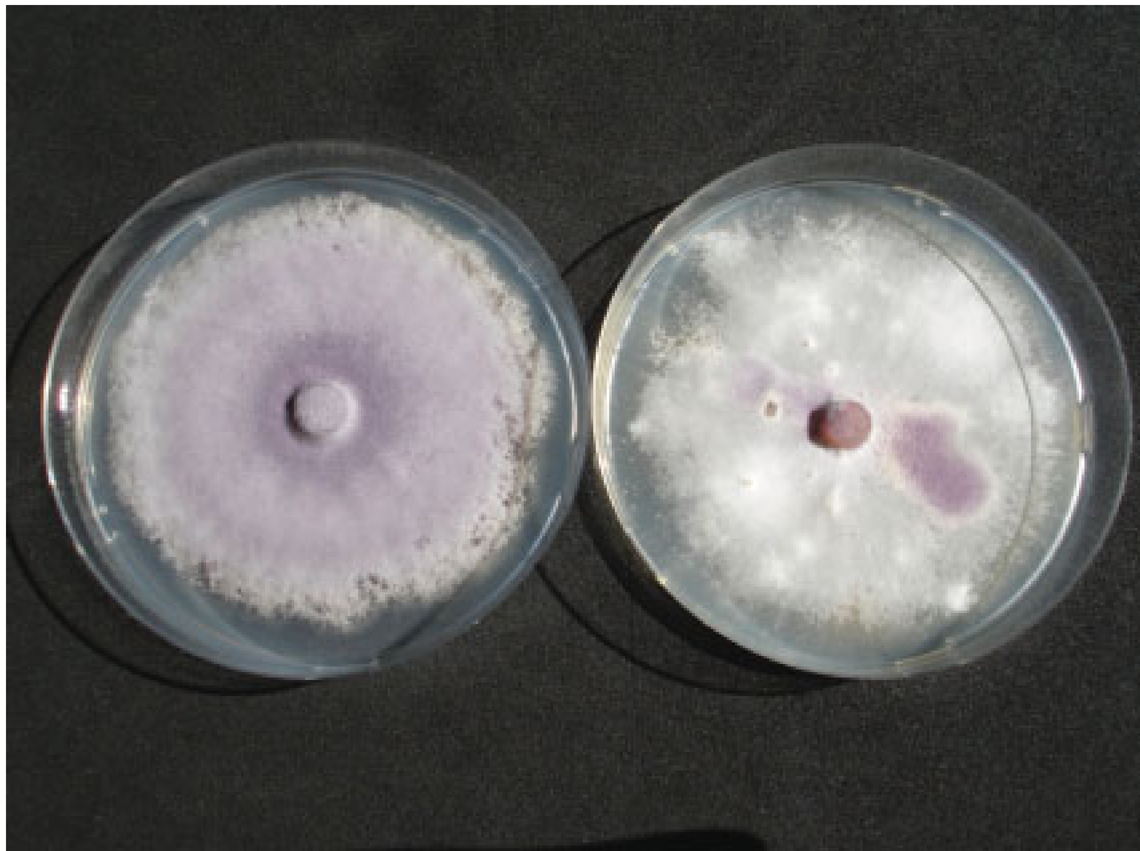

Figure 5.4: Mycelia of *Fusarium oxysporum* f.sp. *orthoceras* (Foo) (left) and *F. oxysporum* (FOG) (right)

6 General discussion

In the last decade, broomrape infestation has increased in German tobacco growing areas (Wegmann, 1999; Buschmann, 2004). The *Phelipanche* soil seed-bank was steadily built up because of monoculture of Virgin tobacco varieties. The common control method applied at the moment is the utilisation of the herbicide glyphosate at a very low dose. However, this is associated with some problems such as certain phytotoxic effects on the host plant (Lolas, 1986). Furthermore, only young attachments can be effectively controlled. Due to continuous *Phelipanche* seed germination throughout the season (Joel *et al.*, 2007), glyphosate has to be applied at least 3-4 times (Billenkamp pers. comm.).

The idea of using fungal pathogens against broomrape infestation has a long history (Bozoukov & Kouzmanova, 1994), and several isolates especially of the genus *Fusarium* have been identified so far (Bedi & Donchev, 1991; Bozoukov & Kouzmanova, 1994; Amsellem *et al.*, 2001b; Boari & Vurro, 2004; Alla *et al.*, 2008). Therefore it was not surprising that a further *F. oxysporum* strain (named FOG) has been found while looking for a highly virulent fungal pathogen for the *Phelipanche ramosa/Nicotiana tabacum* system.

This novel isolate was able to affect all developmental stages of *Phelipanche ramosa* (hypogeal and epigeal), however, a reduction of germination only occurred in the presence of fungal propagules. When sterilising *Phelipanche* seed surfaces after a seven-day preconditioning period with a spore suspension, seed germination did not differ to the untreated control. In contrast, Thomas *et al.* (1999a) and Sauerborn *et al.* (1996) observed a complete destruction of the seed content after inoculation of *O. cumana* and *Striga hermonthica* (Delile) Benth. seeds with *Fusarium* spp. respectively. Therefore, further investigations are needed to find out whether FOG is only able to reduce seed germination by releasing inhibiting compounds, as it was observed by Andolfi *et al.* (2002) and Zonno and Vurro (2005), or whether the hyphae penetrate the seed testa after an incubation period longer than seven days.

In studies conducted in the greenhouse, the highest reduction in total number and biomass (DM) of the parasite's shoots was 95% and 96%, respectively.

This control efficacy could not be consistently achieved in all experiments, but the combination of BION® and the biocontrol agent resulted in a more reliable *Phelipanche* control than single treatments. Similar results were achieved by Müller-Stöver *et al.* (2005) with *O. cumana* and sunflower. As very variable broomrape control levels were achieved under natural conditions by working either with biocontrol agents (Sauerborn *et al.*, 2007) or BION® (Perez-de-Luque *et al.*, 2004), the combination of both control approaches seemed promising. Müller-Stöver *et al.* (2002) assumed that the improved control efficacy could be related to distinct defence mechanisms in sunflower triggered by *Fusarium* and BTH. Yu & Muehlbauer (2001) also mentioned that BTH and *Fusarium* induced different pathways of defence in wheat. However, in field an enhanced effect of combining the two control agents was only noticed at the beginning of the season. This is corroborated by results of Müller-Stöver (unpublished data) investigating the combination of BION® and a different biocontrol agent against *O. cumana* in sunflower under field conditions. It seems that a defence-related response was not or only weakly induced by FOG under field conditions, since the single BTH treatment alone was effective against broomrape. This could be due to the strongly decreasing concentration of the fungus in the soil by up to 80% of the initial population. Fuchs *et al.* (1999) observed a greater protection against *Fusarium* wilt of tomato by induced resistance when applying a higher concentrated conidia suspension of a non-pathogenic *F. oxysporum* strain compared to a lower concentration.

The control efficacy of over 90% under defined environmental conditions could not be reached under field-grown situations. Nevertheless, the sole application of the fungus reduced number and biomass (DM) of *Phelipanche* shoots between 50% and 70% in three consecutive years. The tobacco yield was not increased in the first year, but tobacco biomass was significantly higher in fungus treated plots (with the exception of the sole alginate treatment) in the second season. Cagan & Toth (2003) did neither detect any correlation between the number of *P. ramosa* shoots and the yield of tomato fruits. However, even in cases where no yield increase of the present crop can be measured, a strong positive impact of the biocontrol agent must be seen in the reduction of the future seed population via the reduction of emerging shoots (Lolas,1994).

In the field experiments, *Phelipanche* shoots collected from fungus-treated plots showed a very limited seed production compared to shoots from control plots (Appendix 4). Thomas *et al.* (1998) also reported a lower *O. cumana* seed production from pot trials inoculated with a *F. oxysporum* isolate.

