Aus dem Institut für Zuckerrübenforschung
Göttingen 56/2022

Alan Storelli

Investigation of resistance against *Ditylenchus dipsaci* on sugar beet

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Dissertation

to obtain the Ph.D. degree

in the Faculty of Agricultural Sciences,

Georg-August-Universität Göttingen, Germany

by

Alan Storelli

born in Monthey (CH)

Zollikofen (CH), July 2021

1. Referee: **Prof. Dr. Anne-Katrin Mahlein**

   Head of Institute of Sugar Beet Research / Institut für Zuckerrübenforschung (IfZ)

   An-Institut der Georg-August-Universität Göttingen

2. Referee: **Prof. Dr. Michael Rostás**

   Georg-August-Universität Göttingen

   Professor for Agrarentomology

3. Referee: **Dr. Andreas Keiser**

   Berner Fachhochschule - Hochschule für Agrar-, Forst- und Lebensmittelwissenschaften HAFL

   Professor for Ackerbau und Pflanzenzüchtung

Date of submission for examination: 23.07.2021

Date of disputation: 28.10.2021, Göttingen

This dissertation is registered with the persistent identifier DOI: 10.53846/goediss-24050

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1 Introduction

1.1 *Ditylenchus dipsaci*

The stem and bulb nematode *Ditylenchus dipsaci* (Kühn, 1857) Filipjev 1936 is an obligate endoparasite of the family *Anguinidae*. It belongs to the top ten economically important plant-parasitic nematodes worldwide (Jones et al. 2013). The adult nematodes measure up to 1,460 µm long and 28 µm wide (Esquibet et al. 1998; Kühn 1857; Barraclough and Blackith 1962). *Ditylenchus dipsaci* is prevalent in a wide range of climatic conditions and was observed in Africa, America, Asia, Europe, and Oceania (Janssen 1994; Schluter 1973; Kasimova 1969; Steiner 1938; Stirling 1972). Up to 500 plant species are known as hosts for *D. dipsaci,* including vegetables, field crops, and weeds from over 40 angiosperm families (Subbotin et al. 2005). Ritzema Bos (1888) was the first to observe numerous host plant species for *D. dipsaci* by grouping several plant diseases under the same common pathogen, *Tylenchus devastatrix* (Kühn, 1857), the former name of *D. dipsaci*. Onion (*Allium cepa* L.), garlic (*Allium sativa* L.), alfalfa (*Medicago sativa* L.), clover (*Trifolium* spp.), faba bean (*Vicia faba* L.), and narcissus (*Narcissus* spp.) are cash crops where *D. dipsaci* has the highest economically impact (Ritzema Bos 1888; Godfrey and Scott 1935; Beyerinck 1883; Ormerod 1886). Besides, the host range of *D. dipsaci* includes several weed species, such as the chickweed (*Stellaria media* L.), the cleavers (*Galium aparine* L.), the annual purple dead-nettle (*Lamium purpureum* L.), and the scarlet pimpernel (*Anagallis arvensis* L.) (Staniland 1945; Johnson 1938; Goodey 1947).

Ditylenchus dipsaci penetrates juvenile plants and feeds on the host plant cells (Hooper 1971). Enzymes injected into the host cells dissolve the middle lamellae leading to plant hormone imbalance, cell hypertrophy, and intercellular cavities (Duncan and Moens 2013). Stunted growth, as well as swelling of hypocotyl and epicotyl, are common symptoms of infected plants (Caubel et al. 1994). On garlic, *D. dipsaci* infection leads to bloating and worn-out foliage (Sonmezoglu et al. 2020). Shorter internodes and curled leaves are observed on infected lucerne (Mouttet et al. 2014). Cracking of the epidermis is reported on *D. dipsaci* infected narcissus (Winfield 1970). Early infection of plants may lead to a low emergence rate and

death of young seedlings (Caspary 1976; Storelli et al. 2021). *Ditylenchus dipsaci* infection on juvenile plants impacts even after harvest by decomposing infested onion bulbs in storage (Macias and Brzeski 1967).

According to their host preferences, up to 30 biological races of *D. dipsaci* are identified, representing a species complex (Sturhan et al. 2008; Seinhorst 1957; Sturhan and Brzeski 1991; Bovien 1955). Eriksson (1965) defined biological races as populations distinguished by host preference but not morphologically. Indeed, the red clover (*Trifolium pratense* L.) biological race can not multiply on alfalfa (Whitehead et al. 1987). As the onion and tulip races, some biological races are polyphagous and can reproduce on a high range of host plant species (Webster 1967). According to Whitehead et al. (1987), the sugar beet race is polyphagous but can not multiply on lucerne, while the lucerne race can reproduce on sugar beet.

1.2 *Ditylenchus dipsaci* in sugar beet

Ditylenchus dipsaci is an important pest for European sugar beets (*Beta vulgaris* L.) (Leipertz 2007; Dewar and Cook 2006). Damages on sugar beets were reported in Denmark, France, Germany, Netherlands, Spain, Switzerland, and United Kingdom (Castillo et al. 2007; Graf and Meyer 1973; Salentiny 1959; Vergnaud 2001; Hansen 2012; Whitehead et al. 1987; Niere and Schlang 2006). While *D. dipsaci* infested sugar beets are rarely reported in most countries mentioned above, three sugar beet growing regions in Germany and Switzerland are heavily affected: Franconia (DE), Rhineland (DE), and Seeland-Broye (CH).

Ditylenchus dipsaci life cycle comprises an egg stage, four juvenile stages, and the adult stage (Fig. 1) (Decraemer and Hunt 2013). The first juvenile stage (J1) takes place exclusively in the egg, and the first molt occurs 5-6 days after the egg deposition (Yuksel 1960). The second-stage juvenile (J2) hatches from the egg seven days later and is directly considered an infective stage. The nematode starts its second molt 2-3 days after hatching, followed by the third molt 3-3.5 days later. The adult stage occurs 4-5 days later, completing a life cycle ranging from 19 to 23 days depending on the environmental conditions (Yuksel 1960). Mating male and female are essential for reproduction. Yuksel (1960) and Griffith et al. (1997) reported first egg deposition 3-7 days after the final molt. According to Yuksel (1960), a female *D. dipsaci* produces 207 to 498 eggs during its life duration ranging from 45 to 73 days, meaning a daily production of

8 to 10 eggs on onions. However, *D. dipsaci* reproduction capacity varies greatly depending on environmental conditions and host tissue (Barbercheck and Duncan 2004). Indeed, Griffith et al. (1997) reported a daily production of 0.8 and 3.1 eggs on white clovers (*Trifolium repens* L.) at 10 and 20°C.

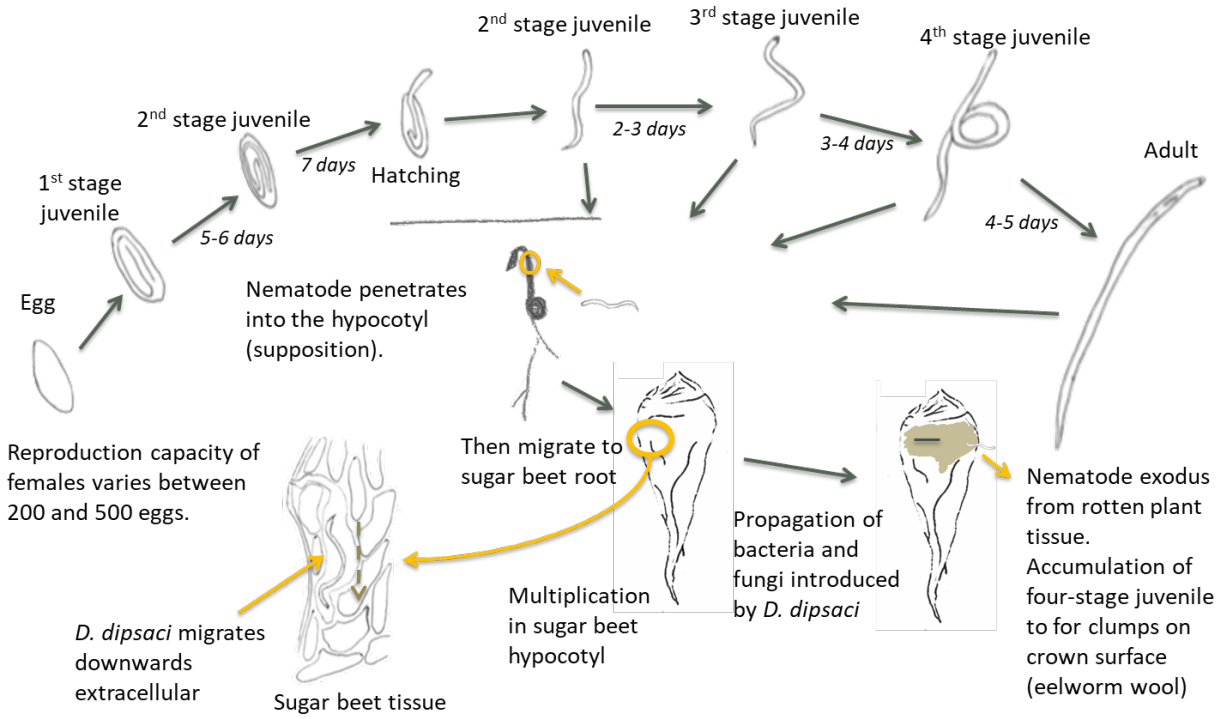

Fig. 1 Life cycle of *Ditylenchus dipsaci* on sugar beet (adapted from Yuksel (1960) and Hooper (1971))

At the beginning of the growing period, *D. dipsaci* follows a water gradient and migrates to the soil surface after rainfall (Wallace 1962). To date, *D. dipsaci* penetration pathway into sugar beet seedlings is not known. Storelli et al. (2021) assumed *D. dipsaci* penetration into freshly germinated sugar beet before plant emergence (Fig. 1). Wallace (1962) reported an increase of *D. dipsaci* in the soil surface near oat (*Avena sativa* L.) seedlings. No nematode was observed on the bare ground soil surface, assuming plant influence on nematode soil migration. Spiegel et al. (2003) validated this statement by reporting the influence of onion root exudates on the chemotactic behavior of *D. dipsaci*. Nematode penetration on sugar beet seedlings leads to swollen hypocotyls and distorted leaves (Storelli et al. 2021). Later in the growing season, soil-borne pathogens introduced by *D. dipsaci* conduct sugar beets to crown decomposition (Storelli et al. 2020;

Kühnhold 2011; Hillnhütter et al. 2011). As *D. dipsaci* is an obligate endoparasite and can not feed on fungi, rotten plant tissue leads to the nematode exodus (Hajihassani et al. 2017; Schomaker and Been 2013). The fourth-stage juveniles (J4) migrate out of the senescing plant tissue and aggregate to form clumps (Fig. 2), known as eelworm wool, on the hypocotyl surface (Hooper 1971; Wharton 2004). J4 *D. dipsaci* is characterized as the dauer larva allowing nematodes to survive in the absence of a host plant. In *D. dipsaci* J4, nematode permeability is reduced to minimize water loss under unfavorable conditions (Wharton 1996). During this state of anhydrobiosis, intestinal cells of the non-feeding nematode are altered to contain a large number of lipids and proteins (Wharton and Barrett 1985). J4 *D. dipsaci* can survive in this state for many years until favorable conditions appear again. Fielding (1951) recorded J4 *D. dipsaci* anhydrobiosis lasting up to 23 years. As soon as conditions are favorable, the recovery period begins. Barret (1982) and Wharton et al. (1985) reported nematodes movement 2-3 hr after immersion of desiccated J4 *D. dipsaci* in water. *Ditylenchus dipsaci* recovery is not dependant on the stress duration but the severity of the desiccation (Wharton and Aalders 1999).

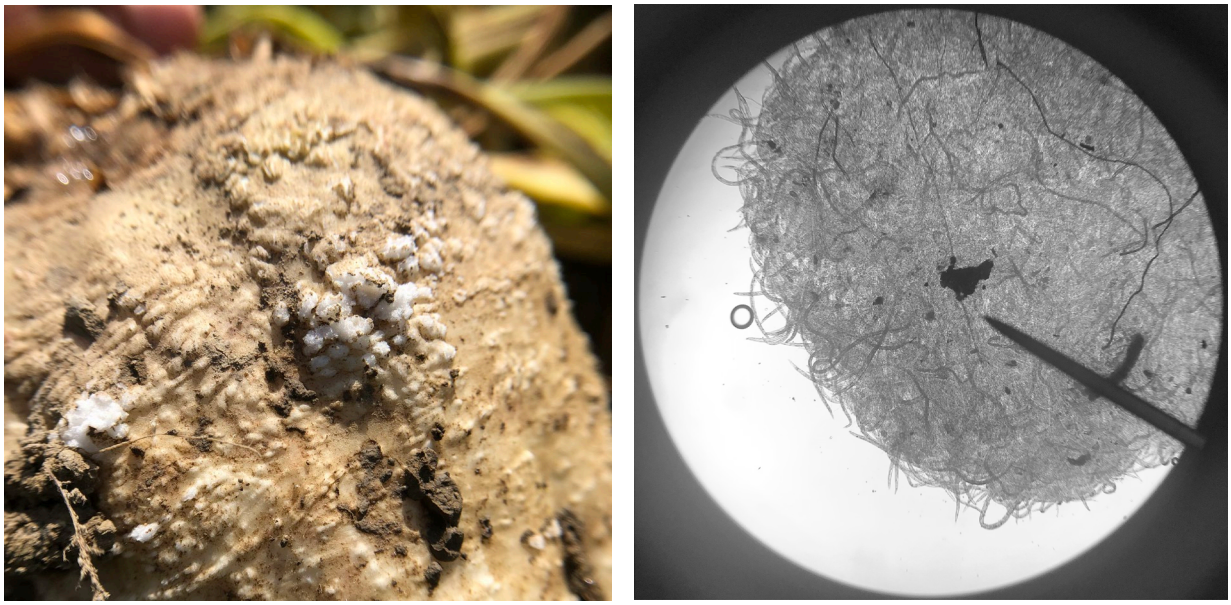

Fig. 2 Nematode clumps, known as eelworm wool, on a sugar beet hypocotyl surface (left) and accumulation of fourth-stage juvenile (J4) *Ditylenchus dipsaci* forming eelworm wool at 40x magnification (right).

Since the nematicide aldicarb withdrawal, no effective direct management has been available for control of *D. dipsaci*. LaMondia (1999) reported low effects of abamectin B1 on *D. dipsaci* development even after

four applications on creeping phlox (*Phlox subulata* L.), while Becker (1999) observed a high rate of garlic bulbs free of *D. dipsaci* when abamectin B1 was applied. Up to 99% nematode mortality was achieved when hydrogen cyanide was applied on *D. dipsaci* infested garlic (Zouhar et al. 2016). These techniques are used on seeds and bulbs to prevent nematode transmission through seed material. However, they are not available to reduce *D. dipsaci* in soil. Despite the efficacy of fluopyram on *Meloidygne incognita* and *Rotylenchus reniformis*, it was not effective at reducing *D. dipsaci* reproduction (Storelli et al. 2020; Faske and Hurd 2015). Storelli et al. (2020) reported the effects of fluopyram at reducing *D. dipsaci* penetration into sugar beet seedlings and fungal development in the plant tissue. However, fungicide effects of fluopyram are limited to ascomycetes and did not reduce the development of basidiomycetes, such as *Rhizoctonia solani* (AG-2IIIB) (Storelli et al. 2020; Veloukas and Karaoglanidis 2012).

As alternatives to nematicide, Sherwood and Huisingh (1970) observed a reduction of *D. dipsaci* reproduction on alfalfa grown in high calcium content soils, assuming a potential influence of liming on *D. dipsaci* development. Soil solarization preceding onion cultivation reduced *D. dipsaci* initial population and bulbs damage (Greco et al. 1985). At the same time, hot water treatment on narcissus bulbs showed contrasting efficacy on *D. dipsaci* mortality and bulbs quality (Winfield 1970, 1972; Qiu et al. 1993). Hay and Bateson (1997) reported effective control of *Verticillium balanoides* inoculated white clover seed on *D. dipsaci* reproduction in the plant.

The broad range of host plants for *D. dipsaci* hinders successful crop rotation strategies (Jones et al. 2013). Additionally, the capacity of *D. dipsaci* to survive many years in soil without a host plant makes the use of break crops ineffective. However, agronomists recommend avoiding successions of crops with a high *D. dipsaci* reproduction potential, such as onion or rye (*Secale cereale* L.). Weed control is also recommended since *D. dipsaci* reproduces in a broad range of weeds (Johnson 1938; Goodey 1947; Staniland 1945). Marigold (*Tagetes patula* L.) is known to reduce *Pratylenchus penetrans* population in the field (Pudasaini et al. 2006; Kimpinski et al. 2000). Nematicide effect of *Tagetes* sp. exudates on *D. dipsaci* was reported *in-vitro* (Uhlenbroek and Bijloo 1958). However, marigold nematicide activity on *D. dipsaci* under field conditions was not reported in the literature.

Due to the lack of effective measures to control *D. dipsaci*, the breeding of resistant sugar beet cultivars remains an adequate long-term control approach.

1.3 Resistance to nematodes

In plant nematology, resistance is described as the plant's ability to suppress the development and consequently the reproduction of the nematodes (Roberts 2002; Trudgill 1991). Here, the author distinguishes between resistance towards penetration, which prevents nematode from invading sugar beets, and resistance towards reproduction, suppressing nematode reproduction in plant tissue and de facto in soil. Tolerance is defined as the plant's ability to compensate for a nematode infection with little or no yield loss (Roberts 2002; Cook 1974). As *D. dipsaci* damages are closely related to bacterial and fungal pathogens, tolerance described the plant ability to reduce swellings and rotting propagation. Susceptibility is related to resistance, whereas sensitivity to tolerance. The term virulence refers to the reproduction ability of a nematode population in a plant. Pathogenicity refers to the level of damage inflicted to the host plant (Müller 1989; Perry and Moens 2013; Shaner et al. 1992).

1.3.1 Resistance towards *Ditylenchus dipsaci*

To date, no sugar beet cultivar was reported to be resistant towards *D. dipsaci*. Kühnhold (2011) observed variations of susceptibility among sugar beet cultivars to *D. dipsaci* penetration and reproduction. However, less susceptible cultivars only showed a partial resistance, characterized by a limited nematode reproduction, and did not succeed for a complete resistance, which entirely prevents nematode reproduction (Parlevliet 1979; Mideros et al. 2007). Knuth (2007) and Leipertz and Valder (2020) reported variations of tolerance among cultivars on field trials. Nevertheless, no cultivar could avoid a complete absence of a soil-borne pathogen infection associated with the nematode. Cultivar sensitivity to *D. dipsaci* was dependant on years and locations, assuming different nematode population pathogenicities.

Resistant cultivars towards *D. dipsaci* were reported in clover, alfalfa, faba bean, potatoes, and oat (Starr et al. 2013; Stanton et al. 1984; McDaniel and Barr 1994; Peng and Moens 2003; Mwaura et al. 2015). A garlic

cultivar was resistant to *D. dipsaci* but failed for commercialization due to low yield potential (Koch and Salomon 1994). Despite nematode reproduction variations among onion cultivars, no complete resistance was observed (Yavuzaslanoglu 2019). No evidence of *D. dipsaci* resistant narcissus cultivar has been yet documented. *Ditylenchus dipsaci* resistance is monogenic on alfalfa and polygenic on faba bean, oat (*A. ludoviciana* L. and *A. sativa* L.), and red clover (*T. pratense* L.) (Plowright et al. 2002). However, Grundbacher and Stanford (1962), validated by Elgin (1979), reported a minority of alfalfa lines with polygenic resistance involving genes with minors effects. Resistant oat cultivars inhibited *D. dipsaci* reproduction in plant tissue but not its initial entry (Griffiths et al. 1957; Blake 1962). A similar resistance mechanism was observed on faba beans and potatoes (Abbad Andaloussi 2001; Mwaura et al. 2015). The rapid spread by stolons of white clover reduced *D. dipsaci* penetration (Cook et al. 1992). Cook et al. (1995) detected formononetin accumulation in meristems on *D. dipsaci* infected resistant white clover cultivars. However, the direct effect of this flavonoid on nematodes was not reported in the litterature. Formononetin is known to have fungicide properties (das Neves et al. 2016). Therefore, Edwards et al. (1995) suggested accumulation of formononetin in *D. dipsaci* resistant cultivars to prevent secondary infection by fungal pathogens.

Although resistant cultivars are available for these different plant species, the resistance is specific to certain *D. dipsaci* populations or biological races. *Ditylenchus dipsaci* populations have been shown virulent to cultivars of alfalfa, white clover, and faba bean, previously described as resistant towards other *D. dipsaci* populations (Plowright et al. 2002; Elgin et al. 1977; Whitehead 1992).

1.3.2 Resistance mechanisms towards migratory plant-parasitic nematodes

Resistance mechanisms towards *D. dipsaci* were not yet described. Studies dealing with resistance towards *D. dipsaci* focused on cultivars' screening. In the following, a review of resistance and tolerance mechanisms to migratory plant-parasitic nematodes is provided.

Steenkamp et al. (2010) identified for the first time a resistant peanut (*Arachis hypogaea* L.) cultivar towards *Ditylenchus africanus*. Resistance to *Ditylenchus angustus* was observed on different rice (*Oryza sativa* L.)

cultivars (Plowright et al. 1996; Latif et al. 2011; Khanam et al. 2016). Mondal and Miah (1987) reported lower *D. angustus* penetration in early maturing rice cultivars. Chlorogenic acid and sakuranetin production was observed in the resistant cultivar after nematode penetration (Plowright et al. 1996). Khanam et al. (2018) reported the influence of salicylic acid, jasmonic acid, and ethylene at preventing *D. angustus* reproduction on a resistant rice cultivar. Indeed, hormone-deficient plants contained a significantly higher amount of nematodes per plant 20 days after nematodes inoculation. OsPAL1, a salicylic acid biosynthesis gene, showed a possible role in resistance response by a consistent up-regulation of OsPAL1 in infected resistant plants. Mwaura et al. (2015) observed potato cultivars resistant to *Ditylenchus destructor*, where nematodes could invade the plant but not reproduce. Transgenic sweet potatoes (*Ipomoea batatas* L.) overexpressing oryzacystatin, a proteinase inhibitor, showed tolerance to *D. destructor* (Gao et al. 2011). Oryzacystatin is known to inhibit proteinase activity in the insect intestine and reduce *Globodera pallida* cyst formation (Ryan 1990; Rahbé et al. 2003; Urwin et al. 1995). Fan et al. (2015) suggested high resistance to *D. destructor* penetration in transgenic sweet potatoes expressing siRNA targeting unc-15 gene, which affects the nematode muscle protein paramyosin.

Plant resistance to migratory plant-parasitic nematodes is frequently linked to biochemical reactions induced by the pathogen penetration (Peng and Moens 2003). *Pratylenchus zeae* penetration on sugarcane (*Saccharum officinarum* L.) did not vary between susceptible and resistant clones towards *P. zeae* multiplication (Kathiresan and Mehta 2002). Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities increased in resistant sugarcane clones inoculated with *P. zeae*, while no activity elevation was found on susceptible clones or non-inoculated resistant clones (Kathiresan and Mehta 2005). Additionally, non-inoculated resistant sugarcane clones showed a higher level of PAL and TAL activities than susceptible clones. Mutant spinachs (*Spinacia oleracea* L.) overexpressing 20-hydroxyecdysone, an ecdyteroids controlling insect molt, showed reduced invasion of *Pratylenchus neglectus* (Soriano et al. 2004). Baldridge et al. (1998) reported a higher level of the phytoalexin medicarpin in alfalfa cultivars resistant towards *Pratylenchus penetrans* reproduction. Marigold root exudates are known to have nematicide effects on several plant-parasitic nematodes, such as *Meloidogyne hapla*, *Heterodera schachtii,*

and *P. penetrans* (Riga et al. 2005). Pudasaini et al. (2006) observed *P. penetrans* soil population decreasing by 90% following marigold cultivation. The nematicide compounds terthiophene and 5-(3-Buten-1-ynyl)-2,2'-bithiophene were identified in marigold (Tang et al. 1987; Marotti et al. 2010; Margl et al. 2002).

Valette et al. (1997) observed a lower penetration rate of *Radopholus similis* on banana (*Musa* sp.) cultivars. Nematodes present in resistant cultivars were limited to the cortical parenchyma. A high level of condensed tannins was observed in a banana cultivar resistant to *R. similis* (Collingborn et al. 2000). Hölscher et al. (2014) demonstrated nematicidal effects of phenylphenalenone anigrofurone produced in response to *R. similis* infection on resistant banana cultivars. Kuroda et al. (1991) observed inhibition of *Bursaphelenchus xylophilus* migration and reproduction in the loblolly pine (*Pinus taeda* L.), known to be resistant to the pinewood nematode. Nematicide compounds were found in resistant pine species, *P. massoniana* L., *P. strobus* L., *P. palustris* L. (Suga et al. 1993). Pinosylvin monomethyl ether and 3-O-methyl-7,8-dihydropinosylvin were determined as nematicidal for *B. xylophilus* (Yamada et al. 1999). Exogenous jasmonic acid, salicylic acid, and ethylene application on rice led to a significant reduction of nematode number in plants (Nahar et al. 2011). In contrast, exogenous abscisic acid treatment increased the nematode population in rice plants (Nahar et al. 2012).

