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Investigation on host-parasite interaction between the stem nematode *Ditylenchus dipsaci* and sugar beet *Beta vulgaris* and their importance for development of alternative integrated management strategies

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Abstract

Investigation on host-parasite interaction between the stem nematode *Ditylenchus dipsaci* and sugar beet *Beta vulgaris* and their importance for development of alternative integrated management strategies

The re-occurrence and rapid spread of *D. dipaci* in German sugar beet production areas enhanced the necessity for a holistic management strategy of this pathogen to secure sustainable sugar production.

To evaluate sources of resistance or tolerance in available germplasms a standard screening protocol was developed. The inoculation of 14 days old sugar beet seedlings between the leaf axils proved to be the most efficient infestation procedure. Different geling agents were tested as nematode carriers. Carboxymethyl cellulose provided the highest increase of nematode penetration rates by promoting a longer penetration time frame. The developed standard inoculation protocol was used to compare different artificial screening procedures to field screening results. The highest correlation was detected between *D. dipsaci* rating results of heavily infested fields and the evaluation of symptoms after artificial inoculation under controlled greenhouse conditions. Therefore a reliant protocol for a high throughput screening of sugar beets against *D. dipsaci* was developed.

Penetration time frame of *D. dipsaci* into sugar beets was evaluated. The highest penetration rate occurred when nematode suspension was applied when the seedlings were germinated but remained in soil. A protection of the short period between germination and emergence from ground could be protected by a nematicidal seed coating.

The reproduction capability of *D. dipsaci* in sugar beets was investigated. Different sugar beet varieties showed significantly varying nematode reproduction. The initial nematode density at time of inoculation, ranging from 10 -200 nematodes per plant had no significant impact on symptom development 54 days after inoculation.

Geostatistical analyses were used to evaluate the site specific management potential of *D. dipsaci* and *Heterodera schachtii*. The cluster size of *D. dipsaci* was smaller and the distribution of rot symptoms was very heterogenic. Therefore it could be concluded that site specific management is rather suitable for *H. schachtii* where also a high correlation between initial nematode densities and occurring yield decrease is known.

Zusammenfassung

Untersuchungen zur Wirt-Parasit Beziehung zwischen dem Stängelnematoden *Ditylenchus dipsaci* und Zuckerrüben *Beta vulgaris* und ihre Bedeutung für die Entwicklung von alternativen integrierten Bekämpfungsansätzen

Das erneute Auftreten und die schnelle Verbreitung von *D. dipsaci* in deutschen Zuckerrübenanbaugebieten hat die Notwendigkeit eines integrierten Managementkonzeptes für diesen Schädling unterstrichen, um auch weiterhin eine nachhaltige Zuckerproduktion zu ermöglichen.
Um genetische Quellen von Resistenz oder Toleranz identifizieren zu können, wurde ein System zur künstlichen Inokulation entwickelt. Die Inokulation von *D. dipsaci* Larven zwischen die Blattachseln von 14 Tage alten Zuckerrüben Keimlingen war der erfolgreichste Ansatz. Zusätzlich wurden verschiedene gelartige Trägersubstanzen miteinander verglichen, wobei Carboxymethyl Cellulose die höchste Erhöhung der Nematoden Penetrationsraten erzielte. Das entwickelte Standard Inokulationsprotokoll wurde verwendet, um verschiedene artifizielle Screeningsysteme mit den Ergebnissen von Feldversuchen zu vergleichen. Die höchste Korrelation wurde zwischen Fäulnisbonituren von artifiziell inokulierten Zuckerrüben und Feldversuchen mit sehr starkem *D. dipsaci* Befall festgestellt. Somit wurde ein effektives Verfahren entwickelt, welches eine zuverlässige Sortenbewertung mit hohem Durchsatz ermöglicht
Weiterhin wurde der Penetrationszeitraum von *D. dipsaci* in Zuckerrübenkeimlinge wurde untersucht. Dabei stellte sich heraus, dass die höchsten Penetrationsraten erzielt wurden, wenn die Nematodensuspension in dem Zeitraum appliziert wurde, wenn die Zuckerrüben bereits gekeimt waren, allerdings noch nicht die Bodenoberfläche durchbrochen hatten. Diese Ergebnisse legen den Schluss nahe, dass eine nematizide Beizung eine hohe Effektivität habe würde.
Untersuchungen der Vermehrungsfähigkeit von *D. dipsaci* in Zuckerrüben zeigten signifikant unterschiedliche Vermehrungsraten in verschiedenen Zuckerrübensorten. Die Anzahl an inokulierten Nematoden hatte allerdings keinen signifikanten Einfluss auf die Fäulnissausprägung 54 Tage nach der Inokulation.
Geostatistische Verfahren wurden verwendet um des Potential einer teilflächen-spezifischen Bekämpfung von *D. dipsaci* und *Heterodera schachtii* zu bewerten. Da sowohl die Größe der einzelnen Nester von *D. dipsaci* geringer war, als auch die Schadensschwelle viel niedriger ist der Ansatz einer teilschlagspezifischen Bekämpfung eher für *H. schachtii* geeignet.

1 Introduction

Today agricultural production systems are under higher economic pressure than ever before. Globalisation increases price competition between farmers in different countries and a rising world population requires affordable and safe nutrition. Limited availability of raw materials also will increase the cost of input factors of crop production such as fertilizers, fuel, machinery and plant protection products. Global warming threatens many production areas through extreme changes in climatic conditions. Especially pests and pathogens whose damage potential is strongly dependent on environmental factors will gain in importance, because increasing temperatures will allow them to complete more generations per year. Invasive species are transported by worldwide trade and their spread also will be promoted by climatic change. These introduced pests are often able to spread rapidly, because no natural enemies are present. Plant pathology therefore will play a major role in securing the nutritional basis of the world population. The importance of plant parasitic nematodes for agricultural production systems also will increase in the future. Under natural conditions, the dispersal of nematodes in soil varies from a couple of centimetres to meters per year. However, anthropogenic spread through increased regional and worldwide trade will strongly increase the damage potential of these soil borne pests in new uninfested areas where natural enemies do not exist.

It is therefore obvious that many control measure modifications and development of new tools for crop health management are needed to maintain present levels of crop production as well as increase overall yields in this ever changing world. Integrated pest management (IPM) is an umbrella term for the use of a combination of strategies targeted at reducing the impact of pests and diseases while simultaneously reducing the amount of pesticides needed to minimize the impact of a pest or disease on crop production (Börner, 1990). IPM is a modern ecologically acceptable alternative to the historical reliance on a single control measure, which in the past was the use

of chemical control. Which control measures should be chosen for IPM requires an understanding of the biology of the pest and the development of the host crop as well as of the cropping system in use. Only if the host-parasite interrelation is understood adequate control measures can be chosen for effective IPM.

Present day production of sugar beets in Europe faces new challenges. Due to the new regulations in the sugar market, the price of beets will decrease by 40% and the price of sugar by 36% in the near future (Anonym, 2007). Narrow profit margins will increase economic pressure on the farmers to reduce inputs and this will include reducing levels of IPM. Sugar beet pests and diseases, therefore will more than likely cause increased levels of economic damage to the crop due to this reduced pest control.

Under these unfavourable conditions a long known pathogen of sugar beet, the stem nematode *Ditylenchus dipsaci,* has re-emerged as an ecomonically important pest of the crop. This nematode is again causing severe damage and yield losses in sugar beet production areas in Germany and in other European countries (Julier et al., 1996, Kühnhold et al., 2006). Crop losses of 50% and more have been observed and the quality of the beets is diminished due to crown rot symptoms of the tap root (Schlang, 2003, Schlang, 2004, Hilnhütter et al.,2010).

In North-Rhine-Westphalia, disease symptoms, which were not seen for almost 3 decades as a result of proper IPM measures, were again observed in a single field in 1992. After the first detection the pest spread rapidly in the years following (Fig 1.1). At present, no effective control measures are available in Germany to prevent the disease damage due to the nematode nor to prevent its spread. Fumigant or non fumigant nematicides are no longer available, are being phased out on the European level and others have not been registered for use due to the size of the market place for nematode control in sugar beet. The typical crop rotation in this region, also called the

"Rheinische Fruchtfolge", includes sugar beet, barley and wheat (Klapp, 1967). The narrow time frame of three years between host crops that support very high pest reproduction leads to a fast rise in soil populations above the threshold. Therefore, an IPM strategy needs to be developed for the control of the stem nematode *D. dipsaci* to secure high yields and insure the economic survival of the beet processing industry in this region. The number of tools used for IPM of plant parasitic nematodes is long. The main control measures for nematode management are described below as outlined by Sikora et al., (2005).

1. Quarantine (to prevent entry of invasive species)

2. Hygiene measures (preventing spread through soil movement or the introduction of nematodes on infested planting material or seeds)

3. Cultural practice (crop rotation with sufficient time gap between growing of a host crop, fallow, trap crops, weed control, biofumigation, adjustment of planting and harvesting time)

4. Physical methods (solarisation, steam treatment, hot water treatment of planting material and flooding)

5. Biological control (use of antagonistic organisms as soil or seed treatment)

6. Chemical control (fumigant/ non-fumigant, soil or seed treatment)

7. Host crop resistance (use of resistance, tolerance or transgenic plants)

8. Precision farming (use of remote sensing to detect nematode clusters for targeted control)

1.1 *Ditylenchus dipsaci*

In 1857 Julius Kühn was the first to describe *Ditylenchus dipsaci* as a pest of *Dipsacus fullonum* in Bonn, Germany. The pathogen occurs worldwide in temperate regions, but has never been discovered in the tropics. *Ditylenchus dipsaci* was considered to be a species complex with up to 30 different races with specific host crop spectra (Eriksson, 1974, Eriksson and Granberg, 1969) . However, new phylogenetic studies showed that most nematode isolates from agricultural, ornamental and several wild plants including an isolate from a German sugar beet field formed one clade and should therefore be considered as *D. dipsaci sensu stricto*, with diploid chromosome numbers (Subbotin et al.2005).

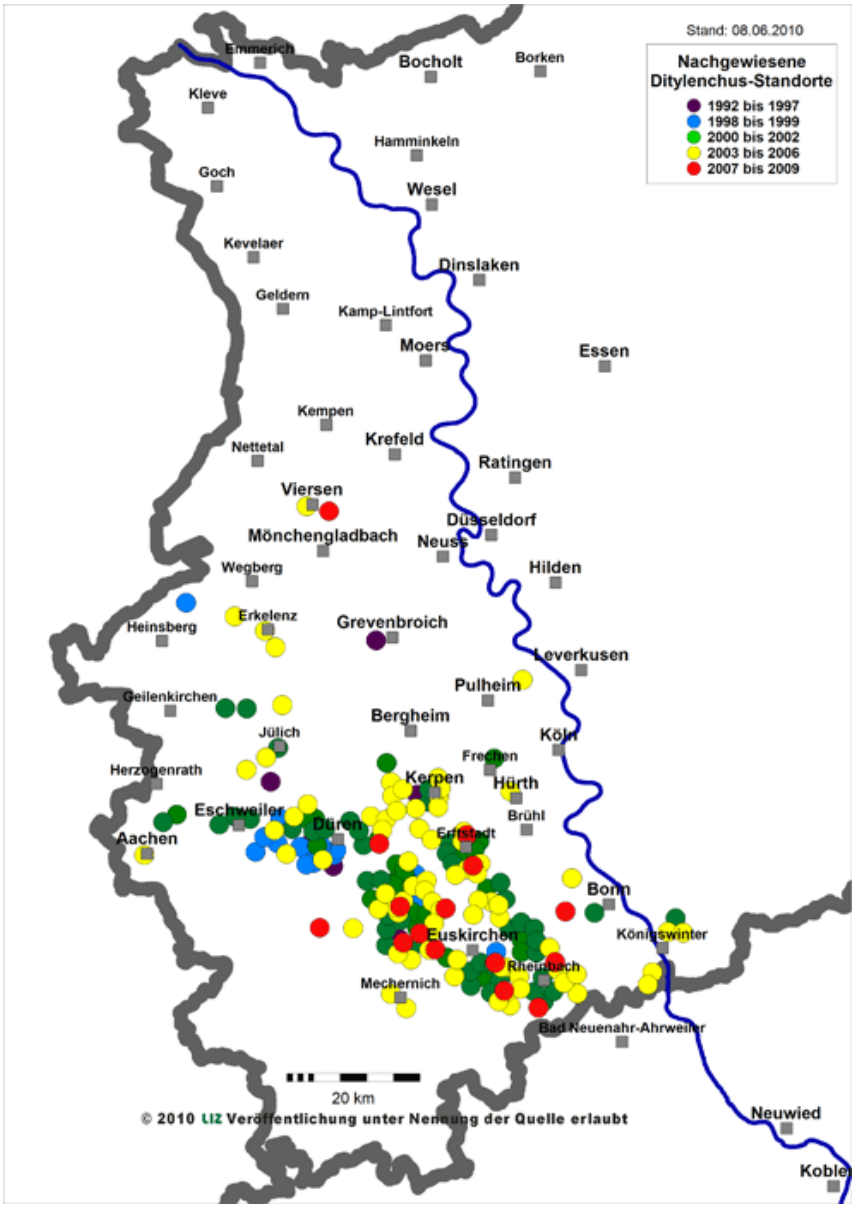

Figure 1.1 *Spatial and temporal spread of* Ditylenchus dipsaci *in the Rhineland, Germany (LIZ, 2010)*

For reproduction, both sexes are compulsory. The total duration of the life cycle of *D. dipsaci* varies between 19 and 23 days (Gubina, 1982), and longevity ranges from 45 to 73 days. The J4 stage of this nematode can slow down its metabolism and falls into a hypobiotis when environmental conditions are unfavourable. Survival for up to 20 years in an anhydrobiotic state was observed (Sayre, 1962, Sturhan and Brzeski 1991). The

reproduction capacity of one female is up to 500 eggs (Yuksel, 1960), and matricidal phenomena occur frequently. Egg production is linearly related to temperature, with 0.158 eggs per day degree produced under optimal conditions (Griffith et al., 1997). These data elucidate the very low threshold of *D. dipsaci* being 1-2 juveniles per 250ml of soil (Pfister and Mittnacht, 1992).

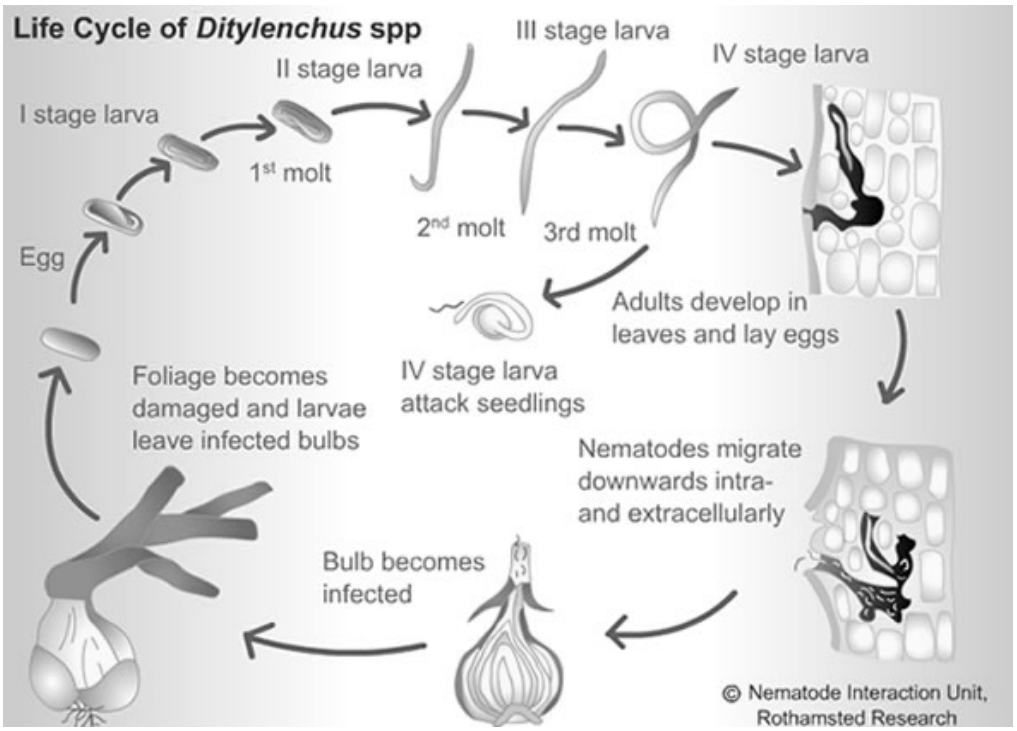

Fig. 1.2 *Life cycle of* Ditylenchus *species on onion (www.rothamsted.ac.uk/.../ ditylifecycle.htm)*

First visible symptoms of damage caused by this plant parasitic nematode are swelling and distortion of primary leaves and petioles, occurring several weeks after sowing. These symptoms are rarely observed in the field because they only appear at very high infestation levels and under favourable environmental conditions. These symptoms disappear quickly in beets due to the compensating growth of the beets. Thereafter, symptoms are not visible until the middle of August.

Fig. 1.3 *Early symptoms caused by high* Ditylenchus dipsaci *densities in soil and favourable environmental conditions for infestation on sugar beets*
left: deformation of leaves, right: swelling of tuber tissue

The first symptoms include unsynchronized emergence, followed by stunted growth of sugar beet seedlings. High numbers of up to 10,000 *D. dipsaci* per gram of fresh tissue can lead to severe stunting and distortion. Later in the season, these symptoms tend to disappear due to compensational growth of the plant. Between the middle of July and the beginning of August, white specks of callus can appear at the soil surface and underneath the soil surface on the epidermis of the beets. Later in the season, crown rot symptoms can be observed and rotting spread throughout the whole taproot. Depending on the type of secondary microbial infection, dry rot as well as bacterial wet rot can develop.

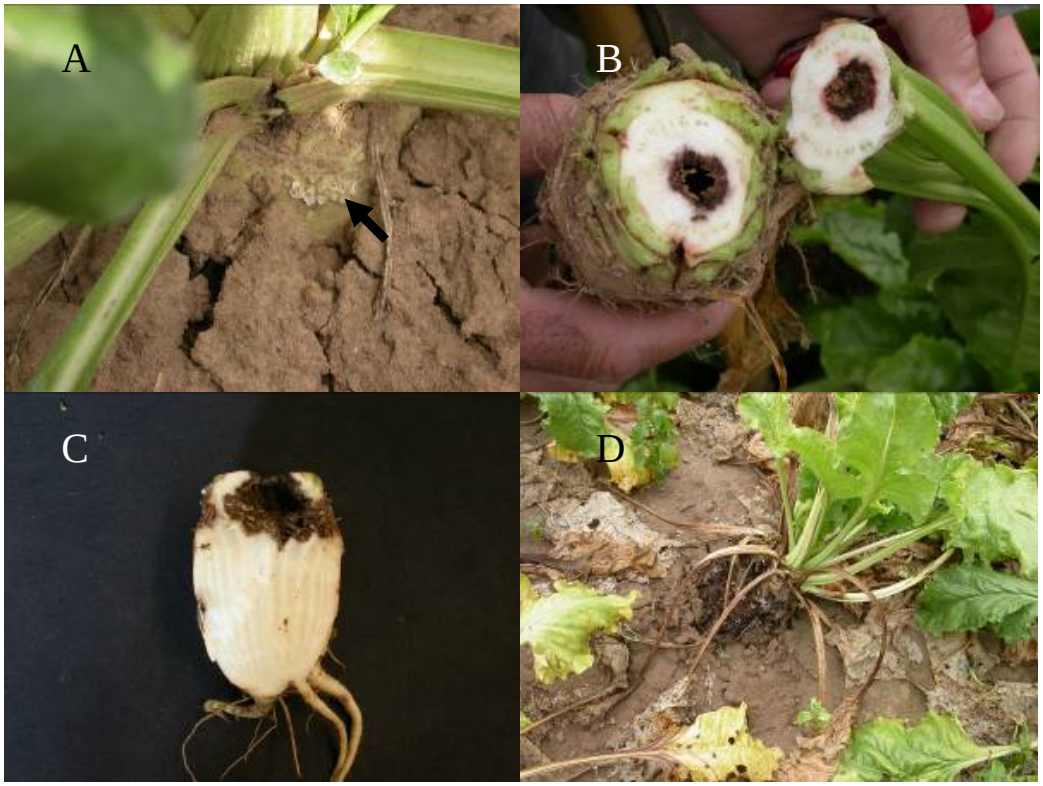

Figure 1.4 Ditylenchus dipsaci *symptoms on sugar beets developing in July until the end of the growing season. A: White pustules of callus tissue, B: Initial symptoms of crown rot, C: Outward spread of crown rot symptoms in the tap root, D: Final crown rot stage showing intact laves on completely rotten tuber*

The highest numbers of *D. dipsaci* are found in the healthy tissue close to the necrotic areas. The largest proportion (75%) of the nematode life-stages in plant and soil was the fourth-stage juvenile (Julier et al., 1996). Since the transport tissues of the xylem and phloem are seldom affected by *D. dipsaci* up until the complete degradation of the tissue occurs, the leaves appear green and healthy even if major parts of the taproot are rotten (Fig.1.4, D). It is known that *D. dipsaci* migrates out of degrading tissues due to toxic water soluble metabolites associated with rot (Robertson, 1928). However, intra nematode behaviour also plays a role since nematode migration per hour is a linear function of the total population per plant (Griffith et al., 1997).

The degree of infestation is dependent on the initial soil infestation level and the activity of *D. dipsaci* at the time of sowing (Seinhorst, 1956). Therefore, the sudden increase in the importance of *D. dipsaci* in Germany is probably

related to the lack of registered nematicidal seed treatments with Aldicarb, Carbofuran or other nematicidal compounds in Germany, which were in use in the past. These nematicides were banned due to their high toxicity and are no longer available in Germany. Nematicide treatments can lead to yield increases between 87 and 100% compared to non treated controls in a highly infested field (Pfister and Mittnacht, 1992). Crop rotation alone usually will not lead to a eradication of *D. dipsaci* in infested fields due to the wide host range of the stem nematode which includes 500 plant species and many weeds (Subbotin et al., 2005;Salentiny, 1959), which makes the species one of the most polyphagous nematodes (Sturhan et al., 2008). Also the fact that biological races of *D. dipsaci* with different host ranges occur, complicates the use of crop rotation in IPM (Sturhan et al., 2008).

1.2 *Heterodera schachtii*

Sugar beet production and nematode problems have a long history in Germany and other European countries. In 1876, about 24 sugar factories were forced to close down their operation because of a decline in beet production at the farm level. Losses were considered to be due to what was then called “Rübenmüdigkeit” caused by *Heterodera schachtii* (Schmidt et al., 1985). Sugar Beet Cyst (SBCN) nematode *H. schachtii* still causes severe crop losses in sugar beet growing areas across Europe. It is one of the most important diseases of sugar beet and causes crop losses up to 25% (Schlang, 1991). The cysts can contain up to 300 eggs and 2-3 life cycles can be completed per year under temperate conditions. The infective stage is the second stage juvenile (J2), which penetrates the root and induces a syncytium. The roots of infected plants are less efficient and therefore the plants have decreased efficiency in removal of water and nutrients from soil. Also recovery after a period of water stress is adversely effected (Gierth, 2004). The economic threshold value of this nematode is ≥ 500 eggs and J2 per 100 ml of soil (Müller J., 1990). The most effective control measure to

manage *H. schachtii* is the use of nematode resistant intercrops, which results in a population reduction up to 80% (Steudel and Müller, 1983). The most common resistant intercrops are cultivars of mustard (*Brassica alba*). However these cultivars were recently found to be good host crops for *D. dipsaci* (Knuth, 1995) and wild sources of genetic material are known to have high reproduction potential. However the nematode suppressive intercrop *Phacelia tanacetifolia*, which is also widely sown for *H. schachtii* control, was not a host of *D. dipsaci* (Knuth, 1995). Therefore, monitoring of *D. dipsaci* infestation in new crops used in sugar beet rotations for *H schachtii* control as well as for new cultivars of a rotation crop is necessary so that new IPM strategies for the beet cyst nematode remain effective even when both parasites are present.