Regarding the differences in biocontrol efficacy between field and greenhouse, several biotic and abiotic reasons may play a role. Additional laboratory and greenhouse experiments were set up to investigate the behaviour of the biocontrol agent under different environmental conditions. The fungus reacted quite sensitively to increase alkalinity in the laboratory experiments and therefore it was assumed that soil pH is an important factor influencing biocontrol efficacy. However, the pH of the soil from the experimental site was between 5.6 - 6.0, which was in the range of the fungus' best performance *in vitro*. Non-optimal climatic conditions may have contributed to a reduction and variation of biocontrol efficacy in the field. It was observed that the fungus thrived better under relatively dry conditions (20% water saturation), as already reported for various *Fusarium* spp. (Stover, 1953a; Oritsejafor, 1986). Cook (1981) suggested for some *Fusarium* species that the higher the temperature is in the range of 15°C to 36°C, the lower should the optimal water potential be for optimal growth. This might partially explain the low control efficacy (20-30%) of the sole pesta treatment in 2008 and 2007 compared to 2006 (50%). In 2006, the average temperature from May to September was 21°C, *i.e.* almost 3°C higher than in the following years. The average soil moisture content was nearly the same in 2006 and 2008 (about 8% (w/w)) but was higher in 2007 (11% (w/w)). It can, therefore, be concluded that the climatically growth conditions for the fungus were best in 2006 (Appendix 1, Appendix 2, Appendix 3).
The soil of the field site was found to have certain fungistatic properties according to the definition of Fravel *et al*. (1996), because the control efficacy of the biocontrol fungus was markedly higher after substrate sterilisation in greenhouse experiments. Furthermore, the soil at the experimental site has very low organic matter content (1-2%), but it was observed in greenhouse trials that the fungus constantly provided a better control efficacy against *Phelipanche ramosa* in a substrate with a higher organic matter content (10%).

Although in many studies (Tilston *et al.*, 2002; Termorshuizen *et al.*, 2006; Perez-Piqueres *et al.*, 2006), compost had a significant negative influence on soil borne pathogens, Bonanomi *et al.* (2007) suggested that an occasional positive affect could be associated with their saprophytic growth promoted by organic matter.

Among the application techniques tested, broadcasting application (50 cm width) of the inoculum gave the best results for *Phelipanche* control at the beginning of the season, but the effect did not last until the end of the experiment. The better efficacy of this treatment could either be due to the better distribution of the fungal propagules in the soil or to the absolutely higher amount of inoculum being applied, which probably led to a high number of cfu in soil at the beginning of the season. Bailey *et al.* (1998) and Gracia-Garza and Fravel (1998) had observed a fast outgrowth of biocontrol agents out of pesta granules, whereas from alginate pellets the release of cfu was retarded. It was therefore hypothesised, that a constantly high supply of fungal propagules achieved by combining a fast-release formulation (pesta) with a slow-release formulation (alginate) will lead to an endurable *Phelipanche* control throughout the whole season. The integration of pesta and alginate indeed constantly performed better than the sole formulations, whereas there was no marked difference in the efficacy of the single applications. However, in the sole pesta treatment the control effect set in later compared to the alginate treatment, which seems to exert an early control effect that at a certain point does not result in a further reduction of emerging *Phelipanche* shoots. No consistent relation could be drawn between biocontrol efficacy of the different formulations and the level of cfu in soil obtained with the dilution-plate method, as already reported by Lewis and Papavizas (1985) and Fravel *et al.* (1995). However, since the underground infection mechanism of FOG is not known and since estimating soil populations of sporulating organisms by dilution plate techniques does not distinguish between active hyphal growth and spore production (Thornton, 2008), the obtained cfu from the soil samples may not correctly reflect the infectious potential occurring in the different treatments.

Risk assessment of field releases of microorganisms is becoming a public concern in terms of allergenicity to humans or animals, competitive displacement, toxicity of metabolites and pathogenicity to non-target organisms (Cook *et al.*, 1996; Boss *et al.*, 2007a). Therefore, every biocontrol agent has to be tested regarding its host specificity, persistence in the environment and its toxin production potential, the latter of which has not been completed for FOG yet.

From the data of the host range experiment it can be concluded that the fungal isolate is very host specific, as it has also been reported for various other *Fusarium* spp. pathogenic against *Phelipanche ramosa* (Amsellem *et al.*, 2001a; Boari & Vurro, 2004; Nanni *et al.*, 2005). Disease symptoms occurred neither on the closely related *Orobanche* species *O. cumana* and *O. crenata* nor on the host plant tobacco, even after field release. Concerns have been expressed that the host specificity of the *F. oxysporum* strains might be overcome via mutation, parasexuality or horizontal gene exchange with pathogenic strains (Amsellem *et al.*, 2001b). Amsellem *et al.* (2001b) support the hypothesis of a divergent origin for broomrape-pathogenic strains than for other crop-pathogenic *F. oxysporum formae speciales*. After the cluster analysis in the studies with 99 RAPD markers, the two broomrape-pathogenic *F. oxysporum* isolates (FOG and Foo) were clustered in the same branch, which also indicates a close relatedness of these strains. Elzein *et al.* (2008) suggest two *F. oxysporum* isolates (Foxy 2, PSM 19) pathogenic to *Striga* to be a new *forma specialis* due to a unique ITS sequence and their high host specificity. Investigations on genealogies in the *F. oxysporum* species complex indicated that even some of the wilt- and rot-causing pathogenic forms are not of monophyletic origin (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000). Therefore Baayen *et al.* (2000) argued that commercialised non-pathogenic *F. oxysporum* biocontrol agents, replacing pathogenic strains in carnations, are most unlikely to convert into pathogenic strains.

It was concluded from the field studies that the capacity of dispersal and persistence of FOG in the environment was rather low. Every year the population of the applied fungus declined in the soil (by up to 90% of the initial population) in a three-month period. Only one isolate obtained from the control plots was genetically identical to FOG.

No identical isolates could be found in fields in a distance of 10 m and just one isolate trapped from the air had the same RAPD-fingerprint as FOG. It was therefore assumed that FOG has a limited mobility in soil and is not well adopted to air-dispersal, as it is generally reported for *Fusarium* species (Burgess, 1981). However, isolates from diseased *Phelipanche ramosa* shoots from control plots showed the same RAPD fingerprints as FOG. This indicates a spread of the disease over short distances, although it is not clear how the plants finally got infected, as FOG was generally not detectable in the soil samples of the control plots. Infection may have occurred by rain-splash or via animal or human activity. These aspects greatly lower the risk of dispersal to non-target cropping systems.

In conclusion, FOG has shown its biocontrol efficacy under field-grown conditions. Especially the biocontrol agent's potential to reduce the seed production even in symptomless plants should be further tested. Since it became obvious, that abiotic and biotic conditions as well as its formulation and application have a great impact on the performance of the fungus, future research is needed to better understand the interactions in that complex system, and in order to be able to optimise and stabilise biocontrol efficacy.

Summary

The obligate root parasite *Phelipanche ramosa* L. (branched broomrape) occurs in many parts of the world. Branched broomrape has a broad host range and causes serious damage to economically important crops. It is becoming an increasing problem in oilseed rape (*Brassica napus* L.) in France, while in Germany it mainly infests tobacco (*Nicotiana tabacum* L.) and hemp (*Cannabis sativa* L.). In the last decade broomrape infestation has extremely increased in German tobacco growing areas. Application of conventional control methods against *Orobanche* L. and *Phelipanche* Pomel species is limited due to their complex biology, *i.e.* the plant reproduces by mean of tiny and long-living seeds, its very close affiliation with the host plant, and the fact that the plants can be hardly detected before they have irreversibly damaged the crop. Management strategies should focus on reducing the soil seed-bank and interfere with the parasite's early development stages. In this context, the idea of using fungal pathogens - especially of the genus *Fusarium* – as biocontrol agents against broomrapes has a long history. The application of a soil borne phytopathogenic fungus has several advantages: it can be very host-specific and able to destroy also the underground stages of parasitic plants, including the seeds, which can contribute to decrease the soil seed-bank every year.

The objectives of the present study were to investigate the impact of a novel *Fusarium oxysporum* (Schlecht.) Snyd. & Hans. isolate (FOG) for controlling *P. ramosa* in tobacco fields, with regard to its efficacy and host specificity under controlled environmental conditions. Subsequently, biocontrol efficacy was assessed under field conditions in Germany, using different formulation and application techniques. RAPD (Random Amplification of Polymorphic DNA) marker-based assay was conducted in order to identify the biocontrol agent from other *F. oxysporum* strains, to verify the utilised monitoring methods based on morphological criteria and to obtain information about the potential spread of the biocontrol agent into the environment.

The fungus affected all developmental stages of the parasite. Already *Phelipanche* seed germination was significantly reduced by 40% in the presence of fungal conidia *in vitro*.