2 Research objectives

The broad range of host plants for *D. dipsaci* does not currently allow to control nematodes through crop rotation. Specific cultural techniques, such as liming or solarization, have shown a low potential of efficacy and are not systematically applicable to sugar beet. Moreover, the current ecological transition of agriculture compromises the registration of new active ingredients to control nematodes.

The development of resistant cultivars towards *D. dipsaci* on clover, alfalfa, faba bean, potato, and oat provided new perspectives for nematode control on sugar beet. Kühnhold (2011) and Leipertz (2007) revealed the potential of the genetic diversity of sugar beets regarding their level of resistance and tolerance to the nematode. For this reason, this thesis aims to investigate resistance against *D. dipsaci* on sugar beet.

The rare number of *in vivo* bioassays investigating *D. dipsaci* interaction with sugar beet led to the use of test systems performing nematode inoculation directly onto the leaf-axil of the first pair of true leaves. The first aim of this thesis is to develop a new *in vivo* protocol through soil inoculation to investigate sugar beet resistance towards *D. dipsaci* penetration. The most suitable inoculation time point, inoculum level, and positioning is determined. The nematode rearing process on carrot discs is as well optimized. Once the new test system is established, the second objective of the thesis is to identify sugar beet pre-breeding populations and breeding lines with resistance towards *D. dipsaci* penetration and reproduction. A wide range of sugar beet genotypes is screened *in vivo* to determine their resistance level towards *D. dipsaci* penetration. Based on this screening, a more in-depth investigation of the best candidates is conducted *in vivo* to determine their potential resistance to *D. dipsaci* reproduction. The resistance level of the candidates is then validated under semi-field conditions by conducting microplot experiments.

Ditylenchus dipsaci represents a species complex with different plant-pathogen interactions depending on the geographic location of the nematode population. The third objective of the thesis is to determine the virulence and pathogenicity of four representative *D. dipsaci* populations from France, Germany, and Switzerland. Here, the influence of the nematode population on *D. dipsaci* penetration rate, reproduction rate, and pathogenicity on sugar beet is determined *in vivo*.

At the end of this thesis, the current limitations of the test system are presented. The perspectives of resistance breeding for the control of *D. dipsaci* are evaluated. Finally, alternatives to *D. dipsaci* control on sugar beets are discussed.

3 Manuscript 1

Development of a new in vivo protocol through soil inoculation to investigate sugar beet resistance towards *Ditylenchus dipsaci* penetration

Alan Storelli [1*], Andreas Keiser [1], Sebastian Kiewnick [2], Matthias Daub [2], Anne-Katrin Mahlein [3], Werner Beyer [4] And Mario Schumann [4]

1) School of Agricultural, Forest and Food Sciences (HAFL), Bern University of Applied Sciences BFH, Länggasse 85, 3052 Zollikofen, Switzerland

2) Julius Kühn Institute, Federal Research Center for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Messeweg 11/12, 38104 Braunschweig, Germany

3) Institute for Sugar beet Research IfZ, Holtenser Landstrasse 77, 37079 Goettingen, Germany

4) KWS SAAT SE & Co. KGaA, Grimsehlstrasse 31, 37574 Einbeck, Germany

Published in Nematology

Received: 20 July 2020; revised: 28 October 2020

Accepted for publication: 29 October 2020

Online publication date: 06 January 2021

DOI 10.1163/15685411-bja10069

Summary – The stem nematode, *Ditylenchus dipsaci*, causes severe damage in sugar beet. To date, nematode inoculation through the leaf axil has been used as the standard method to investigate *D. dipsaci* interaction with sugar beet under *in vivo* conditions. To get as close as possible to field conditions, we established a new screening mechanism to perform soil inoculation. The most suitable inoculation time point, inoculum level and positioning on sugar beet, as well as rearing process on carrots, were determined. At a 15:8°C day:night temperature regime, penetration rates of *D. dipsaci* were at maximum following soil inoculation at plant emergence. Up to 115 nematodes penetrated sugar beet seedlings 22 days post-planting with an inoculum level of 1000 nematodes into the soil at plant emergence. *Ditylenchus dipsaci* penetration rate was higher in plants with soil inoculation than with inoculation on to the leaf axil. High soil moisture increased nematode migration into seedlings when *D. dipsaci* inoculation was carried out in four holes 1 cm from the plant base. Rearing the nematodes for 35 days at 20°C on carrot discs resulted in an infective inoculum containing up to 50% eggs. We recommend a soil inoculation of 1000 freshly extracted nematodes per pot at plant emergence. The nematode suspension has to be previously reared for 35 days on carrot discs to obtain active *D. dipsaci* inoculum. This system will allow for the selection of suitable sugar beet genotypes that suppress nematode penetration, in support of breeding for resistance against *D. dipsaci*.

Keywords – *Beta vulgaris*, carrot disc rearing, inoculum, plant breeding, seedling test system, stem nematode.

Introduction

The stem nematode *Ditylenchus dipsaci* (Kühn) Filipjev is a migratory endoparasite affecting up to 450 plant species, ranked in the top ten plant-parasitic nematodes worldwide (Subbotin *et al*., 2005; Jones *et al*., 2013). Commonly known as a pathogen for onion (*Allium cepa* L.) and garlic (*Allium sativum* L.), *D. dipsaci* also affects sugar beet. Early in the growing season, *D. dipsaci* migration into the parenchymal tissue leads to a swelling of the upper part of the hypocotyl and distortion of primary leaves and petioles (Griffin, 1983). Later in the season, *D. dipsaci* infection leads to the development of bacterial and fungal organisms characterised by crown rotting and commonly known as the secondary attack (Hillnhütter *et al*., 2011). The capacity of *D. dipsaci* fourth-stage juveniles (J4) to survive for many years in the soil or plant debris (Fielding, 1951; Sturhan *et al*., 1991) and the wide range of host plant species (Caspary, 1976) restricts the ability to control the stem nematode by crop rotation. Due to the withdrawal of many nematicides, the breeding of resistant cultivars remains an adequate long-term control approach. The breeding process of resistance against *D. dipsaci* on sugar beet is hampered due to the lack of a proper test system under *in vivo* conditions. The rare number of *in vivo* studies investigating the interaction between *D. dipsaci* and sugar beet led to the exclusive use of test systems where nematodes were directly inoculated onto the leaf axil of the first pair of true leaves (Griffin, 1983; Kühnhold *et al*., 2006; Hillnhütter *et al*., 2011; Kühnhold, 2011). Experiments conducted on onion, yellow pea seedlings (*Pisum sativum*), chickpea (*Cicer arietinum*), large green seed lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), spring wheat (*Triticum aestivum*), oilseed rape (*Brassica napus*), garlic, creeping thistle (*Cirsium arvense*) and white clover (*Trifolium repens*) were all conducted using a shoot inoculation method by placing a *D. dipsaci* suspension on the leaf axils of seedlings (Griffith *et al*., 1997; Hajihassani *et al*., 2016; Yavuzaslanoglu, 2019). Hajihassani *et al*. (2017a) used a *D. dipsaci* inoculation at the base of the hypocotyl of yellow pea seedlings. Sherwood & Huisingh (1970) inoculated *D. dipsaci* on alfalfa (*Medicago sativa* L.) by placing 40 nematodes on the bud of a young plant. Watson & Shorthouse (1979) dispersed 1000 *D. dipsaci* on to the apical meristems of emerged shoots of creeping thistle. The influence of the root exudate on the chemotactic behaviour of *D. dipsaci* (Spiegel *et al*., 2003) suggests that a soil inoculation would be sufficient to identify a resistance mechanism against the

stem nematode. Kühnhold *et al*. (2006) reported an unsuccessful *D. dipsaci* penetration into sugar beet when 200 *D. dipsaci* J4 and adults were inoculated on the soil surface around the basal stem of the seedlings. Soil inoculation was, however, achieved on germinated seeds of several host crops, such as common bean, runner bean (*Phaseolus coccineus* L.), cucumber (*Cucumis sativus* L.), field pumpkin (*Cucurbita pepo* L.), and onion (Hesling, 1972). Several nematodes were found in *Gladiolus hybridus* when the soil was infected with dried *D. dipsaci*-infected narcissus (Goodey, 1952). On potato (*Solanum tuberosum*), 2 weeks after planting, 2000 *D. dipsaci* individuals were inoculated successfully in four 4-cm-deep holes surrounding the plant (Mwaura *et al*., 2015). Soil inoculation systems for *Heterodera* spp. and *Meloidogyne* spp. on sugar beet are already well established and successful. A suspension of second-stage juveniles (J2) and eggs is inoculated in holes surrounding young sugar beet seedlings (Toxopeus & Lubberts, 1970; Wong & Mai, 1973; Campos *et al*., 2012).

Our study aimed to determine a suitable *in vivo* screening mechanism to assess sugar beet resistance towards *D. dipsaci* penetration. To get as close as possible to field conditions, we aimed to determine the optimal conditions for performing a soil inoculation. The most suitable inoculation time point, inoculum level and positioning on sugar beet, as well as the rearing process on carrot, were determined. Finally, the new screening mechanism was validated on diverse sugar beet genotypes.

Materials and methods

NEMATODE INOCULUM

The *D. dipsaci* population used was extracted using Oostenbrink dishes (European and Mediterranean Plant Protection Organization, 2013) from infested sugar beets ('Samuela' KWS SAAT SE & Co. KGaA, Einbeck, Germany) collected in the Seeland region (CH) in 2015. Using a fine needle, 2000 J4 and adult nematodes were hand-picked after morphological identification of the tail, median bulb, stylet and lip shape. After suspending the nematodes in an antibiotic solution containing 0.1% streptomycin sulphate (w/v) and 0.1% amphotericin-B (w/v) for 30 min, nematodes were inoculated on carrot cylinders (2.5 × 5 cm) and incubated for 50 days at 20°C in the dark (Kühnhold *et al*., 2006). After extraction from the carrot cylinders, nematodes were stored at 6-8°C in the dark until further use.

GENERAL METHODS

The sugar beet seeds of 'Belladonna' (KWS), highly susceptible to the bacterial and fungal infection introduced by *D. dipsaci* (Leipertz & Valder, 2020), were sown in 200 ml plastic pots filled with 180 ml non-sterile sieved soil: compost mixture (1:1 (v/v)). The plant was regularly watered to maintain a suitable soil moisture for nematode movement during the entire experiment. The cultivation plant pots were covered with a Plexiglas miniglasshouse to maintain relative humidity above 95%. All experiments were set up at 15:8°C and a photoperiod of 18:6 h day:night in a growth chamber or a glasshouse simulating central European climatic conditions in April (Agrometeo, 2018). At 22 days post-planting (dpp), the sugar beet seedlings were removed from the pots, gently washed and stained in a plastic beaker containing a 0.1% acid fuchsin/lactic dye solution by boiling them twice in a microwave oven for 1 min (Kühnhold *et al*., 2006). Stained seedlings were then rinsed to remove the staining solution. The total number of nematodes per seedling was counted using a stereomicroscope at ×10 magnification after maceration in 30 ml tap water using an Ultra Turrax blender (T25 basic, IKA Labortechnik).

EXPERIMENT 1: INOCULATION TIME POINT

Eight inoculation time points (1, 3, 5, 6, 7, 8, 9, 10 dpp) were evaluated by inoculating 250 μl nematode suspension in four 1-cm-deep holes each (3 mm diam.) approx. 1.5 cm from the sown seed. A thousand nematodes were inoculated into each pot. The same nematode suspension was used for each inoculation time point. The standard leaf axil inoculation (Kühnhold *et al*., 2006) was used as a control by placing 10 μl nematode suspension with 200 *D. dipsaci* onto the leaf axils of the first pair of true leaves 14 dpp. Identical reproduction of the method performed by Kühnhold *et al*. (2006) motivated the leaf axil inoculation of 200 *D. dipsaci* individuals instead of the 1000 nematodes used for the soil inoculation. The experiment was performed with 15 replicates and conducted twice in a growth chamber (KBWF 720, Binder).

EXPERIMENT 2: INOCULUM LEVEL AND POSITIONING

Three different positionings of inoculum were tested in the pot: the first positioning ('4 holes') was implemented by inoculating a 250 μl nematode suspension in four 1-cm-deep holes each (3 mm diam.) approx. 1.5 cm from the sown seed. The second positioning ('surface') was set up with a surface inoculation of 1 ml nematode suspension into the centre of the pot. The third positioning ('mix') was a combination of the two, first positioning the inoculum by applying 200 μl nematode suspension into each of the four holes, and 200 μl nematode suspension was applied on the centre of the pot. For each positioning, 1 ml nematode suspension was used in each pot. Two different inoculum levels (200 and 1000 nematodes pot^{-1}) were investigated for each of the three positionings. The standard leaf axil inoculation (Kühnhold *et al*., 2006) was used as a control by placing 10 μl nematode suspension with 200 *D. dipsaci* onto the leaf axils of the first pair of true leaves 14 dpp. Identical replication of the method performed by Kühnhold *et al*. (2006) justified the leaf axil inoculation of 200 *D. dipsaci* individuals instead of 1000 nematodes used for the soil inoculation. Soil and leaf axil inoculations were conducted at 8 dpp (after considering the results of Experiment 1) and 14 dpp, respectively. Experiments were performed with ten replicates and conducted once in a glasshouse and replicated in a growth chamber (KBWF 720, Binder) with similar temperature, humidity, and light settings described in general methods.

EXPERIMENT 3: REARING OF *DITYLENCHUS DIPSACI* SUSPENSION ON CARROT DISCS

Further to the evaluation of inoculation time point and positioning, the influence of the inoculum level and the incubation period on the composition of development stages of *D. dipsaci* reared on carrot discs was investigated. After callus formation on carrots, 50, 75 or 100 axenic *D. dipsaci* adults were inoculated on individual carrot discs as described by Kühnhold *et al*. (2006). One 500 ml reagent bottle consisted of three carrot discs. The carrot discs were incubated for 35, 45 or 55 days in the dark at 20 ± 1°C. After the incubation period, nematodes were extracted from carrot discs for 24 h using a modified Oostenbrink dish (European and Mediterranean Plant Protection Organization, 2013). The resulting nematode suspension was passed through a 20 μm mesh sieve. The numbers of eggs, second- and third-stage juveniles (J2 and J3), and J4 and adults were determined using an optical microscope at ×40 magnification. Each treatment was replicated four times and the experiment was conducted twice.

EXPERIMENT 4: EFFECT OF NEMATODE REARING ON *DITYLENCHUS DIPSACI* INFECTIVITY ON SUGAR BEET

The nine *D. dipsaci* suspensions, previously tested in Experiment 3, were used in this experiment to determine the influence of the rearing process on the number of *D. dipsaci* penetrating sugar beet seedlings. At 8 dpp, the nematodes reared on carrot discs at different incubation periods and levels were inoculated into the soil. Either 500 or 1000 nematodes pot^{-1} were inoculated in four 1-cmdeep holes (3 mm diam.) approx. 1.5 cm from the sown seed. Experiments were performed with ten replicates and conducted once in a glasshouse and replicated in a growth chamber (KBWF 720, Binder) with similar temperature, humidity and light settings described in general methods.

EXPERIMENT 5: TEST SYSTEM VALIDATION ON DIFFERENT GENOTYPES

The experiment aimed to validate the results obtained in the previously described experiments on different sugar beet genotypes. The genotype selection consisted of seven hybrid lines and two inbred lines (Table 1). The inbred lines ‘DIT_05’ and ‘DIT_06’ are the paternal lines of the hybrid lines ‘DIT_02’ and ‘DIT_01’, respectively. ‘DIT_01’ (‘Belladonna’; KWS SAAT SE & Co. KGaA) is known to be highly susceptible to the bacterial and fungal infection introduced by *D. dipsaci* on the field (Leipertz & Valder, 2020). ‘DIT_02’

('Beretta'; KWS SAAT SE & Co. KGaA) and 'DIT_09' ('Celesta' KWS SAAT SE & Co. KGaA) showed a specific resistance to the bacterial and fungal infection introduced by *D. dipsaci* on the field (Leipertz & Valder, 2020). 'DIT_03' and 'DIT_04' were known as cultivars with a high sugar content and a high yielding cultivar, respectively (KWS SAAT SE & Co. KGaA, pers. comm., 2019). 'DIT_07' was known to be tolerant to *Cercospora beticola* (KWS SAAT SE & Co. KGaA, pers. comm., 2019). 'DIT_08' was a *Rhizoctonia solani* (AG 2-2IIIB) tolerant cultivar (KWS SAAT SE & Co. KGaA, pers. comm., 2019). At 9 dpp, 1000 nematodes previously reared for 45 days on carrot discs were inoculated into four 1-cm-deep holes (3 mm diam.) surrounding the pot's centre at a distance of approximately 1.5 cm. The experiment was conducted in a growth chamber (KBWF 720, Binder) with 15 replicates and conducted twice.

Table 1 Genotype selection used in Experiment 5. Description according to KWS SAAT SE & Co. KGaA (pers. comm., 2019).

Genotype	Trade name	Line	Comments
DIT_01	Belladonna	Hybrid	Susceptible to the bacterial and fungal infection introduced by *Ditylenchus dipsaci*
DIT_02	Beretta	Hybrid	Tolerant to the bacterial and fungal infection introduced by *Ditylenchus dipsaci*
DIT_03	/	Hybrid	high sugar type
DIT_04	/	Hybrid	high yield type
DIT_05	/	Inbred	Paternal line of DIT_02
DIT_06	/	Inbred	Paternal line of DIT_01
DIT_07	/	Hybrid	*Cercospora beticola* tolerant
DIT_08	/	Hybrid	*Rhizoctonia solani* (AG 2-2IIIB) tolerant
DIT_09	Celesta	Hybrid	Tolerant to the bacterial and fungal infection introduced by *Ditylenchus dipsaci*

DATA ANALYSIS

All experiments were arranged in a completely randomised design (CRD). Data for the number of nematodes penetrating sugar beet seedlings were pooled after confirming the absence of effect of the experiment replications. Due to the significant effect of the experiment replications, data from Experiments 2 and 5 were not pooled. To obtain normality of distribution data were transformed using the square root function $g(y) = \sqrt{y}$, in R-Software. An analysis of the variance of the one-way layout was then conducted in Experiment 5. Problems with normality and homogeneity of variances led to using a Kruskal- Wallis Rank Sum Test in Experiments 1 and 2. An analysis of the two-way layout was performed in Experiment 3. A non-parametric three-way ANOVA for trimmed means (Wilcox, 2012) was performed to determine the effect of nematode rearing on *D. dipsaci* infectivity on sugar beet, where the inoculum level on carrots, the incubation period on carrots, and the inoculum level on sugar beets were considered as the three independent variables. Tukey test of multiple comparisons of means was performed as posthoc tests (Lenth, 2016). Values show the mean of the standard deviation ($\pm$ SD). Statistical analysis was performed using the software R.

Results

EXPERIMENT 1: INOCULATION TIME POINT

The inoculation time point significantly affected *D. dipsaci* penetration rate on sugar beet seedlings ($P < 0.0001$, Fig. 1). At 1 dpp, the soil inoculation led to the presence of 17.7 ± 4.9 nematodes in the sugar beet seedlings and declined to 1.9 ± 0.8 after 6 dpp. A significantly higher penetration rate ($P < 0.05$) than the other soil inoculation time points was found with a soil inoculation after 8 or 9 dpp with 56.1 ± 10.7 and 81.3 ± 12.0 nematodes, respectively. The leaf axil inoculation led to 31.1 ± 6.6 nematodes penetrating the sugar beet seedlings, significantly lower than soil inoculation occurring 9 dpp ($P < 0.001$). In each treatment, all *D. dipsaci* development stages were found in sugar beet seedlings. The plant emergence rate was higher than 85% for all treatment except when soil inoculation occurred at 1 dpp (50%).

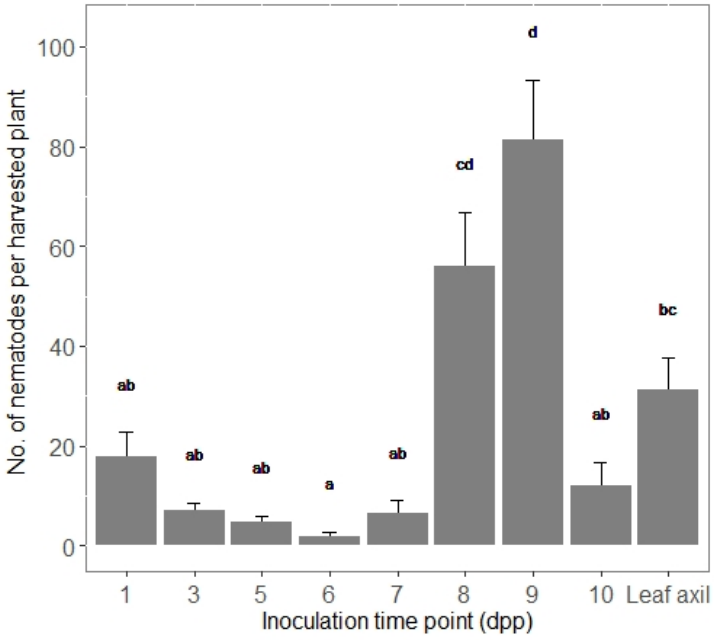

Fig. 1 Effect of the inoculation time point on the number of *Ditylenchus dipsaci* penetrating sugar beet seedlings 22 days post-planting (dpp). Different letters over the bars indicate a significant difference at $P < 0.05$, according to the Tukey test of multiple comparisons of means.

EXPERIMENT 2: INOCULUM LEVEL AND POSITIONING

Inoculating 1000 *D. dipsaci* into the soil resulted in the highest penetration rate and did not significantly differ among inoculum positionings (Fig. 2). In the first experiment replication, all three treatments that were inoculated with 1000 nematodes pot^{-1} contained a significantly higher number of *D. dipsaci* at 22 dpp (115 ± 19 nematodes) compared to the treatments that were inoculated only with 200 nematodes pot^{-1} (16 ± 4) ($P < 0.0001$). The leaf axil inoculation yielded an average of 17 ± 4 nematodes penetrating the sugar beet seedling. In the second experiment replication, the surface inoculation of 1000 nematodes led to the highest *D. dipsaci* penetration (80 ± 30 nematodes). It was significantly higher than in the four treatments, where 200 nematodes pot^{-1} were inoculated ($P < 0.0001$). The two other treatments with inoculation densities of 1000 nematodes ('4 holes' and 'mix') did not lead to a significantly higher *D. dipsaci* penetration compared to the four treatments inoculating 200 nematodes pot^{-1} ($P > 0.05$).

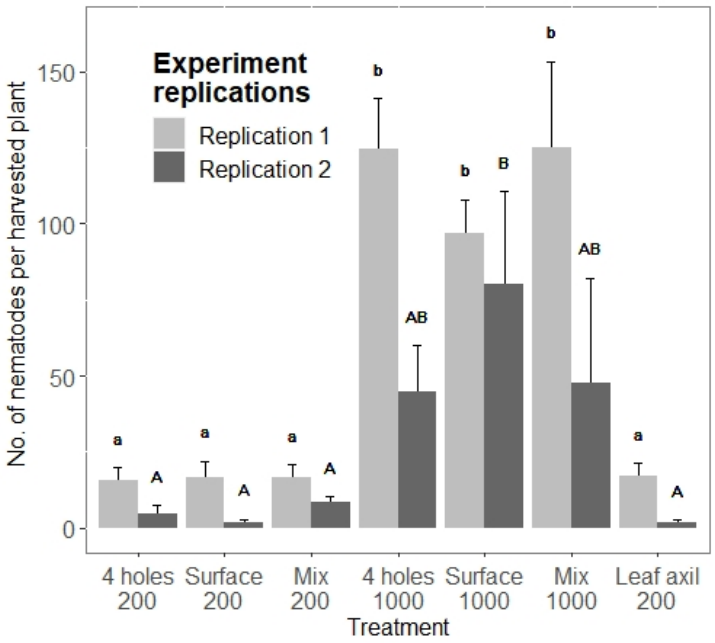

Fig. 2 Effect of inoculum level and inoculum positioning (4 holes; surface; mix; see Materials and methods) on the number of *Ditylenchus dipsaci* penetrating sugar beet seedlings 22 days post-planting. Different letters over the bars indicate a significant difference at $P < 0.05$, according to the Tukey test of multiple comparisons of means.

All *D. dipsaci* development stages were found in sugar beet seedlings. Inoculating 1000 *D. dipsaci* led to the formation of swollen leaf axils at 5 days post-inculation (dpi). The plants inoculated by the '4 holes' inoculation method swelled 1 day later.

EXPERIMENT 3: REARING *DITYLENCHUS DIPSACI* SUSPENSION ON CARROT DISCS

The incubation period on carrots significantly influenced the stage composition of the nematode suspension ($P < 0.0001$, Table 2).

