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Assessing and Monitoring
the Impacts of Genetically modified plants on Agro-ecosystems

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Collaborative Project

Assessing and Monitoring the Impacts of Genetically Modified Plants on Agro-ecosystems

Work Package	**4**
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Task	**4.3: Impact on soil nematodes: Baselines of their diversity and physiological response to GM plants as potential stressors**
Task Leader	**Ewen Mullins**
Deliverable	**4.4: Report of GM effects on soil focal species of nematodes**
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ABSTRACT

Nematodes are among the most abundant metazoan organisms in soil and they are considered good biondicators of soil health. Our study investigates the morphometric and the biological parameters of selected nematodes as triggered by genetically modified crops, i.e., a cisgenic potato with fungal resistance and a transgenic maize resistant to insect pests. As an outcome of a previous task of the AMIGA project, three focal species were chosen as non target organisms: (1) the polyphagous root-knot nematode *Meloidogyne incognita*, (2) the potato cyst nematode *Globodera pallida,* which is an obligate, biotrophic pathogen of numerous plant species, and (3) the cosmopolitan and wide spread fungal feeding *Aphelenchus avenae.* The first two species have complex interactions with their plants, but there are differences in regard to their parasitic cycles. In contrast, *A. avenae* is free-living and sustains itself by consuming fungi.

Residing in the rhizosphere, nematodes have a close association with plant roots and their secreted exudates. As a result, they represent potential candidates to study the impact of GM crop cultivation on non-target organisms (NTO). Hence, the specific objective addressed in this report was to determine if *in vivo* bioassays could be employed to determine if the cultivation of the two above-mentioned GM crops impacted significantly on the non-target nematode species in comparison to their non-modified comparators.

This was achieved by comparing biological parameters (e.g., nematode morphology, hatching eggs of cyst nematodes) from soil, plant, tuber, or root samples collected directly from AMIGA sites in Europe and, for potato only, also by comparing biological parameters (e.g. longevity, fecundity) in laboratory bioassays. No statistical differences were identified for the majority of parameters studied. However, it was observed that the number of juvenile nematodes hatching from *G. pallida* females, *M. incognita* egg masses and reproductive capacity of *A. avenae* all had lower values following exposure to the GM potato material. The reasons for these differences could not be investigated in this study and, thus, would deserve further investigations to assess if they are directly dependent on the presence of the GM variety or due to other variables, i.e., temporal (seasonal), and bio-geographical scales.

1. INTRODUCTION

Monitoring the impact of GM crops on soil micro-fauna can be problematic due to the complexity of the biological and chemical processes involved in the rhizosphere. Furthermore, the determination of isolated substances by traditional analyses has a limited environmental application, since it does not detect the effects on the organisms: Neither does it inform about the possible interactions between the substances or the complex interactions among them. In this sense, the necessity to apply biological methodologies in order to obtain an ecosystems approach is an important consideration. In a biological system, the sequential order of alterations promoted by the presence of stressors, which may include genetically modified plants (GM plants) or their recombinant products, extends from the molecular or biochemical level to the physiological or individual level, and, further up, via the single-species level to populations and the ecosystem level (Vanaverbeke et al., 2004; Souza & Fontanetti, 2011; Fontanetti et al., 2011; Melo and Ruvkun, 2012; Cunha, 2012). Due to the inter-connectivity of ecosystem processes, analytical approaches that identify impacts at the lower levels of the biological framework are considered more constructive (Nascimento et al., 2008; Perez and Fontanetti, 2011; Souza et al., 2011; Höss et al., 2013), since they provide an opportunity to identify concerns at a stage which can mitigate a potentially greater impact on the whole ecosystem.

Nematodes are important components of the soil food web and, as such, it can be hypothesised that they could be potential bio-indicators in assessing the impact of GM crop cultivation on soil ecosystems. Nematodes are the most abundant soil inhabiting animals. They have reduced motility, often susceptibility to stresses and include species with different dietary behaviours. Moreover they are considered as potentially holistic indicators of soil processes as they are active within the soil throughout the year. They occupy several functionally different positions as primary and intermediate consumers in soil food webs (Bongers & Ferris, 1999) and they provide important ecosystem functions, including biocontrol of potential pathogens. Thus, it is crucial to assess whether GM cultivation could potentially directly or indirectly cause their suffering.

1.1. Importance of nematodes as non-target-organisms (NTO) for the risk assessment of genetically modified plants

Nematodes contribute to numerous important ecosystem functions and services and thus must not be damaged unintentionally, as non-target organisms (NTO) by GM crops or their products. Nematodes have been studied in investigations on non-target effects of different GM plants (Vauramo et al., 2006) including maize (Manachini and Lozzia, 2002; Griffiths et al., 2005, 2006) and potato (Griffiths et al., 2000; Cowgill et al., 2004, O'Callaghana et al., 2008).

Particularly the plant feeding nematodes (mainly root-feeding nematodes) can be useful to detect any potential deleterious effect of GM plants. Among nematodes, root-feeding nematodes are of special importance (Bonkowski et al., 2009). To find and invade the root and induce a feeding site root nematodes rely on an arsenal of secreted molecules and signalling pathways. Migrating nematodes can locate their target by sensing chemical gradients, plant cell—specific surface determinants or electrical signals (Bonkowski et al., 2009). Therefore any changes in the biology, biochemistry and physiology as caused by a genetically modified plant can not only have direct effects but also indirect effects on the behaviour and on the biology of the nematodes.