The number of underground developmental stages of the weed was reduced by 55% in root chambers compared to the non-treated control. In pot experiments, soil application of a granular formulation of the fungus resulted in a reduction of number and biomass (DM) of *Phelipanche* shoots by more than 90%. Spraying of a conidial suspension on aboveground *Phelipanche* shoots caused the death of 75% of them within two weeks. In greenhouse experiments, the *Fusarium* treatment combined with the resistance-inducer BTH resulted in the lowest rate, in number and biomass (DM), of emerged *Phelipanche* shoots. Data from initial host-range experiments indicate that the isolate is very host-specific, not even attacking shoots of other *Phelipanche* species.
Under field conditions, FOG reduced number and biomass (DM) of *Phelipanche* shoots between 50% and 70% in three consecutive years (2006–2008) and decreased seed production from emerged shoots in fungus-treated plots. However, in contrast to the results of the greenhouse experiments, no further reduction could be observed when the biocontrol agent was combined with the resistance inducer BTH in the field. Accompanying greenhouse studies revealed some fungistatic effects of the field soil which partly explain the reduced efficacy (-40%) in the field compared to results obtained under controlled conditions. Also climatic conditions seemed to have a great influence in this regard. Laboratory experiments showed a better fungal survival rate under comparatively dry soil conditions (20% of water saturation) and a temperature optimum between 25-28°C. This could be an explanation for the higher control efficacy of the pesta treatment in the first-year field experiment, when climatic conditions correlated best with optimal growth conditions for FOG.
In-furrow application and broadcasting of the inoculum after tobacco planting as well as pre-planting subsoil application decreased the number of emerged *Phelipanche* shoots, but did not differ among each other at the end of the experiment. Broadcasting was more effective at the beginning of the field trial. The application of pesta granules did not show consistent results throughout the seasons, meaning that the 50% reduction of *Phelipanche* biomass (DM) in the first year could not be repeated in the following years (20-30%). An alginate formulation introduced in the second year performed better than the pesta formulation.

However, the combination of pesta granules with alginate pellets had the highest reliable control efficacy (60-70%) of all treatments in two seasons compared to the untreated control. FOG populations declined strongly (70-90%) over a three months period and fungal population counts in soil samples did not show a close correlation to the actual biocontrol efficacy. Tobacco yield was not increased in the first year, but tobacco biomass (DM) was significantly higher in most of the fungal treatments in 2007.

FOG revealed fragment patterns clearly differentiable from another *Fusarium oxysporum* strain (pathogenic to *Orobanche cumana*) when tested with 20 RAPD primers. The RAPD technique was further applied to compare the original FOG isolate with 45 *Fusarium* isolates collected from necrotic *Phelipanche* shoots and soil samples obtained from fungus-treated and untreated plots in the field experiments, as well as from a field in 10 m distance. Thirty isolates showed the same fingerprints as the original FOG isolate. All isolates gained from soil of fungus-treated plots were genetically identical to FOG, whereas in soil samples of the control plots, FOG could be only detected to a very limited extent. All isolates collected from plants or soil of the neighbour field were clearly differentiated from the biocontrol agent. Results verified the population counts obtained with the dilution plate method and indicate that the biocontrol agent has only a limited mobility and persistence in soil. However, since seven out of eight *Fusarium* isolates from *Phelipanche* shoots from control plots were 100% identical with FOG, a short-distance aboveground spread seems to occur, possibly by rain-splash or human/animal activity.

As a conclusion, it can be stated that FOG has a potential for controlling *P. ramosa*. Moreover, the fungus has the ability to reduce the parasite’s seed production. Since it became obvious that abiotic and biotic conditions as well as its formulation and application have a great impact on the performance of the fungus, future research should try to better understand the interactions in that complex system, in order to be able to optimise and stabilise biocontrol efficacy.

Zusammenfassung

Das parasitische Unkraut *Phelipanche ramosa* L. (verzweigte Sommerwurz) verursacht in vielen Kulturpflanzen in Europa, Nordafrika und im Mittleren Osten große Ertrags- und Qualitätsverluste. Unter den veränderten klimatischen Bedingungen in Westeuropa befällt *P. ramosa* zunehmend Tomaten (*Solanum lycopersicum* L.), Tabak (*Nicotiana tabacum L.*), Hanf (*Cannabis sativa L.*) und Raps (*Brassica napus* L.). Im letzten Jahrzehnt ist der Befallsdruck von *P. ramosa* im Tabakanbau in Deutschland stark angestiegen. Trotz intensiver Forschung wurde bisher keine zufriedenstellende Kontrollmethode für das parasitische Unkraut gefunden. Die außergewöhnliche Biologie der Parasiten und die physiologisch enge Assoziation mit der Wirtspflanze sowie die lange Überdauerung der Samen im Boden reduziert die Effizienz der meisten Kontrollstrategien auf ein Minimum. Bekämpfungsmethoden sollten sich auf die frühen Stadien des Parasiten und die Reduzierung der Samenbank im Boden konzentrieren.

Für viele *Orobanche*- und *Phelipanche*-Arten wurden bereits pilzliche Antagonisten, zumeist aus der Gattung *Fusarium*, gefunden, deren Entwicklung zum Mykoherbizid viele Vorteile mit sich bringen würde: die Gegenspieler sind in der Regel wirtsspezifisch und befallen alle unterirdischen Stadien der parasitischen Pflanzen inklusive der Samen, was zu einer stetigen Verringerung der Samenbank führen könnte.

Das Ziel der Untersuchungen war es, einen Beitrag zur Kontrolle des parasitischen Unkrauts *P. ramosa* zu leisten. Dafür wurde der kürzlich isolierte biologische Gegenspieler *Fusarium oxysporum* (Schlecht.) Snyd. & Hans. (Isolat: FOG) unter kontrollierten Bedingungen in Bezug auf seine Wirksamkeit und Wirtsspezifität, getestet. Überprüft wurden als dann die Kontrolleffizienz unter Freilandbedingungen in Deutschland mittels verschiedener Applikationsverfahren sowie Formulierungen. Ein weiteres Ziel war es, mit Hilfe von RAPD (Random Amplification of Polymorphic DNA) Markern eine Methode anzuwenden, die eine eindeutige Unterscheidung des pilzlichen Antagonisten von anderen *Fusarium* spp. ermöglicht.

Dadurch sollten die im Feld verwendeten Monitoring-Methoden, die auf morphologischen Unterscheidungskriterien beruhen, verifiziert, sowie die potentiellen Verbreitungsmöglichkeiten von FOG evaluiert werden.

Alle Entwicklungsstadien von *P. ramosa* zeigten sich anfällig gegenüber FOG. Bereits die Keimung der *Phelipanche*-Samen *in vitro* war in Anwesenheit der pilzlichen Konidien um 40% verringert. Die Anzahl unterirdischer Tuberkel des Unkrauts war in Wurzelgefäßen im Vergleich zur unbehandelten Kontrolle um mehr als 55% reduziert. In Topfversuchen verringerte die Bodenapplikation einer Festformulierung (Pesta) des Pilzes die Anzahl der aufgelaufenen Sprosse und die Biomasse des parasitischen Unkrauts um über 90%. Eine Nachauflauf-Sprühapplikation der Konidien führte zum Absterben von mehr als 75% der Sprosse innerhalb von zwei Wochen. Die Kombination des Pilzes mit BTH in Gewächshausversuchen resultierte in den geringsten Auflaufraten der *Phelipanche* Sprosse verglichen mit den Alleinbehandlungen. Da andere *Orobanche*-Arten sowie alle getesteten Kulturpflanzen nach der Inokulation mit FOG symptomfrei blieben, lässt sich eine hohe Wirtsspezifität des Antagonisten vermuten.

Unter Freilandbedingungen verringerte der pilzliche Gegenspieler die Anzahl und die Biomasse der aufgelaufenen *Phelipanche*-Sprosse um 50% bis 70% im Vergleich zur unbehandelten Kontrolle in drei aufeinanderfolgenden Jahren (2006 – 2008). Im Gegensatz zu den Ergebnissen aus Gewächshausversuchen erbrachte die Kombination von FOG mit dem Resistenzinduktor BTH jedoch keine zusätzliche Steigerung des Kontrolleffektes.

Begleitende Gewächshausstudien offenbarten gewisse fungistatische Einflüsse des Bodens der Feldversuchsstation, was teilweise die verringerte Wirksamkeit (-40%) des biologischen Gegenspielers im Feld verglichen mit Gewächshausversuchen erklärt. Auch klimatische Bedingungen scheinen in dieser Beziehung eine Rolle zu spielen. Laborexperimente zeigten ein verbessertes Pilzwachstum unter relativ trockenen Bodenbedingungen (bei 20% Wassersättigung) und bei Temperaturen zwischen 25 und 28°C. Dies könnte eine Erklärung für die höhere Wirksamkeit der Pesta-Formulierung im Feldversuch von 2006 sein, da in diesem Jahr die klimatischen Bedingungen am ehesten den optimalen Wachstumsbedingungen des Pilzes entsprachen.