1.3 Precision farming

Precision farming is a tool of modern agricultural production systems. Precision technology is used to reduce input and operating costs based on the use of site specific information of the field site. Precision agriculture (PA) substitutes information and knowledge for physical inputs and hence is inextricably linked to sustainability (Bongiovanni and Lowenberg-Deboer, 2004). Information technology is developing rapidly and costs of recording and processing devices are declining. Due to the spread of global positioning system (GPS) technologies all forms of information can be spatially referenced. The most common data used in PA for plant production is the distribution of soil types on soil maps as they are consistent over many years. This created the term “farming by soil” as a synonym for PA (Robert, 1993). However, plant nutrient content, weed distribution and plant diseases can be mapped and used for site specific management strategies (Bongiovanni and Lowenberg-Deboer, 2004). The spatial referenced information can be displayed using geographical information systems (GIS). The obtained maps are used to apply appropriate doses of fertilizer, pesticides and herbicides to

specific sites (Oerke et al., 2010). Also a reduction in workforce and energy use can be achieved, since GPS steering minimizes the amount overlapping. Yield mapping systems for cereals are widely applied. The generated data is difficult to be evaluated and can seldom be used for site specific physical inputs since many factors determine final yields. However, heterogenic yield data often provides valuable information for the use of site specific management. Also basic online systems, which can record data, process the information and conduct adapted applications, like the Yara-N-Sensor, are already commercially available. However, economic benefits alone will not increase the future acceptance of these new technologies in practical agriculture. Consumer awareness of environmentally save production, reduced pesticide use, product traceability and new policies will however, increase the demand for PA technologies (McBratney, 2005).

Nematodes are soil borne pests that are mostly found in clusters in the field and usually spread slowly from one location to another. Their lack of mobility and spread makes them prime targets for site specific management strategies. It is also known that nematode occurrence and damage is often related to textural properties of the soil. Decades ago, a high positive correlation between soil types with more than 30% of clay and *D. dipsaci* attack was demonstrated in onion production areas of the Netherlands (Seinhorst, 1956). Today, soil cards can be easily created by measuring soil electronic conductivity with the EM 38 (Domsch and Giebel, 2004, Mertens et al., 2008, Neudecker et al., 2001, Oerke et al., 2010). A more efficient tool was not available in the past to create such precise and affordable soil maps. Up to 50 ha can be mapped in one hour with the appropriate equipment.

In Germany, the stem and bulb nematode *D. dipsaci* and the sugar beet cyst nematode *H. schachtii* are the predominant pests in sugar beet. As recently reported, *D. dipsaci* is causing increasing levels of damage in Germany (Kühnhold et al., 2006). Other European countries also have encountered a re-emergence of the pest (Castillo et al., 2007). The farmers are facing

potential yield losses of 50% and more (Schlang, 2003, 2004). In addition *H. schachtii* is still a major concern in European sugar beet production areas, even though management strategies like nematode resistant sugar beet varieties and intercrops are available. These two major pest problems are likely to increase due to lower profit margins, which will lead to more intensive sugar beet cultivation around the processing factories.
For the above mentioned reasons, the potential use of site specific technology for the management of these two nematode species was investigated in sugar beet fields.

The irregular distribution of *D. dipsaci* in a field was considered important when soil sampling methodology was developed (Müller et al., 1993).The aggregation behaviour of other species of nematodes has also been investigated, but generally the main focus of the research was to detect correlations between edaphic factors and nematode densities (Koenning et al., 1996, Wallace et al., 1993). Furthermore, the spread of *D. dipsaci* in lucerne field was analysed using aerial photography, showing a fast spread of initially infested areas (Atkinson and Sykes, 1981).
Remote sensing methods have not been use to date to determine aggregation of *D. dipsaci* for use in site specific managment.

Population densities of the root-knot nematode *Meloidogyne incognita* were inversely related to the percentage of clay and silt, whereas, the reniform nematode *Rotylenchus reniformis* was favoured by moderate levels of clay and silt (Koenning et al., 1996). The use of remote sensing using soil texture as criteria for aggregation has been shown to be effective in the management of this root-knot nematode on cotton (Mueller, 2010).

Important is the fact that aggregated occurrence of *H. schachtii* is well known. The clustered distribution of other cyst nematodes such as *H. glycines* (Avendano et al., 2003, Avendano et al., 2004c, Avendano et al., 2004b,

Avendano et al., 2004a), *Globodera pallida* and *G. rostochiensis* (Evans et al., 2002) and also is well studied. In addition the interaction of plant parasitic nematodes with other root pathogens which increases overall damage to the sugar beet crop (Avendano et al., 2007, Back et al., 2002) gives remote sensing of the nematodes involved importance for reduction of synergistic interactions. If a strong aggregation of *H. schachtii* can be confirmed with remote sensing then a heterogenic application system of management for infested fields could be developed to reduce the impact. The use of remote sensing for cyst nematode control is increasing and promising approaches have been developed to detect and quantify soybean cyst nematode population densities (Nutter et al., 2002). However, these findings are based on unspecific plant reactions. No specific signature of plant stress caused by nematodes was recorded. However, it was concluded that plant stress caused by nematodes can be detected. To allocate this unspecific damage to nematode incidence standard sampling methods also are required.

However, nematode data is always impacted by random variability of sampling points (Wallace and Hawkins, 1994). Commercially applied sampling grids are likely to produce misleading application maps (Evans et al., 2002). Some authors applied sampling grids with a scale of 20 cm and detected strong aggregations under crop rows (Rossi et al., 1996). Based on this information it is evident that the influence of edaphic factors on the spatial distribution of *D. dipsaci* and *H. schachtii* needed more investigation to determine the potential of site specific control of these pests.

1.4 Main study objectives

The main objective was to elaborate an integrated approach to manage *D. dipsaci* populations in crop rotations that included sugar beet. As part of this strategy the EM38 was used to map edaphic factors correlated to nematode densities.

The potential of site specific control for the sugar beet nematodes *H. schachtii* and *D. dipsaci* as part of an IPM strategy also was analysed. As host crop resistance should be the foundation upon which all management strategies are built (Sikora et al., 2005), it was one of the main aspects of the present research.

The following aspects were studied experimentally:

- Development of an *in vivo* bioassay to identify sugar beet resistance to the stem nematode *D. dipsaci*
- Detection of tolerance and resistance in sugar beet cultivars and breeding lines against *D. dipsaci*
- Analyse the parasite host interrelationship between the two nematodes and sugar beet to determine weak links for effective control measures.
- Determine the spatial distribution and dispersal of *D. dipsaci* and *H. schachtii* in German sugar beet fields for site specific management

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2 Development of an *in vivo* bioassay to identify sugar beet resistance to the stem nematode *Ditylenchus dipsaci*

2.1 Introduction

The stem nematode, *Ditylenchus dipsaci* (Kühn, 1857) Filipjev 1936, is of great economic importance worldwide on specific hosts plants. The nematode is found under a wide range of climatic conditions from temperate to tropical where cool moist weather regimes enable nematode infection, reproduction and dispersal (Plowright et al., 2002, Subbotin et al., 2005). *Ditylenchus dipsaci* is an important pest of lucerne, clover, pea, bean, and bulbous species of Liliaceae such as tulips, as well as Alliaceae such as garlic and onion. Furthermore, cereals, in particular, oats and rye, are affected. The numerous host races and populations of *D. dipsaci* were regarded as a species complex (Sturhan, 1970) and recent investigations on the phylogeny of *D. dipsaci* by Subbotin *et al*. (2005) confirmed the existance of this species complex. In their studies, they showed that DNA sequences of *D. dipsaci* from sugar beet belong to the largest cluster which includes specimens from all cultivated plants, some ornamental and wild plants, and is therefore considered as *D. dipsaci sensu stricto* (Subbotin *et al.*, 2005). In addition, their data do not support the theory of the presence of biological races within the species *D. dipsaci*.

In the recent past, *D. dipsaci* damage has increased significantly in German sugar-beet-growing areas (Pfister and Mittnacht, 1992, Schlang, 2003). In *D. dipsaci*-infested fields, beet yield and sugar content are often reduced by 50% or more (Schlang, 2004, Pfister and Mittnacht, 1992). Storage of damaged beets results in even greater losses because processing quality is affected. As chemical control is no longer available and crop rotation is complicated by the wide host range of this nematode (Knuth, 1995), resistant cultivars could play an important role in nematode management. The importance of resistance as a management tool for nematodes has been described for

crops where severe economic loses by nematodes occur, like soybean (Koenning, 2004, Chen et al., 2001), banana (Martin et al., 2000) and grapes (Cousins and Walker, 2001). Although sources of resistance in sugar beet germplasm towards *Heterodera schachtii* are known (Schmidt et al., 1985), resistance to *D. dipsaci* is only known for lucerne, clover, rye, bean and oat (Plowright *et al.*, 2002) and is still lacking for sugar beet.

Breeding for resistance to *D. dipsaci* requires reliable *in vivo* bioassay methods and a good source of monoxenic nematode inoculum of consistent quality and infectivity. The two methodologies in combination allows for quick evaluation of germplasm for sources of resistance. Monoxenic culture methods, such as a culture on lucerne callus, have been developed for *D. dipsaci* (Hooper, 1986) and are ideal for providing inoculum for screening. Plowright *et al.* (2002) favoured culturing *D. dipsaci* on lucerne callus. However, culturing *D. dipsaci* on lucerne callus is rather laborious and sterility of the monoxenic culture is often problematical.

The goal of the present study was to establish a simple and efficient protocol for production of *D. dipsaci* inoculum of a population specific to sugar beet and suitable for an *in vivo* bioassay screening programme to test sugar beet germplasm for resistance. Therefore, the objectives were to 1) determine if a *D. dipsaci* population obtained from sugar beet reproduces on carrot disks (*Daucus carota*), for use in optimising the production of inoculum, and 2) to develop a bioassay for efficient testing of sugar beet germplasm for resistance.

2.2 Materials and methods

D. DIPSACI INOCULUM PRODUCTION

A population of *D. dipsaci* was obtained from a sugar beet field near Birgel, Germany, with a known history of severe *D. dipsaci* infestation. Soil samples were collected in February 2003 and nematodes were extracted using a modified Oostenbrinck technique (Hooper *et al.*, 2005). *Ditylenchus dipsaci* were hand-picked with a 5 *µ*l micropipette using a stereo microscope at 40× magnification and transferred to an excavated glass block. A mixture of approximately 1000 fourth-stage juveniles (J4) and adult *D. dipsaci* were transferred to sterile centrifuge tubes. Nematodes were spun down at 1500 *g*, washed twice with sterile demineralised water and re-suspended in an antibiotic solution containing 0.1% streptomycin sulphate (w/v) and 0.1% amphotericin-B (w/v) followed by incubation at room temperature for 30 min. The nematode suspension was adjusted to 1000 *D. dipsaci* ml^{-1} and aliquots of 100 *µ*l were transferred to carrot disks to establish an initial culture. To ensure a monoxenic culture of *D. dipsaci*, nematodes were again reselected at the first subculture as described above and transferred to fresh carrot disks.

IN VITRO CULTURING ON CARROTS

Carrots obtained from a local supplier were surface sterilised by soaking in a 0.5% NaOCl-solution for 30 min. Thereafter, the carrots were carefully rinsed with sterile demineralised water and aseptically transferred to a clean bench. Carrots were peeled using a sterile surgical blade and cut into 4 cm thick disks. Disks were then aseptically transferred to sterile 250 ml wide-mouth flint glass bottles (three disks per bottle) and incubated in the dark at 20 ± 1◦C. Disks were inoculated as soon as callus was visible as white specks on

the carrots, in general after 10-14 days. After the first nematodes egressed from the callus and accumulated on the wall of the glass bottle, the nematodes were collected in sterile water before being used for inoculation of new carrot disks. Prior to the inoculation of new disks, the nematode suspension containing all stages of *D. dipsac*i was spun down and surface sterilised using a 0.1% streptomycin sulphate and 0.1% amphotericin-B solution for 30 min as described above.

To determine the optimum number of *D. dipsaci* as initial inoculum per disk, carrot callus was inoculated with 50, 75, 100, 125 and 250 nematodes. Nematodes were inoculated in 100 *µ*l sterile water with three drops of suspension on top of and near the central cylinder of the carrot disk. Afterwards, carrot disks were incubated at 20 ± 1◦C in the dark. Each treatment consisted of five replicate bottles with three carrot disks each. When the first nematodes egressed from the disks, the number of days after inoculation was recorded. *Ditylenchus dipsaci* was extracted from individual disks using a modified Baermann funnel technique (Hooper *et al.*, 2005). Carrot disks were cut in small pieces and nematodes were collected over a period of 4 days. The resulting nematode suspension was passed through a 20 *µ*m mesh sieve to reduce the volume and the number of extracted nematodes per carrot disk was determined under a microscope using a 2 ml counting slide. The same procedure was applied to extract *D. dipsaci* from naturally infested sugar beets from commercially managed fields.

The number of days until the first nematodes egressed, the percentage of extractable carrot disks (those not prematurely degraded due to unwanted microbial contamination) and the number of *D. dipsaci* per disk were analysed by regression. When the lack of fit test revealed that data analysis by regression was not possible, analysis of variance (ANOVA) followed by mean comparison using a least significant difference test was used to determine the effects of inoculum density.

INVITRO CULTURING ON ALFALFA

To sterilise the alfalfa, the seeds were placed at the bottom of 100ml glass beaker

and covered with 4% sodium hypo chloride solution for one hour. Every ten minutes the seeds were agitated with a glass stirring rod. Afterwards, the sodium hypochlorite was removed and the seeds were washed 4-times with sterile water. They were then placed for 2-3h in water at room temperature to swell. From here on all steps were conducted under sterile conditions. To remove tannins, the water after swell was poured off again and the seeds were again washed with sterile water. After these purification steps, the swollen seeds were placed in Petri dishes with nutrient agar supplemented with antibiotics. The Nutrient Agar (6.9 g Bacto Peptone, 5.1 g sodium chloride and agar 13g/l) was used to provide optimal conditions for bacteria growth, so that contamination was made visible.

The seeds were placed in an incubator at 20°C. After two days, the contamination free seedlings were transferred to 30ml test tubes with agar and further kept at 16h photoperiod and 20°C. Once the plants were in the 3-4 leave stage, they were cut off at the hypocotyl and transferred onto callus inducing medium in Petri dishes. The callus inducing medium B51 was prepared by mixing the nutrient medium Gamborg B5 (3051g / l) pH 5.8, with 2.4 Dichlorphenolacid(2mg / l), Kinetin (0.5 mg / l), sucrose (20g / l) and agar (8g / l).

With a pair of forceps, the entire plant was gently wounded to induce callus production. Two to three seedlings were placed into each Petri dish and maintained in the dark for approximately 14 days at 20°C. The resulting callus was then transferred to new Petri dishes with B51 and kept at 20°C in the dark. The fastest growing callus was selected to stimulate nematode reproduction.

CULTURING ON COURGETTE

The courgettes (*Cucurbita pepo* L.) were purchased in local shops with a length of 20cm and a diameter of 5cm. Before inoculation with *D: dipsaci*, the courgettes were washed with a 70% alcohol saturated paper towel under a transfer hood. The inoculated nematodes were obtained from carrot callus as described above. From now on, all steps were performed under a sterile transfer hood. The *D .dipsaci* juveniles were sterilized with 0.1% Amphotericin B and 0.1% Streptomycinsulfate for 30min and washed with sterile water three times. To inject the nematodes into the courgettes, a 10ml syringe with a 10cm long and 0.8 mm wide needle was used. To determine whether or not the nematodes survived the procedure, a 0.1 ml aliquot was taken from the syringe and examined under g a light microscope. This initial examination showed that all nematodes were viable and had no apparent injuries.

Per courgette, three injections in to the central cylinder were made. After the injection, the points of injection were sealed with liquid wax and the courgettes were stored at 18-20°C in the dark. Nematode extraction was performed then
after 8, 12 and 16 weeks storage. The courgettes were cut into 0.2-0.5 cm wide slices and macerated in a blender. Afterwards, the material was weighed and placed on a modified Baermann funnel overnight at 20-25°C.

IN VIVO INOCULATION SYSTEM

Two different nematode inoculation techniques were evaluated: 1) indirect inoculation of the potting soil or 2) precise application to the leaf axils of the seedlings. These two delivery methods were evaluated for their effectiveness to achieve high rates of *D. dipsaci* penetration into susceptible sugar beet seedlings as determined by staining the nematodes in the plant tissue.

The addition of the carriers: agar (Applichem, Darmstadt, Germany), carboxymethyl cellulose (CMC) and stockosorb powder (SSP, Stockhausen, Krefeld, Germany) were added to the nematode inoculum to facilitate adhesion to the plant surface and to reduce nematode death due to drying on the soil or leaft surface and to increase overall consistency of penetration as compared to the use of water alone.

Fig. 2.1 *Inoculation droplet with carboxymethyl cellulose containing 200* Ditylenchus dipsaci *juveniles placed between the leaf axils of an 14 day old sugar beet seedling*

Sugar beet seeds of the cv. Monza, highly susceptible to *D. dipsaci*, were planted in 3 cm diam. plastic pots filled with a previously sterilised field soil:sand mixture (1:1, v:v). Pots were kept in a growth chamber at 20 ± 1°C with a 16 h light period and were watered daily. Two weeks after planting, 200 *D. dipsaci* were inoculated onto the seedlings by placing a 10 *µ*l nematode suspension in the leaf axils of the first pair of true leaves with the aid of a pipette (Figure 2.1). Nematodes were inoculated in a suspension containing either 0.1% (w/v) agar, 1% (w/v) CMC or 0.1% (w/v) SSP as a gelling agent. Nematodes applied in tap water served as the control. To determine the *D. dipsaci* penetration rate, the seedlings were removed from the pots 10 days

after inoculation and gently washed free of soil. The whole seedling was transferred to 125 ml plastic beakers containing 25 ml of a 0.1% acid fuchsin/lactic acid solution (Byrd *et al*., 1983) and boiled in a microwave oven for 2 min. After removing the staining solution by rinsing the seedling over a 20 *µ*m sieve with tap water, the seedlings were macerated in 30 ml tap water using an Ultra Turrax disperser (T25 basic, IKA Labortechnik, Staufen, Germany). The number of *D. dipsaci* per plant was determined by counting stained nematodes in a 10 ml aliquot under a stereo microscope at 40× magnification using a 10 ml winding-track counting tray (Hooper et al, 2005).

For soil inoculation, approximately 200 nematodes were applied in a volume of 200 *µ*l of the three suspensions described above. The suspensions were applied to the soil surface around the basal stem of the seedlings using a pipette. Nematodes applied in tap water served as the control. To ensure sufficient moisture for a 2-3 day period after inoculation, the pots were placed in plastic trays and covered with clear plastic covers. The experiment was performed in ten replicates and conducted twice. Nematodes in tap water served as the control. Each experiment was performed with ten replicates and conducted twice. Data from two experiments were pooled after confirming homogeneity of variances and analysed by ANOVA. Mean comparison was done using a least significant difference test.

Fig. 2.2 *Symptoms induced after inoculation with 200* Ditylenchus dipsaci *between the leaf axils in different gelling agents (A) no symptoms, (B) swelling of leaf axils and (C) swelling and bursting of seedling tissue*

PLANT PART INOCULATION

Sugar beet seeds of the cultivar Dorena known to be highly susceptible to *D. dipsaci*, were planted in 3 cm diam. plastic pots filled with a previously sterilised field soil:sand mixture (1:1, v:v). Pots were kept in a growth chamber at 20 ± 1°C with a 16 h light period and were watered daily. Two weeks after planting, the plants were removed from soil and cut into three different parts. The leaves, the leave axils and the stems of the plants were then transferred into a Petri dish filled with fine sand and completely buried. Afterwards the sand was moistened with autoclaved tap water and a 2ml suspension containing 5000 *D. dipsaci* was used to inoculate the different plant parts. The Petri dishes were kept in a growth chamber at 20 ± 1°C with a 16 h light period for 48 h. Afterwards the plant parts were removed from sand and gently rinsed with tab water, to ensure that recently penetrated nematodes were detected. Nematode number was then determined by staining and counting as described previously.

Morphometrics and infectivity of in vivo and in vitro nematode populations

The nematodes used in these studies were initially isolated from a sugar beet field population. Thereafter they were reared on carrot callus for many generations. In order to exclude possible shifting effects this population maintained in vitro for two years was compared to *D. dipsaci* freshly extracted from infected sugar beets. To compare typical morphometric characteristics 15 nematodes of the in-vitro and 10 nematodes of the in vivo populations were examined. Nematodes were extracted over a Baermann funnel and immobilized by heating the nematode suspension over 60°C. Afterwards they were fixed and mounted in TAF under a cover slip. Following characteristics were measured: total Length (L), relation of L to maximal width (a), realation of L to length of the oesophagus (b), relation of L to length of the tail and distance of the vulva from anterior end (V).

Infectivity of the two different nematode origins was examined using the inoculation procedures described in in vivo inoculation system.

2.3 Results

IN VITRO CULTURING ON CARROTS

Table 1. *Effect of initial* Ditylenchus dipsaci *inoculum density per carrot disk on the number of days of incubation at 20◦C until the first egress, percent extractable disks and average number of nematodes per extractable carrot disk.*

Inoculum density/ carrot disk	Days until first egress of *D.dipsaci* (range)	% extractable carrot disks (±S.I	Number of extracted *D.dipsaci* / carrot disk (±
50	79.3 (63-101)	50.0 (± 22.4)	117009(± 42995) ab
75	72.4 (52-101)	83.3 (± 10.5)	124165(± 33957) ab
100	63.7 (58-71)	54.7 (± 19.7)	62200(± 10427) ab
125	58.0 (24-79)	60.0 (± 24.5)	21312(± 10367) ab
250	37.7 (28-52)	58.0 (± 19.1)	10321(± 6202) b
R^2	0.426***	n.s.	

R^2 = regression coefficient for the exponential curve ($y = \exp(4.5712 - 0.0041x)$) fitted to inoculum density per carrot disk *vs.* days until first egress of *D. dipsaci*. ∗∗∗significant at $P = 0.001$. n.s. = non-significant. Means in one column followed by the same letter are not significantly different at $P < 0.05$.