1.2. Selection of focal species

Focal species are, according to the EFSA ERA guidance document (EFSA, 2010), defined as species with a high potential exposure linked to a significant functional importance in soils under cultivation of a respective crop (in this case mainly potato and maize). In any crop field, many thousands of species and species groups of NTO may occur. Not all of them can be or must be tested for the potential impacts of GM crops. The choice however must be based on scientific reasons in order to allow evaluations of potential risks (Hilbeck et al., 2008).

Research on the response of nematodes to GM plants mostly focuses on changes in genera/species composition in field (e.g. (Manachini and Lozzia, 2002; Griffiths et al., 2005, 2006) and/or laboratory biological assay common laboratory species, typically using the bacterial feeding nematode *Caenorhabdits elegans* (Höss et al., 2013). However this species is not relevant in arable field soils and therefore may only be of limited value for an ecosystem-oriented risk analyses. It is much better to respect the association of the non target species with the relevant crops or

parts of them. The criteria for association with the crop are geographic distribution, habitat specialization during the crop cycle, prevalence on the crop, abundance on the crop, phenological (temporal) overlap of the crop and species or taxon, and trophic connections to the crop (Pham Van Toan et al., 2008; EFSA, 2010). In the AMIGA project, the main indicator nematodes are selected considering their functional groups, and then listed and ranked to identify suitable assessment endpoints following the methodology proposed by Pham Van Toan et al. (2008). Potential exposure of the different nematode guild to the transgene product or its metabolites is considered in Table 1.

Table 1. Ranking of nematodes feeding type for potential GM plants. High rank = 1, Medium rank = 2, Low rank = 3. A = link to aboveground plant residues that fall onto soil or are incorporated into soil; B = Link to root residues in soil or incorporated into soil; C = Link to root exudates; D = Link to root cells; E = Significance as a soil health indicator, F = Pest/weeds control, G = Pathogens control, H = Regulation of microbial community, I = decomposition of organic matter and regulation of nutrient cycling processes, L = significance for the functioning of the cropping system (according to the indications of Pham Van Toan et al. (2008))

Indicator organisms	**Potential Exposure**					**Potential Significance**							**Summary**	
	A	B	C	D	Mean	E	F	G	H	I	L	Mean	Sum	Rank
Plant feeding	2	2	1	1	1.50	2	2	2	3	2	1	2.00	3.50	1
Fungal feeding	2	2	2	2	2.00	1	2	1	2	2	1	1.50	3.50	1
Bacterial feeding	3	3	3	3	3.00	3	3	1	1	2	3	2.16	5.17	4
Omnivores	2	2	3	3	2.50	2	3	2	1	2	2	2.00	4.50	2
Predators	3	3	3	2	2.75	1	1	3	2	3	1	1.83	4.58	3
Entomopathogenic	3	3	3	3	3.00	2	1	3	3	3	1	2.17	5.17	4

Plant feeding and fungal feeding nematodes provide the most appropriate indicator organisms followed by omnivores and predators (Table 1).

In context of the AMIGA project, a literature survey was conducted to identify potential focal nematode species for each feeding type with major attention to the plant and fungal feeding nematodes with high functional relevance in the crop and in the soils at the different European AMIGA sites in Ireland, The Netherlands, both for potato, and Sweden and The Netherlands for maize. The literature sources indicate the occurrence of nematodes species in Netherland, Ireland, Sweden, and Denmark. However, while in the case of plant feeding nematodes indication on the abundance for the specific selected crop (potato and maize) are available, it is more difficult to find data regarding the other feeding type groups which occur in the different

geographical regions with the respective crops. Data in Table 2 were therefore derived from the nematofauna recorded for potato and maize also from other areas (e.g. Manachini and Lozzia, 2002; Griffiths et al., 2002, 2005, 2006; Niere and Unger, 2012) and then combined with the species recorded at the AMIGA regions under other cropping systems.

Table 2. Ranking of nematode species genus or family for potential GM plants according to their abundance and potential impact in the cropping system. High rank = 1, Medium rank = 2, Low rank = 3. Ir = Ireland, N = The Netherlands, De = Denmark, Sw = Sweden; ° species recorded in the areas but no data are available for the specific crop.

Indicator organisms	**Potato**		**Maize**	
	Ir	N	De	Sw
Plant feeding				
Globodera pallida	*1*	*1*	-	-
Globodera rostochiensis	*2*	*2*	-	-
Meloidogyne spp.	*1*	*1*	*3*	*3*
Meloidogyne minor	*1*	*1*	-	-
Meloidogyne incognita	-	*3*	-	-
Meloidogyne chitwoodi	-	*2*	-	-
Ditylenchus destructor	*3*	*3*	-	-
Tylenchorhynchus dubius	-	-	*2*	*2*
Heterodera spp	*3*	*3*	*1*	*1*
Pratylenchus penetrans	3	3	2	2
Filenchus	3	3	3°	3°
Other phytophagous	3	3	3°	3°
Fungal feeding				
Aphelenchus avenae	1	1	1°	1°
Aphelenchoides spp.	1°	1	1°	1°
Other species	3°	3°	3°	3°
Omnivores				
Eudorylaimus spp.	*2°*	*2°*	*2°*	*2°*
Mesodorylaimus spp.	*2°*	*2°*	*2°*	*2°*
Other species	2°	2°	2°	2°
Predators				
Mononchus spp.	3°	3°	3°	3°
Clarkus spp.	3°	3°	3°	3°
Other species	3°	3°	3°	3°
Bacterial feeding				
Rhabditidae	1°	1°	1°	1°
Panagrellus spp.	3°	3°	3°	3°
Acrobeloides spp.	2°	2°	2°	2°
Chephalobinae	1°	1°	1°	1°
Other species	3°	3°	3°	3°
Entomopathogenic				
Heterorhabditis spp.	2°	2°	2°	2°
Steinernema spp	2°	2°	2°	2°