Die drei Applikationstechniken des zu “Pesta“ Granulaten verarbeiteten Inokulums - schmales Band, breites Band und Pflanzloch - verringerten den Auflauf von *P. ramosa*, zeigten aber keine Behandlungsunterschiede am Versuchsende. Die Breitbandapplikation war am Anfang tendenziell wirksamer als die anderen Ausbringungstechniken.

Die Wirksamkeit der “Pesta“-Formulierung gegen *P. ramosa* blieb nicht konstant (2006: 50%, 2007: 30%; 2008. 20%). Die im zweiten Versuchsjahr eingeführte Alginat-Formulierung schnitt einzeln appliziert zwar besser ab, die höchste und zuverlässigste Kontrolleffizienz (60-70%) aller Behandlungen ließ sich allerdings durch die Kombination von “Pesta“-Granulaten mit Alginat-Pellets erzielen. Die Entwicklung der FOG-Population im Boden in den verschiedenen Behandlungen korrelierte kaum mit der Wirksamkeit des Pilzes. In allen Behandlungen wurde bereits während der Vegetationsperiode ein starker Rückgang der pilzlichen Population um 70 bis 90% in allen drei Jahren beobachtet.

Im ersten Jahr wurde kein erhöhter Tabakertrag erzielt, aber die Tabakbiomasse (TM) war in fast allen Behandlungen in 2007 im Vergleich zur Kontrolle erhöht. Ein weiterer positiver Einfluss des biologischen Gegenspielers ist die Reduzierung der Samenbank durch weniger *Phelipanche*-Auflauf. Zudem wurde eine deutliche Reduktion der Samenproduktion von *Phelipanche*-Sprossen in pilzbehandelten Zellen beobachtet.

In molekularbiolgischen Untersuchungen konnte FOG deutlich von einem weiteren *F. oxysporum*-Isolat (pathogen gegen *O. cumana*) abgegrenzt werden. 20 RAPD Primer wurden daraufhin auf 45 *Fusarium*-Isolate angewendet, die von nekrotischen *Phelipanche*-Sprossen und aus dem Boden von pilz-behandelten und unbehandelten Kontrollparzellen sowie von benachbarten Tabakfeldern (10 m Entfernung) isoliert worden waren. Dreißig der 45 waren genetisch identisch mit FOG. Alle Banden der Isolate aus dem Boden pilzbehandelter Parzellen stimmten zu 100% mit denen von FOG überein, während der Antagonist in Bodenproben aus den Kontrollbehandlungen nur in einem sehr begrenzten Umfang entdeckt wurde. Alle Isolate aus *Phelipanche*-Sprossen oder dem Boden des Nachbarfelds unterschieden sich deutlich vom Original-Isolat.

Die Ergebnisse bestätigen die mit Hilfe der Agarplattenmethode gewonnenen Daten zur Entwicklung der Pilzpopulation im Boden und legen zudem nahe, dass FOG im Boden nur begrenzt beweglich ist. Da aber sieben von acht *Fusarium* spp. von *Phelipanche*-Sprosse aus den Kontrollparzellen mit dem Original-Isolat identisch waren, scheint eine oberirdische Verbreitung über kürzere Entfernungen möglich, die wahrscheinlich durch Regenspritzer oder Tätigkeiten von Menschen und Tieren erfolgt war.

Die Ergebnisse veranschaulichen das große Potential des Antagonisten für die Kontrolle von *P. ramosa.* Besonders seine Fähigkeit zur Reduktion der Samenproduktion der Parasiten sollte weiter geprüft werden. Da es offensichtlich geworden ist, dass abiotische und biotische Bedingungen, sowie Formulierung und Applikationstechniken einen großen Einfluss auf die Effizienz des biologischen Gegenspielers haben, sollten zukünftige Forschungen das Verständnis der Wechselwirkungen in diesem komplizierten System weiter vertiefen und dadurch seine Anwendbarkeit unter Feldbedingungen verbessern.

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Appendices

Appendix 1: Climatic data from 2006-2008

2006)

Date	Soil temperature minus 10 cm [°C]			Solar radiation Dgt [W/m²]	Relative humidity [%]	Air temperature 1 m [°C]			Precipitation [mm]
	average	minimum	maximum	average	average	average	minimum	maximum	sum
MAY	18.0	10.4	21.3	170.2	64.5	15.8	2.4	27.8	42.2
JUNE	22.1	11.3	27.8	308.2	74.2	19.5	2.7	33.6	77.6
JULY	25.6			313.6	63.1	25.0			4.0
AUGUST	19.6			245.3	78.8	17.4			136.8
SEPTEMBER	20.1	14.7	26.8	222.4	82.0	18.3	8.4	28.8	28.6
	Φ 21.1					Φ 19.2			Σ 289.2

2007)

Date	Soil temperature minus 10 cm [°C]			Solar radiation Dgt [W/m²]	Relative humidity [%]	Air temperature 1 m [°C]			Precipitation [mm]
	average	minimum	maximum	average	average	average	minimum	maximum	sum
MAY	18.9	17.3	22.1	208.5	69.9	16.2	4.6	27.8	96.0
JUNE	19.1	12.6	30.0	284.8	78.1	17.0	7.6	30.6	95.8
JULY	18.2	11.9	30.0	277.7	76.8	16.9	5.9	33.4	83.6
AUGUST	18.1	12.6	22.7	281.5	74.1	17.6	5.0	29.5	64.0
SEPTEMBER	16.5			144.9	81.4	13.5	3.9	25.2	81.4
	Φ 18.2					Φ 16.2			Σ 420.8

2008)

Date	Soil temperature minus 10 cm [°C]			Solar radiation Dgt [W/m²]	Relative humidity [%]	Air temperature 1 m [°C]			Precipitation [mm]
	average	minimum	maximum	average	average	average	minimum	maximum	sum
MAY	17.6	12.1	25.4	265	74	17.2	8.3	27.8	34.2
JUNE	18.5	13.2	25.4	317	78	16.6	3	30.6	79.8
JULY	19.7	13.9	28.9	305	76	17.7	6.1	33.4	55.6
AUGUST	19.5	13.3	23.6	242	83	18.3	0	29.5	75.4
SEPTEMBER	13.0	6.5	20.3	109	86	11.3	-0.7	27.4	34.4
	Φ 17.7					Φ16.2			Σ 279.4

Appendix 2: Soil water content in 2006-2008

2006

Date	Water content %(w/w)		
	average	minimum	maximum
6 w.a.t..	8.0	6.2	12.3
9 w.a.t.	5.3	3.6	7.7
12 w.a.t.	4.7	3.0	6.0
15 w.a.t.	10.1	8.8	12.9
19 w.a.t.	11.7	10.8	13.0

2007

Date	Water content %(w/w)		
	average	minimum	maximum
3 w.a.t.	14.8	12.9	17.7
4 w.a.t.	10.3	8.7	10.8
6 w.a.t.	11.0	8.2	13.1
7 w.a.t.	14.1	15.5	12.0
8 w.a.t.	12.1	9.5	14.0
9 w.a.t.	8.4	6.7	9.4
11 w.a.t.	7.6	5.9	9.4
12 w.a.t.	13.8	10.6	16.3
14 w.a.t.	8.4	7.6	10.1
15 w.a.t.	6.2	5.1	8.1

2008

Date	Water content %(w/w)		
	average	minimum	maximum
2 w.a.t.	11.2	10.4	11.8
5 w.a.t.	9.4	8.1	11.6
10 w.a.t.	6.2	5.5	7.7
12 w.a.t.	6.3	5.5	7.5
14 w.a.t.	10.4	7.3	14.3
16 w.a.t.	6.7	4.5	10.0
18 w.a.t.	9.5	8.3	11.8

w.a.t. = weeks after tobacco planting.