The initial inoculum density greatly influenced the reproduction of the *D. dipsaci* from sugar beet on carrot disks (Table 1). The density also had a significant effect on the number of days until the first nematodes egressed from the carrot disks (Table 1). On average, egress of nematodes was detected 79, 72, 64, 58 and 38 days after inoculation with 50, 75, 100, 125 or 250 *D. dipsaci* per disk, respectively (Table 1). The number of days until the first egress of nematode decreased exponentially as inoculum density increased ($R^2 = 0.426$, $P = 0.001$). During the course of the experiment, several carrot disks showed signs of rapid tissue decay due to breakdown of the cells and contamination with bacteria. The percentage of individual carrot disks that actually yielded nematodes was highest, but not significantly different to others, at 83.3% when 75 nematodes were used as inoculum (Table 1). The number of *D. dipsaci* per carrot disk was greatest (124 165 ± 33 957) after inoculation with 75 nematodes (Table 1). This number was significantly higher compared to an inoculum density of 250 nematodes, but was not different from the other treatments. The reproduction factor for *D.*

dipsaci was greatest with an initial inoculum density of 50 nematodes (2340±464) compared to 75 nematodes per disk (1279 ± 189; data not presented). Inoculum densities of 100, 125 and 150 nematodes resulted in reproduction rates of 897 ± 158, 170 ± 44 and 38 ± 16, respectively.

IN VITRO CULTURING ON ALFALFA CALLUS

The procedure outlined for the production of callus resulted in few uncontaminated Petri dishes with callus. However, the callus growth that was obtained was not able to produce sufficient amounts of tissue for nematode culturing. The largest callus only grew to a size of 0.5 cm diameter. Since a reliable reproduction system with carrot callus was established, no further effort was invested in finding alternative host tissue. THIS BELONGS IN RESULTS.

CULTURING ON COURGETTE

Six courgettes were inoculated three times by syringe injection with 1000 *D. dipsaci* each. None of the inoculated courgettes showed signs of contaminations. However, sometimes shrinking of tissue was observed. At none of three dates a significant increase of nematode populations was observed. In only one courgette *D. dipsaci* juveniles could be extracted. However, only 334 of the 3000 inoculated nematodes were detectable in this sample.

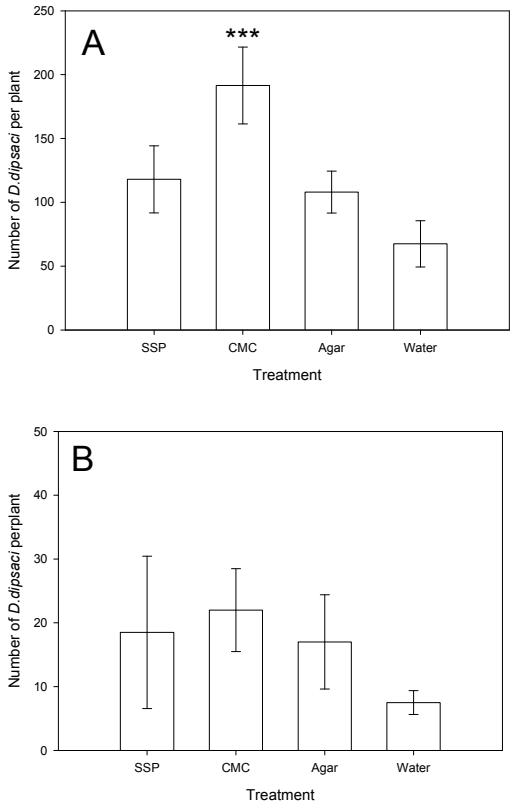

Fig. 2.3 *Effect of inoculum delivery through leaf axils (A) and soil (B) and the carriers stockosorb powder (SSP), carboxymethyl cellulose (CMC), agar and water on the number of* Ditylenchus dipsaci *per sugar beet seedling 10 days after inoculation with 200 nematodes. Vertical lines represent individual standard errors of the means.* ∗∗∗*significantly different compared to the water control at* P < *0.001 (n = 20).*

On average, lower numbers of *D. dipsaci* invaded the sugar beet seedlings after inoculation through soil compared to leaf axils (Fig. 1A, B). Delivering inoculum through the leaf axils resulted in penetration rates ranging from 34% for the water control to 96% for the carrier CMC, which corresponded to 68 (±18) and 191 (±30) *D. dipsaci* per plant, respectively (Fig. 1A). The number of nematodes per plant with the carrier CMC (191) was significantly greater than with SSP (118 ± 26), agar (108±16), and the water control (68±18). No significant differences among treatments (*P* = 0.05) were found when inoculum was delivered to soil (Fig. 1B). The number of *D. dipsaci* per plant

was lowest with tap water (7 ± 2) compared with the carriers CMC, SSP and agar, which resulted on average with 22 (±6), 18 (±12), and 17 (±7) *D. dipsaci* per sugar beet seedling (Fig. 1B). These numbers corresponded to penetration rates of 4% for the water control and 11, 9 and 8% for the carriers CMC, SSP and agar, respectively).

PLANT PART INOCULATION

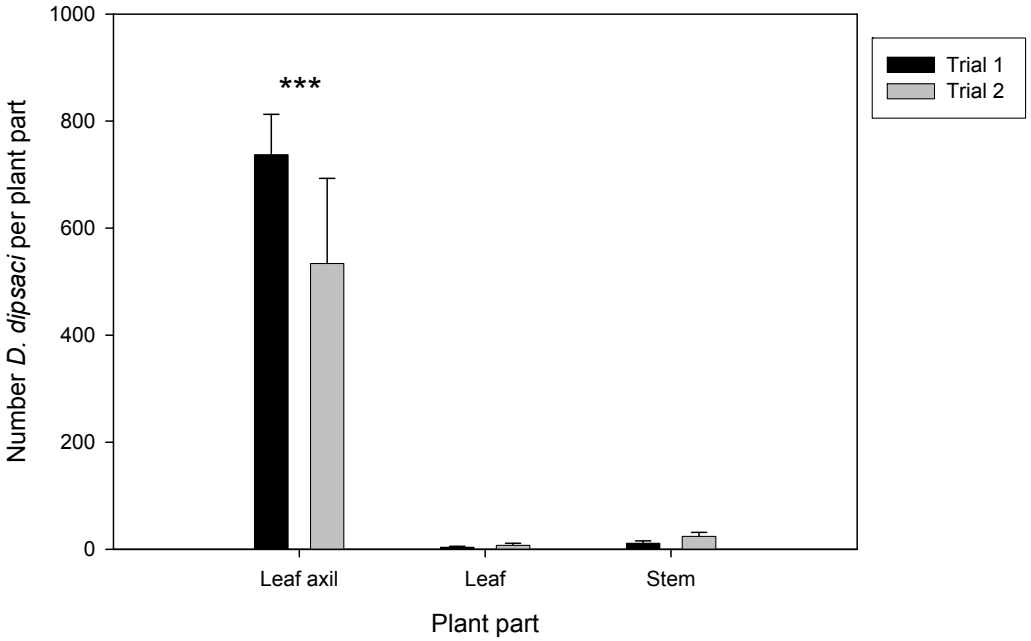

Fig. 2.4 *Number of penetrated* Ditylenchus dipsaci *per excised leaf axil, leaf or stem of 14 day old sugar beet plant parts covered with sand after inoculation with 5000 nematodes per seedling. Vertical lines represent individual standard errors of the means. *** significantly different at P < 0.001 (n = 8).All Pairwise Multiple Comparison Procedures ,Tukey Test.*

On average, 635 nematodes of 5000 inoculated could be found in the leaf axils of the seedlings (Fig. 2.4) within 24h after inoculation. This number was significantly higher than the numbers of penetrated nematodes per stem (18) and leaf (6), respectively. No significant difference occurred between the penetration rates into the stems and leaves.

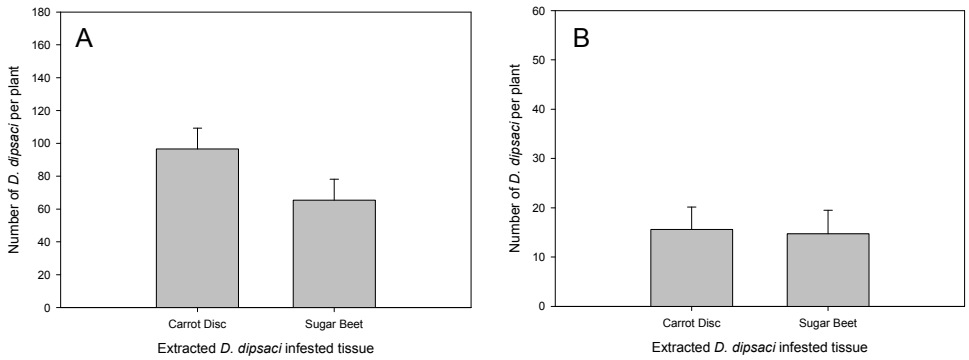

Fig. 2.5 *Effect of the rearing method and different delivery through leaf axils (A) and soil (B) on the number of* Ditylenchus dipsaci *per sugar beet seedling 10 days after inoculation with 200 nematodes. Vertical lines represent individual standard errors of the means.*

Populations of *in-vitro* reared plant pathogens are always at risk to loose their infectivity through gene shifting after multiple generations outside their hosts. Therefore the laboratory *D. dipsaci* population used in these investigations was compared to *D. dipsaci* extracted from naturally infected sugar beets. On the susceptible variety Dorena the source of nematode inoculum had no effect on Penetration rates. No significant difference was found between penetration rates of a naturally reproducing field population of *D. dipsaci* and populations maintained *in-vitro* for two years.

Table 2. *Morphometric differences between in vivo and in-vitro populations of* Ditylenchus dipsaci (L: length, a: relation of L to width, b: relation of L to oesophagus, c: relation of L to tail, V: distance of vulva to mouth (% of L)

	Carrotdisc (n=15)			Field poulation (n=10)		
	min.	max.	avg.	min.	max.	avg.
L (mm)	1.07	1.31	1.19	1.06	1.34	1.19
a	36.0	52.5	43.0	39.2	52.2	43.8
b	5.5	7.2	6.5	5.91	7.14	6.8
c	10.6	16.9	13.3	13.2	20.1	16.0
V	66.2	91.1	79.4	80.3	87.5	83.3

The populations of *D. dipsaci* maintained in-vitro for many generations did not develop unusual morphometric characteristics. All the measured properties were in a normal range of the species and corresponded well with the characteristics of the field population.

2.4 Discussion

In the past, researchers used inoculum coming from natural soil infestations of *D. dipsaci* or infected plant tissue when screening for resistance (Plowright *et al.*, 2002). However, distribution of nematodes in infested soil is heterogeneous and the population dynamics of *D. dipsaci* is strongly influenced by environmental conditions in the field; thus, the results of resistance screening can vary greatly from year to year. Inoculum production of *D. dipsaci* on carrot disk cultures proved to be a reliable and fast method for generating high amounts of nematode inoculum with a low input of time and material.

In the present investigations, and in contrast to Chitambar (2003), surface sterilisation of the nematodes before inoculation was critical to minimise the risk for contamination. The results obtained demonstrate that, in addition to culturing *D. dipsaci* populations from garlic, lupin and narcissus, the carrot disk culture can be used multiply to a *D. dipsaci* population attacking sugar beet. Based on the presented data, the standard protocol now implemented in our laboratory uses an initial inoculum density of 75 nematodes per carrot disk followed by incubation at 20 ± 1◦C for 50-60 days. Although 50 nematodes per disk resulted in a significantly higher reproduction rate compared to 75 nematodes per disk, on average only 50% of the carrot disks could be used for extraction. Because only nematodes from flint glass bottles containing carrot disks without any contamination are used as inoculum for screening purposes, an initial density of 75 nematodes per disk is routinely used in our laboratory. Inoculum densities of 100 or greater resulted in more rapid production of inoculum, but the percentage of carrot disks showing decay and contamination increased significantly.

With the described protocol, 350,000 - 500,000 nematodes per flint glass bottle (three carrot disks) can be produced. In addition, the nematode

suspension obtained after extraction can easily be stored for 4-6 weeks at 4°C, without loss of viability or infectivity (unpubl.). Research by Hooper and Cowland (Hooper and Cowland, 1988) recommended injecting *D. dipsaci* directly into courgette marrow as a cheap and rapid method to produce *D. dipsaci*. My attempts to use courgette failed to produce significant numbers of *D. dipsaci* and in most cases no reproduction occurred.

The results of the research presented here on inoculum delivery systems confirmed previous work on screening for resistance in alfalfa, beans, clover and pea (Plowright *et al.*, 2002). A density of 200 nematodes per seedling was sufficient to achieve high penetration rates with seedling inoculation. The source of the produced nematode inoculum had no effect of the number of *D. dipsaci* that penetrated the sugar beet seedling. This confirmed that virulence of the population was not affected by the *in-vitro* production system using carrot disks. Similarly, Plowright *et al.* (2002) suggested an inoculum density of 30-100 nematodes per plant. Griffin demonstrated that inoculum densities ranging from 25 to 200 per seedling resulted in reproduction of the nematode in sugar beet (Griffin, 1983a). The addition of 1% CMC as a gelling agent significantly increased the penetration rate of *D. dipsaci*. Other gelling agents such as SSP or agar were not efficient enough to increase significantly the penetration rate when compared to tap water. In contrast to other screening systems (Plowright *et al.*, 2002), the loss of inoculum was minimized by using CMC as gelling agent; therefore, repeated inoculation of seedlings was not necessary to minimize the risk of escapes.

Soil inoculation proved unsuitable, with only 4-11% of the inoculum penetrating the sugar beet seedling. Therefore, the leaf axils are suggested as the main point of entry for the nematode. However, the exact location of penetration of *D. dipsaci* into the sugar beet seedlings and other factors influencing the penetration of the nematode warrant further research. In conclusion, the presented culture technique enables a rapid screening for resistance in sugar beet to *D. dipsaci*. Furthermore, intercrops such as oil-

radish or yellow mustard commonly used for control of the sugar beet cyst nematode, *Heterodera schachtii* (Schmidt), that recently have been shown to be host plants for *D. dipsaci* (Knuth, 2004), could be tested for resistance to *D. dipsaci*. These results enable scientists to evaluate the correlation between our screening technique and field experiments in which the root-rot of different sugar beet cultivars and breeding lines are tested in naturally infested field sites.

2.5 References

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3 Comparison of greenhouse and field screening for resistance towards *D. dipsaci* in sugar beet

3.1 Introduction

The stem nematode *Ditylenchus dipsaci* causes severe problems in European sugar beet (*Beta vulgaris* L.) production areas (Julier et al., 1996). Yield losses in some cases exceed 50% (Schlang, 2004, Schlang, 2003) and to date no effective management strategies are available. Due to the wide host range of the nematode, including many common weeds, cultural practices are unlikely to prevent sugar beet yield losses, since nematode populations can persist on many non-host crops in the field (Sturhan et al., 2008). Crop rotation is also hindered by the fact that *D. dipsaci* can remain in the infective J4 stage in soil for many years (Miyagawa and Lear, 1970). Therefore, the routinely applied rotation interval of sugar beets every three years is too short to be effective as a management tool unless resistant varieties are found. Since host resistance should be the foundation upon which other management strategies build (Sharma et al., 1994), a bioassay was developed to evaluate sugar beet for resistance toward *D. dipsaci* (Kühnhold et al., 2006).The political and economic pressures affecting sugar beet production will increase in Europe due to the removal of subsidies (Anonym, 2007). This will narrow profit margins and enforce the need for host resistance the most cost effective management tool available to IPM.

Due to the higher cost for seeds and the lower yield potential of resistant varieties, implementation of these varieties in a nematode management strategy is only applicable if the initial field infestation exceeds a certain economic threshold. The fact that the damage and the economic threshold of *D. dipsaci* in sugar beets is as low as 1-2 nematodes per 250ml of soil Pfister and Mittnacht (1992) and Seinhorst (1956), emphasised the advantages of resistant varieties.

In the particular for *D. dipsaci*, resistant varieties should be planted whenever nematode infestation is detected.

Because the pathogen is strongly affected by soil moisture at time of sowing and spatial distribution in the field is often patchy, the results from field trials are likely to vary from year to year. This underscores the statement of Plowright (2002) that in field trials resistant/tolerant and susceptible reference genotypes are needed within the same field to measure the level of infestation.

Resistance screening methods were developed for many important crops (Martin et al., 2000) and resistant varieties were able to reduce nematode impact on yields (Simpson and Starr, 2001, Chen et al., 2001, Koenning, 2004). However, most sources of nematode resistance have been developed for sedentary nematodes and are monogenetic. Resistance toward the sugar beet cyst nematode (SCN) *Heterodera schachtii,* the most important pest of sugar beet, also is known (Müller, 1998, Schmidt et al., 1985) and routinely applied.

Conversely, migratory plant-parasitic nematode resistance is mostly polygenetic. Nevertheless in some crop plants accessions immune to both the stem nematode as well as the leaf and bud nematodes have been found quite frequently (Peng and Moens, 2003).

However resistance is highly specific, usually only being effective against a single species or even one race of a species (Sharma et al., 1994, Chen et al., 2001).This and the fact that *D. dipsaci* only attacks the epicotyls, while *H. schachtii* attacks the hypocotyls and root tissue (Griffin, 1983b) underlines the need of new tests for resistance towards *D. dipsaci*. Furthermore, trap crop varieties used to reduce the population density of *H. schachtii* were found to be good host crops for *D. dipsaci* (Knuth, 1995) these crops will also need new test systems.

Objectives of these studies were to identify sugar beet varieties or lines with resistance or tolerance toward *D. dipsaci.* The evaluation of the resistance level was based on the reproduction rates of *D. dipsaci* and the rating of tolerance was based on crown rot/root-rot symptom development on sugar beets induced by the nematode. To evaluate the accuracy and reliability of these screening systems they were compared to on farm trials for resistance screening, conducted in fields with a known history of infestation by *D. dipsaci*.

3.2 Material, Methods and Experimental Design

The *D*. *dipsaci* population used was obtained from infested soil of sugar beet fields near Düren (North-Rhine-Westphalia, Germany) and reared on carrot disks as described in Chapter 2 and published by Kühnhold et al. (2006). A mixture of juveniles and adults (mainly J4) was obtained 50-60 days after initial inoculation of the cultures. The inoculum was kept in tab water in the dark at 8°C and was used for inoculation after a passage through a modified Baermann funnel at 20 ± 1°C over 16h to insure viability (Hooper et al., 2005).

Climate chamber screens to detect resistance to *D. dipsaci* penetration

An *in vivo* bioassay was used to evaluate sugar beet germplasms for resistance towards the stem nematode *D. dipsaci* (Kühnhold et al., 2006). In the first screening experiment, 11 varieties were evaluated for their resistance against *D. dipsaci*. In the second set of experiments, 38 varieties were screened under controlled conditions. The high yielding variety Dorena was used as the susceptible control. The experimental design was a complete randomized block design with ten replications and two replications.

Tab. 3.1 Characteristics of sugar beet varieties screened for resistance (Source: Bundessortenamt, 2008).

Variety	Registration Year	*Rhizoctonia* tolerance	Tolerance towards *Heterodera schachtii*	Resistance towards *Heterodera schachtii*	*Cercospora* tolerance	Mildew	Yield
Alabama	2003				4	3	8
Ballade	2003				4	5	7
Belinda	2002				4	5	5
Calida	2005	x			5	7	6
Casino	2002				-	-	-
Dorena	2000				-	-	-
Fabiola	2001	x			-	-	-
Famosa	2001				4	4	6
Felicita	2003				4	5	5
Imperial	2004				3	4	6
Josephina	2004	x			-	-	-
Lessing	2004				4	5	7
Lucata	2004				4	3	7
Marietta	2003				-	-	-
Mars	2001				-	5	7
Miranda	2002				-	-	-
Modus	2002				4	6	7
Monza	2002				3	3	6
Nauta	2005	x			3	7	6
Pauletta	2005	x		x	4	7	7
Paulina	2000	x	x		5	5	4
Premiere	2001	x			-	-	-
Prestige	2005	x			4	7	5
Simenia	2004				4	3	7
Solea	2002	x			-	-	-
Syncro	2002	x			4	-	-

(Characteristics ranging from 1 for low to 10 for high, or x for present, - non present).

Approximately 200 nematodes per seedling were inoculated in 10 µl of 1% carboxymethyl cellulose (w/v). The inoculation droplet was placed between the first pair of true leaves of the sugar beet seedlings. The plants were kept into a growth chamber at 20 ± 1°C with a 16h photo period and watered daily.

Fourteen days after inoculation, the seedlings were removed from the pots, washed free of substrate and transferred to a plastic beaker. After adding 25ml of a 0.1% acid fuchsin/lactic acid solution seedlings were boiled in a micro wave for 1 minute. To remove the staining solution, the seedlings were then decanted over a 20µm mesh sieve followed by maceration in 30ml tap water using an Ultra Turrax blender (T25 basic, IKA Labortechnik, Staufen, Germany). Total numbers of *D. dipsaci* per plant were determined by counting an aliquot of 10 ml out of 30ml using a binocular at 20x magnification and a winding track counting tray (Hooper et al., 2005).

The second screening experiment was conducted under modified conditions to improve nematode penetration. In order to prevent rapid drying of the inoculation droplets after placing them into the leaf axils, the seedlings were covered with a transparent plastic hood, to ensure 95% rel. humidity. The humidity was recorded every 5 min. using a data logger (Testo 175, Testo AG, Lenzkirchen, Germany). This procedure ensured prolongation of the penetration time frame for at least 8h. Each screening experiment was conducted twice. Data from two experiments was pooled and analysed by the Kruskal-Wallis one way analysis of variance on ranks followed by multiple comparisons versus the control variety Dorena (Dunn's Method).

Greenhouse screens for resistance towards *D. dipsaci*

To evaluate the level of resistance of sugar beet varieties in the greenhouse, the highly susceptible variety Dorena (KWS, Einbeck, Germany) and the partially tolerant variety Syncro (Syngenta Seeds, Bad Salzuflen, Germany) served as controls in all experiments. Sugar beet seeds were sown in 2L plastic pots filled with a sterile sand/substrate (50:50) mixture and kept in a growth chamber (20 ± 1°C) and 16h light period. Fourteen days after planting (DAP), 200 nematodes were inoculated in 10 µl of 1% carboxymethyl cellulose (w/v). The inoculation droplet was placed in the leaf axils of the first

pair of true leaves of the sugar beet seedlings. Plants were covered with a plastic hood to ensure 95 % relative humidity, preventing drying of the inoculum droplet and favouring nematode penetration. After 4 days in the growth chamber at 20 ± 1°C the plants were transferred to a greenhouse (25 ± 5°C). Pots were irrigated daily and fertilized on a weekly basis with 10ml 14-10-14 N-P-K fertilizer solution (2g/L). To obtain reliable data on reproduction rates, plants were kept for 50 days after inoculation (DAI) in order to enable at least two nematode life cycles. The experimental design was completely randomized with ten replications per tested variety. The experiment was conducted twice.