Table 2 Effect of the incubation duration and inoculum level of *Ditylenchus dipsaci* on carrots on the number (± SD) of produced nematodes and on the percentage (mean ± SD) of eggs, second- (J2), third- (J3), fourth-stage juveniles (J4) and adult stages in the suspension.

No. of nematodes inoculated /carrot disc	Incubation duration on carrot (d)	Eggs (%)	J2-3 (%)	J4-A (%)	Nematodes in the suspension
50	35	55.73 ± 6.9 a	25.36 ± 8.02 a	18.91 ± 6.58 a	34,883 ± 20,115 a
75	35	48.89 ± 6.63 ab	27.04 ± 4.25 a	24.07 ± 5.7 ab	69,336 ± 28,387 ab
100	35	50.83 ± 9.16 ab	25.28 ± 7.16 a	23.89 ± 6.48 ab	86,746 ± 54,384 ab
50	45	36.82 ± 11.81 bc	26.09 ± 3.68 a	37.09 ± 10.89 bc	95,351 ± 40,746 ab
75	45	28.2 ± 14.47 cd	24.64 ± 6.19 ab	47.16 ± 15.51 cd	93,822 ± 34,473 ab
100	45	25.14 ± 9.72 cd	31.45 ± 7.41 ab	43.41 ± 9.34 c	102,591 ± 44,181 ab
50	55	13.07 ± 12.39 de	23.04 ± 11.76 abc	63.89 ± 21.51 de	78,304 ± 31,954 ab
75	55	6.12 ± 4.76 e	13.86 ± 4.76 bc	80.02 ± 7.22 e	107,487 ± 44,797 b
100	55	4.45 ± 2.95 e	13.12 ± 2.83 c	82.42 ± 2.68 e	117,687 ± 52,630 b
Nematode/carrot disc		< 0.01	> 0.05	< 0.01	0.0225
Incubation period on carrots		< 0.0001	< 0.0001	< 0.0001	< 0.01
Interaction		> 0.05	0.0158	> 0.05	> 0.05

A short period of incubation led to a high percentage of eggs in the suspension. A long period favoured the development of J4 and adults. Nematode suspensions incubated for 35 days on carrots contained a

significantly higher percentage of eggs ($P < 0.05$) and a lower percentage of J4 and adults ($P < 0.05$) than *D. dipsaci* suspensions incubated for 55 days on carrots. Nematode suspensions incubated for 35 days on carrots showed a significantly higher percentage of J2 and J3 than the nematode suspensions incubated for 55 days, with an inoculum of 75 or 100 nematodes on individual carrot discs.

The inoculum level on carrot discs significantly influenced the proportion of eggs, J4 and adults in the suspension ($P < 0.01$) but did not affect the percentage of J2 and J3 in the suspension ($P > 0.05$). After incubation for 35 days, the carrot discs inoculated with 50 *D. dipsaci* adults disc^{-1} led to the highest percentage of eggs (56%) and the lowest percentage of J4 and adults (19%) in the suspension. Inoculation with 100 *D. dipsaci* disc^{-1}, followed by incubation for 55 days, gave the highest percentage of J4 and adults (82%) and the lowest percentage of eggs (4%) in the final suspension. Nematode migration (egress) out of the carrot discs was visible at 45 and 55 dpi. Nematode motility observed under the microscope decreased once egress was visible. The inoculum level ($P < 0.05$) and the incubation period ($P < 0.01$) had a significant effect on the nematode reproduction rate after incubation on carrots. Long incubation resulted in a higher number of reared nematodes. The carrots inoculated with 100 *D. dipsaci* disc^{-1} and incubated for 55 days yielded the highest number of reproduced nematodes. *Ditylenchus dipsaci* suspension incubated for 35 days, with an inoculum level of 50 nematodes, contained a significantly lower number of nematodes than the *D. dipsaci* suspension incubated for 55 days, with an inoculum of 75 or 100 nematodes disc^{-1}.

EXPERIMENT 4: EFFECT OF NEMATODE REARING ON *DITYLENCHUS DIPSACI* INFECTIVITY ON SUGAR BEET

The inoculum level and incubation period on carrots and the inoculum level on sugar beets significantly influenced the number of nematodes penetrating the sugar beet seedlings ($P < 0.001$, Fig. 3). Nematode suspension incubated for 35 days, with an inoculum level of 100 nematodes disc^{-1}, showed a significantly higher *D. dipsaci* penetration potential when we doubled the inoculum level on sugar beet seedlings ($P < 0.01$).

An incubation period of 35 or 45 days on carrot discs resulted in a higher penetration rate on sugar beet seedlings (Fig. 3). A high inoculum level on carrot (100 nematodes disc^{-1}) was required to reach a high *D.*

dipsaci infectivity when nematodes were incubated for 35 days on carrots, whereas a low inoculum level on carrot (50 nematodes disc^{-1}) was required to reach a high *D. dipsaci* infectivity when nematodes were incubated for 45 days on carrots. All *D. dipsaci* development stages were found in sugar beet seedlings.

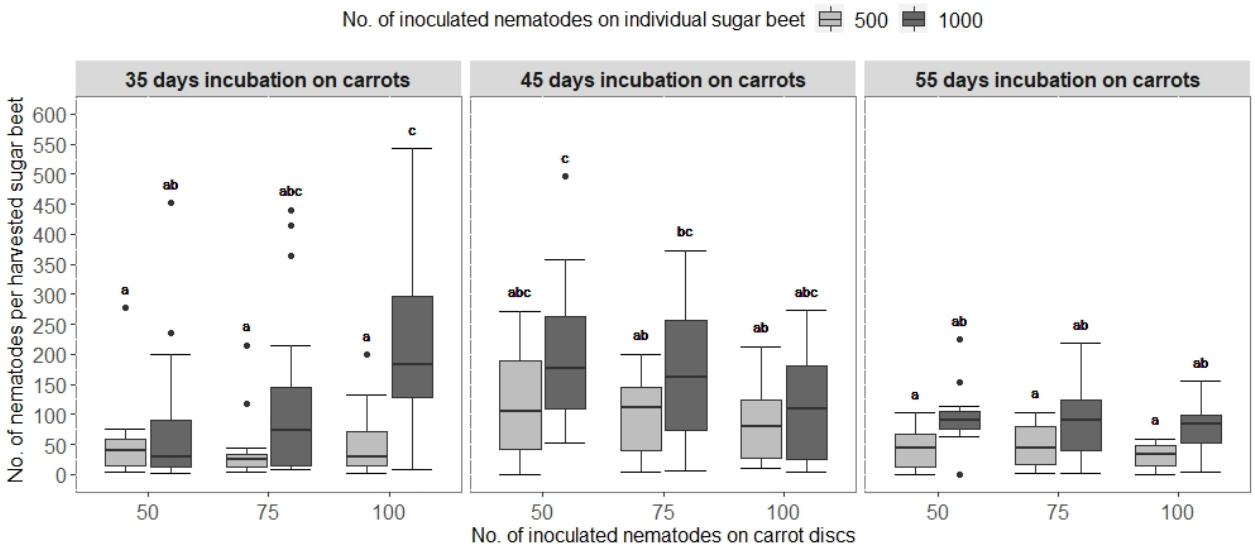

Fig. 3 Effect of the incubation period (days; days post-inoculation (dpi)) and inoculum level on carrot discs and the inoculum level on sugar beets on *Ditylenchus dipsaci* penetration at 22 dpi. Different letters over the boxplots indicate a significant difference at P <0.05, according to the Tukey test of multiple comparisons of means.

EXPERIMENT 5: TEST SYSTEM VALIDATION ON DIFFERENT GENOTYPES

The genotype significantly influenced *D. dipsaci* infectivity on sugar beet seedlings (Fig. 4). At 22 dpp, seedlings of 'DIT_08', a *R. solani* (AG 2-2IIIB) tolerant cultivar, contained a significantly lower number of penetrated nematodes than 'DIT_02', considered as tolerant to the bacterial and fungal infection introduced by *D. dipsaci*. No differences were observed among the highly susceptible cultivar, Belladonna '(DIT_01)', and 'DIT_02' and 'DIT_09', known to be tolerant to the bacterial and fungal infection introduced by *D. dipsaci*. The penetration rate was significantly higher (P <0.0001) in the first experiment replication, where 183 ± 69 nematodes penetrated 'DIT_09' compared to the second experiment replication, where 76 ± 60 nematodes penetrated 'DIT_09'.

All *D. dipsaci* development stages were found in sugar beet seedlings.

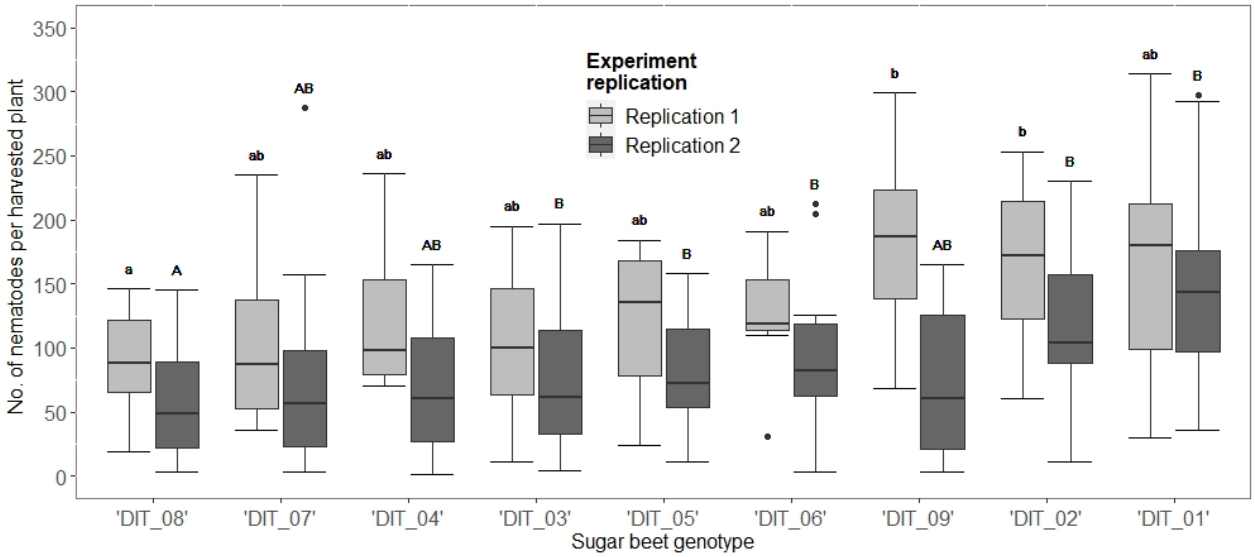

Fig. 4 Effect of sugar beet genotypes on *Ditylenchus dipsaci* penetration at 22 days post-planting. Different letters over the boxplots indicate a significant difference at $P < 0.05$, according to the Tukey HSD test.

Discussion

This study demonstrated the potential of a soil inoculation to investigate the interaction between *D. dipsaci* and sugar beet. The inoculation time point affected the infectivity of *D. dipsaci*. The highest *D. dipsaci* penetration rates coincided with the time point of seedling emergence at 8 and 9 dpp, when most of the plants emerged through the soil surface. This observation suggests that *D. dipsaci* penetration occurs below-ground or at the transition between the soil surface and the hypocotyl. An above-ground penetration on the leaf axil (Wallace, 1962) seems unlikely since the penetration rate decreased when *D. dipsaci* was inoculated at the time point when the major part of the plant had already emerged through the soil surface. The hampered seedling emergence of sugar beet inoculated at 1 dpp also supports the theory of a belowground infection. The below-ground *D. dipsaci* infection on freshly germinated seeds significantly reduced seed viability, leading to 50% of plants dying before emergence. This low emergence rate was also observed in severely infested fields (Caspary, 1976). The decrease of the inoculum quality may explain the lower *D. dipsaci* penetration potential when the inoculation time point occurred between 3 and 7 dpp. As the same suspension was used for each inoculation time point, the first inoculation time point contained a highly active nematode suspension. *Ditylenchus dipsaci* activity might decrease with time due to lower energy reserves. The development of the hypocotyl coincided with the sudden increase of *D. dipsaci* infectivity at 8 dpp. As a consequence of a low plant emergence rate (Caspary, 1976) and a *D. dipsaci* penetration rate similar to field conditions (Storelli *et al*., 2020), an inoculation at 1 dpp, therefore, may be appropriate for the development of a new test system to determine the interaction between *D. dipsaci* and sugar beet. However, here we aim to establish a system to investigate sugar beet resistance towards *D. dipsaci* penetration. The high proportion of non-emerging plants when nematodes are applied at 1 dpp does not allow for a proper investigation of *D. dipsaci* penetration in sugar beet. A high emergence rate is required to obtain enough plant material to determine the number of nematodes in the plant tissue. An early nematode application at 1 dpp may be appropriate to investigate the soil migration and penetration pathway of *D. dipsaci*, but not for penetration resistance. An inoculation at 8 or 9 dpp allows a high *D. dipsaci* penetration while maintaining a high plant emergence rate optimal for investigating resistance.

The positioning of the inoculum did not affect the infectivity of *D. dipsaci*. From the three different positionings of the inoculum, *D. dipsaci* was always able to penetrate the host. The highest *D. dipsaci* number observed in the plant with a '4 holes' inoculation confirms that *D. dipsaci* migrates below-ground into its host (Spiegel *et al*., 2003). The observed 1-day delay at developing swollen leaf axils when the nematodes were inoculated in four holes suggests that *D. dipsaci* required 1 day to migrate into its host. The success of *D. dipsaci* migration below-ground into sugar beet plants enables a better understanding of the interaction between the nematode and its host. The experiment replication conducted in a growth chamber resulted in a higher *D. dipsaci* penetration rate, whereas the glasshouse results showed high heterogeneity. The uncontrolled higher temperature range and the sunlight in the glasshouse may play a role in the variation between the experiment replications (Castillo & Jiménez-Díaz, 1995).

In Experiments 2 and 4, the inoculum level did affect *D. dipsaci* penetration rate on sugar beet seedlings. As expected, inoculating 1000 *D. dipsaci* led to a significantly higher number of nematodes penetrating sugar beet than the 200 and 500 in Experiments 2 and 4, respectively. However, the penetration rate (number of nematodes in plant/number of inoculated nematodes) was the highest in the plant inoculated with 500 nematodes, where the penetration rate was up to 30%. The penetration rate for 200 and 1000 inoculated nematodes was 8% and up to 20%, respectively. Inoculating a high number of nematodes allowed us to avoid seedlings containing no nematodes. By soil inoculating each plant with 200 *D. dipsaci*, we obtained a similar penetration rate as Kühnhold *et al*. (2006) on sugar beets and Yavuzaslanoglu (2019) on onions. The penetration rate in our experiments was lower than the above-ground inoculation conducted on yellow pea (Hajihassani *et al*., 2017a). The leaf axil inoculation used as a control in our experiments was lower than for Kühnhold (2011). The dominance of J4 and adults in the suspension used by Kühnhold (2011) suggests that only J4 and adults may penetrate the leaf axil.

Ditylenchus dipsaci inoculum level and incubation period on carrots did affect the stage composition of the reared suspension. This experiment confirms the high reproduction capacity of *D. dipsaci* (Yuksel, 1960). As demonstrated by Kühnhold *et al*. (2006), the inoculum level greatly influenced the reproduction of *D. dipsaci* on carrots. A short incubation period gave highly active suspensions dominated by eggs and juvenile

nematodes. As we prolonged the incubation period, the nematode suspensions were aging and dominated by J4 and adults. Egress was a good indicator of the presence of J4 and adults in the suspension. Once egress was visible, nematode motility declined as food supply decreased (Perry & Wright, 2013). Longer incubation periods did not lead to a continuous increase in nematode numbers as the carrot discs lost their nutritive capacity (Moody *et al*., 1973; Verdejo-Lucas & Pinochet, 1992; Hajihassani *et al*., 2017b). *Ditylenchus dipsaci* inoculum level and incubation period on carrot discs also affected the nematode penetration rate on sugar beet seedlings. Inoculation of nematode suspension containing a high proportion of eggs and J2-J3 on sugar beet resulted in a higher penetration potential. This suggests an essential role of eggs and J2-J3 for a successful *D. dipsaci* penetration into sugar beet. This result, combined with the presence of all D. dipsaci development stages in sugar beet seedlings, does not support the hypothesis that only J4 overwinter and penetrate the host during the spring.

Experiment 5 aimed to validate the success of soil inoculation on different sugar beet genotypes. *Ditylenchus dipsaci* successfully invaded all tested sugar beet genotypes in Experiment 5. Although no cultivar was found to be immune, a variation in penetration rate did exist among genotypes. To date, no sugar beet cultivar is resistant to *D. dipsaci* penetration. Therefore, high penetration heterogeneity is observed. Penetration rates of nematode on sugar beet often led to high heterogeneity within the cultivars and across the experiment replication (Kühnhold, 2011; Westphal, 2013) due to the strong influence of environmental conditions and nematode population on the virulence of the inoculum.

This study demonstrates that soil inoculation is an efficient method to investigate *D. dipsaci* penetration in sugar beet, though penetration rates remain variable. However, a stable penetration rate through soil inoculation is challenging when sugar beet interacts with plant-parasitic nematodes (Westphal, 2013). Soil humidity and temperature are two critical parameters for high *D. dipsaci* virulence (Duncan & Moens, 2013). By simulating Central European climatic conditions occurring in April (Agrometeo, 2018), we achieved penetration rates of *D. dipsaci* and symptom expressions near to field conditions (Storelli et al., 2020). Therefore, we recommend a soil inoculation of 1000 freshly extracted nematodes per pot at the time point of plant emergence. The nematode suspension has to be previously reared for 35 days on carrot discs to

obtain active *D. dipsaci* inoculum. Thus, the developed soil inoculation offers new opportunities to investigate the interaction between *D. dipsaci* and sugar beet. The plant's influence on the chemotactic behaviour of *D. dipsaci* and below-ground infection can now be investigated under in vivo conditions.

Acknowledgements

The authors thank the seed company KWS SAAT SE & Co. KGaA, Germany, the sugar factory Schweizer Zucker AG, Switzerland, and Bern University of Applied Sciences, Switzerland, for their financial support. We would like to further acknowledge the working group of Nematology at Agroscope, Switzerland for providing the initial *D. dipsaci* population.

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4 Manuscript 2

Screening of sugar beet pre-breeding populations and breeding lines for resistance to *Ditylenchus dipsaci* penetration and reproduction

Alan Storelli[1*], Alexandra Minder[1], Andreas Keiser[1], Sebastian Kiewnick[2], Matthias Daub[2], Anne-Katrin Mahlein[3], Mario Schumann[4], Werner Beyer[4]

1) School of Agricultural, Forest and Food Sciences HAFL, Bern University of Applied Sciences BFH, Länggasse 85, 3052 Zollikofen, Switzerland

2) Julius Kuehn Institute, Federal Research Center for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Messeweg 11/12, 38104 Braunschweig, Germany

3) Institute of Sugar Beet Research IfZ, Holtenser Landstrasse 77, 37079 Goettingen, Germany

4) KWS SAAT SE & Co. KGaA, Grimsehlstrasse 31, 37574 Einbeck, Germany

Published in Journal of Plant Diseases and Protection

Received: 20 December 2020

Accepted for publication: 21 May 2021

Online publication date: 30 May 2021

DOI 10.1007/s41348-021-00483-6

Summary – *Ditylenchus dipsaci* is an economically important plant-parasitic nematode affecting European sugar beets. To date, no sugar beet cultivars carrying resistance against *D. dipsaci* are available to farmers. To find potentially resistant sugar beet lines restricting reproduction and penetration of *D. dipsaci*, three consecutive *in vivo* bioassays were carried out. The first experiment determined the penetration rate of *D. dipsaci* in 79 breeding lines and 14 pre-breeding populations. Based on these results, *D. dipsaci* penetration and reproduction resistance of eight genotypes was intensively investigated. It could be demonstrated that none of the genotypes showed resistance towards *D. dipsaci*. However, a high variation of the penetration rate by *D. dipsaci* was observed among the genotypes. The breeding line ‘DIT_119’ effectively reduced *D. dipsaci* penetration (34.4 ± 8.8 nematodes/plant at 22 days post-planting) compared to the susceptible control (109.0 ± 16.9) while ensuring a yield comparable to non-inoculated plants. However, the breeding line ‘DIT_119’ did not reduce *D. dipsaci* reproduction. The paternal line of the cultivar BERETTA KWS, demonstrating a high tolerance to *D. dipsaci* crown rot symptoms, did not reduce penetration and reproduction. Thus, no correlation can be established between reduced penetration rates, reproduction, and tolerance to *D. dipsaci*. This study provides an essential basis for the development of resistant sugar beet cultivars to *D. dipsaci*. The variations observed among genotypes now need to be confirmed with larger-scale screenings.

Keywords – Breeding line, In vivo, Penetration, Pre-breeding population, Reproduction, Resistance breeding

Introduction

The stem and bulb nematode *Ditylenchus dipsaci* (Kuhn 1857) Filipjev 1936 is a migratory endoparasite affecting up to 450 plant species worldwide (Duncan and Moens 2013; Seinhorst 1956). This nematode pest has emerged as an economically threatening plant-parasitic nematode in the European sugar beet (*Beta vulgaris* L.) production (Dewar and Cook 2006; Leipertz 2007; Subbotin et al. 2005). The penetration early in the growing season leads to swollen hypocotyls and distorted leaves and cotyledons (Griffin 1983). Later in the season, bacterial and fungal infection, such as *Rhizoctonia solani* (AG 2‑2IIIB) and *Verticillium albo-atrum*, introduced by *D. dipsaci* leads to the crown’s rotting (Hillnhutter et al. 2011; Vrain 1987). Since the withdrawal of the nematicide aldicarb, no effective direct management has been available for control of *D. dipsaci*. The broad range of host plants of *D. dipsaci* hinders crop rotation strategies for successful management of this nematode (Jones et al. 2013). The fungicide fluopyram, a succinate dehydrogenase inhibitor (SDHI), effectively reduced the fungal and bacterial infection introduced by the stem and bulb nematode (Storelli et al. 2020). However, no long-term effect on *D. dipsaci* population development was observed. Therefore, breeding for sugar beet cultivars’ resistance is a sustainable management approach (Schomaker and Been 2013).

Roberts (2002) described resistance as the plant’s ability to suppress the development and consequently the reproduction of nematodes. The latter author further described tolerance as the plant’s ability to compensate for nematode infection with little or no yield loss. To date, no sugar beet cultivar with resistance against *D. dipsaci* is although available. Kuhnhold (2011) observed variations in *D. dipsaci* penetration and reproduction rates depending on the breeding line or cultivar tested. However, *D. dipsaci* penetration and reproduction rates in less susceptible genotypes were still high (Kuhnhold 2011). Some sugar beet cultivars are tolerant to the fungal and bacterial infection introduced by *D. dipsaci* in the field (Leipertz and Valder 2020). Resistance towards *D. dipsaci* penetration may prevent nematode penetration and, consequently, the introduction of fungal and bacterial pathogens. However, resistance towards *D. dipsaci* reproduction may reduce nematode population development but does not avoid introducing fungal and bacterial pathogens.

Monogenic or polygenic resistance towards the sugar beet cyst nematode *Heterodera schachtii* (Schmidt) is identified since a long time (Blok et al. 2018; Golden 1959; Savitsky 1975).

Resistance towards *D. dipsaci* has been observed in clover (*Trifolium* spp.), lucerne (*Medicago sativa* L.), faba bean (*V. fabaea* L.), and oat (*Avena* spp.) cultivars (McDaniel and Barr 1994; Stanton et al. 1984; Starr et al. 2013). *Ditylenchus dipsaci* resistance is monogenic on lucerne, and polygenic on faba bean, wild oat (*A. ludoviciana* L.), and red clover (*T. pratense* L.) (Plowright et al. 2002). Resistant oat cultivars successfully reduced the reproduction of *D. dipsaci* but not the penetration of this nematode pest (Blake 1962; Griffiths et al. 1957). Plowright et al. (2002) reported high resistance against *D. dipsaci* in a faba bean line, which has been used to develop resistant cultivars for North Africa. Despite variations in the nematode reproduction rate among onion (*Allium cepa* L.) cultivars, Yavuzaslanoglu (2019) found no resistance towards *D. dipsaci*.

Caubel et al. (1994) demonstrated a positive relationship between symptom expression at 3 weeks postinoculation and *D. dipsaci* reproduction at 10 weeks postinoculation on red clover. Cook and Evans (1988) reported no correlation between leaf size of white clover (*T. repens* L.) and tolerance towards *D. dipsaci* infection. The development of forage crops resistant to *D. dipsaci* was based on the characterization of symptoms on seedlings (Caubel et al. 1994). Variations in the resistance levels of lucerne species depending on the *D. dipsaci* population used were observed (Leclerq and Caubel 1991; Whitehead 1992). The resistance of a host plant to *D. dipsaci* is highly dependent on the geographic origin of the nematode population, which hinders any breeding programme.