Appendix 3: Radial growth of FOG on PDA at different temperature regimes

	25°C		10°C		day 35°C/night 25°C	
D.a.i.	1. trial diameter (cm)	2.trial diameter (cm)	1. trial diameter (cm)	2.trial diameter (cm)	1. trial diameter (cm)	2.trial diameter (cm)
1	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0
3	2.4	1.4	0.0	0.0	1.5	0.0
4	3.0	2.4	0.0	0.0	1.6	1.2
5	4.0	3.2	0.0	0.0	1.7	1.7
6	4.6	4.1	1.0	1.2	1.8	2.1
7	5.7	5.1	1.0	1.2	2.2	2.5
8	6.3	6.3	1.3	1.5	2.5	2.7
9	7.6	6.9	1.5	1.9	2.8	3.1
10	8.5	7.5	2.0	2.1	3.3	3.3
11	P.d. full	7.7	2.1	2.3	3.4	3.6
12		8.0	2.3	2.6	3.6	3.9
13		8.3	2.4	2.9	3.9	4.1
14		P.d. full	2.6	3.0	4.2	4.5
15			2.8	3.3	4.6	4.8
16			3.0	3.4	5.0	5.0
17			3.2	3.6	5.2	5.2

	25°C		20°C		15°C	
D.a.i.	1. trial diameter (cm)	2.trial diameter (cm)	1. trial diameter (cm)	2.trial diameter (cm)	1. trial diameter (cm)	2.trial diameter (cm)
1	0.0	0.0	0.0	0.0	0.0	0
2	0.0	0.0	0.0	0.0	0.0	0
3	1.7	2.0	0.0	1.5	0.0	0
4	2.6	2.9	0.9	2.5	0.0	1.2
5	3.3	4.0	1.8	3.4	0.0	2.3
6	4.4	4.9	2.8	4.0	1.5	2.7
7	5.2	5.5	3.5	4.6	2.2	3.1
8	6.2	6.6	4.3	5.1	2.5	3.5
9	7.2	7.6	5.0	5.7	3.1	4.0
10	7.6	8.0	5.7	6.2	3.6	4.1
11	8.2	8.4	6.0	6.8	3.8	4.7
12	P.d. full	P.d. full	6.5	7.7	4.3	5.2
13			7.0	8.1	4.5	5.7
14			7.6	8.2	5.0	5.9
15			8.1	8.4	5.4	6.2
16			8.6	P.d. full	5.8	6.6

D.a.i. = days after inoculation, P.d. = Petri dish completely covered with mycelia, diameter = diameter of the fungal colony on PDA in a 9 cm Petri dish.

Appendix 4: Seed produced by *Phelipanche* shoots from fungus-treated plots and control plots

Treatment	Shoots	Seeds produced (g^{-1} *Phelipanche* shoots)	SE
Control	healthy	9.6	5.53
Control	diseased	3.1	2.06
FOG	healthy	0.5	0.46
FOG	diseased	0.6	0.39

Means (n=3) ± standard error
Phelipanche shoots = dry weight.

Appendix 5: Matrix of RAPD procedure with 20 primer

		A10								A15					
		400	500	600	700	800	850	900	1100	300	400	500	600	800	1100
Foo	1	1	1	0	1	1	1	1	1	0	1	0	0	0	1
FOG	2	0	1	1	1	1	1	1	1	1	1	0	0	0	1
3013	3	0	1	1	1	1	1	1	1	1	1	0	0	0	1
3015	4	0	1	1	1	1	1	1	1	1	1	0	0	0	1
3061-1	5	0	1	1	1	1	1	1	1	1	1	0	0	0	1
3041-1	6	0	1	0	1	1	1	1	1	0	1	1	1	0	1
3029-1	7	0	1	1	1	1	1	1	1	1	1	0	0	0	1
3019-2	8	0	1	1	1	1	1	1	1	1	1	0	0	0	1
949a	9	0	1	1	1	1	1	1	1	1	1	0	0	0	1
14.08.-10	10	0	1	1	1	1	1	1	1	1	1	0	0	0	1
923-1b	11	0	1	1	1	1	1	1	1	1	1	0	0	0	1
952-2b	12	0	1	1	1	1	1	1	1	1	1	0	0	0	1
948a	13	9	9	9	9	9	9	9	9	0	0	1	1	0	1
FF	14	0	1	1	1	1	1	1	1	1	1	0	0	0	1
944c	15	0	1	1	1	1	1	1	1	1	1	0	0	0	1
942c	16	0	1	1	1	1	1	1	1	1	1	0	0	0	1
FGF	17	0	1	1	1	1	1	1	1	1	1	0	0	0	1
945b	18	0	1	1	1	1	1	1	1	1	1	0	0	0	1
3073-2	19	0	1	1	1	1	1	1	1	1	1	0	0	0	1
924-2b	20	0	1	1	1	1	1	1	1	1	1	0	0	0	1
941-1b	21	0	1	1	1	1	1	1	1	1	1	0	0	0	1
954-1b	22	0	1	1	1	1	1	1	1	1	1	0	0	0	1
24.07.-7	23	0	0	0	1	1	1	1	0	0	1	0	0	1	0
952-14	24	0	0	1	1	1	1	1	0	0	1	1	0	0	1
948-1b	25	0	1	1	1	1	1	1	1	1	1	0	0	0	1
949-1b	26	0	0	1	1	1	1	1	1	0	1	1	0	0	1
17.07.	27	1	0	1	1	1	1	1	1	0	1	0	1	0	1
930-2a	28	1	1	0	1	1	1	1	1	0	1	0	1	0	1
3004-3	29	0	1	1	1	1	1	1	1	1	1	0	0	0	1
N22	30	0	1	0	1	0	1	1	1	0	1	0	0	1	0
N42	31	0	1	0	0	0	1	1	0	0	1	0	0	0	1
N12	32	0	1	0	1	1	1	1	0	0	1	0	0	0	1
28.08.-1	33	0	0	1	0	1	0	0	0	0	0	0	1	0	0
922c	34	0	1	1	1	1	1	1	1	1	1	0	0	0	1
N31	35	0	1	0	1	0	1	1	1	0	0	0	1	1	1
31.07.-5	36	0	1	0	1	1	1	1	1	0	1	0	1	0	1
28.08.-4	37	0	0	0	0	1	1	0	0	0	0	0	1	0	0
N32	38	0	1	0	1	1	1	1	1	0	1	0	1	0	1
3001	39	0	1	1	1	1	1	1	1	1	1	0	0	0	1
3024-5	40	0	1	1	1	1	1	1	1	1	1	0	0	0	1
941d	41	0	1	1	1	1	1	1	1	1	1	0	0	0	1
923b	42	0	1	1	1	1	1	1	1	1	1	0	0	0	1
946d	43	0	1	1	1	1	1	1	1	1	1	0	0	0	1
922a	44	0	1	1	1	1	1	1	1	1	1	0	0	0	1
927-1b	45	0	1	1	1	1	1	1	1	1	1	0	0	0	1
942-2b	46	0	1	1	1	1	1	1	1	1	1	0	0	0	1
24.07.-5-1	47	1	1	0	1	0	1	1	1	0	1	0	0	1	1

		A17					A18				A19					
		450	650	700	850	950	500	550	850	900	400	550	650	750	1000	1150
Foo	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	1
FOG	2	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
3013	3	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
3015	4	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
3061-1	5	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
3041-1	6	0	1	1	1	1	0	1	1	1	0	1	1	0	0	1
3029-1	7	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
3019-2	8	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
949a	9	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
14.08.-10	10	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
923-1b	11	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
952-2b	12	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
948a	13	0	1	1	0	1	0	1	0	1	1	1	1	1	0	1
FF	14	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
944c	15	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
942c	16	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
FGF	17	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
945b	18	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
3073-2	19	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
924-2b	20	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
941-1b	21	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
954-1b	22	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
24.07.-7	23	9	9	9	9	9	0	0	0	0	0	0	0	0	1	0
952-14	24	1	1	1	0	1	0	1	0	1	0	1	1	0	0	1
948-1b	25	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
949-1b	26	1	1	1	0	1	0	1	1	0	0	1	1	0	0	1
17.07.	27	1	1	1	0	1	0	1	0	0	0	1	1	0	0	1
930-2a	28	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1
3004-3	29	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
N22	30	1	0	1	0	0	1	1	1	0	0	0	0	0	1	0
N42	31	0	1	0	1	1	0	1	0	1	0	1	0	1	0	1
N12	32	1	1	1	0	1	0	1	0	0	0	1	1	0	0	1
28.08.-1	33	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0
922c	34	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
N31	35	1	1	0	1	1	0	1	0	0	0	1	1	0	0	1
31.07.-5	36	1	1	1	0	1	0	1	0	0	0	1	1	0	0	1
28.08.-4	37	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0
N32	38	1	1	1	0	1	0	1	0	0	0	1	1	0	0	1
3001	39	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
3024-5	40	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
941d	41	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
923b	42	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
946d	43	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
922a	44	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
927-1b	45	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
942-2b	46	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
24.07.-5-1	47	1	1	0	1	1	0	1	0	0	0	1	1	0	0	0