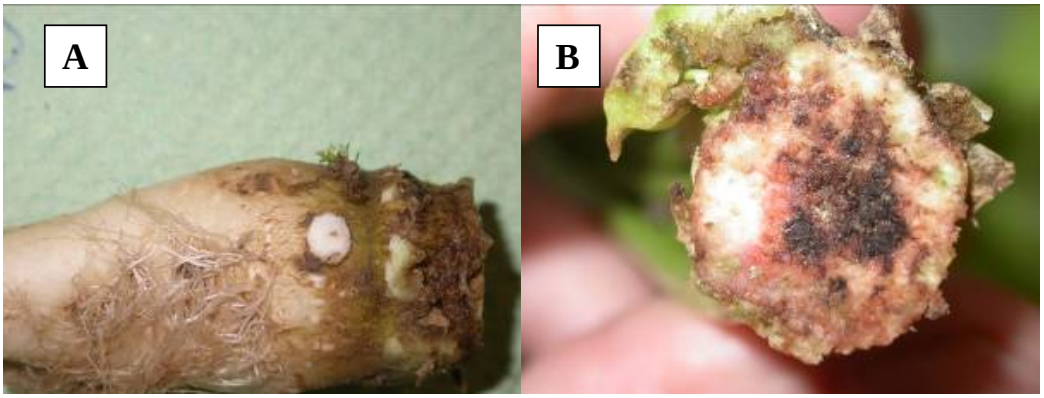

Fig 3.1.a *Symptoms on sugar beet vc. Dorena after inoculation with 200* Ditylenchus dipsaci *per plant determined 50 days after inoculation :(A) White specks of callus (B) Beet crown rot due to nematode damage followed by secondary fungal and bacterial infections*

At termination of the experiment, the reproduction potential was measured by determination of the total number of *D. dipsaci* per plant. To achieve this, the small beets (figure 3.1.a) were cut in 0.5 cm slices and placed on a modified Baermann funnel at 20 ± 1°C overnight (16h). The nematodes were collected by decanting the suspension through a 20µm sieve. Total numbers of *D. dipsaci* per plant were determined by counting three 1 ml aliquots from 15ml total volume using a stereo microscope at 40x magnification. Data from both experiments was pooled and analysed by Kruskal-Wallis one way analysis of variance on ranks followed by Tukey Test.

Assessment of tolerance toward *D. dipsaci*

To optimize the assessment of tolerance of sugar beet varieties against *D. dispaci*, some modifications needed to be implemented for screening a larger set of plants. Instead of a sterile field soil sand mix, non sterile commercial mulch (Terreau Professionnel Gepac “Einheits Erde”: Typ Topf 1.5”) was used as a planting substrate to enhance the development of crown rot symptoms. In these experiments, nematode damage was rated 54 days after inoculation on a scale ranging from 0 = no symptoms, 1 = swelling of the stem base, 2 = strong swelling of the stem base, 3 = first rot symptoms, 4 = heavy rot symptoms to 5 = dead plants (Fig.3.1.b).

Fig. 3.1.b *Rating index of symptoms on sugar beet variety Dorena after inoculation with 200* Ditylenchus dipsaci *per plant.*

At termination of the experiment, the reproduction of *D. dipsaci* was determined as described previously. Sugar beet shoot and root fresh weight was recorded separately. The experiment was conducted twice and data from both experiments was pooled and analysed by Kruskal-Wallis one way analysis of variance on ranks followed by multiple comparisons versus the control variety Dorena using the Dunn's Method.

Screening for resistance under field conditions

During the growing seasons 2004 and 2005 large scale field experiments were conducted near Düren, in NRW, Germany. A 12 row planting machine was used for sowing and each variety was placed in one sowing line. These 12 rows were defined as one block. The between row distance was 45cm and the fields were commercially managed by the farmer. In each block, the susceptible variety Dorena was planted in row 1 and 12, and the partially tolerant variety Syncro was planted in row 7 to serve as controls to evaluate the degree of infestation with *D. dipsaci* in these field plots. In the remaining nine rows the varieties to be tested were planted. Every block was planted on 3 fields in 2004 and 4 fields in 2005, respectively: All the fields used had a known history of *D. dipsaci* infestation. The evaluation of the crown rot symptoms took place in October 2004 and 2005, respectively, just before harvest. In 2004, 29 varieties and breeding lines, respectively, were screened for potential resistance or tolerance sources. The trials were divided into 3 blocks (A, B and C). In 2005, 38 varieties and lines were screened. The trial was split up into 4 blocks (A, B, C and D).

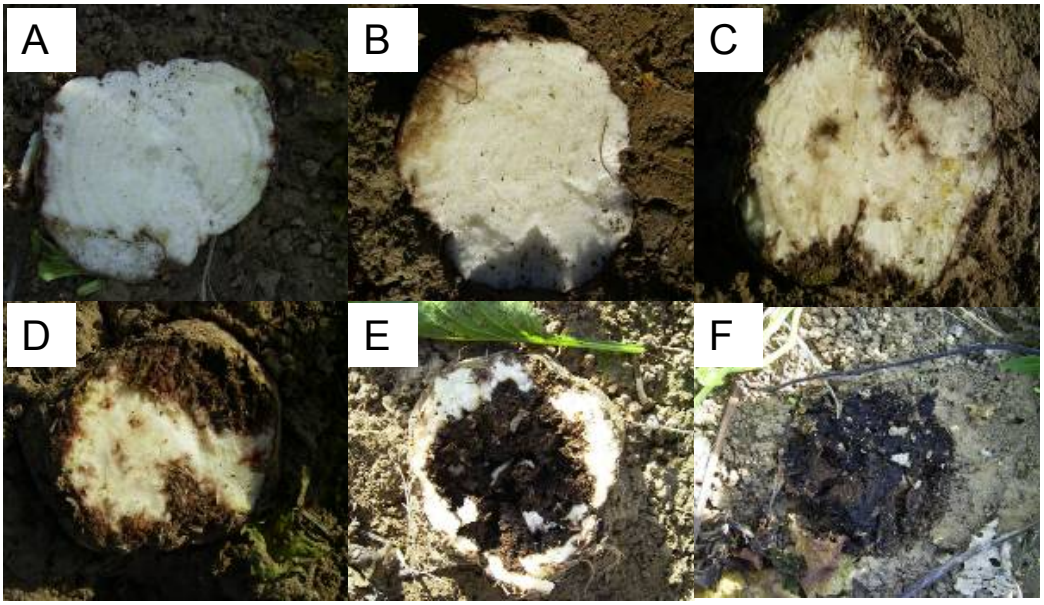

Fig. 3.2 *Disease severity ranking (A) 0%, (B) 20%, (C) 40%, (D) 60%, (E) 80% and (F) 100% rotten beet surface*

On each field, 8 areas with strong crown rot symptoms were identified using the susceptible control as indicator plant. In these areas, a block of 30 beets per variety was examined for symptoms of damage. In this way, up to 960 beets per variety were screened, depending on the number of heavily infested areas that were found. The beets were de-headed until the beet surface with the highest percentage of rot symptoms was detected (Fig.3.2). The percentage of rotten beet surface was used to evaluate *D. dipsaci* resistance/tolerance under field conditions.

Investigations on infection behaviour and disease development

Effect of seedling age and time of nematode infection on disease

Greenhouse tests were conducted to explain biologically the heterogenic distribution of the crown rot symptoms observed in the field. The critical time for nematode penetration as related to beet infection was examined. 50 ml pots were filled with a sterile sand : field soil mixture (1:1, v:v). Commercial sugar beet seeds of the variety *Dorena* were sown at a 2 cm depth. The

seedlings were inoculated at different times after sowing with 1000 *D. dipsaci* that mainly consisted of J4 and adults. The nematode population originated from an infested field in Birgel, the same area studied for the spatial distribution of *D. dipsaci.* The population was reared and maintained as previously described chapter 2.. Plants were inoculated 4, 11 or 18 days after sowing. At the 4th day after planting the germinating seedlings were still completely in the soil, while on the 11th and 18th day after planting the seedlings already emerged through the soil surface. To determine the nematode penetration rate, the seedlings were removed from the pots 10 days after the respective date of inoculation and gently washed free of soil. Each seedling was separately transferred to a 125ml plastic beaker containing 25 ml of a 0.1% acid fuchsin/lactic acid solution ((Byrd et al., 1983) and boiled in a microwave oven for 2 min. After removing the staining solution by rinsing the seedling over a 20 *µ*m sieve with tap water, the seedling was macerated in 30 ml tap water using an Ultra Turrax disperser (T25 basic, IKA Labortechnik, Staufen, Germany). The number of *D. dipsaci* per plant was determined by counting stained nematodes in a 10 ml aliquot under a binocular at 20× magnification using a 10 ml winding-track counting tray.

Effect of inoculum density on disease

The effect of initial inoculum density of *D. dipsaci* on crown rot symptoms was also evaluated. Sugar beet seeds of the *cv. Dorena* which is highly susceptible to *D. dipsaci*, were planted in 3 cm diam. plastic pots filled with 50 cm^3 of the previously mentioned substrate. Pots were kept in a growth chamber at 20 ± 1°C with a 16 h light period and were watered daily. Two weeks after planting, 10, 20, 50, 100 or 200 *D. dipsaci* were inoculated onto the seedlings by placing 10 *µ*l of the nematode suspension in the leaf axils of the first pair of true leaves with the aid of a pipette. Nematodes were inoculated in a suspension containing 1% (w/v) carboxymethyl cellulose as gelling agent as described in chapter 2. Plants were covered with a plastic hood to ensure 95 % relative humidity to prevent drying of the inoculum

droplet and to favour nematode penetration. After a period of 4 days the seedlings were transferred to 2 L pots filled with non sterile pot soil and placed in a greenhouse at 25 ± 5°C. Pots were irrigated daily and fertilized weekly with 10ml 14-10-14 N-P-K fertilizer solution (2g/L). To determine nematode damage, plants were rated 14, 27, 41, and 54 days after inoculation. The rating ranged from 0 = no symptoms, 1 = swelling of the stem base, 2 = strong swelling of the stem base, 3 = first rot symptoms to 4 = plant death. At termination of the experiment nematode density in the plant tissue was evaluated. The beets were cut in 0.5 cm thick slices and placed on a modified Baermann funnel for 16h (Hooper et al.,2005). Plant and the beet fresh weight were also measured.

The one way ANOVA and Tukey test were used for the comparison of symptoms 14 days after inoculation and Kruskal-Wallis test and all pair wise multiple comparison procedure (Dunns method) to compare the development of symptoms 27 and 41 days after inoculation. Nematode penetration from soil was conducted using the non-parametric Kruskal-Wallis test and the Tukey test for all pair wise multiple comparison procedure.

3.3 Results

Climate chamber screens to detect resistance to *D. dipsaci* penetration (2004)

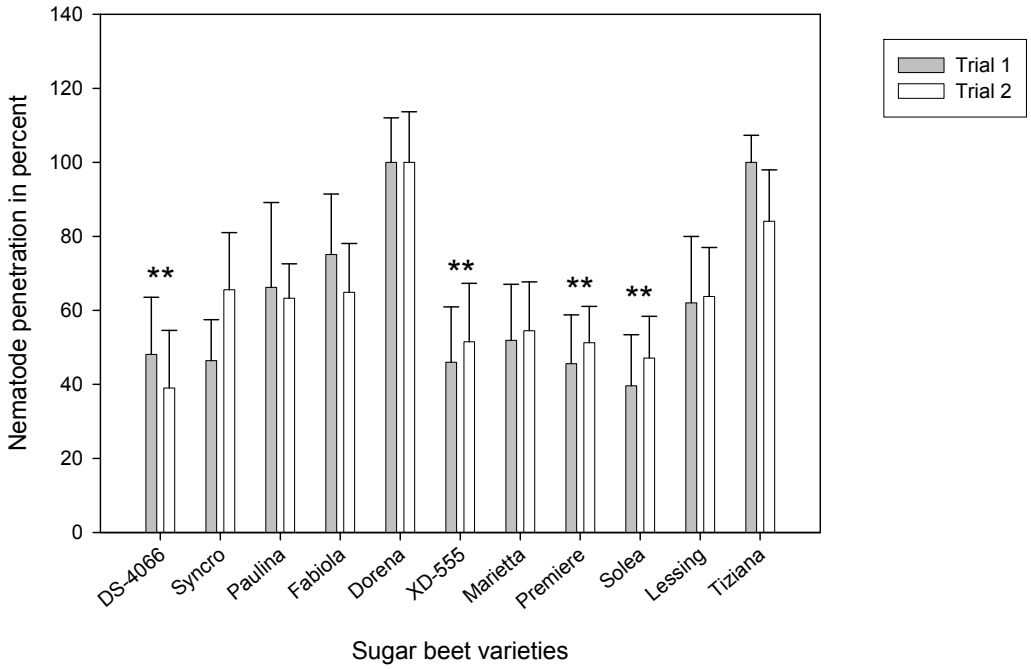

Fig. 3.3 a *Effect of sugar beet varieties and breeding lines on* Ditylenchus dipsaci *penetration compared to the susceptible control Dorena (100%). Vertical lines represent standard error of the means. ** significantly different to control at P<0,01 (n=20).*

The penetration rates of *D. dipsaci* showed significant differences depending on the sugar beet variety or breeding line tested (figure 3.3.a). When compared to the highly susceptible control Dorena, 4 out of 10 tested varieties showed significantly lower penetration rates of *D. dipsaci*. A reduction in penetration of 50% and more compared to Dorena was achieved by the varieties Premiere and Solea and by the line DS-4066. The variety Paulina (KWS, Einbeck, Germany) which is resistant to the sugar beet cyst nematode *H. schachtii* reduced *D. dipsaci* penetration 35% compared to Dorena.

However, the test system did not prove to be completely reliable since nematode penetration rates varied highly between the trials. In the first trial a mean of 15 nematodes per plant were detected in the 110 plants sampled whereas in the second trial this value almost doubled, to 27.

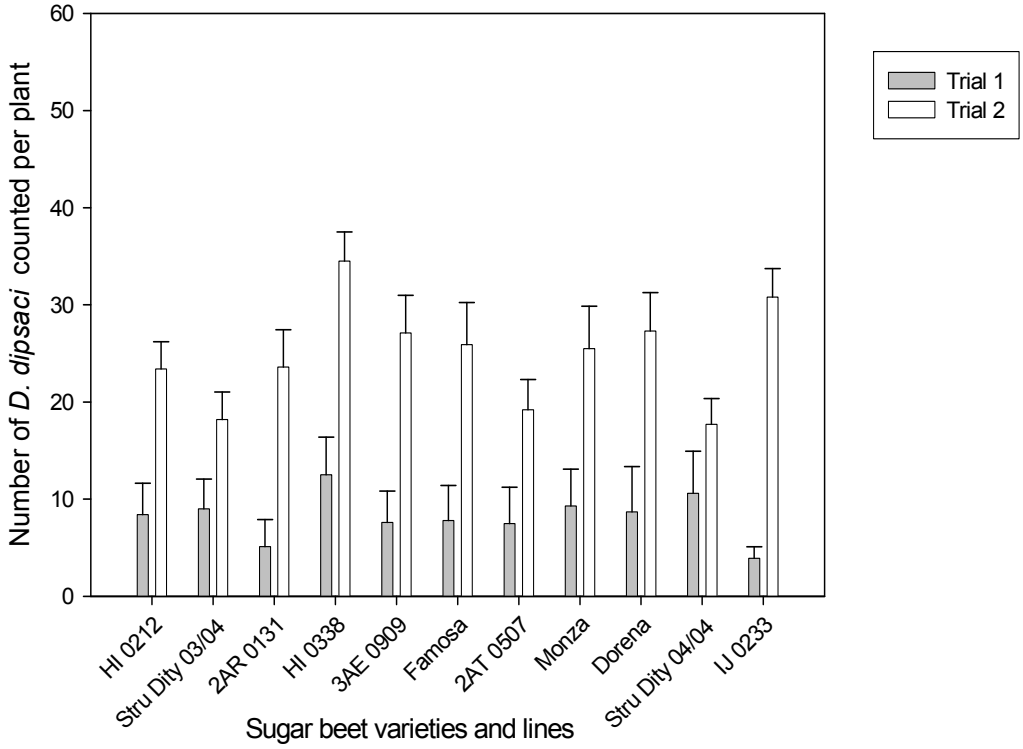

Fig. 3.3.b *Effect of sugar beet varieties and breeding lines on* Ditylenchus dipsaci *penetration compared to the susceptible control variety Dorena. Vertical lines represent standard error of the means.*

The effects of environmental factors on *D. dipsaci* plant penetration were significant. In 2004 a total of 22 vatieties and lines were screened in two different trials with 11 treatments. The second part of this screening (Fig. 3.3.b) was conducted separately from the first because of space and size of the trials.

Inoculation of the first trial failed due to dry conditions in the climate chamber, caused by technical problems. Therefore the data obtained was not

reproducible in by the second trial and was therefore excluded from further analysis.

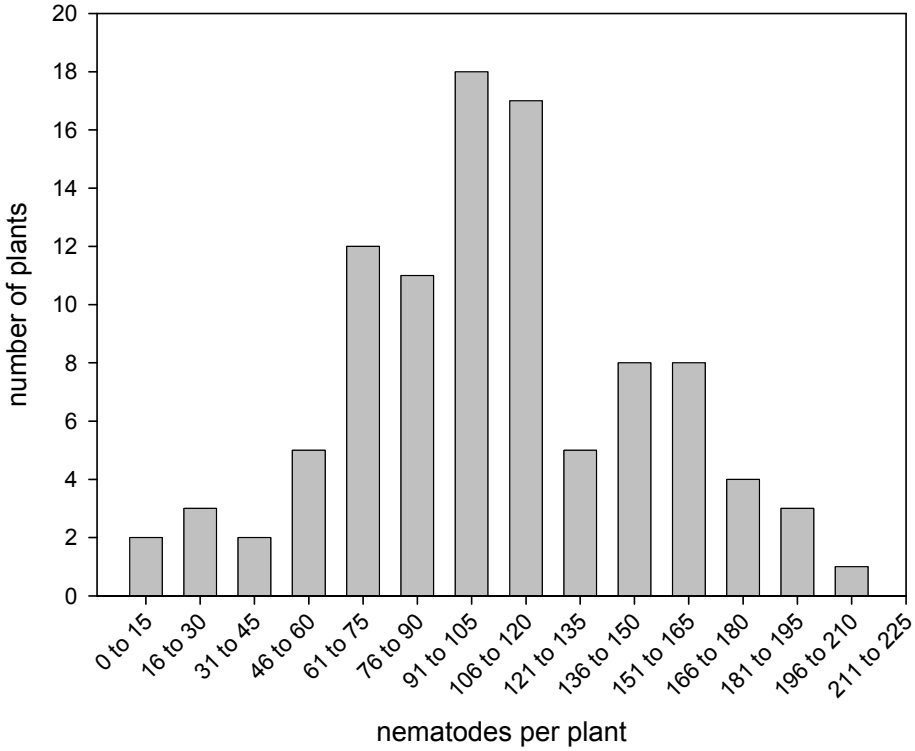

Fig. 3.3.c *Penetration rates of 200 inoculated Ditylenchus* dipsaci *per plant out of 100 inoculated seedlings*

In order to prevent high variation in the level of nematode penetration, the climatic factors were adjusted to ameliorate *D. dipsaci* penetration conditions. High air humidity between 98 and 100% prevented the drying of the inoculation droplet for at least 8h and enhanced nematode penetration. An apportionment of nematodes per plant close to the Gauss distribution was obtained by this modification.

Tab.3.2 *Person product moment correlation values of growth chamber screening data and the crown rot index of 11 sugar beet varieties in five field trials in the year 2004. Correlated* Ditylenchus dipsaci *values are percentages compared to the control variety Dorena.*

	Field A	Field B	Field C	Field D	Field E	Field F	
% Nematode penetration	0.673	0.287	0.646	0.551	0.275	0.543	r^2
	0.0233	0.393	**0.0317**	0.0787	0.413	0.0843	P
	11	11	11	11	11	11	n
Field A		0.664	0.868	0.69	0.715	0.921	r^2
		0.026	**0.000532**	**0.0187**	**0.0133**	**0.0000559**	P
		11	11	11	11	11	n
Field B			0.773	0.345	0.575	0.573	r^2
			0.00525	0.299	0.0645	0.0656	P
			11	11	11	11	n
Field C				0.61	0.618	0.688	r^2
				0.0463	**0.0427**	**0.0192**	P
				11	11	11	n
Field D					0.387	0.673	r^2
					0.24	**0.0232**	P
					11	11	n
Field E						0.666	r^2
						0.0253	P
						11	n

Among the 11 accessions of sugar beet germplasm lines and varieties tested, no resistant genotype was detected. None of the tested genotypes were able to prevent nematode penetration into young seedlings. However, 2 genotypes (DS–4066, XD–555) and 2 varieties (Premiere and Solea) significantly reduced nematode penetration following artificial inoculation of *D. dipsaci*. These germplasms were also able to reduce symptom expression under field conditions. Significant and positive correlations between rot symptoms occurring in tested fields and nematode penetration data were detected. The level of infection in the fields greatly influenced the level of correlation to the growth chamber screening data. The level of infection was evaluated according to the percentage of crown rot symptoms in the control variety Dorena. Therefore in 2004 the Fields A, B, C, D,E, and F had an average

crown rot development of 83, 52, 51, 37, 30 and 20% respectively. The most heavily infested field A also had the highest correlation coefficient an r^2 value of 0,673 to the growth chamber screening. A highly significant correlation could be demonstrated between the penetration rates of artificially inoculated beet varieties under climate chamber conditions and the crown rot symptoms of naturally infested varieties in field trials. The low correlation to field B is due to asymptomatic rating results. In this field 4 varieties had a higher crown rot index than Dorena, probably due to the heterogenic spatial distribution of *D. dipsaci*. Also the correlation of field B to the other fields was low.

Climate chamber screening for reduction of *D. dipsaci* penetration (2005)

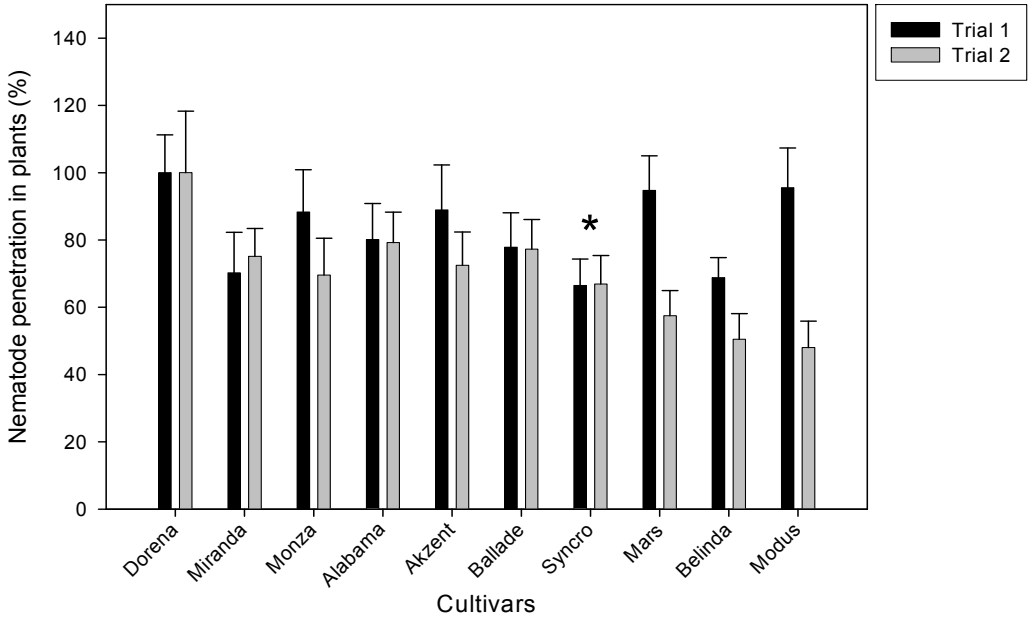

Fig. 3.4.a *Effect of sugar beet varieties on percent penetration of* Ditylenchus dipsac*i compared to the susceptible control variety Dorena. Vertical lines represent standard error of the means. * significantly different to control at P<0,05 (n=20).*

Out of the 2005 commercially grown tested sugar beet varieties invetigated, only the variety Syncro showed a significantly lower rate of nematode penetration (Fig 3.4.a). On average, when compared to the susceptible

control Dorena, penetration rates were lowest in both trials for the variety Syncro. Out of the 200 inoculated *D.dipsaci*, an average of 134 nematodes (100 %) penetrated the seedlings of the variety Dorena whereas 45 % less nematodes penetrated the variety Syncro.