Our study aims were to identify among 14 pre-breeding populations and 79 breeding lines sugar beets with resistance towards *D. dipsaci* penetration and reproduction. A screening of a wide range of genotypes was first conducted to identify potential candidates for resistance towards *D. dipsaci* penetration. Based on this screening, a more in-depth investigation of the genotypes with potential resistance was conducted to determine their potential resistance to *D. dipsaci* penetration and reproduction. The tolerance of the sugar beet genotypes to *D. dipsaci* infection is determined by assessing plant survival and yield.

Materials and methods

NEMATODE INOCULUM

The *D. dipsaci* population used in all experiments was derived from three infested sugar beets tubers (cv. SAMUELA KWS) collected in the Seeland region (CH) in 2015 (47.058154, 7.275107). Nematodes were extracted by Oostenbrink dishes (European and Mediterranean Plant Protection Organization 2013). Fourth-stage juveniles (J4) and adult nematode stages were hand-picked after morphological identification of the tail, median bulb, stylet, and lip shape using an optical microscope at 40x magnification. After suspending the nematodes in an antibiotic solution containing 0.1% streptomycin sulphate (w/v) and 0.1% amphotericin-B (w/v) for 30 min, 50 nematodes were inoculated per surface sterilized (1% NaOCl) carrot disc (2.5 x 5 cm) and incubated for 45 days at 20 °C in the dark (Kuhnhold et al. 2006; Storelli et al. 2021). The nematodes were extracted from the carrot discs, stored in the dark at 6‑8 °C, and after 24 hr the sugar beet plants were inoculated. On average, *D. dipsaci* suspensions used for inoculation contained 43 ± 1.5% eggs, 28 ± 0% second- and third-stage juveniles (J2-3), and 29 ± 1.5% of J4s and adult stages.

PLANT MATERIAL

Investigated plant material included 79 breeding lines and 14 pre-breeding populations from the KWS gene pool (KWS SAAT SE & Co. KGaA, Einbeck, Germany). The pre-breeding populations, compared to breeding lines, were quite heterozygous and less advanced (KWS SAAT SE & Co. KGaA, personal communication). Currently, limited information on genetic variation for resistance or tolerance to *D. dipsaci* in sugar beet has been published (Kuhnhold 2011; Leipertz and Valder 2020). The cvs. BELLADONNA KWS and BERETTA KWS, known to be susceptible and tolerant to the fungal and bacterial infection introduced by *D. dipsaci* in the field, respectively, were used in this study (Leipertz and Valder 2020). The paternal line ‘DIT_006’ of the cv. BELLADONNA KWS and the paternal line ‘DIT_005’ of the cv. BERETTA KWS were used as standards for the experiments.

GENERAL METHODS

The sugar beet seeds were sown in 200-ml plastic pots filled with a 180-ml non-sterile sieved loess soil: compost mixture (1/1) (v/v). The loess soil originated from Einbeck (Germany). Per pot, three seeds of pre-breeding populations or two seeds of breeding lines were sown to compensate for the partially low sugar beet germination rate. After the emergence of the first plant, all following emerging plants were removed each day to ensure only one seedling per pot remained. Due to the large variation of growth rates among genotypes, nematode inoculation was split into two inoculation time intervals. At 8 and 11 days post-planting (dpp), approx. 1.5 cm from the centre of the pot, 500 nematodes (of mixed life stages) were inoculated in 500 µl into two 1-cm deep holes (diam. 3 mm), resulting in 1000 *D. dipsaci* individuals/plant. The plants were regularly watered to maintain a suitable soil moisture allowing nematode movement during the entire experiment.

The resistance of the genotypes towards *D. dipsaci* penetration was determined by the number of nematodes in the whole plant at 22 dpp. The sugar beet seedlings were removed from the pots at 22 dpp, gently washed, transferred to a plastic beaker containing a 0.1% acid fuchsin/ lactic solution, and boiled twice in a microwave oven for 1 min (Kuhnhold et al. 2006). The stained seedlings were then rinsed to remove the staining solution. The total number of nematodes per seedling was counted using a stereomicroscope at 10x magnification after maceration of the whole plant (6500 RPM) in 30 ml tap water using an Ultra Turrax blender (T25 basic/S25 N - 18 G, IKA Labortechnik, Germany). The resistance of the genotypes towards *D. dipsaci* reproduction was determined by the number of nematodes in the whole plant at 60 post-inoculation (dpi). The sugar beet plants were removed from the pots at 60 dpi, washed, weighed (whole fresh plant), and the whole plants cut into 0.5-cm pieces. Nematodes were extracted from the sliced plant material using Oostenbrink dishes for 24 h (European and Mediterranean Plant Protection Organization 2013). The number of *D. dipsaci* individuals per sugar beet plant was determined by counting 3 × 1 ml aliquots from 15 ml total volume using an optical microscope at x40 magnification. The effect of the genotypes on the nematode incidence (%) at the harvest time point (number of plants containing nematodes/number of harvested plants*100) and on the incidence of the symptoms (Fig. 1) (number of swollen plants at 14 dpi/number of

germinated plants at 14 dpi*100) was determined in experiments 2 and 3. The number of harvested sugar beet at 60 dpi in relation to the number of emerged plants at 22 dpp determined plant survival (%).

Fig. 1 *Ditylenchus dipsaci* infected sugar beet seedling at 14 days post-inoculation (dpi) showing swollen leaf-axil

EXPERIMENT 1: SCREENING OF SUGAR BEET LINES FOR *DITYLENCHUS DIPSACI* PENETRATION

To estimate the genetic variation of genotypes belonging to breeding lines (79) or pre-breeding populations (14), they were screened for their response concerning *D. dipsaci* penetration rates. 'DIT_006' was used as the susceptible standard to determine the relative (%) penetration susceptibility (number of nematodes in inbred line 'DIT_xxx'/number of nematodes in 'DIT_006'*100). The experiments were set up at 15 max/8 min °C temperature range and a photoperiod of 18/6 hr day/night and conducted twice in a glasshouse. The investigation of breeding lines and pre-breeding populations was performed with 10 and 15 replicates, respectively.

EXPERIMENT 2: EFFECT OF SUGAR BEET GENOTYPES ON *D. DIPSACI* PENETRATION POTENTIAL

Genotypes selected for their response after inoculation with *D. dipsaci* in the first screening experiment were investigated for their potential to reduce penetration rates of this nematode pest. Only the sugar beet genotypes showing the lowest and the highest *D. dipsaci* penetration values in experiment 1 were further

investigated in experiment 2 with a greater replication number. The experiment was set up as described above regarding the temperature range and photoperiod and conducted twice in a growth chamber (KBWF 720, Binder GmbH, Germany), with 20 replicates.

EXPERIMENT 3—EFFECT OF SUGAR BEET GENOTYPES ON *D. DIPSACI* REPRODUCTION POTENTIAL

Genotypes selected for their response after inoculation with *D. dipsaci* in the first screening experiment were investigated for their potential to reduce reproduction of the species. The experiment commenced in a growth chamber (KBWF 720, Binder GmbH, Germany) under the same temperature and photoperiod conditions as indicated above for experiments 1 and 2. For optimal growth of the sugar beets, the plants were transferred, at 22 dpp, to a glasshouse where a temperature range of 22 max/15 min °C and a photoperiod of 18/6 hr day/night prevailed. The experiment was performed with 10 replicates and conducted twice. For each genotype, 2 x 10 replicates of non-inoculated plants were used as control.

DATA ANALYSES

The investigation of the effect of the breeding lines on the aggressiveness of *D. dipsaci* in experiment 1 was performed in a randomized complete block design. The investigation of pre-breeding populations in experiment 1 and investigations in experiments 2 and 3 were performed in a complete randomized design. In experiment 1, a Friedman rank-sum test was performed to determine the effect of the breeding lines on *D. dipsaci* penetration rate in the whole sugar beet seedlings. The effect of the pre-breeding populations on *D. dipsaci* penetration rate in experiment 1 was determined by using a Kruskal‑Wallis rank-sum test. In experiments 2 and 3, problems with normal distribution led to using a Kruskal‑Wallis rank-sum test to determine the effect of the genotype on *D. dipsaci* penetration and reproduction rate, respectively. Dunn’s multiple comparison tests were performed as post hoc tests. In experiment 3, the effect of *D. dipsaci* inoculation on the fresh biomass of each beet plant at 60 dpi was determined by performing a Wilcoxon signed rank test to compare data for inoculated and non-inoculated plants. Statistical analyses and figures were performed using the software R.

Results

EXPERIMENT 1: SCREENING OF SUGAR BEET LINES FOR *DITYLENCHUS DIPSACI* PENETRATION

No significant difference of *D. dipsaci* penetration into sugar beet seedlings was observed among the breeding lines ($P > 0.05$, Fig. 2). The average number of nematodes penetrating sugar beet seedling at 22 dpp varied from 7.5 to 105.2 nematodes per plant. The standard 'DIT_006' showed an average of 80.5 ± 19.1 nematodes per plant at 22 dpp. 'DIT_119' showed the lowest (10.6%) and 'DIT_144' the highest (129.2%) relative susceptibility to *D. dipsaci* penetration. The breeding line 'DIT_005' showed 60.4 ± 15.3 nematodes per plant at 22 dpp.

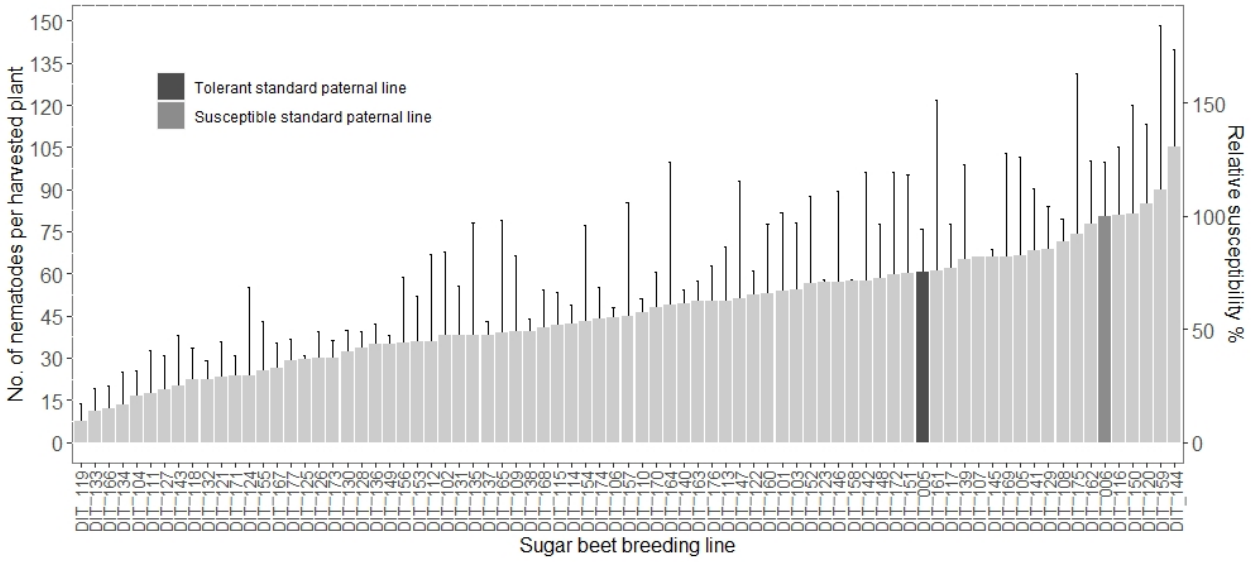

Fig. 2 Effect of sugar beet breeding lines on *Ditylenchus dipsaci* number per harvested plant at 22 days post-planting (dpp), and their relative susceptibility (%) to *D. dipsaci* (average number of nematodes per plant/average number of nematodes in the inbred line DIT_006) in a glasshouse trial. No significant differences among breeding lines according to Friedman rank-sum test (n = 10)

The pre-breeding populations significantly affected *D. dipsaci* penetration into sugar beet seedlings ($P < 0.05$, Fig. 3). The pre-breeding populations 'DIT_207' showed the lowest number of nematodes per plant (12.2 ± 20.9) at 22 dpp.

In contrast, 'DIT_213' contained the highest number of nematodes per plant at 22 dpp (56.7 ± 75.6).

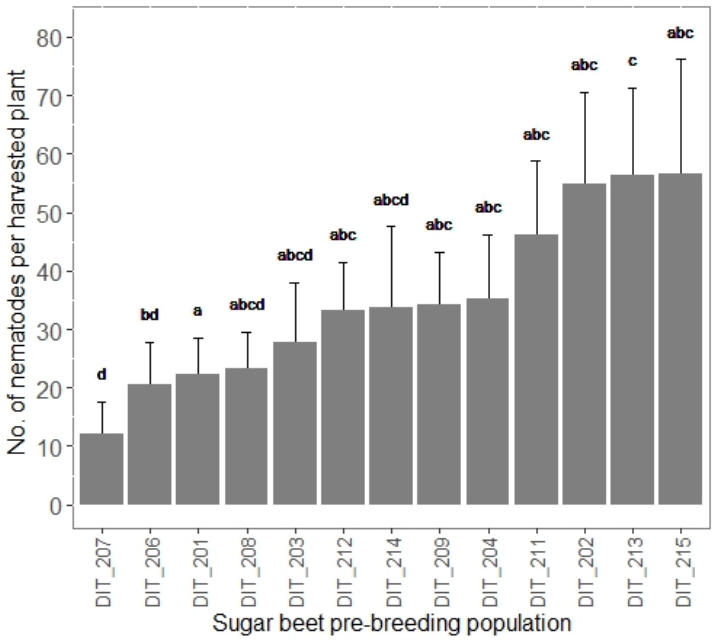

Fig. 3 Effect of sugar beet pre-breeding lines on *Ditylenchus dipsaci* number per harvested plant at 22 days post-planting (dpp) in a glasshouse trial. Different letters above the bars indicate significant differences between pre-breeding lines at $p < 0.05$, according to Dunn's multiple comparison test (n = 15)

EXPERIMENT 2: EFFECT OF SUGAR BEET GENOTYPES ON *D. DIPSACI* PENETRATION POTENTIAL

In contrast to experiment 1, the genotypes tested significantly influenced *D. dipsaci* penetration into sugar beet seedlings ($P \leq 0.0001$, Fig. 4). At 22 dpp, the susceptible line 'DIT_006' contained an average of 109 ± 16.9 nematodes per plant, significantly higher than 'DIT_207', 'DIT_166', and 'DIT_119' with 11.2 ± 2.8, 19.2 ± 5.3, and 34.4 ± 8.8 nematodes per plant, respectively. The pre-breeding population 'DIT_207' did not significantly reduce the number of penetrated nematodes into sugar beet seedling at 22 dpp compared to the pre-breeding population 'DIT_213' (54.1 ± 18.3). All seedlings of the breeding lines 'DIT_005', 'DIT_006', 'DIT_144', and 'DIT_150' contained nematodes at 14 dpi (Table 1). The breeding line 'DIT_166' showed the lowest incidence, with 74% seedlings containing *D. dipsaci* individuals.

Table 1 Effect of the genotype on the incidence of the symptoms induced by *Ditylenchus dipsaci* penetration into sugar beet at 14 days post-inoculation (dpi) (number of swollen plants at 14 dpi/number of germinated plants at 14 dpi*100) and on the *D. dipsaci* incidence (number of plants containing nematodes/number of harvested plants*100 at 14 and 60 dpi) in experiments 2 (n =20) and 3 (n=10)

Line	Symptoms incidence (%)		*D. dipsaci* incidence (%)	
	Experiment 2	Experiment 3	Experiment 2 (14 dpi)	Experiment 3 (60 dpi)
'DIT_005'	70	50	100	100
'DIT_006'	33	58	100	100
'DIT_119'	13	30	91	100
'DIT_144'	30	80	100	100
'DIT_150'	40	70	100	100
'DIT_166'	30	40	74	89
'DIT_207'	13	90	88	100
'DIT_213'	73	100	96	100

Concerning the incidence of the symptoms at 14 dpi (Table 1), the breeding line 'DIT_119' and the pre-breeding population 'DIT_207' showed the lowest percentage of swollen hypocotyls (13%). The pre-breeding population 'DIT_213' contained the highest percentage of swollen hypocotyls (73%).

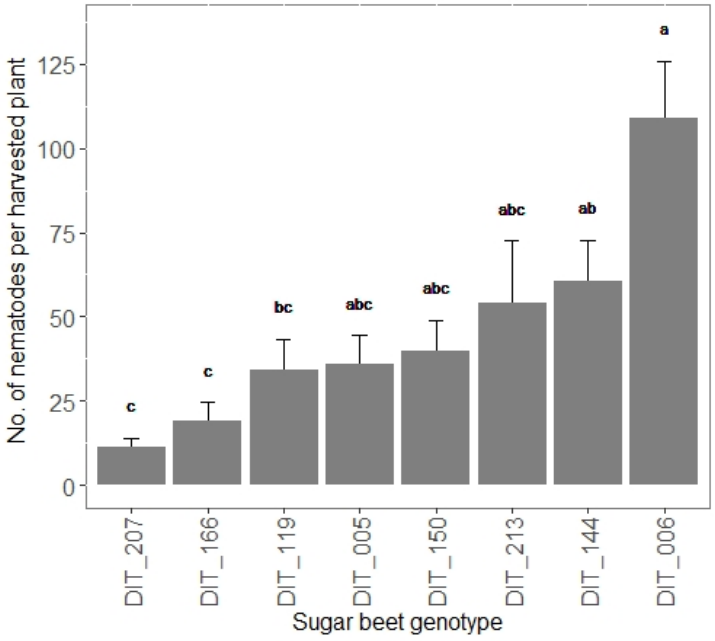

Fig. 4 Effect of sugar beet genotypes on *Ditylenchus dipsaci* number per harvested plant at 22 days post-planting (dpp) in a growth chamber trial. Different letters above the bars indicate significant differences between genotypes at $p < 0.05$, according to Dunn's multiple comparison test (n = 20)

EXPERIMENT 3: EFFECT OF SUGAR BEET GENOTYPES ON *D. DIPSACI* REPRODUCTION POTENTIAL

The genotype tested significantly influenced *D. dipsaci* reproduction in sugar beet ($P \leq 0.001$, Fig. 5). 'DIT_207', 'DIT_150', and 'DIT_006' led to the highest *D. dipsaci* reproduction with 6,097 ± 1,863, 8,255 ± 1,091, and 8,670 ± 3,429 nematodes per harvested plant at 60 dpi. The effect of 'DIT_207', 'DIT_150', and 'DIT_006' on *D. dipsaci* reproduction significantly differed from 'DIT_213', 'DIT_166', and 'DIT_119'. At 60 dpi, the number of nematodes per harvested plant was 1,298.5 ± 985.8, 1,978 ± 895.5, and 2,437.5 ± 687.6 in the genotypes 'DIT_213', 'DIT_166', and 'DIT_119', respectively. The breeding line 'DIT_166' was the only genotype showing plants without nematode at 60 dpi with a nematode incidence of 89% (Table 1).

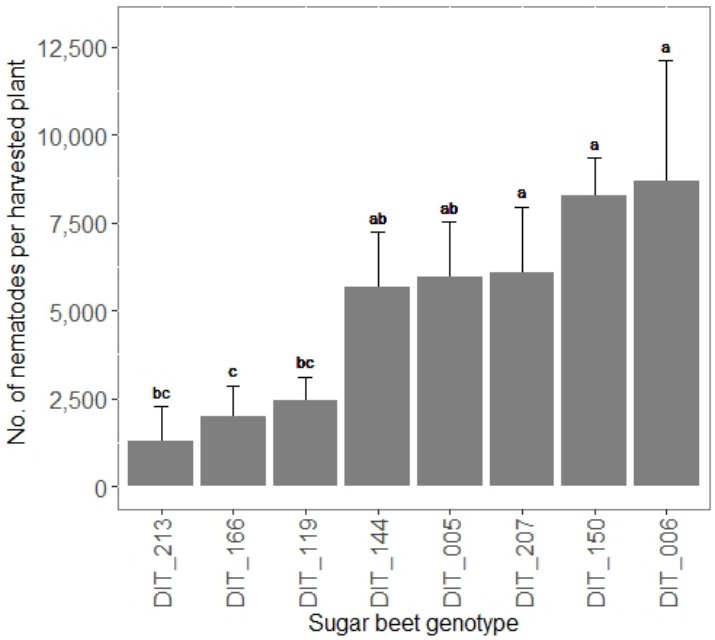

Fig. 5 Effect of sugar beet genotypes on *Ditylenchus dipsaci* number per harvested plant at 60 days post-inoculation (dpi). Different letters above the bars indicate significant differences between genotypes at $p<0.05$, according to Dunn's multiple comparison test (n = 10)

Concerning the incidence of the symptoms at 14 dpi (Table 1), the breeding lines 'DIT_119' (30%) and 'DIT_166' (40%) showed the lowest percentage of swollen hypocotyls. The pre-breeding population 'DIT_213' contained the highest percentage of swollen hypocotyls (100%). For the genotypes 'DIT_006', 'DIT_144', 'DIT_207', and 'DIT_166', after inoculation with *D. dipsaci*, the fresh plant weight was significantly reduced after 60 days ($P \leq 0.05$, Table 2). Whereas, *D. dipsaci* inoculation did not significantly reduce the sugar beet fresh plant weight of 'DIT_005', 'DIT_119', 'DIT_150', and 'DIT_213' compared to the noninoculated plants. The breeding line 'DIT_119' showed the highest survival rate, with 95% harvestable at 60 dpi (Table 2). In contrast, 25% of germinated plants of 'DIT_213' were harvestable at 60 dpi. All *D. dipsaci* non-inoculated plants (100%) survived until harvest (60 dpi).

Table 2 Effect of *Ditylenchus dipsaci* inoculation on the sugar beet fresh weight (g) at 60 days post-inoculation (dpi) and survival (%) (number of harvested plants at 60 dpi/number of germinated plants at 22 days post-planting) in a glasshouse trial

Line	Plant weight (g)[a]		Survival (%)
	Inoculated	Non-inoculated	
'DIT_005'	7.6 (± 2.3)	9.3 (± 1.1)	80 (± 14.1)
'DIT_006'	6.4 (± 4.3)	12.3 (± 3.3)**	60.7 (± 15.2)
'DIT_119'	11.3 (± 4.2)	13 (± 4.6)	95 (± 7.1)
'DIT_144'	4.8 (± 3.4)	10.1 (± 0.8)**	50 (± 28.3)
'DIT_150'	8.7 (± 3.5)	10.3 (± 3.9)	75 (± 21.2)
'DIT_166'	9.5 (± 2.8)	12.5 (± 3.1)*	73.3 (± 9.4)
'DIT_207'	7.5 (± 3)	12.7 (± 3.5)**	75 (± 21.2)
'DIT_213'	9.6 (± 2.6)	9.3 (± 3.2)	25 (± 0)

[a] Wilcoxon signed-rank test *$P < 0.05$, **$P \leq 0.01$; (mean ±SD)

Discussion

This study demonstrated the genetic variation of sugar beet genotypes at reducing *D. dipsaci* penetration into seedlings. The high variation among the genotypes is encouraging from the perspective of finding resistance to *D. dipsaci* penetration. A lower *D. dipsaci* penetration rate in experiment 1 than in experiment 2 suggests a lower inoculation success. Indeed, 'DIT_119' seedlings contained 4.5 times less *D. dipsaci* individuals in experiment 1 than in experiment 2. The low inoculation success in experiment 1 may explain the high variation observed within the genotypes. Despite the lack of significant differences among the breeding lines, some genotypes, such as 'DIT_119' and 'DIT_166', were superior in terms of their low variance of *D. dipsaci* number penetrating sugar beet seedlings. After considering the results from experiment 1, tolerance to *H. schachtii* does not involve resistance to *D. dipsaci* penetration. Due to crosspollination, the pre-breeding populations consist of a mixture of homozygous and heterozygous individuals (Bos and Caligari 2008), explaining the high variation observed.

The second experiment confirms the higher level of resistance of 'DIT_207', 'DIT_119', and 'DIT_166' towards *D. dipsaci* penetration. In contrast, the susceptible breeding line 'DIT_006' attests to its low resistance to *D. dipsaci* penetration. The second experiment suggests that the increase in repetitions (n= 20) significantly improves the probability of observing differences among the genotypes.

In the third experiment, nematode reproduction was observed in the eight genotypes tested. However, the number of produced nematodes varied among the genotypes. 'DIT_119' and 'DIT_166' validated their higher resistance level towards *D. dipsaci* infection. Based on the values obtained in the second and third experiments (number of nematodes at 22 dpp and at 60 dpi), the nematode population in each genotype increased up to 100-fold, suggesting that the mechanisms responsible for resistance occurred during the penetration. *Ditylenchus dipsaci* proved to have a rapid population growth (Abolfazl et al. 2017; Kuhnhold et al. 2006; Storelli et al. 2020). The few nematodes that penetrated sugar beet seedling produced a high number of nematodes at 60 dpi. The low *D. dipsaci* number at 60 dpi observed in the prebreeding population 'DIT_213' was not the effect of resistance towards *D. dipsaci* reproduction. The remaining plants of 'DIT_213' presented rotten hypocotyls at harvest. As *D. dipsaci* is an obligate plant parasite (Duncan and

Moens 2013), the rotting of its feeding site led the nematodes to leave the plant before the time point of plant harvest. Abolfazl et al. (2017) showed the inability of *D. dipsaci* to reproduce on fungi. The low survival and germination of infected plants compared to the non-infected plants suggest a high sensitivity of the pre-breeding population 'DIT_213' to *D. dipsaci* infection.