According to Tables 1 and 2, literature survey, and suitability to testing under laboratory conditions the species: i) *Globodera pallida* (oligophagous plant feeding), ii) *Meloidogyne* sp. and *M. incognita* (high polyphagous plant feeding), iii) *Aphelenchus avenae* (fungal feeding) were finally selected to be included in the standardized laboratory test system.

In the following sections the three focal species will be considered separately due to the differences in their biology and ecology.

1.2.1. Characteristics of the three selected focal species

The most economically important groups of nematodes are the sedentary endoparasites, which include the genera *Globodera* (cyst nematodes) and *Meloidogyne* (root-knot nematodes). Both the cyst and root-knot nematodes have complex interactions with plant hosts but there are characteristic differences in their parasitic cycles.

The potato cyst nematode (PCN), *Globodera pallida* (Stone) Behrens, is a plant pathogenic nematode of plants in the Solanaceae family, infecting primarily potatoes and tomatoes. Non-indigenous to Europe, originating from the Andes region in South America, *G. pallida,* nowadays is prevalent in the majority of European countries where potato is grown. The nematode infects plant roots and tubers leading to plant retardation, water stress, and premature senescence of plants and ultimately yield and quality loss (Greco et al., 2002; 2007a; 2007b). If left uncontrolled, PCN can lead to 80% yield loss in potato fields (Russo and Greco, 2006).

G. pallida is a suitable NTO for GM crops because of its close association with the host plant. In fact juveniles of *G. pallida* enter roots and move to the vascular cylinder, piercing cell walls with their stylets and disrupting cells. Upon reaching the vascular cylinder, the nematode establishes a feeding site, apparently by injecting secretions through their stylets. The formation of a feeding site is characterized by the breakdown of the cell walls between the initial feeding site cell and its neighbouring cells, resulting in the development of a multinucleate syncytium, which provides nutrients for the feeding nematode. Cyst nematodes undergo three molts inside the root before becoming adults. They generally reproduce sexually, and once fertilized, the female becomes full of eggs. After the females die their bodies become protective cysts for the eggs (Greco et al., 2005).

The root knot nematode e.g. *Meloidogyne incognita* is geographically the most widespread and probably also the most serious plant-parasitic nematode pest of potato in tropical and subtropical regions throughout the world (Russo et al., 2007). Being highly polyphagous, it occurs as a pest on a wide range of crops (Manachini et al., 2003; d'Errico et al., 2014). Coffee, cotton, tobacco, fruit-trees, and almost all vegetable and horticultural crops can be damaged. Related species damage cereals, including rice (Sasser and Carter, 1985). In Europe, especially due to reductions in the use of chemical nematicides, *M. incognita* has become increasingly important and detected in recent years by attacks on different crops (Hunt & Handoo, 2009, Giacometti et al., 2010; Wesemael et al., 2011). This nematode species has a short life cycle and, unlike most soil nematodes, populations can increase rapidly within one growing season. Consequently, it can be extremely damaging, with a threshold as low as 60 eggs per kg soil (Di Vito et al., 2003). In contrast to *G. pallida*, the juvenile of the root-knot nematode moves intercellularly after penetrating the root, migrating down the plant cortex towards the root tip. The juveniles then enter the base of the vascular cylinder and migrate up the root. They establish a permanent feeding site in the differentiation zone of the root by inducing nuclear division without cytokinesis in host cells. This process gives rise to large, multinucleate "giant" cells. The plant cells around the feeding site divide and swell, causing the formation of galls or 'root knots' and leading to a reduction in plant vigour (Wyss et al., 1992). *G. pallida* ingests the cytoplasm of the plant-derived giant cells through their stylets and, after three molts, develop into pear-shaped, egg-laying females (Wesemael et al., 2011). Both giant cells and syncytia serve as metabolic sinks that funnel plant resources to the parasitic nematode. Interestingly, Colyer et al. (2008) reported that GM-insect resistant cotton was more susceptible to *M. incognita* than conventional cotton, and this result was, in fact, confirmed by Karuri et al. (2013) who demonstrated that *Bt* cotton is more susceptible to *M. incognita* than its isogenic comparator.