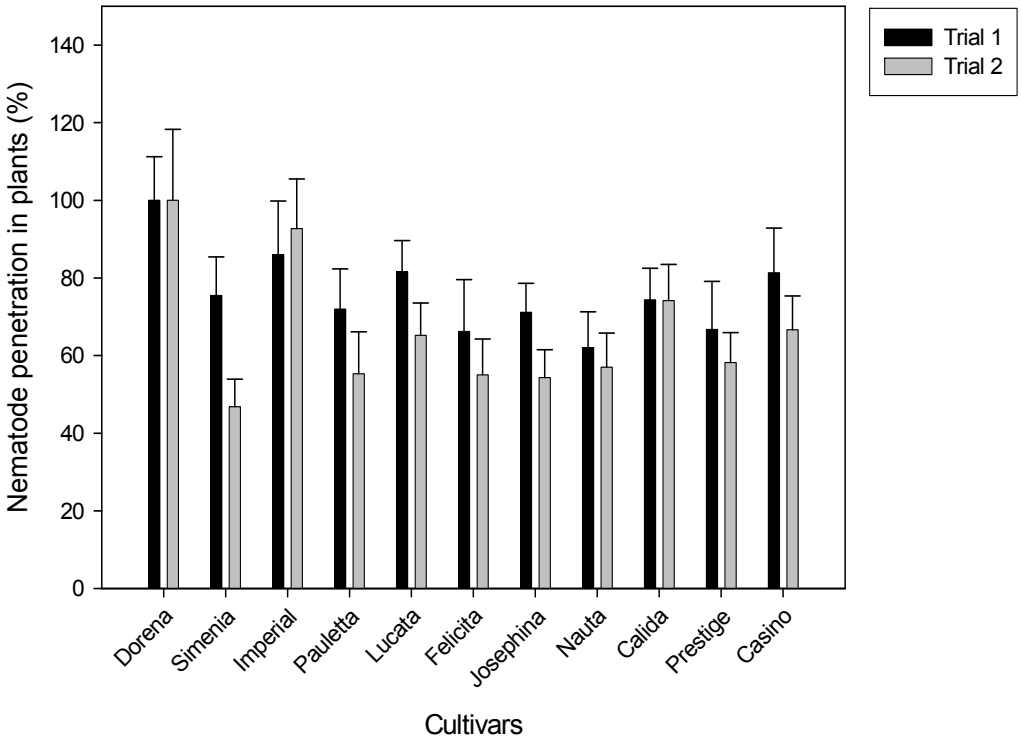

Fig. 3.4.b *Effect of sugar beet varieties on percent penetration of* Ditylenchus dipsac*i compared to the susceptible control variety Dorena. Vertical lines represent standard error of the means (n=20).*

Out of the screened varieties used in the trails that were commercially available to the growers in 2005, none was able to achieve a significant reduction of penetration rates (Fig.3.4.b). The variety Nauta caused a 40 % reduction in the penetration rate when compared to the susceptible control Dorena, but due to the non homogeneous distribution of the data this reduction was not significant.

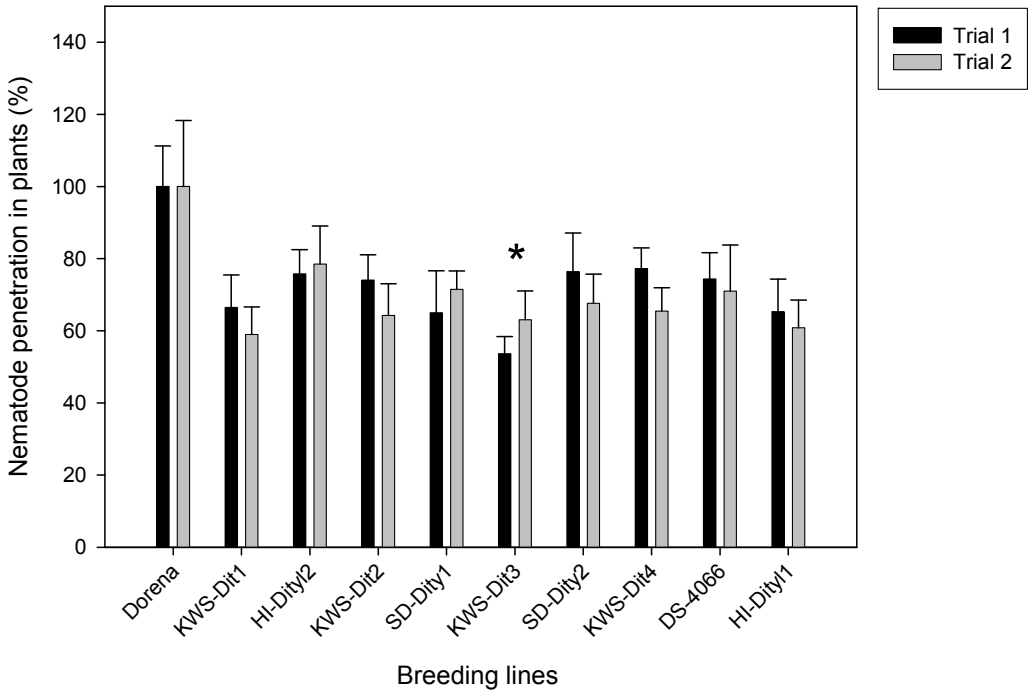

Fig. 3.4.c *Effect of sugar breeding lines on percent penetration of* Ditylenchus dipsac*i compared to the susceptible control variety Dorena. Vertical lines represent standard error of the means. * significantly different to control at P<0,05 (n=20).*

Out of the tested sugar breeding lines, only the variety KWS-Dit 3 caused a significantly lower rate of *_D. dipsaci* penetration (Fig 3.4.c). On average, penetration rates were reduced about 40% by this breeding line when compared to the susceptible control Dorena. Out of the 200 inoculated *D. dipsaci*,

and average of 134 penetrated the seedlings of the variety Dorena whereas only 66 nematodes were recovered from the breeding line KWS-Dit 3, respectively.

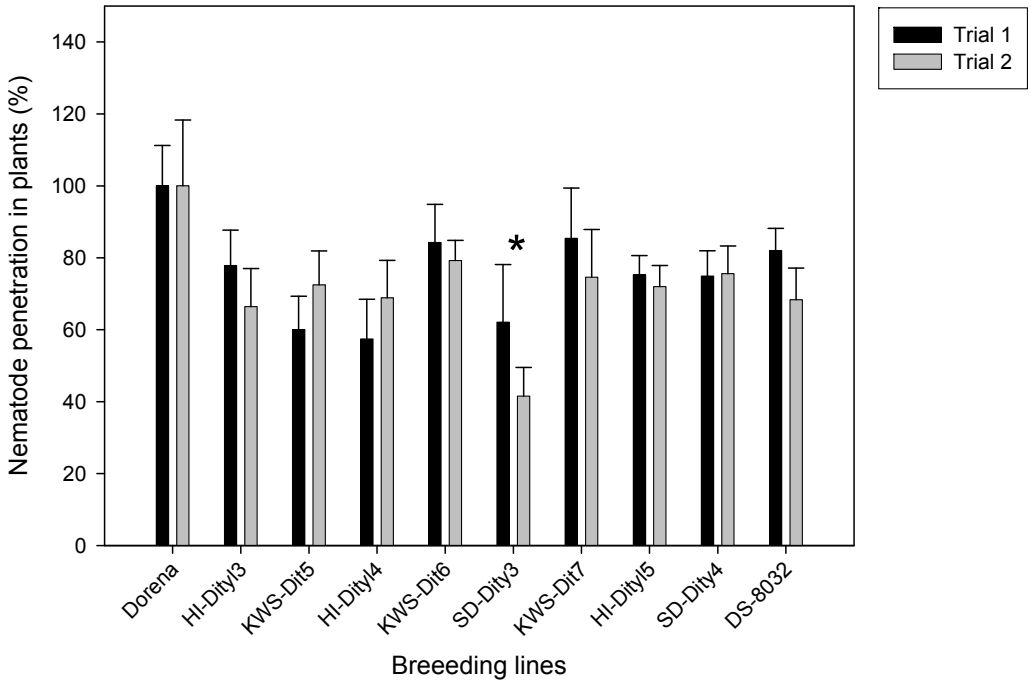

Fig. 3.4.d *Effect of sugar breeding lines on percent penetration of* Ditylenchus dipsac*i compared to the susceptible control variety Dorena. Vertical lines represent standard error of the means. * significantly different to control at P<0,05 (n=20).*

Out of the 9 sugar beet breeding lines tested, only the line SD-Dity 3 showed a significant reduction of nematode penetration rates (Fig 3.4.d). Penetration was reduced 50% when compared to the susceptible control Dorena with this breeding line. Out of the 200 inoculated *D. dipsaci*, an average of 134 nematodes penetrated the seedlings of the variety Dorena verses an average of 59 nematodes in the breeding line SD-Dity 3 respectively.

In the year 2005, 38 varieties and lines were screened in the bioassay for resistance to *D. dipsaci*. The experimental conditions were optimised for higher penetration rates by preventing drying of the inoculum droplet. The time frame available for nematode penetration was at least 8h. Out of the 38 varieties tested, only 3 showed significantly lower penetration rates when compared to the susceptible control Dorena. On average, the variety Syncro and the lines KWS-Dit3 and SD-Dity3 reduced the penetration rates of *D.*

dipsaci by 33, 41 and 49% respectively compared to Dorena. All other tested materials showed no significant difference to the susceptible control.

Greenhouse screens for resistance towards *D. dipsaci* (2005)

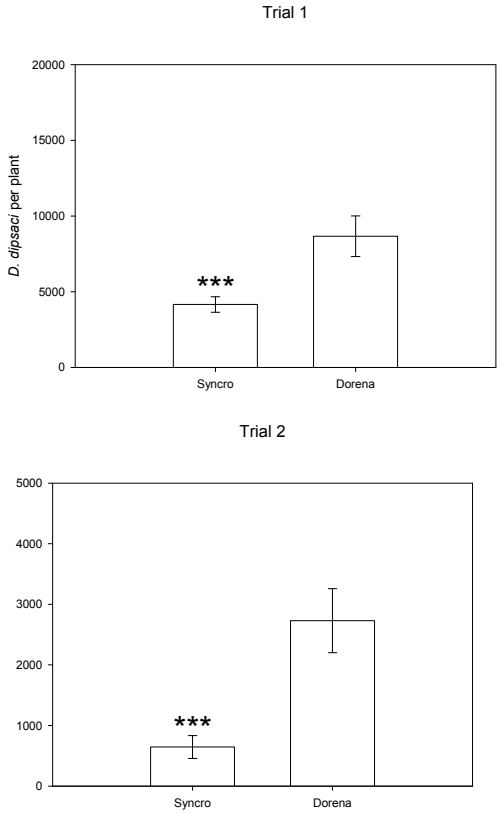

Fig. 3.5 *Reproduction per plant of Ditylenchus dipsaci on the tolerant sugar beet variety Syncro and the susceptible variety Dorena under greenhouse conditions 50 days after inoculation with f 200 nematodes onto two week old seedlings. Vertical lines represent the standard error of the means. *** indicate significant differences between varieties (P=<0.001). Data were pooled from 2 experiments (n=60).*

Reproduction rates of *D. dipsaci* differed significantly between the tested varieties Dorena and Syncro (Figure 3.5). The high yielding and susceptible variety Dorena supported up to 22850 nematodes per plant 50 days after inoculation, which corresponded to a reproduction factor of greater than 100. On average, reproduction rates in the first trial were 43.3 for Dorena and 20.8

for Syncro, respectively. During the second experiment extremely high temperatures had occurred in the greenhouse. The critical daily mean temperature of 27°C, where *D. dipsaci* stops to reproduce, was exceeded during 14 days. Therefore, the reproduction rates were lower when compared to the first experiment and ranged from 13.6 for Dorena to 3.2 for Syncro. Nevertheless, in both experiments the variety Syncro showed significantly lower reproduction rates compared to the susceptible variety Dorena.

Tab. 3.3 *Effect of inoculation of 200* Ditylenchus dipsaci *two weeks after sowing on fresh weight of two sugar beet varieties 50 days after inoculation compared to non-inoculated control. Vertical lines represent standard error of the means. (n=10).*

	Non iculated			Inculated		
	Total plant (g)	Root (g)	Shoot (g)	Total plant (g)	Root (g)	Shoot (g)
Syncro	21.93	6.15	15.78	23.94	5.87	18.07
Dorena	29.02	9.54	19.48	25.69	9.03	16.66

Nematode inoculation had no negative effect on plant growth parameters 50 days after inoculation. No significant differences between the varieties Dorena and Syncro were found when the effect of *D. dipsaci* on plant growth parameters was evaluated under semi-sterile growth conditions. Pustules and rot symptoms occurred due to *D. dipsaci*, but were in an early stage and therefore had no significant impact on plant fresh weight. On average, the variety Dorena produced a significantly greater amount of biomass when compared to Syncro.

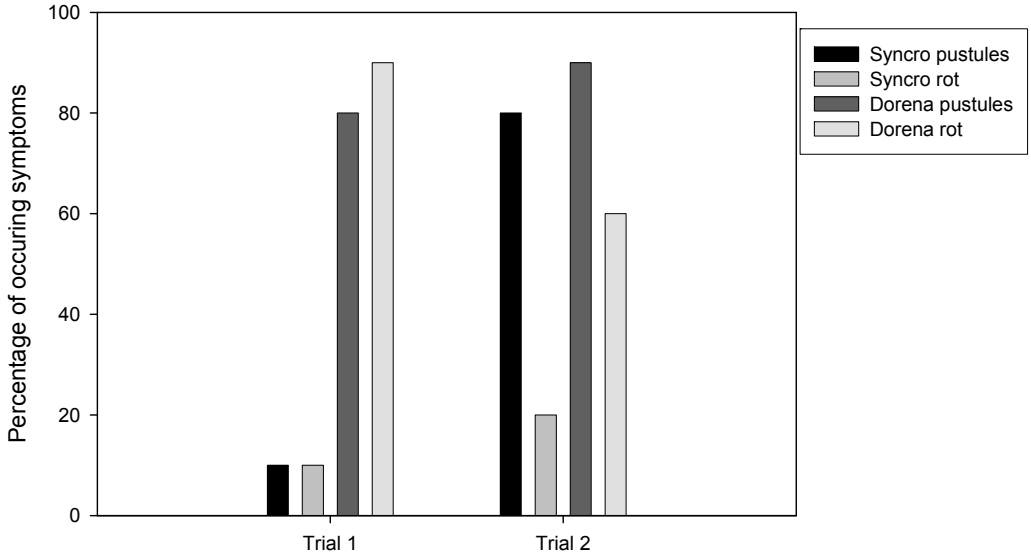

Fig. 3.6 *Effect of inoculation of 200 L4 and adult stages of* Ditylenchus dipsaci *on symptom development of two week old seedlings of the sugar beet varieties Syncro and Dorena 50 day after inoculation. Columns: Percentage of beets showing symptoms of pustules and crown rot. (n=10, binary decision if symptoms are present).*

In trial 1, the variety Syncro showed a significantly lower percentage of beets with pustules symptoms when compared to Dorena. In the second trial this observation could not be repeated. Conversely, the difference between the rot symptoms could be observed in both trials (Figure 3.6). Syncro showed only 10-20% percent beet rot symptoms as compared to 60-90% beets with crown rot in Dorena.

Greenhouse screens for resistance towards *D. dipsaci (*2006)

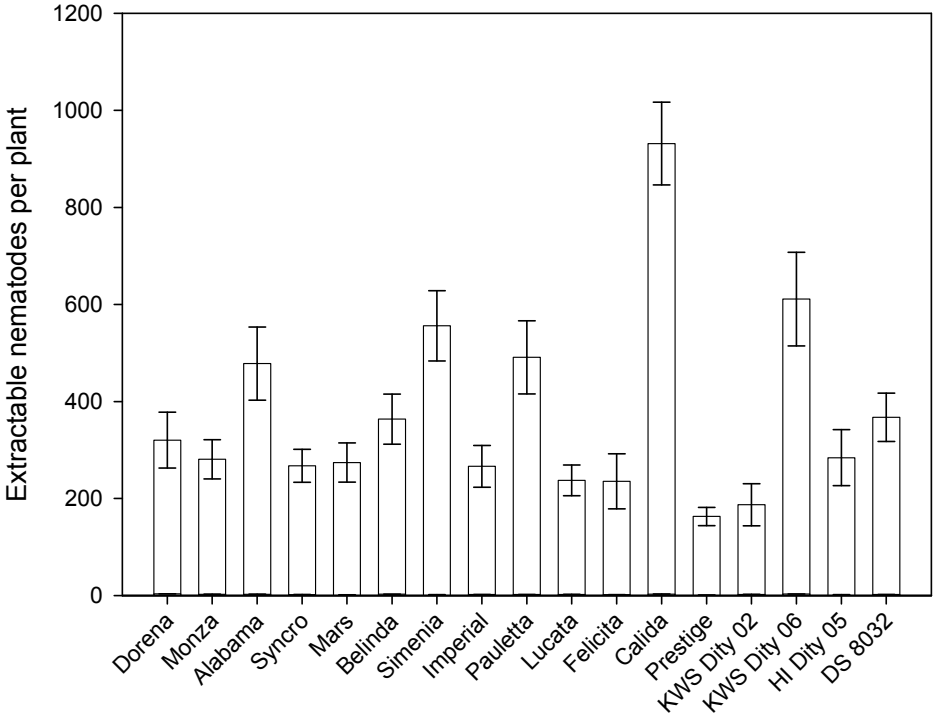

Fig. 3.7 *Effect of sugar beet varieties and lines on* Ditylenchus dipsaci *reproduction When compared to the susceptible control variety Dorena under greenhouse conditions. Vertical lines represent standard error of the means. (n=22).*

In greenhouse experiments, 17 out of the 38 varieties and lines that had been tested under field conditions in the year 2005 were also evaluated for their effects on nematode reproduction rates. The number of nematodes per plant varied highly between the two trials and therefore no clear assessment was possible. This was mainly due to the occurrence of rot symptoms. When *D. dipsaci* sense the presence of water soluble toxic metabolites in rotted tuber tissue they leave the feeding zone and egress to the soil. Therefore varieties with high expression of rot symptoms like Dorena showed significantly lower amounts of extractable nematodes per plant.

Assessment of tolerance toward *D. dipsaci* (2006)

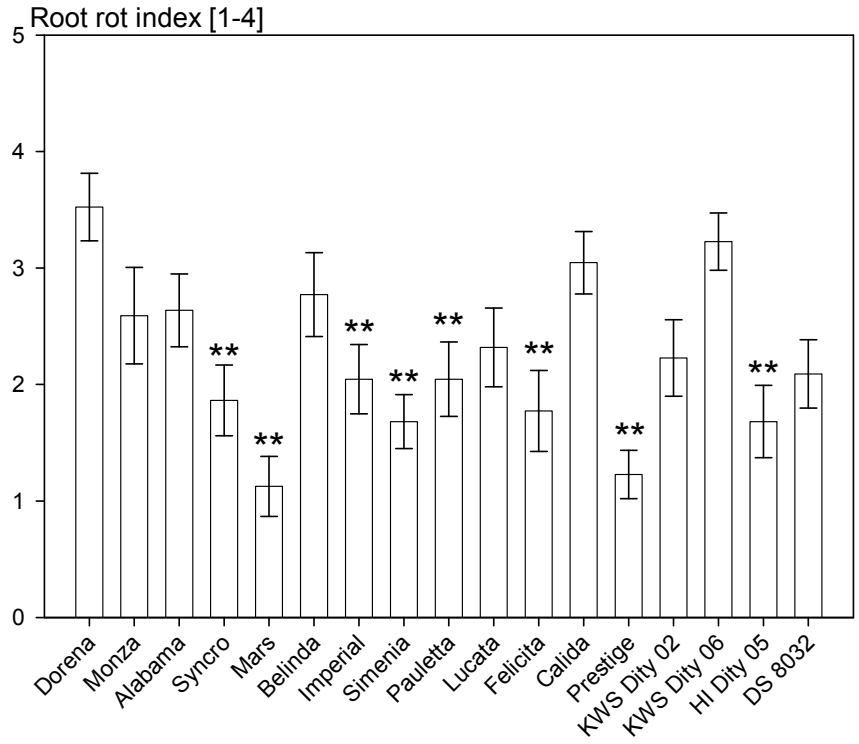

Fig. 3.8 Ditylenchus dipsaci *crown rot development on sugar beet varieties and breeding lines compared to the susceptible control variety Dorena under greenhouse conditions. Vertical lines represent standard error of the means. ** significantly different to Dorena at P < 0,01 (n=22).*

Significant differences in physiological reaction to *D. dipsaci* were detected. The varieties Prestige and Mars reduced disease expression up to 70% compared to Dorena.

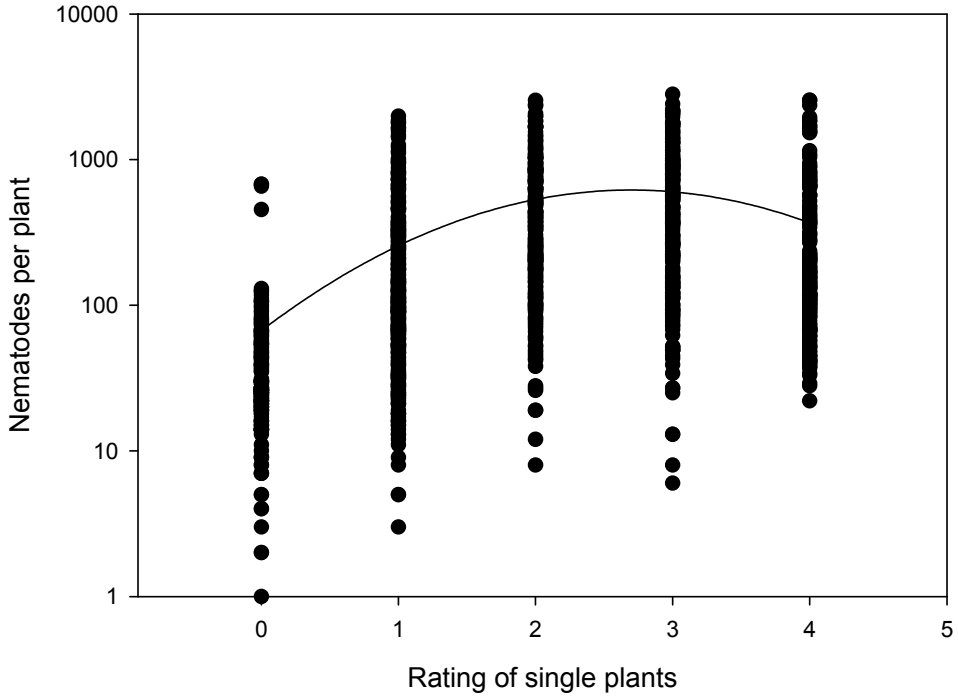

Fig. 3.9 Correlation of nematode numbers per plant with the rating of crown rot index under greenhouse conditions of 17 different varieties (R^2 = 0,151).