The earlier emergence of 'DIT_119' may explain the lowest *D. dipsaci* penetration into sugar beet seedlings. Indeed, it has been observed in the present study that 'DIT_119' emerged earlier compared to the other genotypes. The period of susceptibility of sugar beets to *D. dipsaci* penetration occurs at the emergence of the plant (Storelli et al. 2021). The rapid growth of 'DIT_119' may then reduce this period of susceptibility. It may also explain its lowest amount of swollen hypocotyls and its higher survival rate. Nematode penetration occurs later when the seedlings are already better developed to head to swellings of the hypocotyl. Griffith et al. (1997) indeed reported that symptoms initiation might occur before petioles are fully differentiated. A screening of a large-scale population of early emerging sugar beet genotypes to penetration and infection by *D. dipsaci* may help to validate this statement in the future. The absence of swollen hypocotyls allowed 'DIT_119' to grow with well-developed leaves until 60 dpi. In contrast, a higher proportion of damaged leaf-axils in the other genotypes did not allow for a proper development of the hypocotyl, which later forms the beet. Similar plant biomass measurements between inoculated and non-inoculated plants suggest a higher tolerance of 'DIT_119' to *D. dipsaci* at 60 dpi than other genotypes. However, *D. dipsaci* damages may occur later during the beet storage (Schomaker and Been 2013). The increased amount of nematodes found in the whole plant tissue of 'DIT_119' at 60 dpi suggested no strong resistance towards *D. dipsaci* reproduction.

Many non-swollen hypocotyls contained nematodes at 14 and 60 dpi. These results suggest that *D. dipsaci* can survive in plant tissue without exhibiting symptoms (Cook and Evans 1988). However, this statement is not valid for each *D. dipsaci* host plant. Caubel et al. (1994) positively correlated the lack of symptoms with the absence of nematode in red clover tissue. Resistance can be thus determined by characterizing the symptoms on seedlings, which facilitates the screening of a wide range of plant genotypes (Plowright et al. 2002). However, resistance determination based on expressed symptoms does not work on sugar beet

interacting with *D. dipsaci*. The presence of a low *D. dipsaci* number is sufficient to trigger abnormal morphogenesis (Griffith et al. 1997). In contrast, a high amount of *D. dipsaci* in sugar beet seedling does not automatically lead to a swollen hypocotyl.

Leipertz and Valder (2020) reported tolerance of BERETTA KWS towards the fungal and bacterial infection introduced by *D. dipsaci* in the field. The high susceptibility of 'DIT_005', the paternal line of BERETTA KWS, suggests no direct relation between the resistance of one hybrid component towards *D. dipsaci* development and tolerance of a respective hybrid to the fungal and bacterial infection introduced by *D. dipsaci*.

The nematode inoculation success depends on environmental conditions and the viability of the inoculated nematode population (Storelli et al. 2021). This was also demonstrated by the experiments shown here. Thus, nematode aggressiveness towards sugar beet is highly heterogeneous across the experiments and within the cultivars (Kuhnhold 2011; Westphal 2013). When evaluating inoculation experiments, a possible inoculation failure must be taken into account in any case. After an inadequate inoculation or if the inoculation fails, the subsequently determined infection level may be low, but the plants are not necessarily resistant (Francis and Luterbacher 2003). Additionally, the existence of a few susceptible plants may bias the resistant pre-breeding population's observation since they were not genetically homogeneous (Scholten et al. 2001).

This study did not succeed to find complete resistance in sugar beet genotypes towards *D. dipsaci* penetration or reproduction under glasshouse conditions. However, partial resistance to *D. dipsaci* penetration was observed in some genotypes, where a proportion of plants was less or not affected (Bovien 1955). The lowest incidence of *D. dipsaci* presence in sugar beet seedlings was observed for the breeding line 'DIT_166' suggesting potential candidates with resistance within this line. The pre-breeding population 'DIT_207', with its particular low heterogeneity and low number of *D. dipsaci* per seedling, is a potential candidate for a source of resistance towards *D. dipsaci* penetration, despite the high incidence and reproduction rate of the nematode.

The initial inoculum level used in the experiments was up to 1000-fold higher than an initial natural density occurring in the field at sowing (Storelli et al. 2020). An initial density of 10 *D. dipsaci* individuals per 500

g soil may cause severe damage to onions. The damage was nearly 100% when the initial density of 25 *D. dipsaci* individuals per 500 g soil was reached (Seinhorst 1956). With an increasing initial density of *H. schachtii*, the yield of resistant sugar beet cultivars decreased (Heijbroek et al. 2002). Further trials under field conditions and at lower nematode density may determine the investigated genotypes' real resistance potential.

Khanam et al. (2018) reported a similar penetration of *D. angustus* in the resistant and susceptible rice cultivars, suggesting that host attraction is not linked to resistance. However, *D. dipsaci* damages to sugar beet seedlings encourage resistance development through penetration (Cottage and Urwin 2013). Therefore, screening a large number of pre-breeding populations is recommended as they represent a source of genes for resistance (Tanksley and McCouch 1997). Much of the available germplasm resources remain to be characterized for resistance to nematodes (Starr et al. 2002). Due to the lack of resistant cultivars, high-yielding cultivars, tolerant to the fungal and bacterial infection introduced by *D. dipsaci*, are now the only measure to avoid economic damage. As different fungal and bacterial organisms are introduced by *D. dipsaci*, a case-by-case study is recommended to grow specific cultivars tolerant to the pathogen introduced by the nematode. In a field infested with *R. solani*, the use of *R. solani* resistant cultivars will prevent high yield reduction (Hillnhutter et al. 2011). The use of resistant crops, such as oat, in the rotation to reduce the nematode population has to be further investigated. Thus, tolerant sugar beet cultivars and resistant crops may probably help to maintain a sustainable sugar production in *D. dipsaci* infested fields while resistant cultivars are urgently needed for growers.

Acknowledgements

The authors thank the seed company KWS SAAT SE & Co. KGaA (D), the sugar factory Schweizer Zucker AG (CH), and the Bern University of Applied Sciences (CH) for their financial support. We would like to further acknowledge the working group of Nematology at Agroscope (CH) for providing the *D. dipsaci* population.

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5 Manuscript 3

Virulence and pathogenicity of four *Ditylenchus dipsaci* populations on sugar beet

Alan Storelli[1*], Sebastian Kiewnick[2], Matthias Daub[2], Anne-Katrin Mahlein[3], Mario Schumann[4], Werner Beyer[4], Andreas Keiser[1]

1) School of Agricultural, Forest and Food Sciences HAFL, Bern University of Applied Sciences BFH, Länggasse 85, 3052 Zollikofen, Switzerland

2) Julius Kuehn Institute, Federal Research Center for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Messeweg 11/12, 38104 Braunschweig, Germany

3) Institute of Sugar Beet Research IfZ, Holtenser Landstrasse 77, 37079 Goettingen, Germany

4) KWS SAAT SE & Co. KGaA, Grimsehlstrasse 31, 37574 Einbeck, Germany

Published in European Journal of Plant Pathology

Received: 08 April 2020

Accepted for publication: 24 May 2021

Online publication date: 28 May 2021

DOI 10.1007/s10658-021-02304-w

Summary - The stem nematode, *Ditylenchus dipsaci*, is a severe pest in European sugar beet production. In France, Germany, and Switzerland, *D. dipsaci* damage in sugar beet varies among specific geographic areas. In this study, the reproduction potential of four geographically distinct *D. dipsaci* populations was determined using sterile carrot disc cultures. In addition, virulence and pathogenicity were investigated *in-vivo* using sugar beet. No difference was found in the reproduction potential on carrot discs, as well as penetration rate in sugar beet seedlings. The reproduction rate in sugar beet tissue was significantly affected by the *D. dipsaci* population used. The population from Seeland (CH) showed the highest number of nematodes per plant at 60 dpi (21,071.8±5,340.0), compared to the three other populations contained 3,588.6±3,858.3, 5,136.9±4,950.8, and 3,579.7±5,174.2, respectively. Furthermore, the reproduction rate of *D. dipsaci* was negatively correlated with fresh biomass of sugar beets at 60 dpi. Based on these results, the *D. dipsaci* population "Seeland" is suitable for breeding programs to detect resistance in sugar beet. After selecting candidate genotypes/varieties, these should be further evaluated for their field resistance in their targeted growing regions.

Keywords - *Beta vulgaris*, carrot disc rearing, penetration, reproduction, resistance breeding

Introduction

The stem and bulb nematode *Ditylenchus dipsaci* (Kühn, 1857) Filipjev 1936 is an obligate endoparasite with more than 500 host plants and ranking in the top ten of plant-parasitic nematodes worldwide (Sturhan and Brzeski, 1991; Jones et al., 2013). This nematode is highly pathogenic on onion (*Allium cepa* L.), garlic (*Allium sativum* L.), narcissus (*Narcissus* spp.), alfalfa (*Medicago sativa* L.), and clover (*Trifolium* spp.) (Yuksel, 1960; Siti et al., 1982; Windrich, 1986; Boelter et al., 1985; Grandison, 1965). The host range of *D. dipsaci* varies among biological races which represent a species complex (Subbotin et al., 2005; Sturhan et al., 2008). Ritzema Bos (1888) was the first to observe host preferences of different *D. dipsaci* populations. Later, up to 30 biological races were determined by their host preferences (Seinhorst, 1957; Sturhan and Brzeski, 1991; Bovien, 1955). Eriksson (1965) defined biological races as populations distinguished by host preference but not morphologically. He pointed out the races' ability to interbreed and de facto the impossibility of being considered as different species. While some biological races are polyphagous, some are specific to a limited number of plant species as the red clover race (Webster, 1967). The reproduction of polyphagous races varies greatly among host plant species (Whitehead et al., 1987). Qiao et al. (2013) and Poirier et al. (2019) reported distinct genotypes among *D. dipsaci* populations within a geographic region and between neighboring farms. Esquibet et al. (1998) succeeded to molecularly distinguish the species *D. dipsaci sensu lato* from the giant race *D. gigas*, recently singled out as new species (Vovlas et al., 2011). To date, molecular analyses do not distinguish the biological races of *D. dipsaci,* while the chromosome number may vary among the races (D'Addabbo Gallo et al., 1982). In this study, the term "population" is reflecting different geographic origins without taking host plant references into account.

Fields may be composed of populations with diverging genetic characters that can interbreed (Bovien, 1955). Eriksson (1965) reported fertile polyphagous progenies of interbreeding between the red clover and lucerne races. Interbreeding can occur under natural conditions in plants contaminated by individuals of different biological races (Janssen, 1994). However, the red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), and lucerne races may distinguish themselves from polyphagous races by frequent failure of fertile progenies (Eriksson, 1974).

In Europe, *D. dipsaci* is an important threat for sugar beet (*Beta vulgaris* L.) (Dewar and Cook, 2006; Leipertz, 2007). In Germany and Switzerland, some major sugar beet growing regions are particularly affected by the stem and bulb nematode (Leipertz, 2007; Storelli et al., 2021a). Its penetration on sugar beet seedlings leads to the dissolution of the middle lamellae and swelling of the hypocotyl (Duncan and Moens, 2013; Madani et al., 2015). Later in the growing season, bacterial and fungal pathogens introduced by *D. dipsaci* engender rotting of the sugar beet crown (Storelli et al., 2020; Hillnhütter et al., 2011; Kühnhold, 2011). To date, no resistant cultivars towards *D. dipsaci* are available to farmers (Storelli et al., 2021b). Leipertz (2007) and Kühnhold (2011) reported evidence for tolerance of sugar beet cultivars toward crown rot. Variations in the sensitivity of cultivars towards crown rotting were observed among geographic regions in France, Germany, and Switzerland (Leipertz and Valder, 2017, 2018, 2020).

In this study, the term virulence refers to the ability of a nematode population to reproduce in a plant, whereas pathogenicity refers to the level of damage inflicted to the host plant (Müller, 1989; Perry and Moens, 2013; Shaner et al., 1992). Variations in the pathogenicity of *D. dipsaci* populations challenge breeders in their search for resistant cultivars. *Ditylenchus dipsaci* populations have been shown virulent to cultivars of alfalfa, white clover, and faba bean (*Vicia faba* L.), previously described as resistant towards *D. dipsaci* populations from other geographic origins (Plowright et al., 2002; Elgin et al., 1977; Whitehead, 1992). Therefore, breeders should consider the pathogenicity and virulence of nematode populations from target regions for commercial cultivars right when choosing the reference population at the start of the breeding process.

This study determines the virulence and pathogenicity of four representative *D. dipsaci* populations from the regions of Aisne (FR), Franconia (DE), Rhineland (DE), and Seeland (CH). Initial levels of virulence were assessed using a standardized carrot disc *in-vitro* assay. Furthermore, the *D. dipsaci* populations were evaluated *in-vivo* concerning the penetration rate, virulence, and pathogenicity toward sugar beet seedlings.

Materials and methods

General methods

Carrots used for *D. dipsaci* rearing were surface sterilized by soaking them for 40 min in 1% NaOCl-solution (w/w). Carrots were then rinsed with sterile demineralized water, transferred to a clean bench (HS 18, Kendro Laboratory Products, Germany), and peeled under sterile conditions (Kühnhold et al., 2006). After removing end parts, carrots were cut into three cylinders, which were transferred into a 500-ml sterile glass bottle. After 15 days of incubation in the dark at room temperature, the carrot disc callus was ready for nematode inoculation. A suspension of *D. dipsaci* (juvenile and adult stages) was centrifuged (3500 rpm, 5 min) and washed three times with sterile demineralized water. Afterward, they were re-suspended for 30 min in a sterile antibiotic solution containing 0.1% streptomycin sulfate (w/v) and 0.1% amphotericin-B (w/v), and finally washed twice with sterile demineralized water (Kühnhold et al., 2006). The nematodes were then inoculated on the surface sterilized carrot disc callus with 50 nematodes per cylinder and incubated for 45 days in the dark at 20 ±2 °C (Storelli et al., 2021a). Nematodes were extracted from carrot discs for 24 h using Oostenbrink dishes and collected on a 20µm mesh sieve. The nematodes were then stored in the dark at 6-8°C for 24 h until the start of the new experiment.

Untreated sugar beet seeds were sown in 200-ml plastic pots filled with 180 ml non-sterile sieved loess soil/sand/compost mixture (1/1/1) (v/v/v). The paternal line 'DIT_006' of the cv. BELLADONNA KWS, previously shown to be susceptible to *D. dipsaci* induced crown rot (Leipertz and Valder, 2020), was used to determine the penetration rate and reproduction potential of *D. dipsaci* populations in sugar beet. Two seeds were sown per pot and after the first plant's emergence, all following emerging plants were removed to ensure only one seedling per pot. The plants were covered with a Plexiglas mini-glasshouse and regularly watered to maintain a suitable soil moisture allowing nematode movement throughout the duration of the experiment. At 8 days post-planting (dpp), 1,000 nematodes were inoculated in 500 µl into two 1-cm deep holes (diam. 3 mm) approx. 1.5 cm from the center of the pot (Storelli et al., 2021a).

ISOLATION, EXTRACTION, AND MAINTENANCE OF NEMATODE POPULATIONS

The four *D. dipsaci* populations investigated were originally obtained from either infested soil or sugar beets, respectively. The nematode population from the Seeland region was extracted from the hypocotyl of infested sugar beets (cv. SAMUELA KWS) collected in 2015. Nematodes were extracted for 24 h using the Oostenbrink dish technique (European and Mediterranean Plant Protection Organization, 2013). The three *D. dipsaci* populations from Franconia, Rhineland, and Aisne were extracted in 2018 from infested field soils after the sugar beet harvest as described above. *Ditylenchus dipsaci* was identified with the aid of an optical microscope at 40x magnification using morphological characteristics described in taxonomic literature (European and Mediterranean Plant Protection Organization, 2017). Approximately 300 fourth-stage juveniles (J4) and adult stages of *D. dipsaci* were hand-picked, transferred to 1.5-ml microcentrifuge tubes, and incubated for 45 days on carrot discs after surface sterilization as mentioned above. Nematodes were then extracted for 24 h as mentioned above.

VIRULENCE OF *DITYLENCHUS DIPSACI* POPULATIONS ON CARROTS DISCS

Initial virulence of the four *D. dipsaci* populations was determined using an *in-vitro* assay with carrot discs. Fifty nematodes (J4 and adult stage) were inoculated per carrot disc and incubated for 45 days as described above. After Oostenbrink extraction for 24 h, the numbers of eggs, second-and third-stage juveniles (J2 and J3), J4, and adults in one reagent bottle containing three carrot discs were determined separately using an optical microscope at 40x magnification. Five reagent bottles were prepared per *D. dipsaci* population, and the experiment was conducted twice.

INFECTIVITY OF *DITYLENCHUS DIPSACI* POPULATIONS ON SUGAR BEET

The penetration rate in the breeding line ‘DIT_006’ was determined for all four *D. dipsaci* populations. At 15 days post-inoculation (dpi), the sugar beet seedlings were collected, washed, and stained in a plastic beaker containing a 0.1% acid fuchsin/lactic acid dye solution by heating them twice in a microwave until boiling and subsequently stored at 6 °C until further use. Stained seedlings were rinsed with tap water to remove the dye solution. After a maceration (6,500 rpm, 15 s) of the seedlings in 30 ml of tap water using

an Ultra Turrax blender (T25 basic/S25 N - 18 G, IKA Labortechnik, Germany) (Kühnhold et al., 2006), the number of *D. dipsaci* per seedling was determined using a stereomicroscope at 10x magnification. The experiment was set up in a growth chamber (KBWF 720, Binder GmbH, Germany) at 15/8 °C and a photoperiod of 18/6 h day/night. The experiment was conducted twice with 10 replicates per population.

VIRULENCE AND PATHOGENICITY OF *DITYLENCHUS DIPSACI* POPULATIONS ON SUGAR BEET

Virulence and pathogenicity of the four *D. dipsaci* populations were determined using the breeding line 'DIT_006'. At 15 dpi, the effect of the *D. dipsaci* populations on symptom expression on sugar beet seedlings was determined by assessing the incidence of swollen hypocotyls. At 60 dpi, the plants were harvested, total fresh weight determined, and cut into small pieces. The number of nematodes per plant was determined using an optical microscope at 40x magnification after extraction for 24 h in Oostenbrink dishes (European and Mediterranean Plant Protection Organization, 2013). The experiment was conducted in a growth chamber (KBWF 720, Binder GmbH, Germany) at 15/8°C and a photoperiod of 18/6 h day/night. For secondary growth of the sugar beets, the plants were transferred, at 15 dpi, to a glasshouse at 22/15°C with a photoperiod of 18/6 h day/night. Nematode reproduction, symptom development, biomass and plant survival was determined in 10 plants per *D. dipsaci* population, and the experiment was replicated twice. Non-inoculated plants were used as a control to determine the nematode pathogenicity.

DATA ANALYSES

All experiments were arranged in a complete randomized design (CRD). The data from repeated experiments were pooled after confirming the homogeneity of variances between experiments. Analyses of variance were performed to determine the nematode population's effect on their development on carrot discs and sugar beets. As the normal distribution of data was not given, the Kruskal-Wallis rank-sum test was performed to identify significant effects of population on the number of produced nematodes during the rearing process on carrots. A Friedman test determined the effect of the nematode populations on plant survival and symptoms incidence. Tukey test of multiple comparisons of means was performed as posthoc tests. Values

show the mean of the standard deviation (± SD). Statistical analyses, as well as graphs, were performed with R (4.04).

Results

VIRULENCE OF *DITYLENCHUS DIPSACI* POPULATIONS ON CARROT DISCS

The number of nematodes reproduced per carrot disc did not vary among the four *D. dipsaci* populations testet (Table 1). All *D. dipsaci* populations reproduced well on carrot callus. An average of 111,425.1±10,009.9 nematodes was obtained after 45 days from an initial inoculum of 150 *D. dipsaci* individuals per reagent bottle. The proportion of the different nematode stages varied significantly among the four *D. dipsaci* populations (Table 1). The nematode population from Franconia (DE) produced a greater percentage of eggs ($P < 0.05$) and a lower percentage of J2 and J3 ($P < 0.01$) compared to the populations from Rhineland (DE) and Seeland (CH), respectively.

Table 1 Effect of the *Ditylenchus dipsaci* population on the number of produced nematodes and the percentage of eggs, second- (J2), third- (J3), fourth-stage juveniles (J4), and adult stages (± SD) in a reagent bottle containing three carrot discs incubated for 45 days.

Nematode population	Nematodes per 3 carrot discs	Eggs (%)	J2-J3 (%)	J4-Adult (%)
Aisne	111,231.0 ± 76,452.0	17.8 ± 5.7 ab	29.4 ± 6.2 ab	52.7 ± 8.2
Franconia	85,885.9 ± 68,828.9	21.5 ± 7.9 b	23.7 ± 6.1 a	54.8 ± 10.3
Rhineland	116,083.4 ± 49,111.6	13.9 ± 4.8 a	34.7 ± 7.9 b	51.4 ± 7.3
Seeland	132,500.3 ± 55,954.3	13.8 ± 4.0 a	34.2 ± 7.7 b	52.0 ± 10.0
	n.s.	$P < 0.05$	$P < 0.01$	*n.s.*

Different letters indicate a significant difference at $P < 0.05$, according to the Tukey test of multiple comparisons of means; n.s.= non-significant; (n=10)

INFECTIVITY OF *DITYLENCHUS DIPSACI* POPULATIONS ON SUGAR BEET

The number of nematodes in sugar beet seedlings at 15 dpi did not vary among the four *D. dipsaci* populations tested, with an average of 22.8 *D. dipsaci* per seedling (Fig. 1). After inoculation with the Franconia population, *D. dipsaci* was detected in 100% of the plants at 15 dpi. The remaining populations showed few escapes for the Rhineland and Aisne (F) population (2 plants each) as well as Seeland (1 plant).

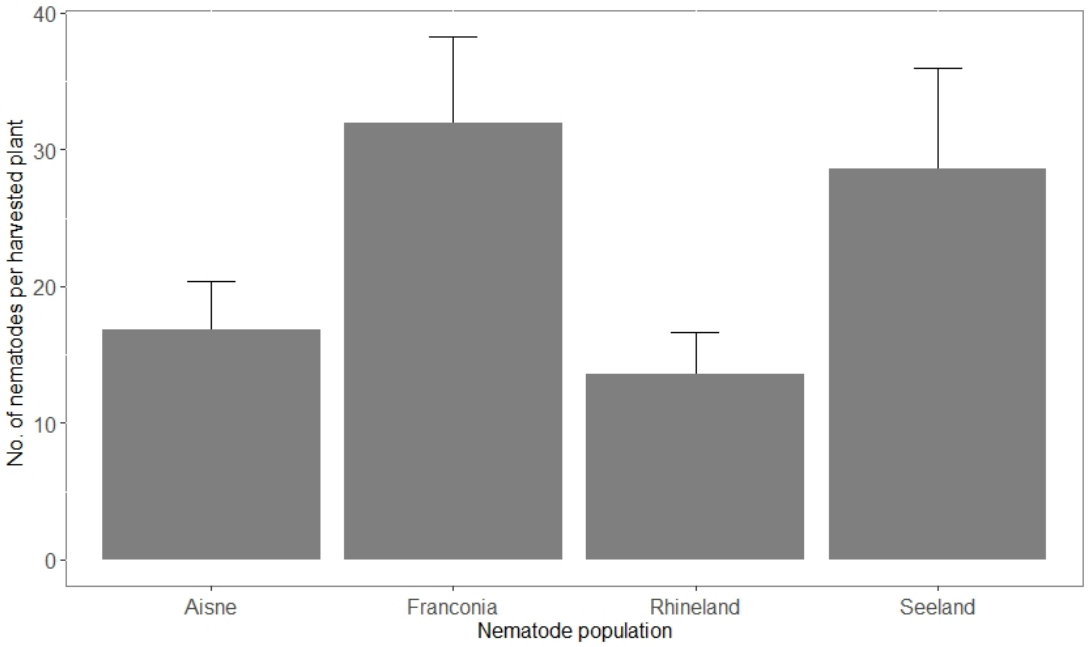

Fig. 1 Number of *Ditylenchus dipsaci* per plant, 15 days post-inoculation (dpi) with the four populations Aisne, Franconia, Rhineland, and Seeland. (n=20)

VIRULENCE AND PATHOGENICITY OF *DITYLENCHUS DIPSACI* POPULATIONS IN SUGAR BEET

The number of nematodes in sugar beets at 60 dpi varied significantly among the four *D. dipsaci* populations (Fig. 2, $P < 0.001$). While all *D. dipsaci* populations could reproduce on sugar beets, reproduction was significantly higher for Seeland nematodes, with 21,071±16,020 individuals per plant. At 60 dpi, the sugar beets inoculated with nematodes from Aisne, Franconia, and Rhineland contained 3,588±3,858, 5,136±4,950, and 3,579±5,174 individuals, respectively. The incidence for plants showing reproduction of *D. dipsaci* was 100%, except for the population Aisne, where one plant contained no nematodes.