The third focal species, *Aphelenchus avenae* Fuchs (Fam. Aphelenchidae) is a free-living parthenogenic nematode, which feeds on fungi. It was chosen because it is a common species in several crop systems including both potato and maize (Manachini and Lozzia, 2002; Celis et al., 2004; Manachini et al., 2004), thus making it very suitable for the crops of the AMIGA project. The nematode species is easy to

rear in the laboratory (Hechlerh, 1962; Barker and Darling 1965). In addition, fungivorous nematodes in general and particularly *A. avenae*, have a biocontrol potential since they can suppress fungal plant diseases (Lagerlöf et al., 2011).

Published reports suggest that this species has life history characteristics that confer tolerance and/or resilience to physical and chemical or other environmental disturbance (Bongers, 1990; Li et al. 2005; Korthals et al., 1998; Fiscus and Neher, 2002). In the laboratory *A. avenae* can be easily cultivated on fungi. Morphological variations of this species were induced and recorded varying diets, e.g. species of fungi or their availability, or under different stress conditions (Pillai and Taylor, 1976; Kline, 1976; Fisher and Davies, 1990; Townshend and Blackith, 1995).

A. avenae was to study GM effects under laboratory as well field conditions. The abundance of *A. avenae* was found to be lower in silty soil cultivated with Bt maize (event Bt 176) compared to isogenic maize (Manachini and Lozzia, 2002). The difference in the proportion of the nematode population belonging to the fungal feeding trophic group was more strongly affected by the variety than by effects of the genetic modifications, as found with potato with the example of *Aphelenchus* (O'Callaghana et al., 2008). On the other hand, abundances were not affected in the soil planted with genetically modified nematode-resistant potato clones that express a cysteine proteinase inhibitor (cystatin) in roots (to control the potato cyst nematode *Globodera* spp.) when compared to the abundance recorded for the non-GM potato variety (Celis et al, 2004; in supplementary data).

For all the above reasons, *A. avenae* can thus be considered as a suitable NTO for non-targeted GM crops, especially for those which exhibit resistance to fungal diseases.

1.3. Selection of endpoints

Sub lethal direct or indirect effects of GM plants on NTO are considered as harm (Hilbeck et al., 2008; EFSA, 2010). To meet this need for risk assessment under chronic exposure conditions, the test systems for such parameters should include growth (expressed as morphometric parameters), reproduction and survival of focal nematode species as main components of their fitness and relevant performance traits, especially for concluding on potential long-term effects and changes in ecological functions (Pey et al., 2014). GM crop risk assessment of nematodes as NTO should therefore be based on growth (expressed as

morphometric parameters), egg production, egg hatching, as well as on survival and development of offspring as measurable endpoints.

The objective of this study was therefore to determine if specific bioassays and morphological analysis could be designed to quantify the potential impact of the above mentioned GM potato and maize on the three species of nematodes, i.e., *G. pallida, M. incognita* and *A. avenae*.

Results on the effect of GM plants on body size-related parameters of nematodes have rarely been published. However body size influences many aspects of animal life such as life history, physiology, energy requirements, as well as biotic and abiotic interactions (Calder 1984; Vanaverbeke et al., 2004). The optimal size of an organism can be linked with both its food input and quality (Kline, 1976). This is especially true for nematodes, because their morphology (expressed, e.g., as width/length ratios) reflects the feeding mode of the organisms and the effect of environment (Tita et al. 1999, Fonderie et al., 2013). Recently Ngo Xuan Quang et al. (2014) demonstrated that nematode lengths showed a significant correlation with numbers of environmental variables, in the case of plant feeding, with the host species (Sheila Shahab, 2014). Therefore, changes in morphometric parameters (estimated according to the species) are expected to be an indicator of different effects.

They were considered as suitable parameters, to detect potential deleterious effects due to the presence of GM plants. Because laboratory bioassays cannot take into account all potential direct and indirect effects of GM plants on NTO, several parameters, especially including the morphometric ones, were compared for the specimens collected directly in the plots sown with the different varieties. On the other hand, laboratory assays can estimate more precisely some other parameters over the life cycle (e.g. fecundity) of the focal species reared on the different plant varieties.

Thus, in this study, potential sub-lethal effects of GM potato and maize compared to isogenic and conventional varieties for two different approaches were carried out including: (i) comparisons of morphometric parameters and, where possible, some biological ones, e.g., morphology, hatching eggs of cyst nematodes of specimens collected from soil/plant/tuber/root samples directly from AMIGA fields and, for potato, in addition (ii) comparisons of biological parameters (e.g. life span, fecundity) recorded from laboratory bioassays.

2. MATERIALS AND METHODS

2.1. Organisms

For potato the plant feeding nematodes *G. pallida, Meloidogyne sp.* were considered. The fungal feeding *A. avenae* was chosen for both maize and potato. The actual *Meloidogyne* species present on potatoes was *M. incognita*. However, it should be noted that several morphometric parameters overlap with the morphometric description of *M. fallax* or similar species. Traditionally, *Meloidogyne* species are described and identified based on the morphology and morphometric of second-stage juveniles and males, and on perineal patterns of adult females (Hunt & Handoo, 2009). Morphological identification of root-knot nematodes can be challenging because of overlapping characters (Jepson, 1987). Indeed solid identifications are best achieved with the integration of morphological-, isozyme- and molecular-based datasets (Wesemael et al., 2011). As it was not possible to complete this level of analyses in this study, the nematodes studied were named *Meloidogyne* sp. In the case of the specimens collected from Italy, the identification was supported by previous molecular analysis done on the populations collected in the same areas (see below in the specific section). Table 3 summarizes from where the nematodes were obtained and for which assays and endpoint were used.