The rating index of the single plants had a higher impact on the number of extractable nematodes than the different genetic background. Plants with high expression of symptoms showed decreasing *D. dipsaci* numbers. Therefore, misleading results would be obtained if only the number of nematodes is considered in determining the level of resistance.

Tab. 3.4 *Correlation of growth chamber results on* Ditylenchus dipsaci *penetration (exp.2) and mean rating (exp.3) data of the crown rot index in the field trials in 2005 (n=17)*

	Penetration mean	Penetration median	Field A	Field B	Field C	Field D	Field E	
Rating mean	0.535	0.452	0.682	0.619	0.458	0.621	0.305	r^2
	< 0.05	**0.068**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	P
	17	17	216	216	184	129	80	n
Penetration mean		0.853	0.591	0.525	0.342	0.466	0.0837	r^2
		< 0.01	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**0.46**	P
		17	216	216	184	129	80	n
Penetration median			0.611	0.561	0.404	0.469	0.0933	r^2
			< 0.01	**< 0.01**	**< 0.01**	**< 0.01**	**0.411**	P
			216	216	184	129	80	n
Field A				0.499	0.332	0.543	0.386	r^2
				< 0.01	**< 0.01**	**< 0.01**	**< 0.01**	P
				216	184	129	80	n
Field B					0.465	0.308	0.357	r^2
					< 0.01	**< 0.01**	**< 0.01**	P
					184	129	80	n
Field C						0.44	0.239	r^2
						< 0.01	**0.0657**	P
						109	60	n
Field D							0.137	r^2
							0.225	P
							80	n

The evaluation of two different standard assay screening methods by comparing them with data obtained under field conditions clearly demonstrated (Tab. 3.4) that the rating index showed a closer correlation to field data in any field than the count data of penetrated nematodes. The mean rating was also significantly correlated to the field assessments in each field. The mean rating under greenhouse conditions produces more reliable data than field trials, which is shown by the fact, that the field data was not significantly correlated among themselves (especially field E).

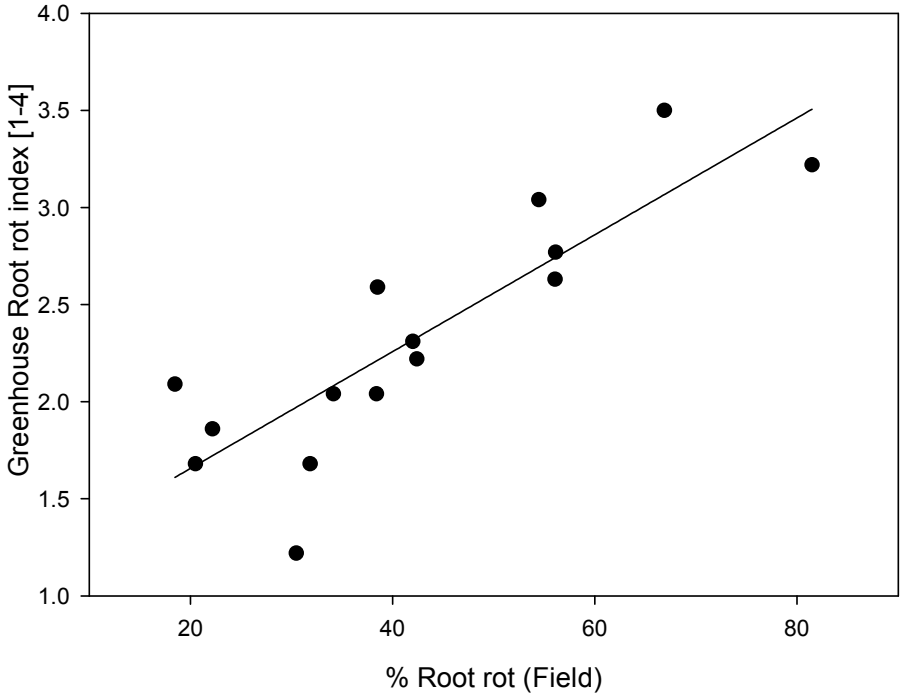

Fig. 3.10 *Correlation between crown rot symptoms from field trials and the crown rot index under greenhouse conditions of 15 different varieties (R^2 = 0.727).*

In the greenhouse test 17 previously field tested varieties were examined according to the here developed rating index (Fig. 3.1.b). Since two varieties showed an asymptomatic high variance of rot expression under greenhouse conditions, they were excluded from this analysis.

Plants raised under controlled greenhouse conditions showed a physiological reaction to *D. dipsaci* inoculation similar to that of plants sown in infested fields. Damage levels occurring at harvest could be simulated by artificial inoculation under greenhouse conditions. Furthermore, the greenhouse rating results were already obtained 50

days after inoculation, due to the high inoculum density used. A clear correlation (R^2 = 0,727) could be demonstrated between the mean crown rot index obtained by artificial inoculation and the mean symptom rating of greenhouse trials.

Investigation on infection behaviour and disease development

Effect of seedling age and time of nematode infection on disease

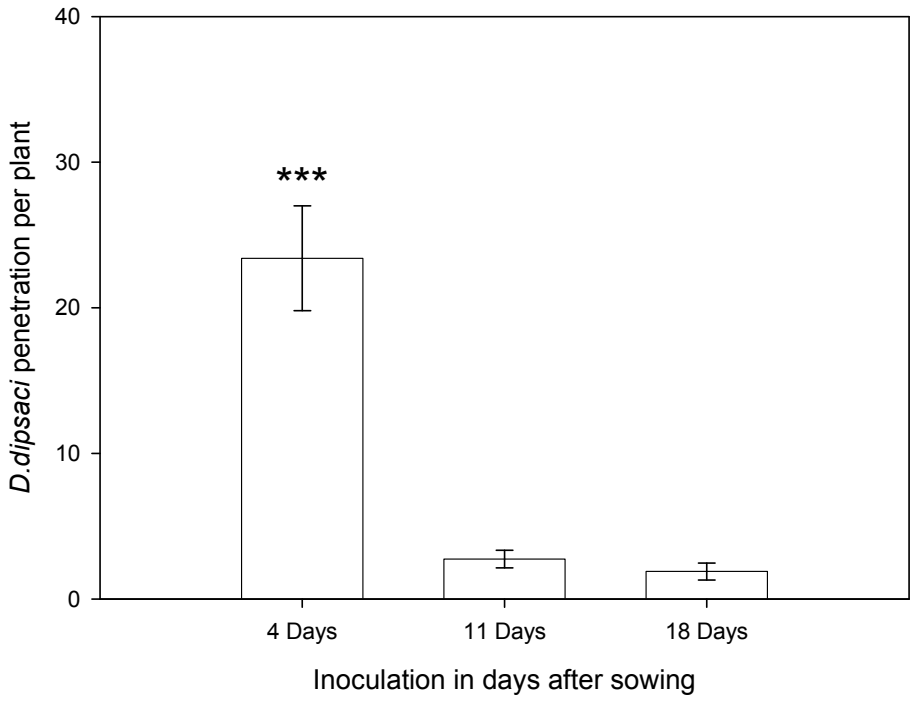

Fig. 3.11 *Penetration of* Ditylenchus dipsaci *into sugar beet seedlings after inoculation at day 4, 11 or 18 after sowing. Penetration rate was recorded 14 days after inoculation. Vertical lines represent standard error of the means. *) significant different at P < 0.001 using non parametric Kruskal-Wallis test and all pairwise multiple comparison procedure (Tukey Test), (n = 35).*

D. dipsaci was detected in all sugar beet seedlings regardless of the inoculation time when inoculated with 1000 nematodes at different times after sowing, (Figure 3.11). When nematodes were inoculated 4 days after planting, and the seedlings were still under the soil surface, the number of *D.*

dipscaci per plant was significantly higher compared to later inoculation times (P ≤ 0,001). No significant difference was observed between penetration rates, when inoculation took place 11 or 18 days after sowing. At these time points cotyledones of the seedlings already emerges above the soil surface.

Effect of inoculum density on disease

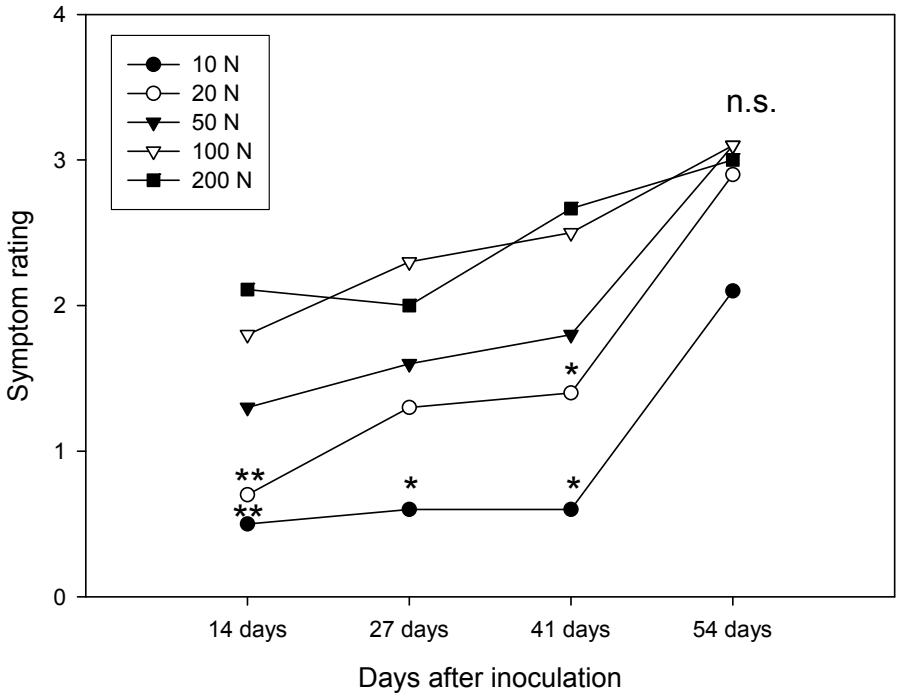

Fig. 3.12 *Effect of increasing inoculum density on the development of crown rot symptoms in sugar beets induced by* Ditylenchus dipsaci. *Two asterisks indicate significant difference using parametric 1-way ANOVA (P < 0.01). *) significant different according to Kruskal-Wallis test (P < 0.05).Legend: Number of inoculated nematodes per plant (n = 10).*

The inoculum density of *D. dipsaci* had a significant effect on crown rot symptoms development when evaluated 14, 27 and 41 days after inoculation (Figure 3.12). Inoculum densities of 50 nematodes or higher resulted in a significant increase in crown rot symptom development. When measured 54

days after inoculation differences between the inoculum densities were no longer significant.

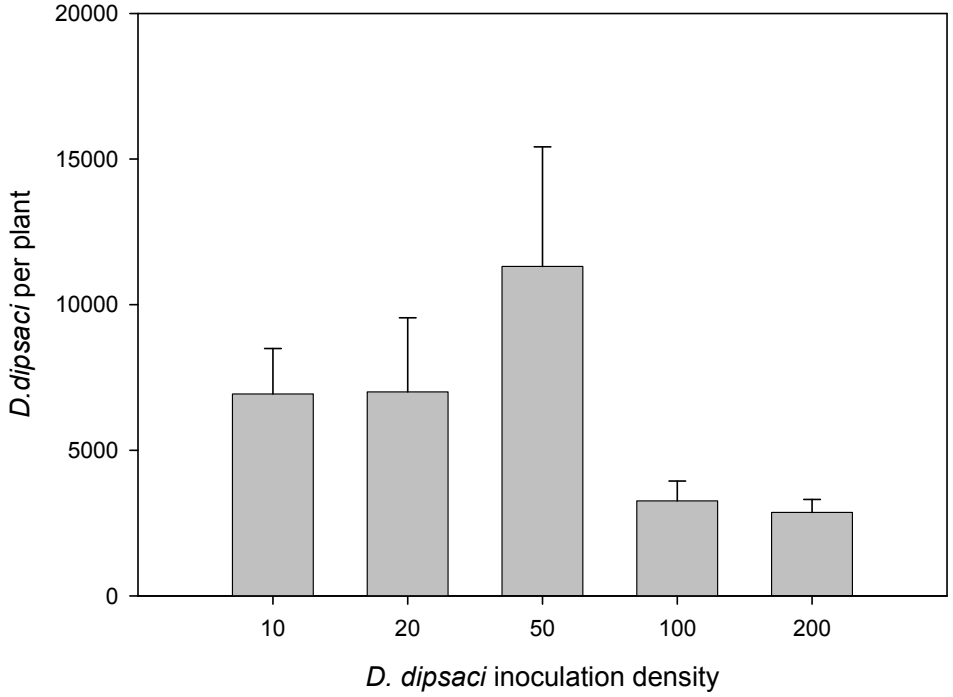

Fig. 3.13 *Effect of increasing inoculum density on the* Ditylenchus dipsaci *number in sugar beets 54 days after inoculation. Vertical lines represent standard error of the means (n = 10).*

Furthermore, the number of extractable *D. dipsaci* per beet, 54 days after inoculation, were not significantly different regardless of the initial inoculation densities. The highest number of extractable nematodes was obtained when the initial inoculation density was 50 nematodes per seedling.

A mean of 11.315 *D. dipsaci* was recorded when the treatments were pooled. At inoculation densities of 100 and 200 nematodes per seedling the lowest number were extracted but they also showed a higher expression of rot symptoms.

3.4 Discussion

In the present study different screening systems were applied and evaluated to improve detection of sugar beet plant resistance to *D. dipsaci*. General differences occurred in the systems develop with regards to input of time, workload and monetary costs. The development of a standard method for a high throughput screening method was the main target of this research program. An important consideration was to use standardized conditions and commonly used materials so that the methods could easily be repeated by any other sugar beet breeding or research program. The results obtained clearly demonstrate that short-term greenhouse testing reliably reflects the data obtained under field conditions.

Climate chamber screens to detect resistance to *D. dipsaci* penetration

In the present study, the interrelationship between *D. dipsaci* and different sugar beet varieties and breeding lines was analysed. As the most critical hurdle limiting stem nematode infection is juveniles penetration into the plants, an artificial inoculation methods were established and the number of nematodes from a defined inoculum that were able to penetrate the plants was used to identify resistance at this early stage of plant nematode interaction.

The growth chamber tests conducted in 2004 yielded a significant reduction in nematode penetration rates in 4 out of 11 tested sugar beet varieties and breeding lines when compared to the susceptible control variety Dorena.

The physiological differences of the varieties that reduced or enhanced nematode penetration needs to be determined.

Other experiments (Figure 3.9 and 3.10) showed that the high yielding variety Dorena promoted nematode reproduction. Since the mean penetration rates

between the two trials varied, further improvements of the conditions affecting penetration were applied during the follow-up screening trials in 2005.

The drying out of the inoculum droplets could be prevented for at least 8h, by using plastic hoods and thereby giving the nematodes a longer time frame for penetration. However the utilisation of these optimized infection conditions not only led to an increase in nematode penetration but also to a decrease in the differences between the test varieties and lines. In 2005 only 3 out of the 37 tested varieties showed significantly reduced penetration rates compared to the susceptible control variety Dorena.

This indicated that there may be a maximum number of nematodes that can penetrate each seedling. This hypothesis is underlined by the fact that the mean number of nematodes per plant in the variety Dorena did not augment as much as the other test varieties and lines between the two repetitions of the trials. Since the number of nematodes per plant in Dorena seedlings was already high in the first experiment, the amelioration of environmental factors influencing penetration in the second trial did not lead to the same percent increase of nematode per plants as in tolerant varieties.
This raised the following questions: 1) how do the physiological differences observed in experiment one correlate with nematode symptoms under field conditions and, 2) is there a difference in the visible physiological reaction between varieties inoculated with high nematode numbers?

The inoculation method developed that used very high *D. dipsaci* pressure could still be used in order to evaluate the host range of this beet nematode population, especially regarding other crops to be tested for use in intercropping and crop rotation systems.

Greenhouse screens for resistance towards *D. dipsaci*

In the greenhouse experiments conducted under partial sterile conditions, *D. dipsaci* penetration did not affect plant growth parameters of sugar beet for up to 50 days after inoculation. Therefore plant-growth parameters can not be used as indicators to determine nematode infestation or the presence of resistance. Although swelling and distortion of the first pair of true leaves occurred the symptoms disappeared due to compensatory growth of the beets. Also under field conditions early infections are often not recognized and damage dimension is only recognized prior to harvest yielding (Leipertz, 2005, Niere et al., 2006). This underlines the statement that damage caused by this nematode is mainly due to increased secondary infections, which are stimulated by initial nematode damage to plant tissue (Wallace, 1962). Nematode feeding and damage to the sclerenchyma seems to open a pathway for secondary fungal and bacterial pathogens. The variety Syncro was selected for these experiments because it produced the lowest level of disease symptoms under field conditions. This variety supported 58% or 42% significantly lower rates of nematode reproduction when compared to Dorena. However, reproduction was not completely inhibited. The reproduction rate between the repetitions varied, due to extremes in environmental conditions occurring between the two trials. During the second trial the mean daily temperature exceeded 27°C on 13 days, which lead to a cessation of *D. dipsaci* reproduction (Griffith et al., 1997). The higher temperature during the second trial might be a reason for the high increase of pustule formation observed for the variety Syncro. This would affirm that pustule formation is an enzymatic, temperature dependent process induced by salivary secretion of *D. dipsaci*. However, under field conditions with a growing period from the end of April until September/October both tested varieties could strongly enhance the nematode infestation of field soil.

Assessment of tolerance toward *D. dipsaci*

Other authors already reported a correlation between symptoms produced and the reproduction rates of *D. dipsaci* on other crops (Sharma et al., 1994).

Their study strongly supports the argument for the need and use of greenhouse screening for *D. dipsaci* breeding programs. The presented data demonstrated clearly that resistance screening systems can be based on the rating of symptoms caused by the nematode. The highest correlation was detected between symptoms in greenhouse tests with the data from field tests under high nematode infestation pressure. Replacing the field trials with the newly developed greenhouse rating system reduces the time frame and the costs of screening in the field. Laboratory work caused by extracting and counting nematodes can be eliminated and therefore a higher number of varieties and lines can be screened for resistance.

One of the main advantages of greenhouse screening is that results are available within 68 days after sowing of the test lines. Therefore more than 5 selections can be carried out in one year, while field testing would only allow one cycle. Crown rot symptoms occurring under greenhouse conditions were the most suitable approach in rating resistance or susceptibility to the stem nematode. Non inoculated controls never showed symptoms similar to those produced in the presence of nematode infection.

Field screening for resistance

In both years, a significant correlation between symptoms produced in the greenhouse and those observed in the field symptoms was demonstrated. The correlation was highest between the growth chamber rating data 54 days after inoculation and fields with high levels of *D. dipsaci* crown rot. Due to the heterogeneous spatial occurrence of *D. dipsaci* and differences in environmental conditions like humidity and temperature, field data can vary from field to field and from year to year (Plowright et al., 2002). The results obtained in the present investigations demonstrated that it is feasible to transfer the entire screening procedure from extensive field trials to controlled conditions in the greenhouse. Factors responsible for variation like climatic conditions, soil heterogeneity and varying pre-plant densities of *D. dipsaci*,

which always occur during field trials, can be excluded using the developed screening protocol.

Investigations on infection behaviour and disease development

Lower penetration rates were detected when *D. dipsaci* was inoculated 11 days after planting, when seedling emergence took place instead of 4 days after planting, when the seedling remained in soil. This data indicates that the main factors influencing the expression of symptoms under field conditions are weather conditions and initial distribution of *D. dipsaci* during the time period in which the seedlings remain under the soil surface. Therefore wet conditions at planting, enabling stem nematode juveniles to penetrate the seedling, will be favourable for infection and disease. Planting after recent rainfall or before expected rain should be avoided if possible.

Nematode population development in a sugar beet plant was exponential and mainly determined by available nutrient and temperature. Since *D. dipsaci* has up to 9 life cycles per year in perennial crops (Griffith et al., 1997) and damage is highly influenced by environmental factors a correlation between the initial infestation and damage can be low. Similar observations were made by Palo (Palo, 1962), who found a positive correlation between the initial inoculum density of *D. dipsaci* and reproduction rates. Nematode development and reproduction is linearly related to the temperature under optimal plant growth conditions (Griffith et al., 1997). Regarding these facts it seems to be a binary situation in the brief time frame after sowing which decides about the infection of single beets. One gravide *D. dipsaci* female, successfully invading the beet at the beginning of the growth season therefore would be able to cause strong crown rot symptoms by the time of harvest. Regarding this brief time frame, chemical control by seed coating or granulates with nematicidal or nematostatical active ingredients could prevent effective protection of the young seedling. The penetration of juveniles could

be inhibited and, thus, the nematode damage and reproduction potential significantly diminished.

3.5 General conclusions

Normal nematode management strategies are based on several approaches to reduce nematode impact under the economic threshold. Avoiding host crops in the crop rotation, or extending the time frame between susceptible crops, to keep nematode densities at a non yield diminishing level is normally the most sustainable management tool. However *D. dipsaci* seems to be adapted to these approaches. The nematode is heterophagous and can reproduce on many plant species including many common weeds like *Galium aparine, Polygonum convolvulus, Matricaria chamomilla, Veronica chamaedrys Capsella bursa-pastoris Alopecurus myosoroides Agropyron repens, and Geranium pratense* (Knuth, 2005). The nematode can survive on dried plant material under hot dry growing conditions until the next crop is planted. It also penetrates new seedlings quickly before they emerge out of the soil, thereby avoiding parasitism by microbial antagonists. Also a distribution by wind in light dried plant material might be possible. For these reasons *D. dipsaci* does not need sugar beets alone as a host crop and vice versa will not be eradicated even if the field is not planted with beets for decades. In addition, the nematodes reproduction capability enables apparently one gravid female invading the beet seedling to damage the whole plant.

All these biological characteristics of *D. dipsaci* lead to the conclusion that only two control tools have the potential to secure sugar beet production on infested fields. 1) resistance or tolerance of the planted varieties or, 2) chemical protection of the young seedling to prevent nematode penetration. Since no nematicides are currently registered in Germany for use in sugar beets, host resistance or tolerance is the only reasonable approach.

The results from the present laboratory tests on screening demonstrated that greenhouse screening can agree with results obtained from field tests.

Similiar results with screen systems were reported for other crops by (Sherwood et al., 1967, Sharma et al., 1994), Greenhouse screen was found to be more reliable and economical for determining resistance or tolerance to sugar beet in the present tests..

Using the here described screening procedures, a large amount of germplasm can be evaluated for sources of tolerance or resistance toward the stem nematode. In 1983 the Sugar Beet Germplasm Committee was founded and provides over 2500 accessions from within the genus *Beta* (Panella and Lewellen, 2007). Applying the screening system developed here would increase significantly the chances of detection of suitable genetic material which could be incorporated into high yielding lines. After several backcrosses, the yield potential of a wild species with nematode resistance can be improved (Simpson and Starr, 2001).