The nematode populations from Franconia, Rhineland, and Aisne showed a significantly greater percentage of plants with swollen hypocotyls compared to the non-inoculated control (Table 2, $P < 0.05$). The nematode populations significantly affected the sugar beet fresh weight at 60 dpi (Table 2, $P < 0.0001$), with the lowest value recorded after inoculation with the Seeland population. In addition, the population of *D. dipsaci* significantly influenced the plant survival at 60 dpi (Table 2, $P < 0.05$), with the nematode populations from Franconia and Seeland leading to the lowest percentage of survivors (70.8%).

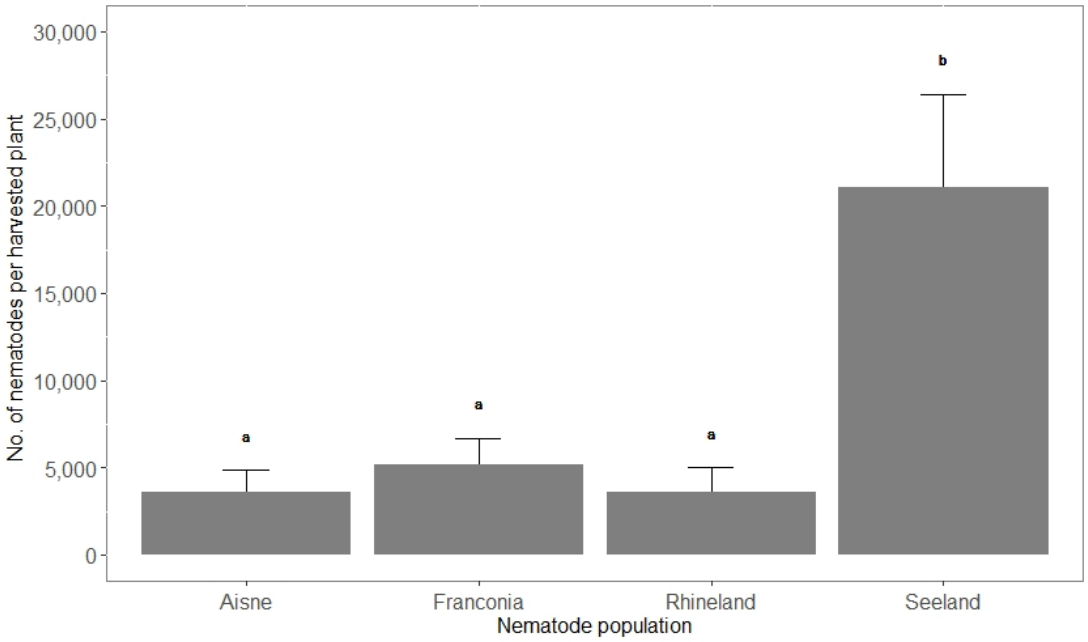

Fig. 2 Number of *Ditylenchus dipsaci* per plant, 60 days post-inoculation (dpi) with the four populations Aisne, Franconia, Rhineland, and Seeland. (n=20). Bars (+/- SD) showing different letters are significantly different according to a Tukey test at P < 0.05 (n=20)

Table 2 Effect of the *Ditylenchus dipsaci* population on the incidence of plants with swollen hypocotyl (%) at 15 days post-inoculation (dpi), plant fresh weight (g), and survivor plants (%) at 60 dpi (± SD).

Nematode population	Swelling incidence (%)	Plant fresh weight (g)	Survivor (%)
Non-inoculated	0.0 ± 0.0 b	12.1 ± 1.8 d	100 ± 0.0 a
Aisne	75.0 ± 31.9 a	9.2 ± 2.5 c	100 ± 0.0 a
Franconia	81.2 ± 23.9 a	6.2 ± 1.6 ab	66.7 ± 31.2 b
Rhineland	87.5 ± 14.4 a	8.1 ± 2.6 bc	87.5 ± 14.4 ab
Seeland	56.2 ± 18.5 ab	5.0 ± 2.7 a	70.8 ± 21.0 b
	$P < 0.05$	$P < 0.0001$	$P < 0.05$

Different letters indicate a significant difference at $P < 0.05$, according to the Tukey test of multiple comparisons of means (n=20).

Discussion

Our study demonstrated variations of virulence and pathogenicity of the different nematode populations on carrot discs and sugar beet. All four *D. dipsaci* populations were able to invade sugar beet seedlings. As the soil inoculation method was applied, sugar beet evidently attracted all four populations. While penetration was similar for the four nematode populations, the Seeland population showed a significantly greater reproduction. This finding correlates with the observation of Elgin et al. (1977), where *D. dipsaci* populations did not differ in their ability to invade the host plant but differed in reproducing in their host. All four *D. dipsaci* populations did not show a significant variation of nematode incidence, defined by the percentage of plants affected by *D. dipsaci*. In contrast, Whitehead (1984) observed a different percentage of infected lucerne among 11 nematode populations from England. The very few non-infected plants in our experiments were probably due to plants escaping inoculation.

The different nematode populations did significantly influence the fresh weight of infected sugar beet. As the non-inoculated plants showed a significantly higher biomass, we can assume that all populations are pathogenic to sugar beet. Inoculation of the Seeland population resulted in the lowest biomass in comparison to the other populations. Additionally, the Seeland population led to a low percentage of plants surviving nematode infection. These results validate the observations in field trials located in the Seeland region, where crown rotting regularly occurs more severely than in other regions (Leipertz, 2011; Leipertz and Valder, 2017, 2018, 2020). However, this may also be enhanced through many host plants in the crop rotation. Indeed, the Seeland region is an important vegetable growing region, where it is common to find sugar beets, onions, and carrots in the crop rotation. Similar penetration and symptoms incidence of all four populations at 14 dpi, followed by a greater number of nematode and a lower biomass and survival rate of plant inoculated with the Seeland population, suggest the presence of a *D. dipsaci* biological race highly adapted to sugar beet in the Seeland.

The *D. dipsaci* population from Seeland may consist of the beet race. As onion is frequently cultivated in the Seeland region, the *D. dipsaci* onion race, which reproduces greatly on sugar beet (Whitehead et al., 1987), may also be the original race of this population. In contrast, the three other populations may come

from different races' interbreeding, reducing their reproduction (Janssen, 1994). Indeed, interbreeding can easily occur under natural conditions (Bovien, 1955). In France, *D. dipsaci* mainly infects faba bean and lucerne (Esquibet et al., 1998; Mouttet et al., 2014). The damages on sugar beets are rare in France and not published. This correlates with our study, where the Asine population showed a survival rate similar to non-inoculated plants and a low impact on sugar beet biomass. We assume that the Aisne region's nematode population does probably not belong to the beet race, and its reproduction is de facto lower on sugar beet. Whitehead et al. (1987) observed a lower reproduction of the lucerne and bean races on sugar beet. A *D. dipsaci* population from the Aisne region proved to be a lucerne race (Whitehead, 1992). Similar can be assumed for the Rhineland population. Indeed, Kotthoff (1950) reported rye (*Secale cereale* L.) as the main host crop for *D. dipsaci* in this region.

All four populations were able to reproduce on carrot callus. The reproduction was very high on carrots, where the population increased by almost 750 times in 45 days. A previous study performed by the authors of this paper obtained a similar reproduction of the Seeland population on carrot discs (Storelli et al., 2021a). The reproduction was higher than reported from a Canadian population observed by Abolfazl et al. (2017), where the population increased up to 250 times in three months. However, the reproduction may vary greatly depending on the experimental settings. Indeed, Kühnhold et al. (2006) observed high variations of the reproduction in a *D. dipsaci* population from the Rhineland region depending on the incubation period and inoculum level on carrot discs. Although all populations were successfully reared on carrot callus, our study cannot state that carrot is a host plant for each *D. dipsaci* population under natural conditions. Eriksson (1965) demonstrated that *D. dipsaci* populations could reproduce on the callus of non-host plants.

Our study demonstrated that pathogenicity and virulence vary among nematode populations. While the penetration was similar among the four populations, the Seeland population showed a greater reproduction and, thus, a higher virulence on sugar beets than populations from other regions. Therefore, the authors recommend using the *D. dipsaci* population from Seeland for the selection of resistant or tolerant sugar beet lines in the breeding process. Due to the uncertainty of a mixed composition of *D. dipsaci* races in field populations and differences in virulence and pathogenicity between populations, it is a prerequisite to

conduct field trials in the target regions to validate resistance or tolerance towards *D. dipsaci*. This study did not aim to determine the biological race of the four *D. dipsaci* populations. A host preference investigation is, to date, the only way to determine a *D. dipsaci* population's biological race (Poirier et al., 2019). An easy method to determine the biological race of field population would help growers to adapt their crop rotation and reduce nematode pressure by avoiding host plant species.

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Microplot screening of sugar beet breeding lines and hybrid cultivars for resistance to *Ditylenchus dipsaci* penetration and reproduction

Alan Storelli[1*], Andreas Keiser[1], Sebastian Kiewnick[2], Anne-Katrin Mahlein[3], Mario Schumann[4], Werner Beyer[4], Matthias Daub[2]

1) School of Agricultural, Forest and Food Sciences HAFL, Bern University of Applied Sciences BFH, Länggasse 85, 3052 Zollikofen, Switzerland

2) Julius Kuehn Institute, Federal Research Center for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Messeweg 11/12, 38104 Braunschweig, Germany

3) Institute of Sugar Beet Research IfZ, Holtenser Landstrasse 77, 37079 Goettingen, Germany

4) KWS SAAT SE & Co. KGaA, Grimsehlstrasse 31, 37574 Einbeck, Germany

Not submitted

Summary – The stem nematode, *Ditylenchus dipsaci*, is a serious pest of sugar beet in Europe. The nematode migrates into the plant in the spring and reproduces in the hypocotyl during the growing season. Fungal and bacterial pathogens introduced by *D. dipsaci* leads to crown root rotting and plant death. To date, no sugar beet cultivars carrying resistance towards *D. dipsaci* are available for sugar producers. This study investigated the resistance and tolerance level of sugar beet breeding lines and hybrid cultivars in microplot experiments. The sugar beet genotype effect on *D. dipsaci* penetration could not be validated based on the results obtained under previous *in vivo* bioassays. The sugar beet genotype did influence nematode population development in plant tissue and soil. However, the genotypes did not show a sufficient tolerance towards the rotting of the plant tissue. Nematode pathogenicity differed depending on experiment locations and years. This study demonstrated variations between *in vivo* and semi-field conditions. Further sugar beet genotype screenings are needed to identify resistance towards *D. dipsaci* penetration and reproduction and sufficient tolerance towards rotting associated with infestation with the nematode.

Keywords – *Beta vulgaris*, microplot experiment, penetration, reproduction, resistance, tolerance

Introduction

The stem and bulb nematode *Ditylenchus dipsaci* (Kühn, 1857) Filipjev 1936 is a migratory endoparasite damaging European sugar beets (*Beta vulgaris* L.) (Dewar and Cook 2006). The Rhineland (DE) and Seeland (CH) regions are two sugar beet growing areas particularly affected by *D. dipsaci* (Leipertz 2007). Nematode penetration into germinating sugar beet leads to the dissolution of the middle lamellae in hypocotyl tissues and abnormal morphogenesis (Duncan and Moens 2013). A reduction in plant emergence was observed in severely infested fields (Caspary 1976). Later in the season, fungal and bacterial pathogens introduced by *D. dipsaci* lead to crown rotting (Hillnhütter et al. 2011; Kühnhold 2011). The nematode reproduces in the hypocotyl until plant tissue dies, leading to the exodus of the obligate phytoparasite *D. dipsaci* (Schomaker and Been 2013). Up to 100% yield losses can be observed on infested patches of various sizes (Storelli et al. 2020). To date, no effective measures are available for sugar beet growers to control population development of *D. dipsaci*. The withdrawal of the nematicide aldicarb and the growing public demand for sustainable sugar production have encouraged researchers to develop resistant cultivars (Starr et al. 2002).

In plant nematology, Roberts (2002) described resistance as the plant ability to suppress the nematode development and tolerance as the plant ability to compensate for a nematode infection with little or no yield loss. Our study distinguishes between resistance towards penetration, which prevents nematodes from invading sugar beets, and resistance towards reproduction, which suppresses nematode reproduction in plant tissue and de facto in soil. As *D. dipsaci* damage in sugar beet is closely associated with secondary infection by soil-borne pathogens, tolerance here described the plant ability to reduce crown rotting propagation. Here we define susceptibility as the terminological counterpart to resistance, whereas sensitivity is the antonym for tolerance.

Leipertz and Valder (2020) reported variations of tolerance towards *D. dipsaci* among cultivars which appear differently among sugar beet growing regions in Germany, France, and Switzerland. Kühnhold (2011) observed variations of susceptibility to nematode penetration and reproduction among sugar beet cultivars and breeding lines. However, he found that no sugar beet genotype was able to prevent nematode penetration

or reproduction. Storelli et al. (2021b) reported variations of susceptibility to nematode penetration and reproduction among prebreeding populations and breeding lines under *in vivo* conditions.

Field and microplot trials allow validating results obtain under *in vivo* conditions (Joalland et al. 2017). However, variations may occur between *in vivo* and field conditions. Plants tend to grow faster under *in vivo* experiments due to stable climatic conditions and artificial light. Additionally, soil composition differs between both systems, where sand and compost mixture are often mixed with field soil under *in vivo* conditions (Storelli et al. 2021a). *Ditylenchus dipsaci* represents a species complex with up to 30 biological races that may differ among geographic regions. Therefore, the nematode population used under *in vivo* conditions may differ from the field population (Storelli et al. 2021c).

Our study aims to validate the *in vivo* observations from Storelli et al. (2021b) under semi-field conditions. Microplot experiments were conducted in the Rhineland and Seeland regions to investigate sugar beet genotype resistance towards *D. dipsaci* penetration and reproduction as well as tolerance towards secondary infection by soil-borne pathogens.

Material and methods

General methods

Microplot systems at two different sites were used to estimate the genetic variations among sugar beet genotypes. The first microplot experiment was located at the Julius Kühn-Institut (JKI) research station in Elsdorf (DE), where twenty buried 230L-pots were available per year (2019 and 2020). In fall 2017, natural *D. dipsaci* infested field soil (top 15 cm) from Elsig in North Rhine-Westphalia (DE) was mixed with 25% (v/v) sand and transferred into the 230L-plots. In 2018, faba beans (*Vici faba* L.), undersown with carrots (*Daucus carota* L.), were grown as host crops to maintain a high nematode level before starting the sugar beet screening in 2019. Mustard (*Brassica juncea* L.) was the previous crop for the experiment in 2020. Drip irrigation was used to maintain constant humidity allowing nematode mobility.

The second microplot experiment was located at the Bern University of Applied Sciences (BFH) in Zollikofen (CH), where 32 buried 130L-pots filled with artificially *D. dipsaci* infested soil were available in 2020. In early October 2019, two sugar beets, naturally infested by *D. dipsaci*, were incorporated in each pot at the Zollikofen site. Additionally, a 5-cm *in vitro* carrot disc (Storelli et al. 2021a), containing approx. 10,000 *D. dipsaci* individuals, was placed onto the surface center of each pot. By the end of October 2019, rye (*Secale cereale* L.) was sown as *D. dipsaci* host crop and incorporated into the topsoil in March 2020. In the absence of drip irrigation, the plants were watered with a watering lance in Zollikofen.

The cultivars BELLADONNA KWS and BERETTA KWS, known to be susceptible and tolerant to soil-borne pathogens introduced by *D. dipsaci* in the field (Leipertz and Valder 2020), were used as standards in all microplot experiments.

The effect of sugar beet genotype on *D. dipsaci* penetration was determined in seedlings collected at plant development stage BBCH 12 (two leaves unfolded), washed, and stained in a plastic beaker containing 0.1% (v/v) acid fuchsin/lactic dye solution. Dye solution containing the seedlings was heated twice in a microwave until boiling and kept at 6 °C until further use (Kühnhold et al. 2006). Stained seedlings were rinsed in tap water to remove the dye solution. The total number of nematodes per seedling was determined using a

stereomicroscope at x10 magnification after maceration in 30 ml tap water using an Ultra Turrax blender (T25 basic/S25 N - 18 G, IKA Labortechnik, Germany) at 6,500 rpm for 10 seconds.

Tolerance of sugar beet genotype towards *D. dipsaci* infection was determined by the percentage of tissues showing rot at a crosssection of the beet head area after harvesting and washing of beets using an index with 0 = no rot visible, 1 = ≤ 10% of rotten root, 2 = 11 to 33% of rotten root, 3 = 34 to 66% of rotten root, and 4 = 67 to 100% of rotten root (Storelli et al. 2020). Resistance of sugar beet genotype was assessed according to the number of nematodes determined in 150 g of hypocotyl tissue using an optical microscope at 40x magnification after extraction of sliced material from the beat head for 24 h in Oostenbrink dishes.

The effect of sugar beet genotype on *D. dipsaci* population development in soil (Pf/Pi) was investigated by determining *D. dipsaci* number in 150 ml of soil sampled at sowing (Pi) and harvest (Pf) time using an optical microscope at x40 magnification after extraction for 24 hr in Oostenbrink dishes. Soil samples were collected by coring the top 15 cm of soil at several locations in the pot and mixed in a 150-ml subsample.

MICROPLOT EXPERIMENT 2019

We aimed to validate the results obtained in a previous unpublished *in vivo* genotype screening, where the cultivar DIT_010 and DIT_017 showed low *D. dipsaci* penetration into seedlings. The microplot experiment, conducted in 2019 in Elsdorf, was set up as a randomized complete block design with four replications and five sugar beet hybrid cultivars. The hybrid cultivar DIT_010 and DIT_017 were compared to the standard genotypes and the cultivar CELESTA KWS, reported as tolerant to soil-borne pathogens introduced by *D. dipsaci* in a 2018 field screening (Leipertz and Valder 2020).

Ten seeds were sown 3-cm-deep in each 230L-pot on April 8, 2019. At 24 days post-planting (dpp), seedlings were taken out and collected to leave five well-distributed plants per pot. The effect of sugar beet genotype on *D. dipsaci* penetration was determined as described earlier. On September 18, 2019, the remaining plants were harvested to determine the effect of sugar beet genotype on *D. dipsaci* reproduction and their tolerance to root rot.

MICROPLOT EXPERIMENTS 2020

We aimed to validate the *in vivo* experiments obtained in Storelli et al. (2021b). The first experiment, conducted in 2020 in Zollikofen, was set up as a randomized complete block design with four replications and eight sugar beet genotypes. The breeding lines DIT_119 showed potential tolerance to *D. dipsaci* under *in vivo* conditions. DIT_301 and DIT_302 were the hybrid progenies of DIT_119. DIT_005 and DIT_006 were the parental lines of DIT_002 and DIT_001. DIT_303 was used as a standard hybrid cultivar available for farmers. The second experiment, conducted in 2020 in Elsdorf, was set up as a randomized complete block design with four replications and the five sugar beet cultivars tested in Zollikofen. The breeding lines were not investigated in Elsdorf due to the lower amount of available pots.

Thirteen seeds were sown 3-cm-deep in each pot on March 27 and April 3, 2020, in Zollikofen and Elsdorf. At 31 dpp, seedlings were taken out and collected to leave four and five well-distributed plants per pot in Zollikofen and Elsdorf, respectively. The effect of the sugar beet genotype on *D. dipsaci* penetration was determined. Sugar beets were harvested to determine the effect of genotype on *D. dipsaci* reproduction in plant tissue and their tolerance to root rot on September 16 and 18, 2020, in Zollikofen and Elsdorf.

DATA ANALYSES

Friedman rank-sum tests were performed to determine differences in *D. dipsaci* penetration, reproduction, and pathogenicity among sugar beet genotypes. Dunn's multiple comparison tests were performed as post-hoc tests.

Results

MICROPLOT EXPERIMENT 2019

In Elsdorf in 2019, the sugar beet cultivar did not significantly influence the number of nematodes in seedlings at 24 dpp, the nematode population development in soil (Pf/Pi), or the root rot index (Table 1). All sugar beet cultivars allowed reproduction of nematodes at Pf/Pi values above 2. The sugar beet cultivars significantly influenced the root rot index ($P < 0.0001$, Table 1), the percentage of marketable beets, and the fresh plant biomass ($P < 0.05$, Table 1). The sugar beet cultivar BERETTA KWS showed the lowest root rot index. In contrast, the cultivars BELLADONNA KWS and DIT_010 showed the highest root rot index and the lowest percentage of marketable beets. The cultivar DIT_010 led to a significantly lower fresh plant biomass than the cultivar BERETTA KWS, CELESTA KWS and DIT_017.

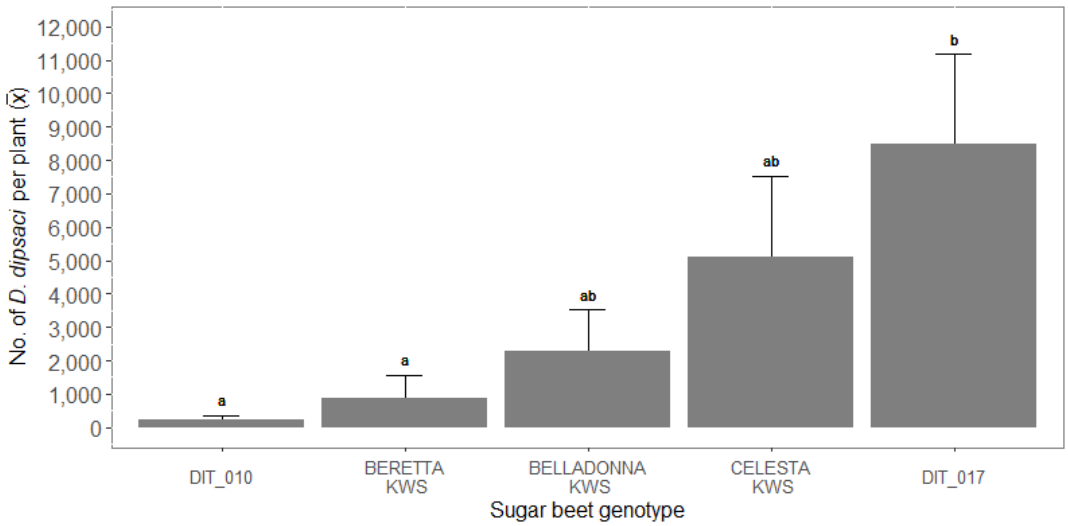

Fig. 1 Effect of sugar beet cultivar in the 2019 microplot experiment in Elsdorf on *Ditylenchus dipsaci* number in 150 g hypocotyl tissue at harvest. Different letters above the bars indicate significant differences among cultivar at $p < 0.05$, according to Dunn's multiple comparison test (n=4)

The initial *D. dipsaci* population at sowing (Pi) did not vary significantly among cultivars with a microplot average of 14.2±10.6 individuals per 150-ml soil sample ($P > 0.05$).

Table 1 Effect of the sugar beet cultivar in the 2019 microplot experiment in Elsdorf on the number of *Ditylenchus dipsaci* individuals per seedlings at 24 days post-plantation (dpp), on the nematode population development in soil from sowing (Pi) to harvest (Pf), on the plant fresh biomass at harvest, on the root rot index and the percentage of marketable beet (root rot index 0-1).

Sugar beet cultivar	No. of nematodes per seedling at 24 dpp	Pf/Pi	Root rot index (0-4)	Marketable beets (%)	Fresh plant biomass (g)	No. of plants per pot at harvest
BELLADONNA KWS	49.5 ±33.7	37.9 ±19.0	3.3 ±0.2 c	5.0 ±5.0 a	967.8 ±108.1 ab	5.0 ±0.0
BERETTA KWS	51.8 ±20.5	5.0 ±1.5	0.6 ±0.1 a	95.0 ±5.0 b	1151.9 ±138.5 a	5.0 ±0.0
CELESTA KWS	33.7 ±10.5	5.3 ±2.5	1.7 ±0.3 b	50.0 ±17.3 b	1199.3 ±98.2 a	5.0 ±0.0
DIT_010	29.5 ±6.1	38.7 ±23.8	3.7 ±0.1 c	0.0 ±0.0 a	767.9 ±128.1 b	4.5 ±0.3
DIT_017	31.0 ±10.5	9.3 ±2.5	1.9 ±0.3 b	23.3 ±8.9 ab	1158.9 ±171.7 a	4.3 ±1.1
Friedman test	$P > 0.05$	$P > 0.05$	$P < 0.0001$	$P < 0.05$	$P < 0.05$	$P > 0.05$

Root rot index: 0 = 0% rotten crown surface; 1=: ≤ 10% rotten crown surface; 2 = 11-33% rotten crown surface; 3 = 34-66% rotten crown surface, 4=: ≥ 67% rotten crown surface. Different letters indicate significant differences among sugar beet cultivar at $p < 0.05$, according to Dunn's multiple comparison test (n=4)

The sugar beet cultivar significantly influenced the number of *D. dipsaci* individuals in 150 g hypocotyl tissue at harvest ($P < 0.01$, Fig. 1). The cultivar DIT_010 (217.0±151.8) and BERETTA KWS (854.0±695.8) contained significantly fewer nematodes in 150 g hypocotyl tissue at harvest than the cultivar DIT_017 (8,498.0±2,665.4).