		L16						L20						M10			
		400	500	650	750	850	900	480	520	700	800	900	1100	550	650	700	800
Foo	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0	1
FOG	2	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
3013	3	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
3015	4	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
3061-1	5	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
3041-1	6	1	1	1	0	0	1	1	0	1	0	0	1	1	1	0	0
3029-1	7	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
3019-2	8	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
949a	9	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
14.08.-10	10	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
923-1b	11	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
952-2b	12	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
948a	13	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0	1
FF	14	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
944c	15	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
942c	16	9	9	9	9	9	9	1	1	0	1	0	1	1	1	0	0
FGF	17	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
945b	18	9	9	9	9	9	9	1	1	0	1	0	1	1	1	0	0
3073-2	19	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
924-2b	20	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
941-1b	21	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
954-1b	22	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
24.07.-7	23	0	1	0	1	1	0	0	0	0	0	0	1	1	0	0	1
952-14	24	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0
948-1b	25	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
949-1b	26	1	1	0	1	1	1	0	1	0	0	1	1	1	1	1	0
17.07.	27	1	0	1	1	0	0	0	1	0	1	0	1	1	1	1	0
930-2a	28	1	0	1	1	0	0	0	1	0	1	0	1	1	1	1	0
3004-3	29	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
N22	30	0	1	1	1	0	0	0	0	0	1	0	1	1	1	0	1
N42	31	1	1	0	1	0	0	0	1	0	0	1	1	1	0	1	0
N12	32	1	0	1	1	1	0	0	1	0	0	0	1	1	1	1	1
28.08.-1	33	0	1	0	1	1	1	0	0	0	1	0	1	1	0	1	1
922c	34	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
N31	35	1	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0
31.07.-5	36	0	1	1	0	0	0	1	0	0	1	0	1	1	1	1	1
28.08.-4	37	1	0	1	1	0	1	0	0	0	0	1	0	1	0	1	1
N32	38	1	1	1	0	1	0	1	0	0	1	0	1	1	1	1	1
3001	39	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
3024-5	40	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
941d	41	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
923b	42	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
946d	43	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
922a	44	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
927-1b	45	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
942-2b	46	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0
24.07.-5-1	47	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0

		M14				M15					M17		
		600	650	750	1000	500	550	600	700	900	500	650	850
Foo	1	1	1	0	1	0	1	0	0	0	0	1	1
FOG	2	1	1	1	1	1	1	0	1	0	1	1	0
3013	3	1	1	1	1	1	1	0	1	0	1	1	0
3015	4	1	1	1	1	1	1	0	1	0	1	1	0
3061-1	5	1	1	1	1	1	1	0	1	0	1	1	0
3041-1	6	1	1	1	0	1	1	1	1	0	1	0	0
3029-1	7	1	1	1	1	1	1	0	1	0	1	1	0
3019-2	8	1	1	1	1	1	1	0	1	0	1	1	0
949a	9	1	1	1	1	1	1	0	1	0	1	1	0
14.08.-10	10	1	1	1	1	1	1	0	1	0	1	1	0
923-1b	11	1	1	1	1	1	1	0	1	0	1	1	0
952-2b	12	1	1	1	1	1	1	0	1	0	1	1	0
948a	13	1	0	1	0	0	1	0	1	0	0	1	0
FF	14	1	1	1	1	1	1	0	1	0	1	1	0
944c	15	1	1	1	1	1	1	0	1	0	1	1	0
942c	16	1	1	1	1	1	1	0	1	0	1	1	0
FGF	17	1	1	1	1	1	1	0	1	0	1	1	0
945b	18	1	1	1	1	1	1	0	1	0	1	1	0
3073-2	19	1	1	1	1	1	1	0	1	0	1	1	0
924-2b	20	1	1	1	1	1	1	0	1	0	1	1	0
941-1b	21	1	1	1	1	1	1	0	1	0	1	1	0
954-1b	22	1	1	1	1	1	1	0	1	0	1	1	0
24.07.-7	23	1	1	0	1	0	1	0	0	0	0	0	0
952-14	24	0	1	0	0	0	1	0	1	0	1	0	0
948-1b	25	1	1	1	1	1	1	0	1	0	1	1	0
949-1b	26	1	1	1	0	1	1	0	1	1	1	1	0
17.07.	27	1	1	1	0	0	1	0	1	0	1	1	1
930-2a	28	1	1	1	0	0	1	0	1	0	1	1	1
3004-3	29	1	1	1	1	1	1	0	1	0	1	1	0
N22	30	0	1	0	0	1	0	0	1	0	1	0	0
N42	31	0	1	0	0	1	0	0	0	0	1	0	0
N12	32	1	0	0	0	1	0	0	1	0	1	0	0
28.08.-1	33	1	0	0	0	1	0	0	0	0	1	0	1
922c	34	1	1	1	1	1	1	0	1	0	1	1	0
N31	35	1	0	0	0	0	0	0	0	0	0	0	1
31.07.-5	36	1	0	0	1	1	1	0	1	0	0	1	0
28.08.-4	37	1	0	0	0	1	1	0	0	0	1	0	1
N32	38	1	0	0	1	1	1	0	1	0	0	1	0
3001	39	1	1	1	1	1	1	0	1	0	1	1	0
3024-5	40	1	1	1	1	1	1	0	1	0	1	1	0
941d	41	1	1	1	1	1	1	0	1	0	9	9	9
923b	42	1	1	1	1	1	1	0	1	0	1	1	0
946d	43	1	1	1	1	1	1	0	1	0	1	1	0
922a	44	1	1	1	1	1	1	0	1	0	1	1	0
927-1b	45	1	1	1	1	1	1	0	1	0	1	1	0
942-2b	46	1	1	1	1	1	1	0	1	0	1	1	0
24.07.-5-1	47	1	1	1	0	1	1	0	0	0	1	0	0

		M18					N12					N14					
		450	600	700	800	1200	450	550	650	850	1000	650	700	850	900	1100	1150
Foo	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1
FOG	2	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
3013	3	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
3015	4	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
3061-1	5	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
3041-1	6	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1
3029-1	7	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
3019-2	8	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
949a	9	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
14.08.-10	10	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
923-1b	11	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
952-2b	12	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
948a	13	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	0
FF	14	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
944c	15	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
942c	16	1	1	1	1	0	0	1	1	1	1	9	9	9	9	9	9
FGF	17	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
945b	18	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
3073-2	19	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
924-2b	20	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
941-1b	21	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
954-1b	22	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
24.07.-7	23	0	0	1	1	0	0	1	0	0	0	1	0	1	1	1	1
952-14	24	1	0	1	0	0	0	1	0	1	1	0	0	1	1	1	0
948-1b	25	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
949-1b	26	1	1	0	1	1	0	1	0	1	1	0	0	0	1	1	0
17.07.	27	1	1	0	1	0	0	1	0	1	1	1	0	1	1	1	0
930-2a	28	1	1	0	1	0	0	1	0	1	1	1	0	1	1	1	0
3004-3	29	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
N22	30	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0
N42	31	1	0	1	0	0	0	1	0	1	1	0	0	1	1	1	0
N12	32	1	0	1	1	0	0	1	0	1	1	1	0	1	1	1	0
28.08.-1	33	1	0	1	1	0	0	1	0	1	0	1	1	0	1	0	0
922c	34	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
N31	35	1	0	1	1	0	1	1	0	1	1	0	0	1	0	0	0
31.07.-5	36	1	0	0	1	0	0	1	0	1	1	1	0	1	1	1	0
28.08.-4	37	1	0	1	1	0	0	0	0	1	1	1	1	0	1	1	0
N32	38	1	0	0	1	0	0	1	0	1	1	1	0	1	1	1	0
3001	39	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
3024-5	40	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
941d	41	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
923b	42	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
946d	43	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
922a	44	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
927-1b	45	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
942-2b	46	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0
24.07.-5-1	47	1	0	1	0	0	0	1	0	0	1	1	1	0	1	1	0