2.2. Plant materials

The origin of the samples and the proposes for which they were used are indicated in table 3.Tubers of potato were received from potato plots grown in 2012 at AMIGA sites in Netherlands and in 2013 from potato plots grown in Ireland. This because in 2012 adequately sized tubers were not available from Ireland due to the small size of the 2012 field study, which arose due to delays in the GM licensing process and in 2013 illegal intervention in the Dutch potato trials by activists, the latter leading to an unforeseen admixture of GM and non-GM tubers. Since this admixture compromised the genetic identity of the cultivated plots the respective tubers were not included in the subsequent analyses.

The varieties used were: the GM cisgenic potato line (GM), the respective isogenic cultivar Desiree (ISO) and the conventional variety Sarpo Mira (Con). The cisgenic potato line was previously generated at the University of Wageningen as part of the DurPh project and contains the cis-gene *Rpi-vnt1*, which confers resistance to the

potato late blight disease pathogen *Phytophthora infestans*. For each of the three potato genotypes, two treatments were applied *in situ*, which included current fungicide practise (CP) and a no spray control (NS). The received tubers were examined and then used for the laboratory bioassay regarding nematode mortality and fertility after obtaining from them *in vivo* plantlets of all potato varieties (GM, ISO, Con). To complete the analysis of the nematodes from potato plots grown in 2012 and 2013 at AMIGA sites, when tubers were not available or infestation was too low, tiny roots or nematodes from soil samples were also used (Table 3).

Only soil samples were received in 2013. These originated from the AMIGA maize plots located in Denmark and Sweden, which included the GM maize event MON810, expressing the insecticidal toxin Cry1Ab derived from *Bacillus thuringiensis* (BT: DKC 3872 YG) and its isogenic variety (ISO: DKC 3871).

1 **Table 3** Selected focal species, including their major characteristic, sources, stages analysed and
2 definition of endpoints

Focal species	***Globodera pallida***	***Meloidogyne sp.***	***Aphelenchus avenae***
Source for morphological bioassays of specimens from fields	Potato plots in Ireland 2013	Potato tubers in Netherlands 2012 Soil and roots Ireland 2013	Soil from maize field of Denmark and Sweden 2013
Source for biological bioassay in laboratory	Specimens from infested potato fields in Italy (see Avezzano, 2013). Potato tubers varieties from Ireland 2013	Specimens from infested tomato fields in Italy (see Chieri, 2012. Potato tubers varieties from Netherlands 2012	Specimens from Denmark and Sweden 2013 (pooled) Potato tubers varieties from Ireland 2013
Natural habitat	Mainly on potato	Polyphagous plant feeding nematode on Solanacea and many other families	Polyphagous fungal feeding nematode wide spread and cosmopolitan.
Growth stages and gender for specimens from fields	Female (cysts), eggs	Juveniles (II) and females, eggs	Females
Growth stages and gender analysed for laboratory bioassay	Juveniles (II, III) and young females and males, eggs	Juveniles, young females, egg masses, eggs	Juveniles and eggs, females
Plant material analysed	Potato varieties (GM cisgenic potato line (GM), isogenic cultivar Desirée (ISO) conventional cultivar Sarpo Mira (Con)	Potato varieties (GM cisgenic potato line (GM), isogenic cultivar Desirée (ISO) conventional cultivar Sarpo Mira (Con)	From fields transgenic maize (BT: DKC 3872 YG) and its isogenic variety (ISO: DKC 3871).
from maize	-	-	Soil
from potato	Soil, tiny roots and tubers	Soil, tiny roots and tubers	For laboratory bioassay potato tubers from Ireland 2013
Endpoints assessed			
For field population	Female body measurement	Female body measurement	Female body measurement
For field population	Juvenile II body measurement	Juvenile II body measurement	-
For field population	Fertility (Eggs/ female cyst)	Fertility (juveniles II/ female	-
For field population	Vitality of offspring (hatched eggs/females)	Vitality of offspring (Mobility)	-
Laboratory bioassay on potato	Life cycle in 100 days (% of instars)	Life cycle in 30 days (average number of instars)	Reproduction (average number of eggs and II Juveniles)
Laboratory bioassay on potato	Number of female (cysts) after 100 days	Number of female (cysts) after 30 days	Mortality (% dead individuals)
Laboratory bioassay on potato	Vitality of offspring (hatched eggs/females)	Fertility (egg masses and eggs)	Female body measurement

3

2.3. Analytical procedures for determination of endpoint measurements

Morphometric parameters, according to the focal species (see details below and in Table 1), were compared for individuals collected directly from the AMIGA field plots sown with the different varieties. The morphometric parameters were chosen in this to compare individuals grown on different plant species /varieties or under different stress (e.g. in Hechlerh, 1962; Kline, 1976; Calder, 1984; Di Vito et al., 2003; Greco et al., 2002; 2005; Li et al., 2005; Sheila Shahab, 2014).

Fecundity was estimated for the specimens of *G. pallida* and *M. incognita* collected directly in the field respectively as number of eggs for female and number of juveniles for female. The fecundity and the survival of the different stages were measured for the populations reared for the laboratory bioassays of all the selected focal species on the different varieties of potato. More details are given for each species in the following sections.