MICROPLOT EXPERIMENTS 2020

In Zollikofen in 2020, the sugar beet genotype did not significantly influence the number of nematodes in seedlings at 31 dpp, the root rot index, the percentage of marketable beetsor the fresh plant biomass (Table 2).

Table 2 Effect of the sugar beet genotype in the 2020 microplot experiment in Zollikofen on the number of *Ditylenchus dipsaci* individuals per seedlings at 31 days post-plantation (dpp), on the nematode population development in soil from sowing (Pi) to harvest (Pf), on the plant fresh biomass at harvest, on the root rot index and the percentage of marketable beet (root rot index 0-1).

Sugar beet cultivar	No. of nematodes at 31 dpp	Pf/Pi	Root rot index (0-4)	Marketable beets (%)	Fresh plant biomass (g)	No. of plants per pot at harvest
BELLADONNA KWS	48.9 ±19.7	4.9 ±2.5 ab	3.2 ±0.3	0.0 ±0.0	270.0 ±125.5	0.3 ±2.5 bc
BERETTA KWS	53.5 ±21.9	9.4 ±3.0 b	3.0 ±0.3	0.0 ±0.0	506.5 ±236.3	3.3 ±0.3 c
DIT_005	52.3 ±10.7	1.9 ±0.7 a	2.2 ±0.4	33.3 ±15.6	193.9 ±70.9	3.8 ±0.3 c
DIT_006	53.3 ±8.4	8.8 ±2.7 b	2.7 ±0.3	12.5 ±12.5	144.4 ±68.2	1.0 ±0.4 a
DIT_119	64.8 ±35.8	1.4 ±0.5 a	1.4 ±0.5	41.7 ±21.0	174.6 ±30.4	2.5 ±0.5 bc
DIT_301	54.5 ±8.1	6.6 ±2.0 ab	2.6 ±0.5	12.5 ±12.5	482.8 ±262.9	3.0 ±0.4 c
DIT_302	Na	1.0 ±0.7 a	2.7 ±0.7	8.3 ±8.3	306.9 ±130.7	1.5 ±0.6 ab
DIT_303	71.6 ±9.4	5.8 ±2.7 ab	2.5 ±0.6	16.7 ±16.7	265.0 ±64.5	2.8 ±0.6 bc
Friedman test	$P > 0.05$	$P < 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P < 0.05$

Root rot index: 0 = 0% rotten crown surface; 1=: ≤ 10% rotten crown surface; 2 = 11-33% rotten crown surface; 3 = 34-66% rotten crown surface, 4=: ≥ 67% rotten crown surface. Different letters indicate significant differences among sugar beet cultivar at $p < 0.05$, according to Dunn's multiple comparison test (n=4). The insufficient number of emerged DIT_302 did not allow to determine the number of nematodes in seedling at 31 dpp.

The initial *D. dipsaci* population at sowing (Pi) did not vary significantly among genotypes, with a microplot average of 64.8±14.6 individuals per 150-ml soil sample. The sugar beet genotype influence the nematode population development in soil (Pf/Pi) ($P < 0.05$, Table 2). The breeding line DIT_006 led to a higher reproduction rate of *D. dipsaci* in soil (Pf/Pi) than the breeding lines DIT_005 and DIT_119 and the cultivar DIT_302.

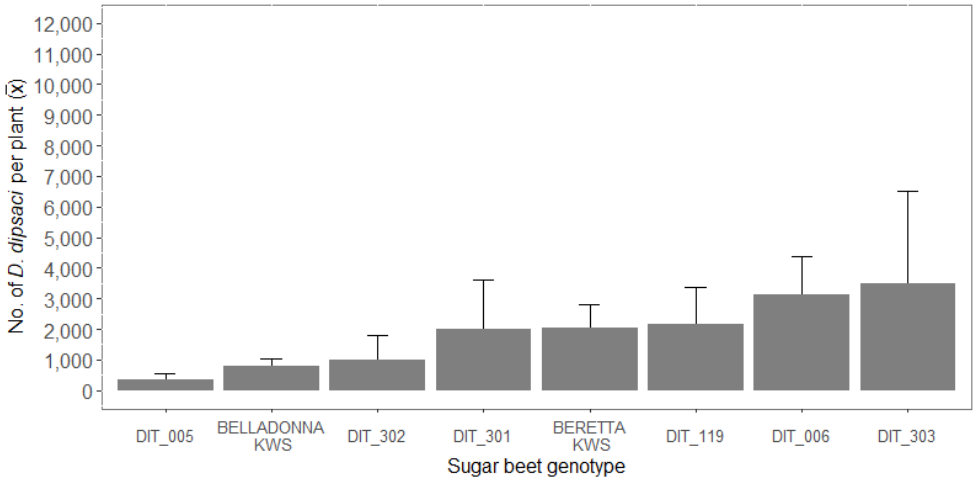

Fig. 2 Effect of sugar beet genotype on *Ditylenchus dipsaci* number in 150 g hypocotyl tissue at harvest (n=4), in the 2020 microplot experiment in Zollikofen.

The nematode number in 150 g hypocotyl tissue at harvest was not significantly affected by the sugar beet genotype (Fig. 2). The average number of nematodes in 150 g hypocotyl tissue at harvest varied from 357.0 to 3493.0 individuals. The standard cultivars BELLADONNA KWS and BERETTA KWS showed 796.5±245.8 and 2,037.0±762.5 nematodes in 150 g hypocotyl tissue. Their respective parental line, DIT_006 and DIT_005, contained 3,148±1223.2 and 357.0±191.4 nematodes. The breeding line DIT_119 and its hybrid progenies, DIT_301 and DIT_302, led to 2,162±1,218.9, 2,016.5±1,594.1, and 980.0±830.8 *D. dipsaci* individuals in hypocotyl tissue at harvest.

In the 2020 experiments in Elsdorf, the sugar beet cultivar did not significantly influence the number of nematodes in seedlings at 31 dpp, the root rot index, or the fresh plant biomass (Table 3). The cultivar DIT_301 significantly increased Pf/Pi in soil compared to the other cultivars ($P < 0.05$, Table 3). The percentage of marketable beets varied significantly among cultivars. The cultivar BELLADONNA KWS showed a lower percentage of marketable beets than the cultivar BERETTA KWS and DIT_302 ($P < 0.05$, Table 3).

Table 3 Effect of the sugar beet cultivar in the 2020 microplot experiment in Elsdorf on the number of *Ditylenchus dipsaci* individuals per seedlings at 31 days post-plantation (dpp), on the nematode population development in soil from sowing (Pi) to harvest (Pf), on the plant fresh biomass at harvest, on the root rot index and the percentage of marketable beet (root rot index 0-1).

Sugar beet cultivar	No. of nematodes at 31 dpp	Pf/Pi	Root rot index (0-4)	Marketable beets (%)	Fresh plant biomass (g)	No. of plants per pot at harvest
BELLADONNA KWS	2.5 ±1.5	81.2 ±36.1 a	1.8 ±0.8	48.8 ±18.5 a	1430.9 ±23.6	4.5 ±0.3 a
BERETTA KWS	17.3 ±8.1	4.5 ±1.6 a	0.5 ±0.2	95.0 ±5.0 b	2299.8 ±781.0	4.0 ±1.0 a
DIT_301	5.1 ±1.5	197.9 ±67.0 b	0.6 ±0.3	80.0 ±14.1 ab	1162 ±79.6	5.0 ±0.0 a
DIT_302	Na	19.3 ±9.1 a	0.3 ±0.3	100.0 ±0.0 b	2057.9 ±322.1	2.0 ±0.7 b
DIT_303	6.3 ±2.3	46.3 ±34.6 a	1.6 ±0.8	58.8 ±19.6 ab	1926.0 ±363.0	4.0 ±0.7 a
Friedman test	$P > 0.05$	$P < 0.05$	$P > 0.05$	$P < 0.05$	$P > 0.05$	$P < 0.05$

Root rot index: 0 = 0% rotten crown surface; 1=: ≤10% rotten crown surface; 2 = 11-33% rotten crown surface; 3 = 34-66% rotten crown surface, 4=: ≥67% rotten crown surface. Different letters indicate significant differences among sugar beet cultivar at $p < 0.05$, according to Dunn's multiple comparison test (n=4). The insufficient number of emerged DIT_302 did not allow to determine the number of nematodes in seedling at 31 dpp.

The nematode number in 150 g hypocotyl tissue at harvest was not significantly affected by the sugar beet genotype (Fig. 3). The standard cultivars BELLADONNA KWS and BERETTA KWS showed 1,126.0±882.2 and 1,715.0±1,499.4 nematodes in 150 g hypocotyl tissue. The cultivar DIT_301, DIT_302,

and DIT_303 led to 805.0±795.7, 1,265.5±1,228.5, and 147.5±103.6 *D. dipsaci* individuals in hypocotyl tissue at harvest.

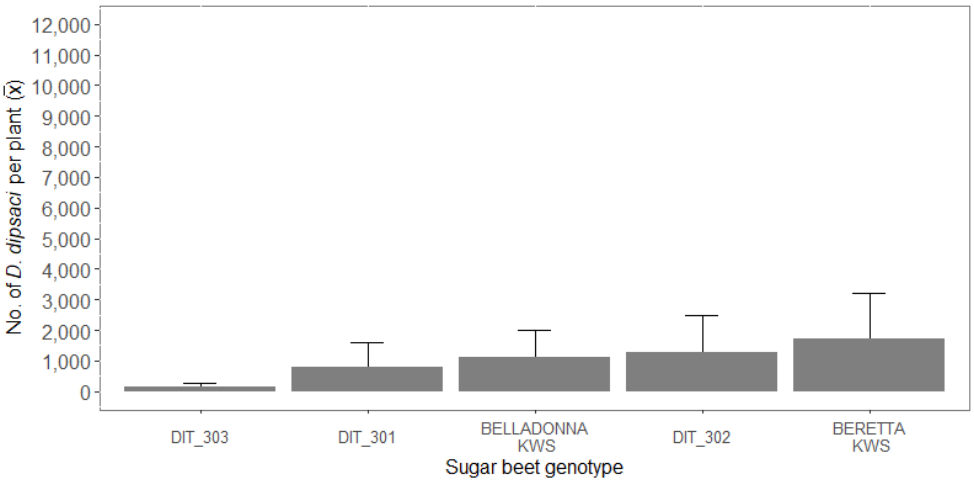

Fig. 3 Effect of sugar beet cultivar in the 2020 microplot experiment in Elsdorf on *Ditylenchus dipsaci* number in 150 g hypocotyl tissue at harvest (n=4).

The initial *D. dipsaci* population at sowing (Pi) did not vary significantly among cultivars, with a microplot average of 5.5±1.4 individuals per 150-ml soil sample.

Discussion

Our study demonstrated variations between *in vivo* and semi-field conditions. Indeed, results obtained in Storelli et al. (2021b) under glasshouse conditions could not be validated in microplot. The lower susceptibility of the breeding line DIT_119 on *D. dipsaci* penetration was not validated in the microplot. This observation contradicts Storelli et al. (2021b), assuming a higher expression of resistance towards *D. dipsaci* at a lower inoculum level. However, the number of nematodes in DIT_119 seedlings was similar in microplot and glasshouse experiments. In contrast, the number of nematodes in other sugar beet genotypes was reduced under semi-field conditions. We, therefore, assume that the lower Pi under semi-field conditions did not allow a higher quantity of nematodes to penetrate the highly susceptible genotypes. The longer emergence time in semi-field conditions may also explain the absence of differences among genotype susceptibility to *D. dipsaci* penetration. Thus, the breeding line DIT_119 lost the advantage of an early emergence observed under *in vivo* conditions.

In contrast to penetration, the effect of the genotype on *D. dipsaci* reproduction was observed in Elsdorf in 2019. Indeed, the cultivars BERETTA KWS and DIT_010 showed fewer nematodes in sugar beet tissue at harvest than the cultivar DIT_017. However, no other difference was observed in all three microplot experiments. The less sensitive control, BERETTA KWS, could not prevent nematode reproduction compared to the highly sensitive control, BELLADONNA KWS. The large number of nematodes in plant tissue at harvest in the cultivar BERETTA KWS suggests no correlation between tolerance and resistance. The low nematode reproduction in Zollikofen may be due to the increased development of fungal and bacterial pathogens. Indeed, as *D. dipsaci* is an obligate phytoparasite, the death of the sugar beet tissue is known to force the nematode to leave the plant (Schomaker and Been 2013). In contrast, in Elsdorf in 2019, the low bacterial and fungal development allowed the nematode to reproduce until harvest. The cultivar DIT_010 was an exception by showing a low nematode number in the plant at harvest but a high rotting index and Pf/Pi. This validates Hajihassani et al. (2017), where *D. dipsaci* can not reproduce on fungi and, therefore, leave the plant tissue. In Elsdorf, the absence of *D. dipsaci* reproduction difference in 2020 was possibly due to a lower Pi level. Indeed, the Pi in 2019 was almost three times higher than in 2020. We can

assume a positive relationship between Pi and root rot symptoms. However, this statement should be taken with caution as Schomaker and Been (2013) reported no influence of the Pi on plant damage.

Despite the effect of the cultivar on nematode reproduction in plant tissue, no effect was observed on Pf/Pi in Elsdorf in 2019. However, a trend could be observed, where the cultivars with high rotting symptoms led to a great number of nematodes in the soil at harvest and thus a great Pf/Pi. In contrast, despite the absence of effects of the genotype on nematode reproduction in the plant tissue, effects on Pf/Pi could be observed in 2020 in Elsdorf. The high Pf/Pi of the cultivar DIT_301 was due to the high number of plants per pot. Indeed, the cv DIT_301 was the only one to count five plants per pot at harvest, which allowed a great nematode reproduction. In Zollikofen, the low Pf/Pi of the cultivar DIT_302 was due to the lower amount of plants in the pots due to a failed emergence. An unpublished experiment on healthy soils demonstrated that the low emergence of DIT_302 was not related to *D. dipsaci* infection. The lower amount of germinated plants led to a smaller supply of plant material required for nematode reproduction during the experiment. In one of the four pots sown with the cultivar DIT_302, no plants germinated, leaving the pot without sugar beets for the entire duration of the trial. The Pf/Pi then represented the natural *D. dipsaci* development during the growing season without host plants. Despite the absence of a host plant, the nematode population did not reduce, which validates Sturhan et al. (2008), stating no reduction of *D. dipsaci* population in the absence of host plant for many years. The cultivar BERETTA KWS showed a high Pf/Pi, whereas its paternal line DIT_005 led to a low Pf/Pi.

We observed a lower Pf/Pi average in Zollikofen than in Elsdorf. However, this is due to high Pi at sowing in Zollikofen, which drives to a lower Pf/Pi rate. Indeed, microplot pots in Zollikofen contained an average of 40 *D. dipsaci* individuals per 100 ml soil at sowing. We assume that rye intercropping allowed a high reproduction of the Swiss *D. dipsaci* population before the sugar beet experiment. In Elsdorf, the Pi was higher in 2019 than in 2020. The previous crop in 2019 was faba bean undersown with carrots. Mustard was the previous crop for the experiment in 2020. We assume that faba beans and carrots are host crops with a higher *D. dipsaci* reproduction rate than mustard for the Rhineland nematode population.

The effect of the sugar beet genotype on the root rot index was only observed in Elsdorf in 2019. The cultivar BERETTA KWS validated its higher tolerance towards rot induced by *D. dipsaci* (Leipertz and Valder 2020). However, CELESTA KWS did not validate its high tolerance to *D. dipsaci* observed by Leipertz and Valder (2018). The usually higher Pi in the soil in microplot than in field experiments may explain the lower tolerance observed. Soil-borne pathogens are triggered in microplot experiments by desired side effects like deep sowing or maintenance of high soil moisture. Additionally, *D. dipsaci* pathogenicity differs depending on the year and geographic location (Leipertz 2011; Leipertz and Valder 2017, 2018, 2020). In Elsdorf in 2020, rotting of sugar beets remained low without significant differences among cultivars accompanied by a low Pi and low reproduction. In Zollikofen, in contrast, the high Pi in soil and the pathogenicity of the Swiss *D. dipsaci* population led to severe damages on sugar beets, even to the less sensitive control BERETTA KWS and DIT_119. Seinhorst (1956) reported a correlation between Pi in the soil and damages to the host crop, where damages on onion were nearly 100% with a Pi of 25 *D. dipsaci* individuals in 500 g soil. Thus, no difference in the root rot index could be observed in Zollikofen. However, DIT_119 tended to show a lower percentage of rotten tissue, validating Storelli et al. (2021b).

To date, no study has demonstrated the relationship between Pi and *D. dipsaci* virulence on sugar beets. It is common to say that the damage threshold is reached when at least one individual is found in a soil sample. However, it has never been determined whether a higher Pi leads to a higher virulence and pathogenicity of the nematode population. Therefore, it would be of interest to study this interaction by carrying out microplot experiments with different nematode densities in the soil at sowing (Pi).

In this chapter, we have discussed Pf/Pi extensively. However, the behavior of the nematode challenges the importance of this parameter in determining the level of resistance of a plant species. Indeed, the fact that nematodes leave the plant at the time of the plant tissue death ends with a significant quantity of nematode in the soil. Therefore, there is a good chance that the Pf will be higher than the Pi. In addition, Sturhan et al. (2008) reported that the nematode population did not decrease if there were no host plants for several years. This implies that a resistant cultivar would not necessarily reduce the nematode population in the soil at harvest. Therefore, another way to evaluate the resistance level of a plant species towards *D. dipsaci* is

needed. It might be more appropriate to evaluate Pf/Pi over several years. Indeed, instead of determining Pf at harvest, one could consider performing a soil sample the following spring, one year after Pi. This would allow time for the nematodes to re-distribute in the soil. This method would also allow for natural population reduction following winter.

The high pathogenicity of the Swiss *D. dipsaci* population also impacted yield. Indeed, the average fresh biomass was lower in Zollikofen compared to Elsdorf. The effect of the sugar beet genotype on yield was only observed in Elsdorf in 2019. In Zollikofen, the absence of difference among hybrid cultivars and breeding lines suggests that the great *D. dipsaci* pathogenicity severely impacted the hybrid cultivars, as observed by (Storelli et al. 2021c). However, caution should be exercised when comparing the two microplot locations. Indeed, both microplot systems differed from each other concerning plot size, depth, soil material, fertilizer, watering regime, nematode population, and climate.

This study demonstrated variations between *in vivo* and semi-field conditions and between experiment locations and years, as demonstrated earlier by Caubel et al. (1994). Microplot experiment has proven to be an effective step at validating *in vivo* results in field conditions by showing similar nematode penetration and reproduction than in the growth chamber and glasshouse. Some parameters, such as the Pf/Pi and root rot index, are challenging to investigate under *in vivo* conditions. Our microplot experiments highlighted results that could not be observed in the greenhouse, where we could not identify candidates with a lower susceptibility towards soil-borne pathogens introduced by *D. dipsaci*.

Resistant cultivars are excellent and sustainable tools to control nematode population development and need to be encouraged in the future (Schomaker and Been 2013; Starr et al. 2013). Further research is required to find a potential sugar beet candidate for resistance towards *D. dipsaci*. The screening of a large number of pre-breeding populations under *in vivo* conditions is recommended as they represent a source of genes for resistance (Tanksley and McCouch 1997). Progenies of potential candidates can then be investigated on microplot experiments before field screenings.

Acknowledgment

The authors thank the seed company KWS SAAT SE & Co. KGaA (D), the sugar factory Schweizer Zucker AG (CH), and the Bern University of Applied Sciences (CH) for their financial support. The authors would like to thank the staff of the Julius Kühn-Institut (JKI) in Elsdorf for their valuable assistance in conducting the trial and their flexibility in times of health crisis.

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7 General discussion

In Europe, *D. dipsaci* is an important threat for sugar beet (Dewar and Cook 2006). The increase of nematode pressure is the consequence of an intensive crop rotation with a shorter time frame between two *D. dipsaci* host crops. Since the withdrawal of aldicarb, no nematicide is available to farmers. The capacity of *D. dipsaci* to survive for many years in the soil or plant debris and the wide range of host plant species restrict the ability to control the stem nematode by crop rotation (Fielding 1951; Sturhan and Brzeski 1991; Caspary 1976). Breeding for sugar beet cultivars resistant towards *D. dipsaci* is required.

In the first manuscript, we implemented a new *in vivo* test system emphasizing soil inoculation of the nematodes. We demonstrated the high efficacy of a nematode soil inoculation compared to an above-ground inoculation used so far (Kühnhold 2011; Hillnhütter et al. 2011; Storelli et al. 2020). This new process is near field conditions and enables the investigation of the below-ground interaction between *D. dipsaci* and sugar beet. In manuscripts 2 and 4, we demonstrated the genetic variation for resistance against *D. dipsaci* in the sugar beet gene pool of KWS SAAT SE & Co. KGaA. Despite the failure of finding a complete resistance against *D. dipsaci*, partial resistance was found. This result may encourage the breeders to investigate a large number of pre-breeding populations as they represent a source of genes for resistance (Tanksley and McCouch 1997). The third manuscript reported variations of virulence and pathogenicity among the four tested nematode populations. We highlighted the importance of the nematode population to use during the breeding process.

An efficient and reliable test system is a prerequisite for investigating *D. dipsaci* interaction with sugar beet. Here, the importance of simulating natural climatic conditions in the growth chamber to obtain a nematode penetration rate on sugar beet seedlings similar to the field was demonstrated. By simulating average temperatures occurring in the Seeland (CH) in April (Agrometeo 2021), this method proved to be effective, showing a similar penetration rate between the *in vivo* and microplot experiments. A low temperature reduced sugar beet growth and increased susceptible stage duration for *D. dipsaci* infection. Hygrometric

conditions also proved to be determinants for the inoculation success. European sugar beet growing regions with *D. dipsaci* contamination present a high relative humidity in spring. Relative humidity higher than 95% was easily maintained in growth chambers, compared to experiments performed in a glasshouse, where a relative humidity higher than 65% was difficult to achieved. Thus, a higher penetration rate was observed in experiments conducted in growth chambers. It is recommended, therefore, the use of growth chambers.

Only the rearing process did not match field observations, where the fourth-stage juveniles (J4) overwinter and penetrate sugar beet seedlings in spring (Duncan and Moens 2013). In the *in vivo* bioassays, it was demonstrated that active young nematode populations, with a high percentage of eggs, were the most infectives, while in the field, a low percentage of eggs in spring was assumed. However, nematodes present in sugar beet seedlings, as well in the semi-field or *in vivo* experiment, were identified as J4 and adults. It can then be assumed that J4 and adults are the infective stages for *D. dipsaci* in sugar beet in growth chambers and fields. The high percentage of eggs in the nematode population indicates a healthy population, and therefore active J4 and adult nematodes. The rearing process plays a key role in the inoculation success and must be defined as part of the experiment and not as an independent task, where nematodes are stored until further use. The inoculum density and incubation duration on carrots and the storage duration after extraction were defined to achieve optimal inoculation conditions on sugar beets. Planning an experiment requires good organization, where the rearing process is included in the time plan.

The development of a new test system emphasizing a *D. dipsaci* soil inoculation offers new opportunities to investigate *D. dipsaci* and sugar beet interactions. Wallace (1962) reported *D. dipsaci* penetration at leaf basis. However, the observations were based on an above-ground inoculation. A nematode inoculation at 1 dpp may be performed to investigate *D. dipsaci* penetration pathway into sugar beet seedlings and understand the effect of nematodes on plant emergence (Caspary 1976). Additionally, in the past, divergences were shown between *in vivo* and field observations. Storelli et al. (2020) observed the effect of fluopyram on reducing *D. dipsaci* infectivity in the field. However, no effect was observed in *in vivo* bioassays performing a nematode leaf-axil inoculation. Faske and Hurd (2015) reported the effect of fluopyram on disrupting

nematode chemotactic behavior. A repetition of the *in vivo* bioassays by replacing the above-ground inoculation with soil inoculation may help better understand the effect of fluopyram on *D. dipsaci* infectivity.

An above-ground *D. dipsaci* inoculation can still be justified for some research objectives. Indeed, if the experiment investigates *D. dipsaci* interaction with the host plant while avoiding the root – nematode interaction, an above-ground inoculation is preferred. Additionally, conditions for soil inoculation success require infrastructures able to maintain low temperatures. Such materials are expensive and may not be available for all research programs. An above-ground inoculation is then justified, as it can be conducted at room temperature (18-22 °C) (Hillnhütter et al. 2011; Kühnhold et al. 2006).

In the first manuscript, the significant efficacy of the soil nematode inoculation at 1 day post-plantation (dpp) was demonstrated. Later, it was decided to perform a soil inoculation at 8 and 11 dpp for the following experiments, as it had a less negative effect on sugar beet emergence. Indeed, the high proportion of non-emerging plants when nematodes were applied at 1 dpp did not allow a decent number of emerging plants to observe resistance to *D. dipsaci* penetration and reproduction. Additionally, polygermic sugar beet genotypes were used and presented a high heterogeneity of the germination rate. A 1dpp inoculation would have led to uncertainties of the origin of a low emergence rate.