		N15				N17						U05				
		500	550	1000	1100	550	650	750	800	1100	1200	500	650	750	850	1100
Foo	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1
FOG	2	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3013	3	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3015	4	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3061-1	5	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3041-1	6	1	1	1	1	1	1	0	0	0	1	1	1	0	0	1
3029-1	7	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3019-2	8	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
949a	9	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
14.08.-10	10	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
923-1b	11	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
952-2b	12	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
948a	13	0	1	1	1	1	1	1	0	1	0	1	1	0	1	0
FF	14	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
944c	15	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
942c	16	9	9	9	9	9	9	9	9	9	9	1	1	1	1	1
FGF	17	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
945b	18	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3073-2	19	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
924-2b	20	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
941-1b	21	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
954-1b	22	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
24.07.-7	23	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1
952-14	24	0	1	1	0	1	1	1	0	1	1	0	1	1	0	0
948-1b	25	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
949-1b	26	0	1	1	1	0	1	1	0	1	1	0	1	0	0	0
17.07.	27	0	1	1	0	1	1	1	0	0	1	0	1	0	0	0
930-2a	28	0	1	1	0	1	1	1	0	0	1	1	1	1	0	0
3004-3	29	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
N22	30	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
N42	31	0	1	1	1	1	0	0	0	1	1	0	1	0	0	1
N12	32	0	1	1	1	1	1	1	0	1	1	9	9	9	9	9
28.08.-1	33	0	0	0	1	1	0	0	0	1	0	1	0	1	1	1
922c	34	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
N31	35	0	1	0	1	0	1	1	0	0	1	0	1	1	0	1
31.07.-5	36	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
28.08.-4	37	0	0	1	1	1	1	0	0	0	0	1	0	1	1	1
N32	38	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3001	39	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3024-5	40	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
941d	41	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
923b	42	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
946d	43	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
922a	44	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
927-1b	45	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
942-2b	46	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
24.07.-5-1	47	1	1	1	0	1	0	1	0	1	1	1	0	1	1	0

		U17		U19				U20				
		1000	1500	550	650	750	900	450	600	700	850	900
Foo	1	0	0	1	1	0	1	1	1	0	1	1
FOG	2	0	1	1	1	1	1	1	0	1	1	1
3013	3	0	1	1	1	1	1	1	0	1	1	1
3015	4	0	1	1	1	1	1	1	0	1	1	1
3061-1	5	0	1	1	1	1	1	1	0	1	1	1
3041-1	6	0	1	1	1	0	0	1	1	0	1	1
3029-1	7	0	1	1	1	1	1	1	0	1	1	1
3019-2	8	0	1	1	1	1	1	1	0	1	1	1
949a	9	0	1	1	1	1	1	1	0	1	1	1
14.08.-10	10	0	1	1	1	1	1	1	0	1	1	1
923-1b	11	0	1	1	1	1	1	1	0	1	1	1
952-2b	12	0	1	1	1	1	1	1	0	1	1	1
948a	13	0	0	0	1	1	1	1	0	0	1	1
FF	14	0	1	1	1	1	1	1	0	1	1	1
944c	15	0	1	1	1	1	1	1	0	1	1	1
942c	16	0	1	1	1	1	1	1	0	1	1	1
FGF	17	0	1	1	1	1	1	1	0	1	1	1
945b	18	0	1	1	1	1	1	1	0	1	1	1
3073-2	19	0	1	1	1	1	1	1	0	1	1	1
924-2b	20	0	1	1	1	1	1	1	0	1	1	1
941-1b	21	0	1	1	1	1	1	1	0	1	1	1
954-1b	22	0	1	1	1	1	1	1	0	1	1	1
24.07.-7	23	0	0	0	0	0	0	1	1	0	0	0
952-14	24	0	0	1	0	0	0	1	0	0	0	0
948-1b	25	0	1	1	1	1	1	1	0	1	1	1
949-1b	26	0	1	1	0	0	0	1	0	0	0	1
17.07.	27	0	1	1	0	0	0	1	0	0	0	1
930-2a	28	0	0	0	1	0	0	1	0	0	0	1
3004-3	29	0	1	1	1	1	1	1	0	1	1	1
N22	30	0	0	1	0	0	1	1	1	1	0	0
N42	31	0	0	0	1	0	0	1	1	1	1	1
N12	32	0	0	1	1	0	0	1	1	1	1	1
28.08.-1	33	0	0	1	0	1	0	0	0	1	1	0
922c	34	0	1	1	1	1	1	1	0	1	1	1
N31	35	1	0	1	1	0	0	0	0	0	1	1
31.07.-5	36	0	0	0	1	0	0	1	1	1	1	1
28.08.-4	37	0	0	0	0	1	1	0	0	1	0	0
N32	38	0	0	0	1	0	0	1	1	1	1	1
3001	39	0	1	1	1	1	1	1	0	1	1	1
3024-5	40	0	1	1	1	1	1	1	0	1	1	1
941d	41	0	1	1	1	1	1	9	9	9	9	9
923b	42	0	1	1	1	1	1	1	0	1	1	1
946d	43	0	1	1	1	1	1	1	0	1	1	1
922a	44	0	1	1	1	1	1	9	9	9	9	9
927-1b	45	0	1	1	1	1	1	1	0	1	1	1
942-2b	46	0	1	1	1	1	1	1	0	1	1	1
24.07.-5-1	47	1	1	1	1	0	1	1	1	0	1	1

0 = absence of amplicon, 1 = presence of amplicon, 9 = no data

Appendix 6:Jaccard’s similarity coefficient

	1	2	3	4	5	6	7	8	9	10	11
1	1,00E+14										
2	6,52E+13	1,00E+14									
3	6,52E+13	1,00E+14	1,00E+14								
4	6,52E+13	1,00E+14	1,00E+14	1,00E+14							
5	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14						
6	6,35E+13	6,71E+13	6,71E+13	6,71E+13	6,71E+13	1,00E+14					
7	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14				
8	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14			
9	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14		
10	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	
11	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
12	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
13	5,84E+13	6,89E+13	6,89E+13	6,89E+13	6,89E+13	5,95E+13	6,89E+13	6,89E+13	6,89E+13	6,89E+13	6,89E+13
14	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
15	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
16	6,32E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,72E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
17	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
18	6,39E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
19	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
20	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
21	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
22	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
23	3,61E+13	3,25E+13	3,25E+13	3,25E+13	3,25E+13	3,43E+13	3,25E+13	3,25E+13	3,25E+13	3,25E+13	3,25E+13
24	5,90E+13	5,88E+13	5,88E+13	5,88E+13	5,88E+13	5,45E+13	5,88E+13	5,88E+13	5,88E+13	5,88E+13	5,88E+13
25	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
26	5,60E+13	6,14E+13	6,14E+13	6,14E+13	6,14E+13	5,75E+13	6,14E+13	6,14E+13	6,14E+13	6,14E+13	6,14E+13
27	5,61E+13	5,98E+13	5,98E+13	5,98E+13	5,98E+13	5,77E+13	5,98E+13	5,98E+13	5,98E+13	5,98E+13	5,98E+13
28	6,25E+13	6,22E+13	6,22E+13	6,22E+13	6,22E+13	6,03E+13	6,22E+13	6,22E+13	6,22E+13	6,22E+13	6,22E+13
29	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
30	3,97E+13	3,49E+13	3,49E+13	3,49E+13	3,49E+13	3,33E+13	3,49E+13	3,49E+13	3,49E+13	3,49E+13	3,49E+13
31	5,13E+13	5,32E+13	5,32E+13	5,32E+13	5,32E+13	5,27E+13	5,32E+13	5,32E+13	5,32E+13	5,32E+13	5,32E+13
32	6,05E+13	6,32E+13	6,32E+13	6,32E+13	6,32E+13	6,16E+13	6,32E+13	6,32E+13	6,32E+13	6,32E+13	6,32E+13
33	3,41E+13	3,78E+13	3,78E+13	3,78E+13	3,78E+13	3,29E+13	3,78E+13	3,78E+13	3,78E+13	3,78E+13	3,78E+13
34	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
35	5,39E+13	4,63E+13	4,63E+13	4,63E+13	4,63E+13	4,93E+13	4,63E+13	4,63E+13	4,63E+13	4,63E+13	4,63E+13
36	6,30E+13	6,27E+13	6,27E+13	6,27E+13	6,27E+13	6,08E+13	6,27E+13	6,27E+13	6,27E+13	6,27E+13	6,27E+13
37	3,70E+13	3,73E+13	3,73E+13	3,73E+13	3,73E+13	3,77E+13	3,73E+13	3,73E+13	3,73E+13	3,73E+13	3,73E+13
38	6,46E+13	6,63E+13	6,63E+13	6,63E+13	6,63E+13	6,25E+13	6,63E+13	6,63E+13	6,63E+13	6,63E+13	6,63E+13
39	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
40	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
41	6,67E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,79E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
42	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
43	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
44	6,55E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,75E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
45	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
46	6,52E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	6,71E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14
47	5,53E+13	5,70E+13	5,70E+13	5,70E+13	5,70E+13	5,88E+13	5,70E+13	5,70E+13	5,70E+13	5,70E+13	5,70E+13