2.4. Globodera pallida

2.4.1. Analysis of specimens collected from field plots

In 2013, potato tubers and roots were provided from Ireland and The Netherlands where they were collected from AMIGA plots grown with the GM cisgenic potato line (GM), its respective isogenic cultivar Desirée (ISO) and the conventional cultivar Sarpo Mira (Con), with varietal plots treated with the current fungicide practice (CP) or unsprayed (NS). For Ireland, tubers were provided from NS plots but because of their low infestation level, the analyses were done using cysts in good conditions collected from roots and soil samples. Three replicates for each variety and treatment (CP and NS) were taken and five mature females for each replicate were analysed.

Morphological parameters, including body length (excluding neck), greatest body diameter, distance from anus to nearest edge of fenestra (anus-vulva) and Granek´s ratio (the vulva – anus distance divided by vulval basin diameter) were assessed. All measurements and observations were taken using a DLMB Leica (Wetzlar, D, Germany) microscope. After morphological analyses cysts were crushed according to Bijloo's modified method (Seinhorst and Den Ouden 1966) and the number of hatched eggs was determined. Also the morphological parameter such as body length, stylet length, hyaline region and tail length of the hatched juveniles (II J) were recorded.

2.4.2. Laboratory incubations

G. pallida were collected from infested potato fields located in the province of Avezzano (Aquila, Italy) in November 2013 with cysts stored in the soil at 7°C until extraction. This additional sampling was done to have a huge amount of viable specimens available at the time when plantlets derived from the received materials were ready to receive the inoculum of nematodes.

For plant material, *in vivo* plantlets of all potato varieties (GM, ISO, Con) were transplanted into pots containing organic potting soil. After thirty days plantlets were transplanted into 14 cm diameter clay pots containing 1,000 cm^3 of steam sterilized sandy soil (89% sand), which was infested with 20 eggs/g soil of *G. pallida*. Plants were then extracted after 40, 60, 80 and 100 days with roots weighed and washed before being cut into 0.5 cm long pieces. Three individual plantlets were analysed for each potato variety and for each period (days). The nematodes were then extracted from roots as per the centrifugation method of Coolen (1979) before being counted and classified into developmental stages. Each stage is expressed as a percentage of the total nematode specimen. The developmental stages recorded, during the bioassays were: juvenile of second stage (II J), juveniles of third stage (III J), young female (IV JF) and young male (IV JM). Moreover, after 100 days, the number of cysts/10 g soil and number of hatched eggs per cysts were also determined. Three replicates of five specimens each were analysed.

2.4.3. Statistical analysis

For data analysis, collated datasets were compared using analysis of variance (ANOVA) with homogeneity of variances performed using Cochran's test prior to ANOVA analysis. The statistical significances of the reproductive output in the various scenarios were calculated by the means of total offspring per cyst in each replicate, or by the average percentage of stages at the same days after inoculum, by one-way ANOVA and Tukey's pairwise comparisons. Comparisons among the cumulative means of the number of different nematode stages during all of the sampling periods (1-100 days) for the potato varieties was made by Kruskal Wallis and post-hoc pairwise tests. Statistical significance was assessed by all applied tests ($p < 0.05$) using Statistica 6.0 (StatSoft, Tulsa, OK, USA).

2.5. Meloidogyne sp.

2.5.1. Analysis of specimens collected from field plots

In 2012, potato tubers were received from the The Netherlands and roots and soil were obtained from the Irish partner: Adequately sized tubers were not available from Ireland due to the small size of the 2012 field study, which arose as a consequence of delays in the GM licensing process. Delivered samples were taken from plots sown with GM cisgenic potato line (GM), its respective isogenic cultivar Desirée (ISO) and the conventional cultivar Sarpo Mira (Con). The root knot nematode was identified as *Meloidogyne* sp.. As such the morphological analysis of *Meloidogyne* sp. female and juvenile nematodes was only completed on tuber samples that were shipped from the Netherlands. While the potato roots received from Ireland in 2012 contained some *Meloidogyne* sp.*,* the roots were deemed unsuitable to complete the planned analysis. Therefore, to compare the effect of GM potatoes directly on the biology of *Meloidogyne* sp.*,* egg masses were collected directly from tubers (Netherlands, collected in 2012, as no tubers were received from Ireland) and roots (Ireland, collected in 2013) by the method of Byrd *et al.* (1972). Therefore the morphometric analyses of the females were carried out only for specimens collected in The Netherlands (2012). The few females and egg masses extracted from the tiny roots and soil from Ireland samples were used only to analyse the mobility and the morphometric parameters of juveniles.

From the *Meloidogyne* sp., collected in 2012 in The Netherlands and in 2013 in Ireland, the resulting second-stage juveniles derived from the egg masses were compared with three replicates (of five egg masses each) per variety considered, with the number of viable juveniles hatching from the egg masses evaluated at 3, 5, 7 days. Moreover, to avoid any effects due to the environmental conditions, the mobility of second-stage juveniles after a 24 h water rinse was also assessed.

To assess adult females and second stage juveniles (Netherlands, tubers collected in 2012), five individuals per replicate were considered with measurements including: length of median bulb (LMB), width of median bulb (WMB), stylet length, neck length, length of vulval slit (LVS), distance from anus to vulval slit (AVS), and anus to tail terminus area (ATT).