Applying resistant varieties is only acceptable if the damage threshold of a pest is exceeded, since their yield potential at below threshold levels does not equal that of high yielding varieties. However, the low threshold described by Seinhorst (1956) to be 10 *D. dipsaci* / 500g of soil and the results of this study show that inoculation density did not have a significant effect on symptom expression 54 days after inoculation.

The high yield loss potential of *D. dipsaci* literally requires that all infested fields should be planted with resistant or at least tolerant varieties.

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4 Spatial distribution of *Ditylenchus dipsaci* and *Heterodera schachtii* in sugar beet fields

4.1 Introduction

The stem and bulb nematode *Ditylenchus dipsaci* has recently caused increasing levels of damage in German sugar beet production areas (Kühnhold et al., 2006). Other European countries also have encountered a re-emergence of the pest (Castillo et al., 2007). The symptoms of damage occur at the end of the growing season without any premonition. Since nematicides are not presently registered for use in sugar beets in Germany, the farmers are facing potential yield losses of 50% and more (Schlang, 2003, Schlang, 2004). In addition *Heterodera schachtii* is still a major concern in european sugar beet production areas, even though management strategies like nematode resistant sugar beet varieties and intercrops are available. Important is the fact that yellow mustard (*Brassica alba*) the successful resistant intercrop used to reduce *H. schachtii* densities (Steudel and Müller, 1983), is a good host for *D. dipsaci* (Heinrichs, 2004).The occurring problems of *D. dipsaci* are serious and therefore new management strategies need to be designed quickly.

Nematodes generally should be a prime target for site specific control strategies because of their limited mobility and spread. The aggregated occurrence of cyst nematodes such as *Heterodera glycines* (Avendano et al., 2004c 2003, Nutter et al., 2002), *Globodera pallida* and *Globodera rostochiensis* (Evans et al., 2002)is well known. The aggregation behavior of other species is also well-investigated, but generally the main focus was to detect correlations of edaphic factors and nematode densities (Wallace et al., 1993, Koenning et al., 1996). The relation between the occurrence of *D. dipasci* disease symptoms on onions and clay content was already observed decades ago (Seinhorst, 1956).

The objectives of this investigation were to quantify the spatial heterogeneity of *D. dipsaci* and *H. schachtii* in the soil in the field. To evaluate the potential of site specific control of the collected data, the SADIE software (Spatial Analyses by Distance Indices) was used to identify spatial patterns (Perry, 1995, Perry, 1996, Perry, 1998).

4.2 Materials and methods

Spatial distribution

The fields surveyed in 2005 and 2006 were in Birgel, near Düren in North-Rhine-Westphalia, Germany. The fields where previously shown to be heavily infested with *D. dipsaci* and known to be infested with *H. schachtii* The fields were also chosen based on their flat topography. To reduce the influence of undesirable stress factors such as nutrient deficiency or weed infestation, all fields were commercially managed and treated uniformly with fertilizers and herbicides.

In June 2005 grid raster sampling was conducted in one field at intervals of 5 m along the rows and 5.4 m between the rows. The position of the sampling area was recorded using a GPS recorder. Soil characteristics were investigated by the contact free EM38 measurements of the apparent electrical conductivity (ECa) (Geonics Limited, Ontario, Canada), to obtain information about soil heterogeneity. The measurement was taken after heavy rainfall since EC_a has the greatest potential to differentiate between soils under moist conditions (Brevik, 2006, Mertens et al., 2008).

At each of the 120 sampling points three soil cores 20cm deep and 1.5 cm in diameter were taken and collected in plastic bags. *H. schachtii* and *D. dipsaci* densities were determined by extracting 100g of soil for each sampling point using a modified Baermann funnel technique (Hooper, 2005) for 16h at room temperature. Nematode numbers in this solution were counted twice under 100 fold magnification after dilution.

The position of the sampling points was recorded using a GPS recorder. To detect possible correlations between nematode numbers and soil properties the sensor EM 38 (Geonics, Ontario, Canada) was used. The sensor was hand pulled on a plastic slay with an interval of the measurements of 3

seconds. The obtained data was geographically referenced and interpolated using ordinary kriging.

The fields analysed in October 2006 were located in the same area and chosen according the previously mentioned factors. The spatial distribution and occurrence of crown rot caused by *D. dipsaci* was monitored in two infested fields. Three neighbouring sowing rows were examined over a distance of 50m and 60m respectively.

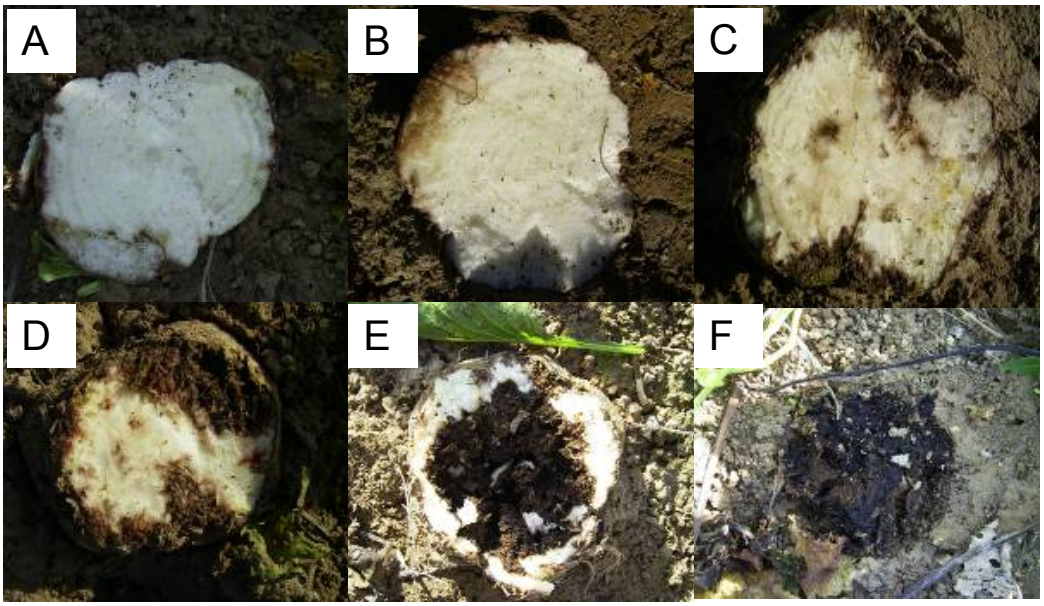

Fig. 4.1 *Disease severity ranking (A) 0%, (B) 20%, (C) 40%, (D) 60%, (E) 80% and (F) 100% rotten beet surface*

The crowns of the beets were cut 2-3cm above ground level. Images were taken with a GPS-linked camera. Each beet was examined and ranked according to the disease severity from 0-5 (no rot symptoms, 20%, 40%, 60%, 80% and 100% rotten beet surface respectively, fig. 4.1) in the field. The disease rating conducted in the field was re-evaluated by repeating the visual evaluation using the stored data. Only 1-2% of misclassification occurred when repeating the visual rating of the symptoms from the pictures taken.

To analyze aggregation and spatial correlation of the nematodes with soil properties, the SADIE- software was used (Perry, 1998, Perry, 1996, Perry, 1995). SADIE, the Spatial Analyses by Distance Indices, was developed to analyse spatial patterns of the individual count data. The program requires two dimensional arranged count data. The algorithm measures the minimal total distance that the observed individuals have to move to achieve hypothetical regular distribution within the investigated area. The higher the distance to regularity is, the more the individuals are aggregated. The index that gives an indication

how aggregated a data set is, is I_a (Index of Aggregation), which is defined by D (the observed distance to regularity) / E_a (the arithmetic mean distance to regularity for the observed values randomly distributed over the sampling grid coordinates). This value allows the comparison of different data sets. By definition values of $I_a > 1$ indicate aggregated pattern in data sets. In addition, the distance to regularity for each data point is given. The index v indicates if a point belongs to a cluster. Therefore it can have a positive (contributing to a patch) or a negative (contributing to a gap) value. The Probability P_a is defined as percentage of the randomisations that resulted in higher I_a than the original data.

The nematode count data and the cluster indices obtained by the SADIE software were displayed using GIS software (Arc-GIS 9.0, ESRI, Redlands, USA). Ordinary kriging was used to interpolate the cluster indices in order to indicate the spatial distribution of patches and gaps.

4.4 Results

4.4.1 Spatial Distribution

4.4.1.1 Soil samples

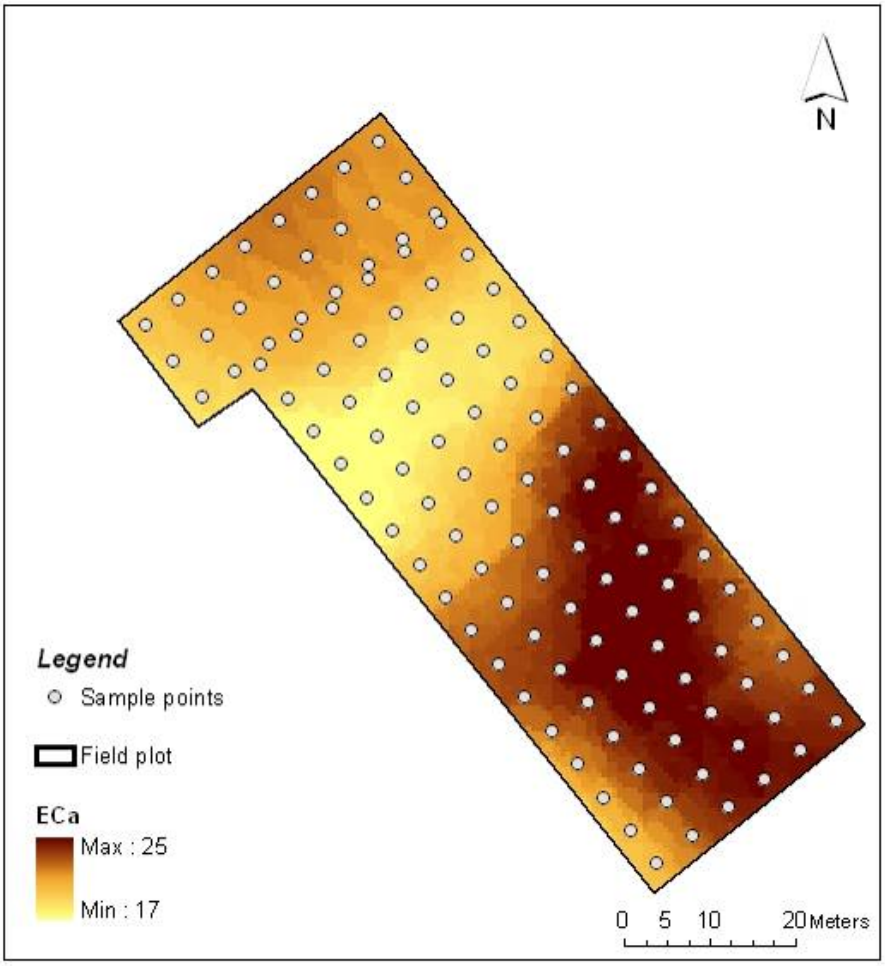

Fig. 4.2. *Sample points and a the apparent electromagnetic conductivity (EC_a) of the sampling area*

The EM38 measurements of the study plot showed only slight variability of the apparent electrical conductivity (ECa). All measured values ranged between 17-25 mS m^{-1} indicating a soil texture of loamy silt (Fig. 1).

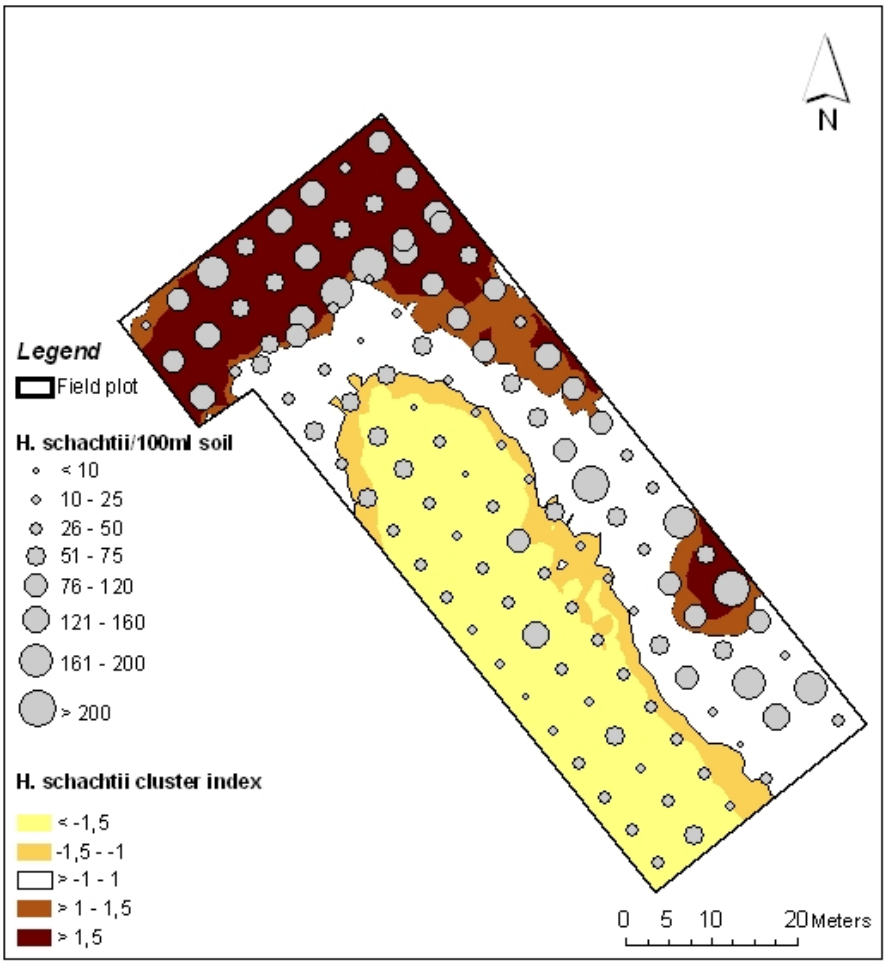

Fig. 4.3.a *Spatial distribution of* Heterodera schachtii *juvenile density counts from 120 sample points in a sugar beet field in Germany 2006 and the interpolated SADIE cluster indices*

The graduated symbols in figure 4.3a show categories of the numbers of *H. schachtii* in the soil samples collected at 120 sampling points in the field, as well as the interpolated cluster indices as derived by SADIE. The map shows that *H. schachtii* occurred at over 95% of all sample points. High number of nematodes, up to 265 juveniles per 100g soil, occurred at the northern and eastern part of the field. The SADIE analysis demonstrates that *H. schachtii* was generally found in clusters (values > 1.5). The observed spatial distribution of *H. schachtii* was aggregated with an I_a of 2.13 and a $P_a < 0.001$. In the investigated field only two large patches appeared. On the other hand

some sample points with high *H. schachtii* values were not considered as clusters (I_a> -1 - <1) or even as gaps (I_a<-1), because they were surrounded by lower numbers of nematode values.

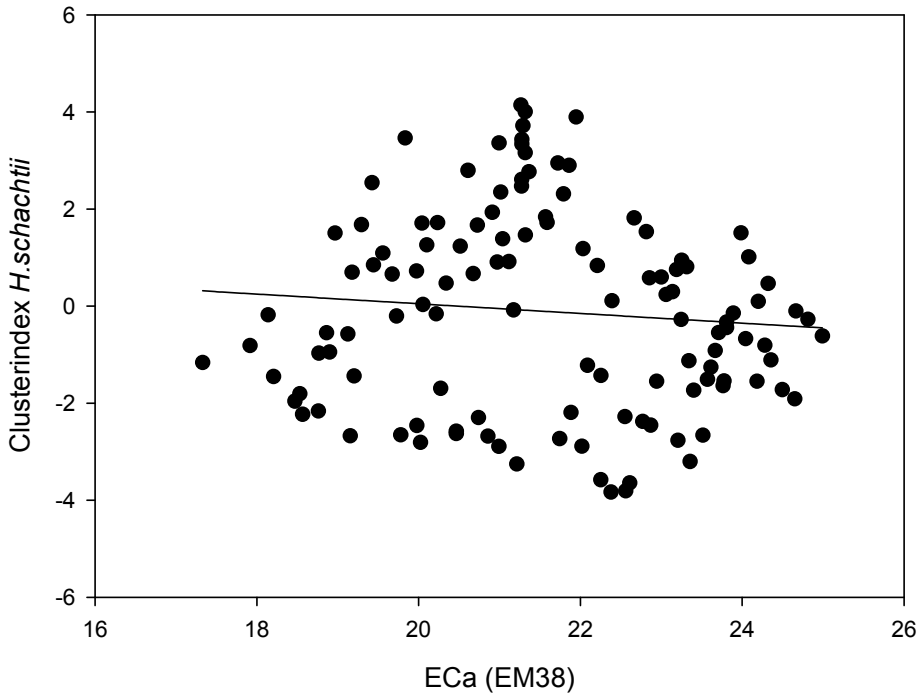

Fig. 4.3.b *Correlation between cluster indices of* Heterodera schachtii *and the apparent electromagnetic conductivity (EC_a) of the sampling area*

No clear correlation could be found between edaphic factors and *H. schachtii* count numbers (Fig. 4.3.b). However a slight tendency of aggregation in sandy soils might be interpretable.

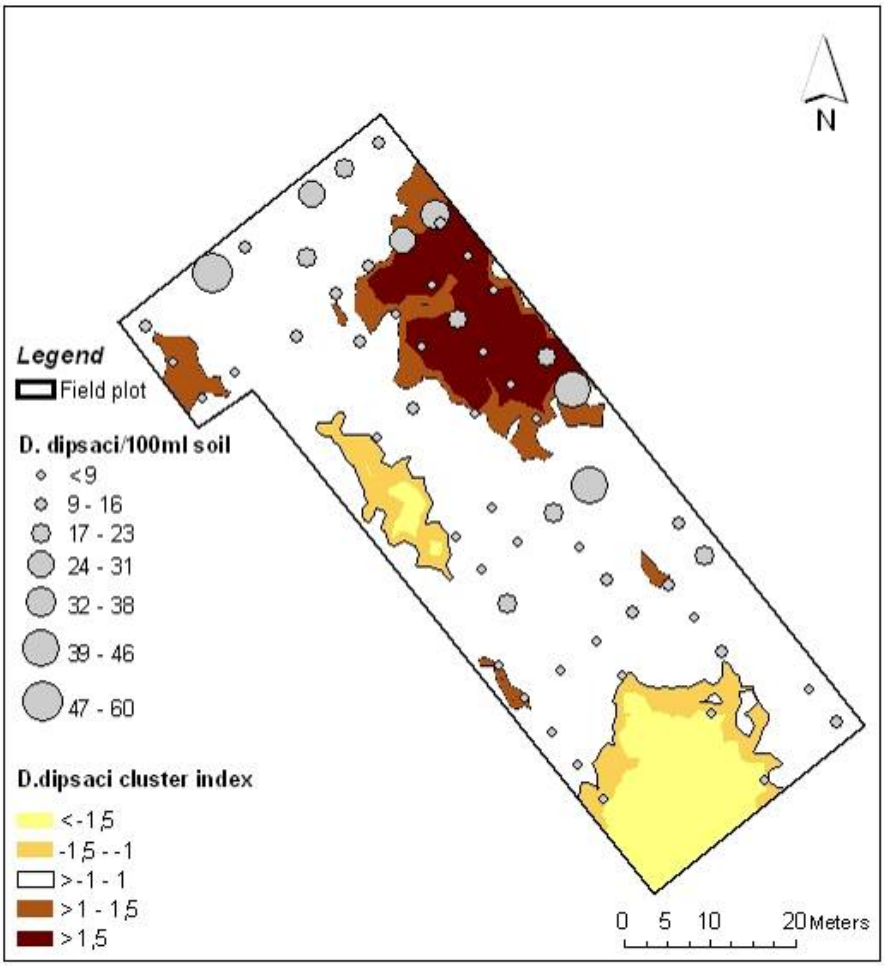

Fig. 4.4.a *Spatial distribution of* Ditylenchus dipsaci *counts and the interpolated SADIE cluster indices*

In contrast, Figure 4.4.a shows the characteristics of distribution for *D. dipsaci,* which indicates that this nematode only occurred at a few sample points (Zero-values are excluded). SADIE analysis showed a cluster of *D. dipsaci* at the north eastern part of the field. The observed spatial distribution of *D. dipsaci* in the field was aggregated with an I_a of 1.78 and a P_a < 0.01. The size of the spatial patterns was small compared to *H. schachtii* and the 4 patches which were detected covered only a small area. In contrast to *H. schachtii* more high *D. dipsaci* values occurred in areas not classified as patches by SADIE.

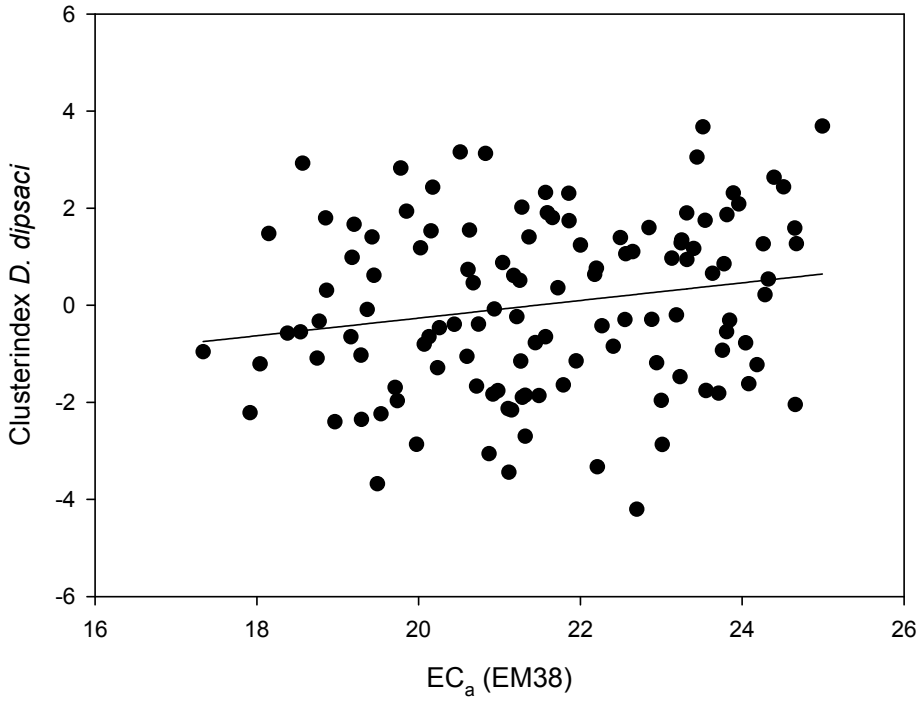

Fig. 4.4.b *Correlation between cluster indices of* Ditylenchus dipsaci *and the apparent electromagnetic conductivity (EC_a) of the sampling area*

Figure 4.4.b shows the correlation between the cluster index of *D. dipsaci* and the EC_a values. Due to the heterogeneous distribution, the high variance of count data of *D. dipsaci* and the fact that high *D. dipsaci* values occurred in areas not classified as patches by SADIE, the correlation is low. Anyhow a slight tendency of higher cluster indices in high EC_a areas could be detected. This indicates that this nematode has a preference for heavier soil types as already mentioned by Wallace (1954).