The emergence time point varied greatly among sugar beet genotypes. The fast-growing genotypes started to emerge at 8 dpp, while the slow-growing genotypes first emerged at 13 dpp. A *D. dipsaci* inoculation at 1 dpp suggested high risks of nematode leaching before the slow-growing genotypes reached the emergence stage. Therefore, it was decided to inoculate nematodes at 8 dpp. After observing a lower inoculation success on slow-growing sugar beet genotypes, it was decided to split the inoculation at 8 and 11 dpp.

The inoculation time point can lead to variations of susceptibility of a sugar beet genotype to nematode penetration. Indeed, in manuscript 2, a lower *in vivo D. dipsaci* penetration in the breeding line DIT_119, a fast-growing genotype, was highlighted. In manuscript 4, DIT_119 did not reduce *D. dipsaci* penetration compared to other tested breeding lines and hybrid cultivars in semi-field conditions. First, it was assumed

that the slower plant growth under semi-field conditions increased the duration of potential *D. dipsaci* infection. Its later emergence in semi-field compared to *in vivo* conditions negatively impacted DIT_119. It was suggested that an 8 dpp soil inoculation was not adequate for fast-growing sugar beet genotypes. However, DIT_119 showed the same amount of *D. dipsaci* individuals in sugar beet seedling in microplot and *in vivo* screenings, confirming the reliability of the test system. The absence of different penetration rates under semi-field conditions is due to highly susceptible genotypes showing lower *D. dipsaci* penetration in microplot than in growth chambers. Lower nematode density in the microplot may be the reason. Indeed, microplot pots in Zollikofen contained an average of 40 *D. dipsaci* individuals per 100 ml soil at sowing. In contrast, nematode inoculation under *in vivo* conditions corresponded to 556 *D. dipsaci* individuals per 100 ml soil (1,000 nematodes inoculated in 180 ml). Additionally, it was assumed that the *in-vitro* rearing process offered healthier nematodes than semi-field conditions, where nematodes were confronted with high-temperature variations and nematophagous fungi.

Similar *D. dipsaci* reproduction in the breeding lines DIT_119 was reported in the glasshouse and the microplot in Zollikofen. Nematode reproduction potential in the other sugar beet genotypes was reduced in the microplot in Zollikofen. It was the consequence of the Seeland *D. dipsaci* population's high pathogenicity, reported in manuscript 3, which led to a high rotting index at harvest. As *D. dipsaci* is an obligate phytoparasite, the sugar beet tissue's death forced the nematode to leave the plant (Schomaker and Been 2013). The experiment duration was shorter in the glasshouse, which did not allow rotting propagation in the entire plant. The nematode could then reproduce longer in the susceptible sugar beet genotypes.

In manuscript 3, the Seeland nematode population proved to be significantly more virulent and pathogenic than the Rhineland population used in the microplot in Elsdorf. This observation was validated in the microplot experiments. Indeed, in 2020, manuscript 4 reported a higher *D. dipsaci* pathogenicity in the microplot in Zollikofen than in Elsdorf. Despite identical nematode reproduction in sugar beets, plant growth in Zollikofen showed a high rotting index. None of the tested genotypes were able to present more than 50% marketable beets. In Elsdorf, where *D. dipsaci* pathogenicity was lower, sugar beets were less damaged, and

the marketable beet rate varied from 48 to 100%. These observations validated manuscript 3, where the Swiss *D. dipsaci* population showed the highest virulence and pathogenicity, as well as the field observations of Leipertz and Valder (2020), where the field experiments in Switzerland showed the highest pathogenicity.

Despite the absence of complete resistance, partial resistance was found. Manuscript 2 reported lower susceptibility of the fast-growing breeding line DIT_119 under *in vivo* conditions. A lower *D. dipsaci* penetration rate and, consequently, a lower number of nematodes in sugar beet tissue at harvest were observed. DIT_119 also showed lower sensitivity to *D. dipsaci* by the absence of swollen seedlings and similar plant fresh biomass compared to the non-inoculated control. In Manuscript 4, despite the lack of resistance to *D. dipsaci* penetration and reproduction, DIT_119 tended to show higher tolerance in the microplot in Zollikofen, where *D. dipsaci* pressure was extremely high. It is evident that the breeding line DIT_119 is not a candidate for complete resistance. However, the breeders may learn from the partial resistance and tolerance showed by DIT_119. The rapid emergence of DIT_119 in the growth chamber reduced *D. dipsaci* infection time. The authors, here, suggest to the breeders to further investigate fast-growing breeding lines. Cook et al. (1992) reported higher susceptibility towards *D. dipsaci* infection in white clover cultivars failing to establish vigorous fast-growing plants. Mondal and Miah (1987) reported lower *D. angustus* penetration in early maturing rice cultivars.

Manuscript 2 demonstrated that resistance breeding based on the characterization of symptoms is not possible with sugar beets and *D. dipsaci* (Plowright et al. 2002). Caubel et al. (1994) reported a positive correlation between the sensitivity of red clover to swellings and its susceptibility to *D. dipsaci* reproduction. In manuscripts 1 and 2, despite the lack of swollen seedlings, the breeding line DIT_119 did not avoid *D. dipsaci* penetration and reproduction in sugar beet tissue. These observations validated the statement of Cook and Evans (1988), attesting *D. dipsaci* capacity to survive in plant tissue without exhibiting symptoms. Additionally, no correlation between tolerance to fungal and bacterial infection introduced by *D. dipsaci* and resistance towards *D. dipsaci* reproduction was demonstrated. The cultivar BERETTA KWS, characterized

by Leipertz and Valder (2018) as tolerant to *D. dipsaci* by expressing lower rotting crown symptoms at harvest, did not avoid *D. dipsaci* penetration and reproduction in sugar beet tissue in manuscript 2 and 4.

Manuscript 2 suggested to breeders focusing on resistance towards *D. dipsaci* penetration into sugar beet seedlings. As mentioned above, resistance to penetration of nematodes from the genus *Ditylenchus* seems related to morphological and growth traits (Cook et al. 1992; Mondal and Miah 1987). In contrast, resistance to the reproduction of nematodes from the genus *Ditylenchus* is induced by phytohormone (Plowright et al. 1996; Khanam et al. 2018). Therefore, the author encourages, as well, the breeders to investigate variations of hormone production among sugar beet genotypes. Khanam et al. (2018) demonstrated the effect of salicylic acid, jasmonic acid, and ethylene on *D. angustus* reproduction on rice.

Additionally, resistant cultivars can respond to nematode infection by increase hormone production compared to non-infested plants (Kathiresan and Mehta 2005). The author also recommends breeders to determine the influence of nematode inoculation on gene expression responsible for various biochemical production in sugar beets.

For a long time, the breeding process of resistance towards *D. dipsaci* on sugar beet was hampered due to the lack of a proper test system under *in vivo* conditions. The development of resistant sugar beet cultivars towards *D. dipsaci* was a secondary breeding objective. It was only conducted in fields on registered cultivars or cultivars in the late registration stages. *In vivo* screening of breeding lines was rare (Kühnhold 2011). This thesis provided significant results to improve resistance breeding towards *D. dipsaci*. The development of the new test system using soil inoculation allowed the breeders to obtain *in vivo* conditions closer to field conditions. Results obtained *in vivo* correlated with observations in microplots. The development of the new *in vivo* test system allowed to investigate a wide range of breeding lines and pre-breeding populations in a short time. Now, breeders have all the resources they need to use this test on a larger number of genotypes.

While waiting for the development of resistant cultivars, tolerant cultivars have a key role. Although they are not effective in reducing nematode pressure, tolerant cultivars still allow sugar production in *D. dipsaci*

infested regions. However, as Leipertz and Valder (2020) and manuscripts 3 and 4 reported, the level of tolerance to crown rotting induced by *D. dipsaci* varies among growing regions. It is therefore essential to determine soil-borne pathogens associated with *D. dipsaci* in each region. Thus, we can target specific tolerance for each field. Indeed, *R. solani* (AG-2IIIB) demonstrated to belong to the soil-borne pathogens introduced by *D. dipsaci* (Hillnhütter et al. 2011). Griffin (1992) observed positive interaction between *D. dipsaci* and *Fusarium oxysporum* f. sp. *Medicaginis*, on alfalfa. Positive correlation between *D. dipsaci* density in the soil at planting and the percentage of *Phoma solanicola* infested potatoes was reported by Hijink (1963). Johnson (1939) and Metcalfe (1940) described *D. dipsaci* as an agent of infection of *Erwinia* spp. by introducing the pathogen from the soil through its created wounds and by spreading the bacteria within the rhubarb (*Rheum rhaponticum* L.) plant. Based on these statements, *Fusarium* spp, *Phoma betae*, and *Erwinia* spp. are assumed as potential secondary pathogens introduced by *D. dipsaci* into sugar beet tissue. Although the literature does not report any studies on the influence of *D. dipsaci* on introducing other fungal and bacterial diseases into sugar beets, a potential interaction between *D. dipsaci* and root rot diseases can be assumed. Sugar beet resistance to *R. solani*, *P. betae*, *Fusarium* spp. and *Erwinia* spp. have been available for several years for farmers (Gaskill 1968; Bugbee and Campell 1990; Asher and Hanson 2006). Monitoring soil-borne pathogens introduced by *D. dipsaci* in fields will guide breeders to develop cultivars tolerant to these pathogens.

Aside from resistance breeding, alternative control measures are required. Storelli et al. (2020) recommended medium-term use of the active ingredient fluopyram to reduce fungal infection introduced by *D. dipsaci*. Although the active ingredient did not reduce *D. dipsaci* population in the soil, fluopyram significantly controlled root rot propagation. However, as fluopyram is a SDHI fungicide against Ascomycota, the active ingredient had a limited effect on *R. solani* (Veloukas and Karaoglanidis 2012). Fluopyram on Swiss sugar beets is registered under conditions (Jenni and Weber 2019). In Germany, fluopyram is not registered on sugar beet due to its use on a high range of crops such as wheat (*Triticum aestivum* L.), rye or corn (*Zea mays* L.) (Proplanta 2021).

Further research into the influence of crop rotation on *D. dipsaci* population is required. As *D. dipsaci* represents a species complex with different plant-pathogen interactions depending on the geographic location of the nematode population (Sturhan et al. 2008), the host plant spectrum has to be investigated for each nematode population. Manuscripts 3 and 4 demonstrated variations of *D. dipsaci* virulence among populations. These observations suggest that different *D. dipsaci* biological races invade European sugar beets. Therefore, different host plant range among *D. dipsaci* populations from Switzerland, Germany, and France are assumed. In this sense, a project aiming to determine *D. dipsaci* host plant among Swiss cash crops began in 2021 at the Bern University of Applied Sciences.

Marigold (*Tagetes patula* L.) is known to reduce *Pratylenchus penetrans* population in the field (Pudasaini et al. 2006; Kimpinski et al. 2000). Nematicide effect of *Tagetes* sp. exudates on *D. dipsaci* was reported *in-vitro* (Uhlenbroek and Bijloo 1958). Further research on field-scale is required and will be pursued, in 2021, at the Bern University of Applied Sciences.

Since 2016, very little damages of *D. dipsaci* on sugar beet has been reported in Switzerland and Germany. The dry springs observed in recent years are the reason for the absence of strong nematode damages. Indeed, *D. dipsaci* migration to the soil surface, where infection occurs, is initiated by rainfall (Barbercheck and Duncan 2004). Thus, regular rainfall in spring just after sowing favors nematode infection. In the Seeland region (CH), a positive correlation can be observed between the rainfall amount in spring and the severity of root rot induced by *D. dipsaci* at harvest. Serious damages to *D. dipsaci* were reported in the Seeland in 2008 and 2012 to 2017 (Jenni 2008, 2012, 2013; Jenni and Cornamusaz 2014; Jenni and Weber 2015, 2016, 2017). During these years, precipitation in April ranged from 100.7 to 309.6 mm (Agrometeo 2021). In contrast, no or few *D. dipsaci* damages were reported in 2009 to 2011 and 2018 to 2020 (Jenni 2009, 2010, 2011; Jenni and Weber 2018, 2019). During these years, precipitation in April ranged from 27.4 to 73.6 mm (Agrometeo 2021). With climate scenarios predicting an increase in the frequency of summer droughts (CH2018 2018), it is perhaps wise to consider the future of this pest in Central Europe. Indeed, the decrease in the risk of attack by the nematode may encourage breeders to interrupt their breeding program against this nematode

pest. However, the same climate scenarios are more hesitant about spring precipitation. Indeed, the report predicts a slight increase in spring precipitation in the future. The high damages induced by *D. dipsaci* in 2012, despite a record low precipitation from June to August (131.8 mm) (Agrometeo 2021), suggest that dry summers do not influence *D. dipsaci* development. In contrast, wet springs are a prerequisite for high *D. dipsaci* infection.

The increase of winter temperatures might favor the cultivation of fall-sown sugar beets in Central Europe in the future. Indeed, scenarios predict higher temperatures and precipitations in winter (CH2018 2018). Castillo et al. (2007) observed in fall-sown sugar beet fields a high incidence of crown-root infections induced by *D. dipsaci*. If future breeding enables the cultivation of fall-sown sugar beet in Central Europe, the increased risk of *D. dipsaci* infections should be considered.

Currently, European sugar beet production is facing critical phytosanitary challenges. The emeregence of *Cercospora beticola* Sacc. isolates resistant to fungicides led to a substantial expansion of the Cercospora leaf spot disease (Ladewig et al. 2018; Rangel et al. 2020). Vogel et al. (2018) reported up to 90% of the German sugar beet area infested by *C. beticola*. Additionally, since the withdrawal of the neonicotinoid seed treatment in 2019, virus yellow diseases have increased dramatically in the European sugar beet production areas. Hossain et al. (2021) detected yellowing virus in 35.9% of collected samples across Europe (n=1,334). Finally, in recent years, Switzerland and Southern Germany have seen the emergence of a new disease, the Syndrome des Basses Richesses (SBR), jeopardizing sugar production in these regions (Schaerer et al. 2019). In Germany, Pfitzer et al. (2020) reported 16,400 ha of sugar beets infested by SBR, of which 8,750 ha were severely impacted. In Switzerland, 3,000 ha of sugar beets were infested by SBR in 2019 (Bussereau et al. 1/19/2021). Considering the emergence of these diseases, breeders have to set priorities in their breeding programs. Although the sugar beet area affected by the stem nematode is much more limited than the diseases mentioned above, breeders must continue their resistance programs towards *D. dipsaci*. However, the limited workforce capacity of the breeding companies does not allow them to invest much time developing resistance towards *D. dipsaci*. Therefore, the yearly field screenings conducted by Leipertz and Valder provide an

intermediate solution by determining the tolerance level of the cultivars. These trials must be, at all costs, maintained in the future.

The current political pressure is reshaping European agriculture (Bystricky et al. 2020). In 2009, the European Parliament and Council established directives to achieve sustainable use of pesticides (European Union 11/25/2009). These directives asked the Member States to adopt national action plans to reduce pesticide use risks and impacts on human health and environment (European Union 7/26/2019). They encouraged the development and introduction of integrated pest management and alternative approaches to reduce pesticide use. In this regard, the German Federal Government adopted in 2013 the National Action Plan for the Sustainable Use of Plant Protection Products (Federal Ministry of Food and Agriculture 2017). In 2017, the Swiss Federal Council launched its National Action Plan for risk reduction and sustainable use of plant protection products (The Federal Council 9/6/2017). Sugar beet production is also concerned by the environmental transition. In Switzerland, the production system "IP Suisse" was extended to sugar beet. This integrated production system gives a bonus of 60 CHF/ tonne sugar for sugar beet growers, excluding fungicide and insecticide. A federal contribution of 400 CHF/ha is also granted for non-use of insecticides and fungicides. For the non-use of herbicides, a contribution of 800 CHF/ha is granted (Arnold 2017).

Faced with these challenges, the development of resistance towards the various pathogens and diseases of sugar beet remains the most effective and sustainable approach (Schomaker and Been 2013). In the future, resistance breeding will increase, along with a growing need for workforce. Collaborations between breeding companies, industry, public research institutes, and universities are prerequisites for a successful research program. This Ph.D. project is a great example, thanks to the collaboration of KWS SAAT SE & Co. KGaA, the Bern University of Applied Sciences (BFH-HAFL), Schweizer Zucker AG, the Julius Kuehn-Institute (JKI), and the Institute of Sugar Beet Research (IfZ).

8 Summary

The stem and bulb nematode *Ditylenchus dipsaci* (Kuhn 1857) Filipjev 1936 is a migratory endoparasite ranked in the top ten plant-parasitic nematodes worldwide. *Ditylenchus dipsaci* has emerged as an economically threatening pest in the European sugar beet (*Beta vulgaris* L.) production. In Germany and Switzerland, some major sugar beet growing regions are particularly affected by *D. dipsaci*. The nematode migrates into the plant in the spring and reproduces in the hypocotyl during the growing season. Soil-borne pathogens introduced by *D. dipsaci* leads to crown root rotting and plant death.

The broad range of host plants of *D. dipsaci* hinders crop rotation strategies for a successful management of this nematode. To date, no sugar beet cultivars carrying resistance towards *D. dipsaci* are available for sugar beet producers, depriving them of effective measures against this nematode. The lack of control measures and the growing public demand for sustainable sugar production have encouraged breeders to develop resistant cultivars. For this reason, this thesis aimed to investigate resistance against *D. dipsaci* on sugar beet.

Before investigating the interaction between sugar beet and the nematode, the development of an *in vivo* test system was required. It aimed to replace above-ground *D. dipsaci* inoculation with a soil inoculation more closely related to field conditions. The most suitable inoculation time point, inoculum level, and positioning on sugar beets, as well as rearing process on carrots, were determined. At a 15:8°C day:night temperature regime, penetration rates of *D. dipsaci* into sugar beet seedlings were at maximum following soil inoculation at plant emergence. High soil moisture increased nematode migration into seedlings when *D. dipsaci* inoculation was carried out in four holes 1 cm from the plant base. The nematode suspension was previously reared for 35 days on carrot discs to obtain active *D. dipsaci* inoculum.

To find potentially resistant sugar beet restricting reproduction and penetration of *D. dipsaci*, *in vivo* bioassays were carried out with 15 pre-breeding populations and 79 breeding lines. It could be demonstrated that none of the genotypes showed complete resistance towards *D. dipsaci*. However, a high variation of the penetration rate by *D. dipsaci* was observed among the genotypes. They also responded differently to the fresh biomass reduction caused by the nematode combined with soil-borne pathogens.

Based on these results, candidates for partial resistance were further investigated in microplot experiments conducted in the Rhineland (DE) and Seeland (CH) regions. The sugar beet genotype effect on *D. dipsaci* penetration could not be validated. The genotypes did not show a sufficient tolerance towards the rotting of the plant tissue. Nematode pathogenicity and virulence differed depending on experiment locations and years.

Finally, virulence and pathogenicity of four *D. dipsaci* populations were investigated under *in vivo* conditions. No difference was found in *D. dipsaci* penetration rate into sugar beet seedlings. However, Seeland (CH) population showed a significantly higher reproduction on sugar beets than the others populations, validating observations obtained in microplot experiments.

Keywords – Breeding line, Nematode population, Pre-breeding population, Resistance breeding, Test system

9 References (Introduction and General discussion)

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10 Publications

Storelli A, Keiser A, Kiewnick S, Daub M, Mahlein A-K, Beyer W, Schumann M (2021a) Development of a new in vivo protocol through soil inoculation to investigate sugar beet resistance towards *Ditylenchus dipsaci* penetration. nematology:1–10. https://doi.org/10.1163/15685411-bja10069

Storelli A, Minder A, Keiser A, Kiewnick S, Daub M, Mahlein A-K, Schumann M, Beyer W (2021b) Screening of sugar beet pre-breeding populations and breeding lines for resistance to *Ditylenchus dipsaci* penetration and reproduction. J Plant Dis Prot, 9 p. https://doi.org/10.1007/s41348-021-00483-6

Storelli A, Kiewnick S, Daub M, Mahlein A-K, Schumann M, Beyer W, Keiser A (2021c) Virulence and pathogenicity of four *Ditylenchus dipsaci* populations on sugar beet. Eur J Plant Pathol, 9 p. https://doi.org/10.1007/s10658-021-02304-w

11 Presentations at conferences or workshops

Storelli A, Keiser A, Kiewnick S, Daub M, Mahlein A-K, Beyer W, Schumann M (2020) Investigation of resistance against *D. dipsaci* on sugar beet. Schweizerische Gesellschaft für Phytomedizin, Fribourg, Switzerland

Storelli A, Keiser A, Kiewnick S, Daub M, Mahlein A-K, Beyer W, Schumann M (2020) Investigation of resistance against *D. dipsaci* on sugar beet. Arbeitskreis Nematologie. Deutsche Phytomedizinische Gesellschaft, Vienna, Austria

12 Acknowledgments

Throughout the writing of this dissertation, I have received a great deal of support and assistance.

I would like first to thank my principal advisor Prof. Dr. Anne-Katrin Mahlein, who gave me the opportunity to complete my thesis under his supervision. I would also like to thank her for her advice throughout the project.

I would also like to thank Prof. Dr. Michael Rostás, who provided me with valuable advice regarding the investigation of the nematode-plant interaction.

I would like to acknowledge Dr. Andreas Keiser, who allowed me to complete my thesis in his research group and sparked my interest in research throughout my studies and beyond.

I would like to thank Dr. Mario Schumann and Dr. Werner Beyer, who welcomed me at KWS SAAT SE & Co. KGaA. I am very grateful for the fascinating discussions about the experiments we had. Without them, this thesis would not have been possible.

I would also like to thank my two supervisors at the Julius Kühn-Institut, for their nematology expertise. I thank Dr. Sebastian Kiewnick for having transmitted to me the enthusiasm for this discipline. I thank Dr. Matthias Daub for his precious support in conducting the microplot experiments and his colossal help proofreading the journal articles.

This thesis would also not have been possible without the financial support of KWS SAAT SE & Co. KGaA, Schweizer Zucker AG, and the Bern University of Applied Sciences.

I would like to acknowledge my KWS colleagues, and in particular, Sören Maulhardt, Luca Scholz, and Britta Ahlswede, for their precious support in assessing the glasshouse experiments. I would also like to thank my colleagues at the Bern University of Applied Sciences, and in particular, Andrea Marti and Stefan Vogel, who courageously dared to brave the autumn rain to help me build our microplot in Zollikofen.

The field trials would not have been possible without their implementation by Stephan Schlichenmaier and Christina Rothkranz-Hansen at KWS SAAT SE & Co. KgaA, and Samuel Jenni and Hans-Jörg Weber at the Swiss Beet Centre (SFZ/CBS).

The keys to a successful thesis are also in private life. That is why I would like to thank my friends in Germany and Switzerland for their support. I would especially like to thank Anthony for all the hours he spent developing Arduino hardware to equip my trials with all kinds of sensors.

I want to thank my uncles Jean-Daniel and Pascal, who sparked my interest in agriculture.

Finally, I want to thank my parents and my sisters for their continuous support. Without them, none of this would be possible.

13 Curriculum vitae

Education

Since 2018:	**Ph.D. candidate in Agricultural Sciences**
	Graduate School Forest and Agricultural Sciences (GFA)
	Thesis: Investigation of resistance of sugar beet against *Ditylenchus dipsaci*
	Georg-August-Universität Göttingen (DE)
2015 - 2017:	**Master of Sciences in Life Sciences**
	Agricultural and Forest Sciences
	Sustainable Production Systems - technical & ecological approach
	Thesis: Sensitivity of *Ditylenchus dipsaci* to fluopyram
	HAFL, Zollikofen (CH)
2012 - 2015:	**Bachelor of Science in Agriculture**,
	Major in crop production with a
	Minor in Management & Leadership
	HAFL, Zollikofen (CH)
2011-2012:	**Vocational baccalaureate specializing in natural sciences**
	Agrilogie, Marcelin (CH)
2008-2011:	**Federal VET Diploma as a farmworker**
	EAV, Châteauneuf-Sion (CH)

Work experiences

Since 01.2021:	**HAFL**, Zollikofen (CH)
	Scientific collaborator, Team Field Crop Production and Plant Breeding
01.2018 – 12.2020:	**HAFL**, Zollikofen (CH); **KWS SAAT SE & Co. KGaA**, Einbeck (DE)
	PhD candidate, Team Field Crop Production and Plant Breeding
03.2017 – 12.2017:	**HAFL**, Zollikofen
	Research Assistant, Team Field Crop Production and Plant Breeding
	Field and laboratory work
07.2014 – 08.2014 :	**Syngenta**, Dielsdorf (CH)
	Training as field trialist

- Organization of trials with a different kind of crop
- Analysis of the trial's results

14 Statutory declaration

I hereby declare that this dissertation was undertaken independently and without any unaccredited aid.

Place, Date: Zöllikofen (CH), 20th July 2021

Signature: Alan Storelli