2.5.2. Laboratory incubations

To standardize the inoculum for the laboratory bioassays, *M. incognita* was collected in December 2012 from infested tomato fields located on sandy soil in a coastal area of southern Sicily (Chieri, Ragusa, Italy). To prepare the host-pathogen interactions, tubers from each of the three varieties (The Netherlands, collected in 2012) were disinfected for 5 min in a 5% sodium hypochlorite solution, thoroughly washed with water and left to sprout. Post-sprouting tubers were planted in pots filled with 5 kg of heat-hygienized soil (sand:loam, 1:1) and three weeks after planting, when roots and shoots had developed, each pot was inoculated with 1000 *M. incognita* eggs and/or juveniles (< 24 h).

The *M. incognita* inoculum was prepared by washing field infected tomato roots before chopping them into 2 cm segments. The galled root segments were processed in 0.05% sodium hypochlorite and extracted eggs rinsed thoroughly before being placed in sterile water. The egg suspension containing the appropriate number of eggs and juveniles was then pipetted evenly around each plant. Plants were harvested after 30 days and the developmental stage of *M. incognita* observed by acid fuchsine staining as described by Byrd *et al.* (1983) with the aid of a stereomicroscope (Wild Heerbrugg, M8 – 377567, Gais, Switzerland). For this assessment, the presence/absence of eggs, dead/alive juveniles were counted with the number of females, males, galls, egg masses and eggs compared across varieties. Roots were scored by visual inspection for the presence of galls with galling severity rated according to the following scale to estimate the root galling index (RI): 0 = no galls; 1 = 1 to 2 galls; 2 = 3 to 10 galls; 3 = 11 to 30 galls; 4 = 31 to 100 galls; 5 = >100 galls per root system (Colyer *et al.,* 2008).

2.5.3. Statistical analysis

Generated data were compared using ANOVA. Prior to ANOVA, the homogeneity of variances was analysed using Cochran's test. Whenever necessary, the data were log-transformed. Depending on the characteristics of the data, statistical significances were assessed either by Student's t-test ($p < 0.05$) or by post hoc Tukey's test for multiple comparisons using Statistica 6.0 (StatSoft, Tulsa, OK, USA).

2.6. Aphelenchus avenae

2.6.1. Analysis of specimens collected from field plots

A. avenae was isolated from soil samples collected in 2013 from Denmark and Sweden, using the nematode extraction of the Baermann funnel method (1917). Soil samples were obtained from plots sown with GM maize and its isogenic variety. Each variety was analysed with ten replicates (50 g of soil/replicate). The extracted nematodes were used for the morphological study and, in part, for the laboratory bioassays. The *A. avenae* abundance was on average 6.45 ±1.29 in 50 g of soil. Three replicates were randomly selected per variety and used to extract five specimens for the morphological analysis. The remaining material was used to rear *A. avenae* for the subsequent bioassays.

For the morphological study, nematodes were heat killed at 70°C, fixed in TAF fixative containing 8% formalin and 2% triethanolamine in distilled water and slide mounted in anhydrous glycerine. Observations and measurements were completed using the DLMB Leica microscope. The measurements considered for female *A. avenae* included: body length, width at lip region, width at anus, width at mid body, a= body length/body width, b=body length/distance from the anterior end to base of median oesophageal bulb, c=body length/tail length, stylet length (µm), tail length (µm), V= (distance of vulva from anterior end) x 100/ body length.

2.6.2. Laboratory incubations

For the bioassays, the maintenance and rearing of *A. avenae* was carried out according to the method described by Hansene et al. (1972) and Ishibashi et al. (2005) using potato tubers collected from the Irish AMIGA plots in 2013. All experiments were conducted using 60-mm petri dishes. *A. avenae* was fed on stock cultures of *Rhizoctonia solani* AG2-1 F56L grown on tuber slides for each variety and on Difco potatoes' dextrose agar (PDA) as a control (CR). The control was chosen according to Fisher and Davies (1990).

Survival, size and reproduction were measured for nematodes grown on the three different potato varieties versus the growth media control. Three biological replicates for each variety were performed with 10 gravid females studied per replicate. Percentage mortality was assessed after 30 days. Death was diagnosed when an individual did not respond to touched with a probe. The resulting nematode populations that developed from the fungal growth on each potato variety were

recorded and the number of new individuals emerging from eggs used to estimate the level of reproduction. Data on reproduction are given as sum of eggs and juveniles because it is not always easy to separate and count eggs from juveniles (Buecher et al., 1974). Moreover three females for each replicate were employed to record body dimensions (same parameters as described above).

2.6.3. Statistical analysis

The data were compared as described above in 2.5.3.

3. RESULTS

3.1. Globodera pallida

3.1.1 Analysis of specimens collected from field plots

The morphometric parameters of the cysts collected from the different plots with the different potato varieties are shown in Table 4. The body lengths of the specimens were consistent and ranged from 390 to 720 μm with an average of 684 μm, while the greatest body diameter ranged from 370 to 600 μm and an average of 505 μm. Female nematodes exposed to the GM variety showed no physical differences compared to the females exposed to the other two varieties. All parameters recorded did not show any statistical difference among the three varieties with respect of the different treatments (sprayed versus unsprayed) ($p > 0.05$).