4.4.1.2. Distribution of disease symptoms

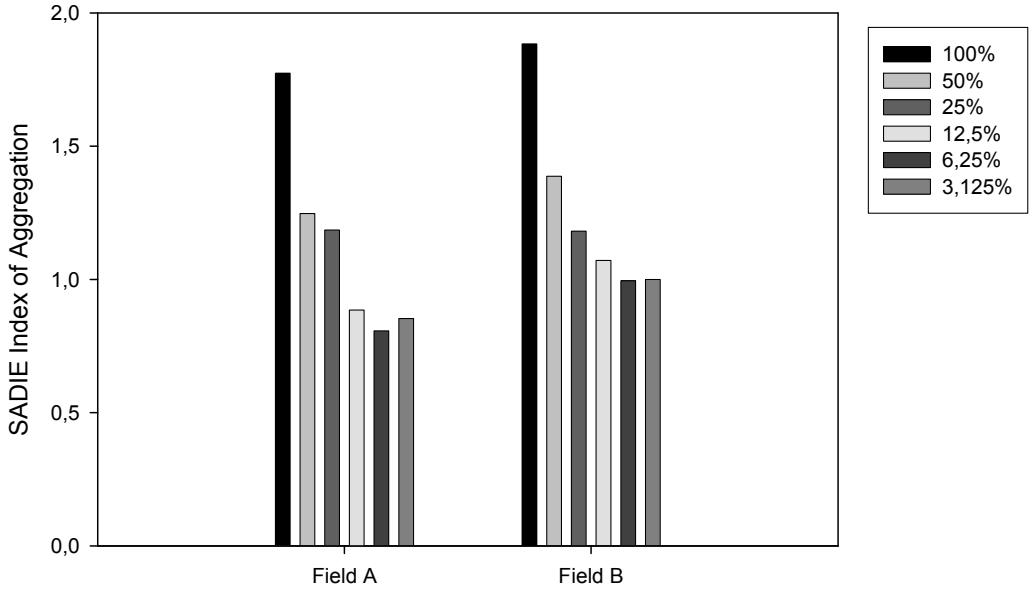

Fig. 4.5 *Development of the SADIE index of aggregation of crown rot symptoms caused by* Ditylenchus dipsaci*, when the sampling grid was enlarged and the amount of evaluated beets decreased. Legend: percentage of rated beets.*

The aggregation of disease symptoms caused by *D. dipsaci* at the end of the 2006 growing season also showed indices of aggregation indicating a patchy distribution. Field A had an I_a of 1.77 and field B an I_a of 1.88, when the disease symptoms of every beet were used for calculation (Figure 4.5). These values demonstrate a strong clustering of *D. dipsaci*. However the observed crown rot patch size at harvest was small. The aggregation index I_a decreased continuously as the number of data points to evaluate beet crown rot decreased. In field A the I_a was reduced from 1.77 to 1.25, 1.19, 0.89, 0.81 and 0.85 if every 2nd, 4th, 8th, 16th and 32nd beet was taken into account. In field B the I_a diminished from 1.88 to 1.39, 1.18, 1.07, 0.99 and 1.00 respectively. If only every second beet would be rated and evaluated by the SADIE software the I_a values approached 1, which would indicate a random distribution of *D. dipsaci* in field.

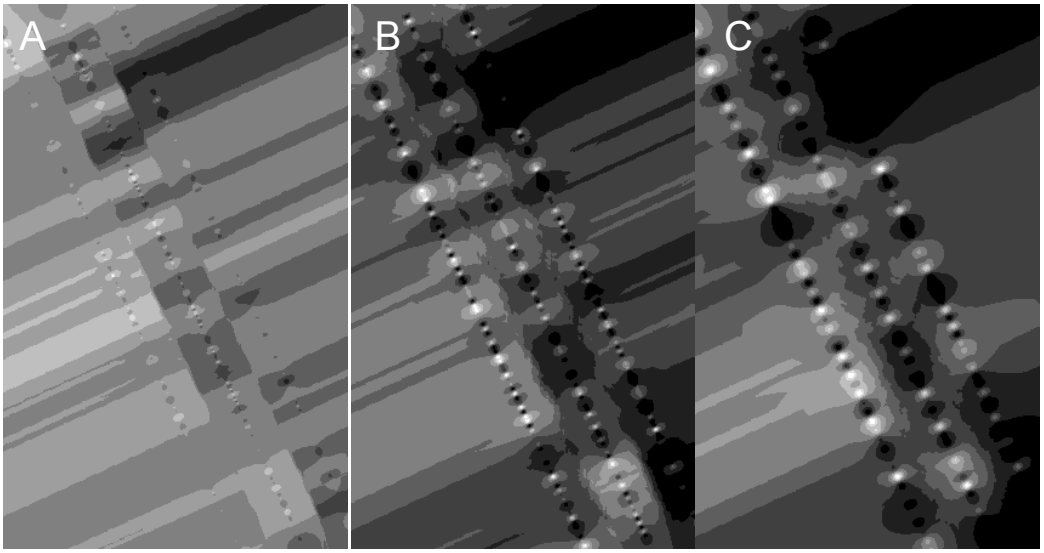

Fig. 4.6.a *Development of maps obtained by ordinary kriging interpolation of crown rot symptoms on sugar beet caused by* Ditylenchus dipsaci*, when the sampling grid was enlarged and the amount of evaluated beets decreased. (A) Every beet, (B) every second beet, (C) every fourth beet was included in the analysis. White no symptoms, black 100%roten beet surface*.

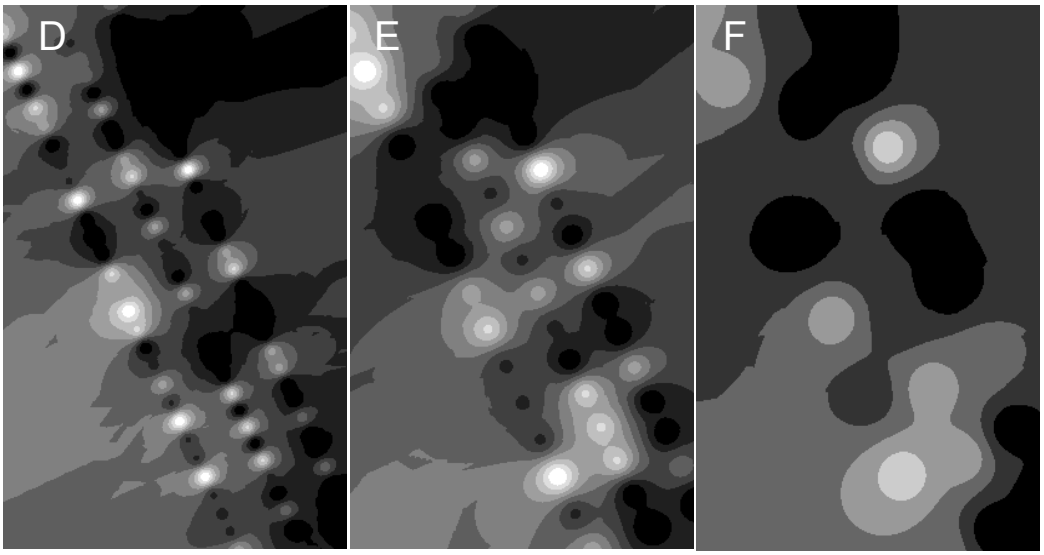

Fig. 4.6.b *Development of maps obtained by ordinary kriging interpolation of crown rot symptoms on sugar beet caused by* Ditylenchus dipsaci*, when the sampling grid was enlarged and the amount of evaluated beets decreased. (D) Every eighth beet, (E) every sixteenth beet, (F) every thirty-second beet was included in the analysis. White no symptoms, black 100%roten beet surface.*

A similar conclusion can be drawn regarding the interpolation by ordinary kriging of maps from field A. An expansion of the sampling grid clearly leads

to misinterpretable maps. Already a reduction to the analysis of every second beet contributes to the occurrence of non existing gaps and clusters.

4.5 Discussion

Geostatistical analysis could be used to reduce the measurement random variability associated with nematode densities (Wallace and Hawkins, 1994). The importance of SADIE in plant pathology is increasing since spatial and temporal variability as well as interactions between different organisms can be analysed and visualized using GIS software. In the recent past, SADIE has been applied successfully to analyse spatial distributions of field pests like insects, diseases and weeds (Blackshaw and Vernon, 2006, Pethybridge et al., 2005, Pethybridge and Turechek, 2003, Thomas et al., 2001, Warner et al., 2003)

The results from this research provide new insights into the spatial distribution of *D. dipsaci* and *H. schachtii* in sugar beet fields. The data collected in 2005 demonstrate that site specific management is an appropriate tool for *H. schachtii* management. This plant parasite has a high economic threshold of 500-1000 eggs and juveniles per 100cm^3 of soil (Müller J., 1990). Therefore, only high densities cause reduced plant growth and yield. There is a direct correlation of initial soil infestation levels to occurring damage since this nematode has 2-3 life cycle per growing season under European climatic conditions (Jones, 1950). Therefore, areas with high initial densities could be treated with nematicides, biocontrol agents or planted with tolerant sugar beet varieties. No clear correlation was found between EC_a values and nematode numbers (Fig. 4.4). The difference of soil properties of the investigated area, derived by the EM-38 measurement with a maximum value difference of 8 mS s^{-1} (Fig.4.1), can be considered as low (Domsch and Giebel, 2004, Neudecker et al., 2001). Neudecker et al. (2001) noted that generally low ECa-values are typical for sandy soils (5-15 mS m^{-1}) and higher values (30-60 mS m^{-1}) represent soils with higher clay content and intermediate ranges are typical for loamy soils.

Field experiments in commercial sugar beet growing areas demonstrated for the first time that the spatial distribution of *D. dipsaci* was irregular. High nematode counts close to counts with very low values indicated a very high spatial heterogeneity. The low threshold of this nematode (Schmidt et al., 1985) complicates the use of site-specific control and if considered should therefore be based on interpolated maps of actual numbers and not on cluster indices.

The data indicate that a sampling grid of 5m x 5.4m is too wide for use in site specific management of *D. dipsaci*. Therefore, smaller sampling grid sizes are needed which of course would increase costs and would not be appropriate for commercially managed sugar beet fields. To determine the actual size of *D. dipsaci* patterns in a field, disease symptoms of every beet in one row were evaluated. In both years the pathogen was significantly more aggregated than expected by chance. However, the size of the patches was small and their distribution irregular. In 2006, the aggregation of symptoms was not detectable when only every fourth beet was rated (Fig.4). The I_a values in 2006 decreased to almost 1.0, indicating random distribution of symptoms. Since this pathogen has up to 9 life cycles per year in perennial crops (Griffith et al., 1997) and damage is highly influenced by environmental factors, a correlation between the initial infestation and damage, is low. The data indicates that the main factors influencing the expression of symptoms are weather conditions and initial distribution of *D. dipsaci* during the time period in which the seedlings remain in the soil. Similar observations were made by Palo (Palo, 1962), who found a positive correlation between the initial inoculum density of *D.dipsaci* and reproduction rates. Lower penetration rates also were detected when *D. dipsaci* was inoculated 10 days after planting when seedling emergence took place instead of directly at sowing. Population growth in a sugar beet plant was exponential and mainly determined by available nutrient and temperature. Nematode development and reproduction is linearly related to the temperature under optimal plant growth conditions (Griffith et al., 1997). One *D. dipsaci* juvenile, successfully invading the beet

at the beginning of the growth season, therefore, can result in a population that causes strong crown rot symptoms at the time of harvest.
In vitro experiments demonstrated that the dispersal of *D. dipsaci* through the soil, reached up to 70 cm per hour and is tenfold higher than that reached by *H. schachtii* (Wallace, 1958). Therefore some areas classified as uninfected regions in the field area could be infected from infested areas during the growing period.

In studies where *D. dipsaci* damage in Lucerne fields was monitored in three subsequent years; a four to five fold increase of the *D. dipsaci* foci, their mean size, and the area of the field that was damaged during the first years of infestation after nematode introduction through infested seeds occurred. Active movement seemed to be the main factor for dispersal. Long distant dispersal however may occur when surface drainage or wind contribute to this process (Atkinson and Sykes, 1981). The reproduction potential for *D. dipsaci* is higher in sugar beets than in Lucerne. In Lucerne only the stem base and the growing points are suitable host tissue for the nematodes (Palo, 1962), whereas the whole storage root tissue of the tuber can be colonized in sugar beet with approximately 4500 nematodes per gram tap root recorded by (Julier et al., 1996). The higher amount of available tissue for nutrition might also contribute to the rapid spread of *D. dipsaci* in narcissus fields (Webster, 1962, Webster, 1964). These above mentioned problems demonstrate that site specific control of *D. dipsaci* is not feasible for most field situations. The most promising approach for management of this plant parasite; at the present time, is a seed coating with nematicides or biopesticides that prevent seedling damage in the period directly after germination (Tenente et al., 1999, Roberts and Matthews, 1995, Whitehead and Tite, 1972). This would provide protection at the critical stage and at the exact location of penetration early in the growing phase.

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5 Conclusions and importance for nematode IPM

The term Integrated Pest Management (IPM) in nematology stands for the combination of different control measures. The present application of nematicides ranges from seed coating and in furrow treatment to soil fumigation of high value crops. Fumigation leads to eradication of plant parasitic nematodes as well as most other living organisms in the top soil layer and e.g. with methyl bromide results in a short term highly effective solution to a nematode problem.

In order to reduce dependence on highly toxic substances many pest control measures are combined to build a sustainable management strategy (Batchelor, 1998). IPM strategies include quarantine of invasive species, hygienic measurements, cultural practice, physical methods, biological control with antagonistic organisms, fumigant/non-fumigant, soil or seed treatment with nematicides,, host crop resistance or tolerance, and precision farming to name a few (Sikora et al., 2005). Based on the literature and on the results of this study a hypothetical IPM strategy for the control of *D. dipsaci* on sugar beet was developed.

Crop rotation

Widening the crop rotation and implementing non host crops is a reliable tool to manage *H. schachtii* in sugar beets. Population densities of this nematode can be reduced up to 80% using nematode resistant intercropping as trap crops and also fallow reduces populations up to 80% (Steudel and Müller, 1983).

D. dipsaci is, however, more difficult to control since this species can prolong longevity through a slowing down its metabolism during times of suboptimum environmental conditions. Furthermore, the fourth stage juveniles are the survival stage and can persist in field soils for many years in the absence of a host (Miyagawa and Lear, 1970). Also many common weeds are stem

nematode hosts and ensure persistence of the pest while fields are planted with non host crops. This has probably been the factor that maintained this nematode in the fields over many years of nematicide treatment, and has lead to the present outbreak of damage after nematicides were taken off the market.

The decrease of subsidies narrows the profit margins of sugar beet cultivation. The high biomass of the tap roots lead to high transportation costs. Hence only in the regions around the processing factories will continue to grow sugar beets and the amount of suitable acreage will decrease in the future. This will lead to intensification of the beet percentage in crop rotations on farms close to the sugar factories. The development of sugar beet production in a global market highlights the need of efficient control measures in the near future.

However, crop rotation is always a very important tool to manage nematode problems. Even if resistant cultivars are found, crop rotation must be performed in order to reduce selection pressure on nematode populations to avoid resistant breaking races (Chen et al., 2001).

Tillage

D. dipsaci penetrates the plants, unlike most other plant parasitic nematodes, near the soil surface. Therefore, a different vertical distribution of this nematode species in soil can be expected when compared to *H. schachtii* for example which is found throughout the soil profile. Rainfall events and the presence of plants resulted in higher stem nematode numbers in the first 5cm of top soil compared to the frictions underneath (Wallace, 1962). Plowing just prior to sowing would bury the aggregated stem nematodes found in the top layer of soil down to a thirty centimeter depth. However, as *D. dipsaci* is extremely mobile this strategy is only likely to be partially successful by reducing some early seedling infection. In addition, the 10 to 14 day time frame for nematode penetration from sowing until emergence of the sugar

beet seedlings is too long for the approach to be highly successful. However, plowing generally improves soil structure and therefore favors plant development. This would increase the chance of seedling escape to nematode penetration by plants outgrowing the pest. In addition, many weeds are controlled by plowing and therefore the number of secondary hosts would be reduced.

Choice of cultivars

The first presumption for a screening system is to develop a reliable screening technique (Boerma and Hussey, 1992). Therefore, in the present investigations, a bioassay was developed to evaluate sugar beet for resistance to the stem nematode *D. dipsaci*.

To produce large numbers of *D. dipsaci* for inoculation of sugar beet seedlings, a protocol for a monoxenic carrot disk culture was established. *D. dipsaci* multiplication was greatest with 125 000 nematodes per carrot disk after 72 days at 20◦C with an initial inoculum density of 75 nematodes. Higher inoculum densities resulted in more rapid decay of carrot tissue and lower numbers of *D. dipsaci*.

Inoculation of 200 *D. dipsaci* in leaf axils of sugar beet seedlings resulted in high penetration rates. The addition of 1% carboxymethyl cellulose (CMC) as a gelling agent resulted in a significantly increased penetration rate of 96% compared to tap water with 34%. Soil infestation was not suitable, with only 4 and 11% of the nematodes penetrating the sugar beet seedlings when inoculum was applied in water and CMC, respectively. The bioassay enabled a rapid screening for resistance in sugar beet to *D. dipsaci*.

Up to 34 sugar beet genotypes were tested in different experiments. Resistance towards *D. dipsaci* was analysed according to reproduction of the nematodes after two life cycles under semi sterile controlled greenhouse conditions. Tolerance was evaluated by rating of crown rot symptoms under

non sterile conditions. The variety Dorena achieved the highest penetration and reproduction rates and the development of the severest crown rot symptoms. The variety Syncro was the most tolerant genotype and reduced reproduction and development of symptoms significantly up to 50% and more.

The term resistance is defined as ability of a plant to inhibit nematode reproduction relative to a plant lacking such resistance. Therefore some of the investigated varieties clearly reduced the reproduction of *D. dipsaci*, even if the reduction of reproduction will not lead to a decrease of nematode soil population. Also the definition of resistance with less then 10% of the reproduction of the susceptible control was not achieved. Screening field trials were conducted meanwhile with the same varieties and showed similar results.

The results of the present studies demonstrate that the most suitable test system for detecting nematode resistance is the evaluation of crown rot symptoms, because here the highest correlations with results from field data were found. Furthermore, it is a less work intensive screening procedure and will allow a high throughput of breeding lines at lowest costs possible. In general, results from laboratory tests correlated well with those from field tests, but laboratory evaluation was found to be more reliable and economical for determining resistance. These findings are also supported by previous studies (Sherwood et al., 1967). Starting a high throughput screening would dramatically raise the chance to find a resistance source from wild beets (Jung et al., 1998). Then the success of rhizomania and beet cyst nematode resistance, where resistant varieties have been commercialized (Panella and Lewellen, 2007), could be repeated for the stem nematode *D. dipsaci*.

Ongoing field screening trials detected new varieties that are less succeptible to *D. dipsaci* damage. The variety Beretta was able to reduce rot symptoms up to 85 %. However *H. schachtii* resistant varieties did not reduce *D. dipsaci* damage sufficiently (LIZ 2010).

Sowing time frame

Weather conditions at sowing are one of the most important factors determining the level of pest incidence and impact. The results of the present studies indicated that only a short time frame, from sowing till emerging of the beet from the soil is favorable for *D. dipsaci* penetration. Moist soil conditions enhance mobility of *D. dipsaci* and therefore also plant penetration of the nematodes (Wallace, 1958). It was also stated that numbers of *D. dipsaci* in top soil layer are highest after a rainfall event (Wallace, 1962). Therefore, whenever possible the sowing of sugar beet under wet conditions in fields with known *D. dipsaci* infestations should be avoided. A delay of sowing also resulted in a decrease of rot symptoms up to 90% at time of harvest (LIZ, 2010). This might be the result of drier conditions at sowing and a reduced nematode propagation time frame.

Site specific control measures

SADIE methodology was applied to compare the spatial distribution of *D. dipsaci* and *H. schachtii,* for use in site specific management. The spatial distribution of the initial population of *D. dipsaci* in soil at beginning of the growing season was aggregated with an Index of Aggregation (I_a) of 1.78. The population of the cyst nematode *H. schachtii* was even more aggregated with an I_a of 2.13. In addition, the sizes of *H. schachtii* infested areas were larger when patches were compared to *D. dipsaci.*

The spatial distribution of crown rot symptoms caused by *D. dipsaci* at the end of the growing period was similar to preseason levels with an I_a of 1.77 and 1.88 respectively for two infested fields. Again the size of the patches was very small. The heterogenic occurrence of the symptoms could be explained using in-vitro tests. The first factor influencing the irregular distribution is the short time frame for nematode penetration which ranges from sowing till emergence of the seedling from ground. During this critical 10

day period, the nematodes must penetrate the beet seedlings to survive and initiate their reproduction cycle.

The second factor influencing the heterogenic occurrence is the high reproduction capability of *D. dipsaci*. Site specific control measures are only feasible if a reliable control of the pest is achieved. The results of the present investigations show a low spatial aggregation of *D. dipsaci* in commercial field soils. This diminishes the probability of targeting most of *D. dipsaci* individuals with partial application of nematicides. The low damage threshold of *D. dipsaci*, which is actually under the detection threshold (Evans et al., 2002) also enforces the need of efficient control measures.

Therefore it has to be concluded that site specific control measures are until today not an appropriate tool for the management of *D. dipsaci* due to the extreme heterogenic cluster distribution and the low economic threshold values.

Chemical control

Today the only registered nematicide in Germany is the Organo-Phosphate Nemathorin© 10g which can only be applied in potatoes. Because of the infection, development and spread of *D. dipsaci* early in the season, the application for §18 permits, to allow the use of nematicides in *D. dipsaci* infested fields, needs to be strongly considered. A good efficacy of the granular nematicides Fenaminphos, Carbofuran and Ethoprop iagainst D. dipsaci was demonstrated in garlic field trials (Andres and Lopeu-Fando, 1996).

Harvest

The heavy crown rot symptoms caused by *D. dipsaci* usually occur close to the sugar beet harvest season. Toxic water solvable metabolites induce a

change of nematode behavior (Robertson, 1928). As crown rot occurs nematode populations have an increasing proportion of fourth stage juveniles (Subbotin et al., 2005). This is the survival stage, which is able to survive many years. These fourth stage juveniles either emerge from the beets to the soil or stay in plant tissue in an anabiotic stage. Early harvest of the beets has several advantages. The rot symptoms would be at an early stage and therefore the quality of the beets would not be affected as much. Furthermore, the nematode field population would not increase as much as the fourth stage juveniles would still be inside the root. This promising approach is only limited by the fact that sugar content of the beets is developed mainly in the last month of the cropping season. This means that an early harvest would diminish one of the most important yield factors. The importance of this management tool is underlined by the fact *D. dipsaci* infested fields are monitored and routinely harvested as first (LIZ, 2010).

IPM in the future

The discussion of the results obtained in the present studies shows that management of the stem nematode is achievable with the proper mixture of tools in an economically appropriate IPM system.

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