	12	13	14	15	16	17	18	19	20	21	22	23
12	1,00E+14											
13	6,89E+13	1,00E+14										
14	1,00E+14	6,89E+13	1,00E+14									
15	1,00E+14	6,89E+13	1,00E+14	1,00E+14								
16	1,00E+14	6,55E+13	1,00E+14	1,00E+14	1,00E+14							
17	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14						
18	1,00E+14	6,76E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14					
19	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14				
20	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14			
21	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14		
22	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	
23	3,25E+13	2,66E+13	3,25E+13	3,25E+13	2,81E+13	3,25E+13	3,10E+13	3,25E+13	3,25E+13	3,25E+13	3,25E+13	1,00E+14
24	5,88E+13	5,52E+13	5,88E+13	5,88E+13	5,16E+13	5,88E+13	5,68E+13	5,88E+13	5,88E+13	5,88E+13	5,88E+13	3,17E+13
25	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
26	6,14E+13	5,49E+13	6,14E+13	6,14E+13	5,69E+13	6,14E+13	5,97E+13	6,14E+13	6,14E+13	6,14E+13	6,14E+13	3,03E+13
27	5,98E+13	5,82E+13	5,98E+13	5,98E+13	5,87E+13	5,98E+13	6,05E+13	5,98E+13	5,98E+13	5,98E+13	5,98E+13	3,17E+13
28	6,22E+13	6,12E+13	6,22E+13	6,22E+13	6,19E+13	6,22E+13	6,32E+13	6,22E+13	6,22E+13	6,22E+13	6,22E+13	3,13E+13
29	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
30	3,49E+13	2,19E+13	3,49E+13	3,49E+13	3,85E+13	3,49E+13	3,38E+13	3,49E+13	3,49E+13	3,49E+13	3,49E+13	3,75E+13
31	5,32E+13	4,49E+13	5,32E+13	5,32E+13	4,92E+13	5,32E+13	5,34E+13	5,32E+13	5,32E+13	5,32E+13	5,32E+13	2,98E+13
32	6,32E+13	6,09E+13	6,32E+13	6,32E+13	5,79E+13	6,32E+13	6,29E+13	6,32E+13	6,32E+13	6,32E+13	6,32E+13	3,79E+13
33	3,78E+13	3,88E+13	3,78E+13	3,78E+13	3,71E+13	3,78E+13	3,55E+13	3,78E+13	3,78E+13	3,78E+13	3,78E+13	2,59E+13
34	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
35	4,63E+13	4,48E+13	4,63E+13	4,63E+13	4,38E+13	4,63E+13	4,47E+13	4,63E+13	4,63E+13	4,63E+13	4,63E+13	2,63E+13
36	6,27E+13	6,32E+13	6,27E+13	6,27E+13	6,19E+13	6,27E+13	6,49E+13	6,27E+13	6,27E+13	6,27E+13	6,27E+13	3,38E+13
37	3,73E+13	4,03E+13	3,73E+13	3,73E+13	3,33E+13	3,73E+13	3,51E+13	3,73E+13	3,73E+13	3,73E+13	3,73E+13	2,32E+13
38	6,63E+13	6,52E+13	6,63E+13	6,63E+13	6,35E+13	6,63E+13	6,62E+13	6,63E+13	6,63E+13	6,63E+13	6,63E+13	3,64E+13
39	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
40	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
41	1,00E+14	6,91E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,43E+13
42	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
43	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
44	1,00E+14	6,86E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,33E+13
45	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
46	1,00E+14	6,89E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	3,25E+13
47	5,70E+13	4,61E+13	5,70E+13	5,70E+13	5,45E+13	5,70E+13	5,63E+13	5,70E+13	5,70E+13	5,70E+13	5,70E+13	2,99E+13

	24	25	26	27	28	29	30	31	32	33	34	35
24	1,00E+14											
25	5,88E+13	1,00E+14										
26	6,82E+13	6,14E+13	1,00E+14									
27	6,88E+13	5,98E+13	7,16E+13	1,00E+14								
28	6,42E+13	6,22E+13	6,25E+13	8,69E+13	1,00E+14							
29	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14						
30	3,48E+13	3,49E+13	3,33E+13	3,10E+13	3,19E+13	3,49E+13	1,00E+14					
31	5,56E+13	5,32E+13	4,79E+13	4,37E+13	4,86E+13	5,32E+13	2,77E+13	1,00E+14				
32	6,51E+13	6,32E+13	6,23E+13	6,52E+13	6,77E+13	6,32E+13	3,91E+13	6,33E+13	1,00E+14			
33	3,24E+13	3,78E+13	3,11E+13	3,06E+13	2,97E+13	3,78E+13	2,93E+13	3,13E+13	4,19E+13	1,00E+14		
34	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14	3,49E+13	5,32E+13	6,32E+13	3,78E+13	1,00E+14	
35	4,26E+13	4,63E+13	4,44E+13	4,43E+13	4,93E+13	4,63E+13	2,81E+13	4,22E+13	5,24E+13	2,97E+13	4,63E+13	1,00E+14
36	5,56E+13	6,27E+13	5,45E+13	6,11E+13	6,86E+13	6,27E+13	3,47E+13	4,93E+13	7,46E+13	3,80E+13	6,27E+13	4,79E+13
37	3,58E+13	3,73E+13	3,24E+13	3,57E+13	3,86E+13	3,73E+13	2,46E+13	3,49E+13	4,35E+13	5,71E+13	3,73E+13	2,92E+13
38	5,54E+13	6,63E+13	5,64E+13	6,08E+13	6,81E+13	6,63E+13	3,51E+13	5,14E+13	7,78E+13	4,03E+13	6,63E+13	5,21E+13
39	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14	3,49E+13	5,32E+13	6,32E+13	3,78E+13	1,00E+14	4,63E+13
40	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14	3,49E+13	5,32E+13	6,32E+13	3,78E+13	1,00E+14	4,63E+13
41	6,08E+13	1,00E+14	6,10E+13	6,00E+13	6,27E+13	1,00E+14	3,42E+13	5,14E+13	6,23E+13	3,73E+13	1,00E+14	4,80E+13
42	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14	3,49E+13	5,32E+13	6,32E+13	3,78E+13	1,00E+14	4,63E+13
43	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14	3,49E+13	5,32E+13	6,32E+13	3,78E+13	1,00E+14	4,63E+13
44	6,05E+13	1,00E+14	6,20E+13	6,03E+13	6,28E+13	1,00E+14	3,46E+13	5,14E+13	6,20E+13	3,72E+13	1,00E+14	4,62E+13
45	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14	3,49E+13	5,32E+13	6,32E+13	3,78E+13	1,00E+14	4,63E+13
46	5,88E+13	1,00E+14	6,14E+13	5,98E+13	6,22E+13	1,00E+14	3,49E+13	5,32E+13	6,32E+13	3,78E+13	1,00E+14	4,63E+13
47	5,14E+13	5,70E+13	4,88E+13	5,06E+13	5,53E+13	5,70E+13	3,11E+13	4,93E+13	5,42E+13	3,07E+13	5,70E+13	4,38E+13

	36	37	38	39	40	41	42	43	44	45	46	47
36	1,00E+14											
37	3,94E+13	1,00E+14										
38	9,52E+13	4,17E+13	1,00E+14									
39	6,27E+13	3,73E+13	6,63E+13	1,00E+14								
40	6,27E+13	3,73E+13	6,63E+13	1,00E+14	1,00E+14							
41	6,18E+13	3,82E+13	6,58E+13	1,00E+14	1,00E+14	1,00E+14						
42	6,27E+13	3,73E+13	6,63E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14					
43	6,27E+13	3,73E+13	6,63E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14				
44	6,15E+13	3,80E+13	6,54E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14			
45	6,27E+13	3,73E+13	6,63E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14		
46	6,27E+13	3,73E+13	6,63E+13	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	1,00E+14	
47	5,38E+13	3,75E+13	5,38E+13	5,70E+13	5,70E+13	5,70E+13	5,70E+13	5,70E+13	5,68E+13	5,70E+13	5,70E+13	1,00E+14

Accession numbers are explained in Table 5.2, 1 = Foo, 2 = FOG.