Table 4. Average body measurements (lower - upper limits) taken from cysts of *G. pallida* from potato varieties grown in Irish AMIGA plots in 2013. Results are given as mean (n = 3 replicates of 5 cysts/replicate). No statistically significant differences were recorded as per *post hoc* Tukey's t-test for multiple comparisons. GM = GM cisgenic Desiree, ISO = Desirée, Con = Sarpo Mira, NS = no fungicide spray, CP = current fungicide practice

	GM NS	GM CP	ISO NS	ISO CP	Con NS	Con CP
Body length (μm)	574 (400-700)	580 (410-720)	570 (390-705)	565 (400-680)	559 (400-705)	570 (400-710)
Greatest body diameter (μm)	530 (400-600)	500 (400-600)	520 (400-620)	480 (370-610)	500 (400-600)	500 (400-600)
Distance from anus to fenestra (μm)	25 (17-45)	22 (16-42)	25 (18-44)	24 (16-42)	23 (15-40)	25 (17-46)
Granek's ratio	2.0 (1.1-3.4)	1.9 (0.9-3.3)	2.2 (1.2-3.3)	2.1 (1.1-3.5)	2.1 (1.2-3.4)	2.2 (1.2-3.6)

The average number of eggs for female (cyst) of *G. pallida* collected from different varieties is shown in Figure 1. The number of eggs/cyst was relatively high, ranging from a minimum of 440 eggs/cyst (GM treated with fungicides) to a maximum of 680 eggs/cyst (in Con treated with fungicides).

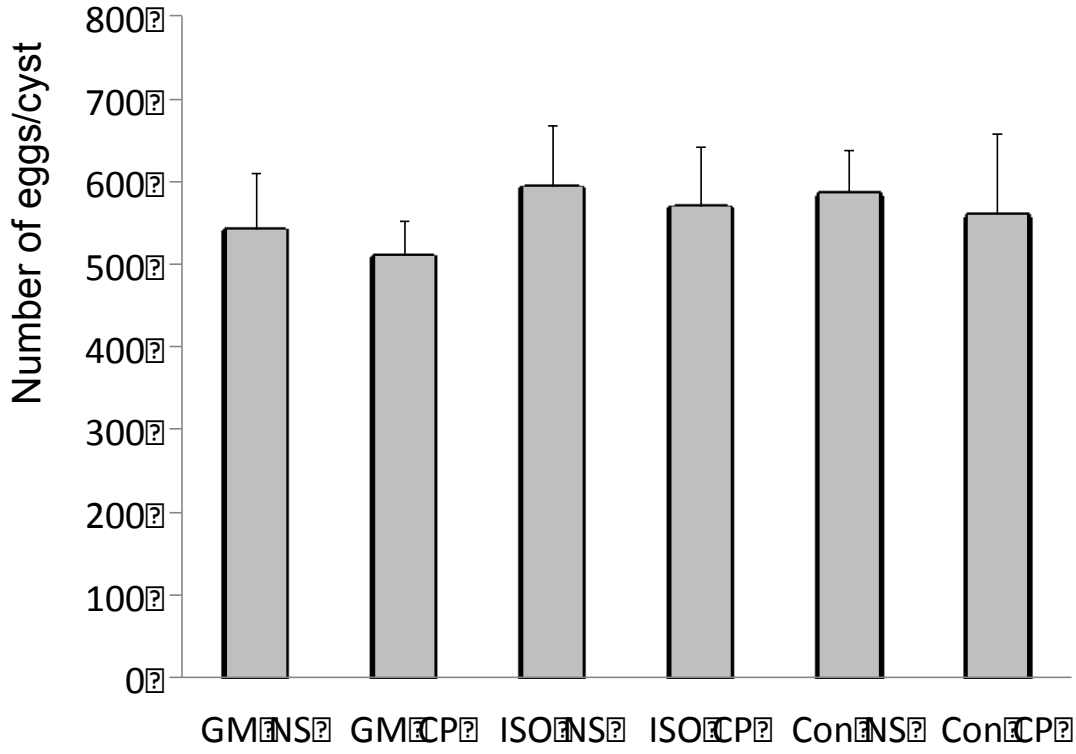

Fig. 1. Average number of eggs for cyst of *Globodera pallida* collected from potato samples taken from Ireland (2013). Results are given as mean (n = 3 replicates of 5 cysts), and standard deviation. Bars with different letter (a) are statistically significant different at post hoc Tukey's t-test for multiple comparisons significant (P < 0.05). GM = GM cisgenic Desiree, ISO = Desirée, Con = Sarpo Mira, NS = no fungicide spray, CP = current fungicide practice.

A lower number of *G. pallida* eggs/cyst were recorded from the GM potato compared to the isogenic and conventional varieties, but the difference was not statistically significant ($p>0.05$), irrespective of the presence/absence of treatment (Fig. 1).

The mean of hatched eggs for females of *G. pallida* collected from different varieties is shown in Figure 2. A significantly lower number of hatched *G. pallida* eggs/cyst were recorded from the GM cisgenic potato variety compared to the isogenic and conventional comparators ($p<0.001$), which was irrespective of the fungicide treatments.

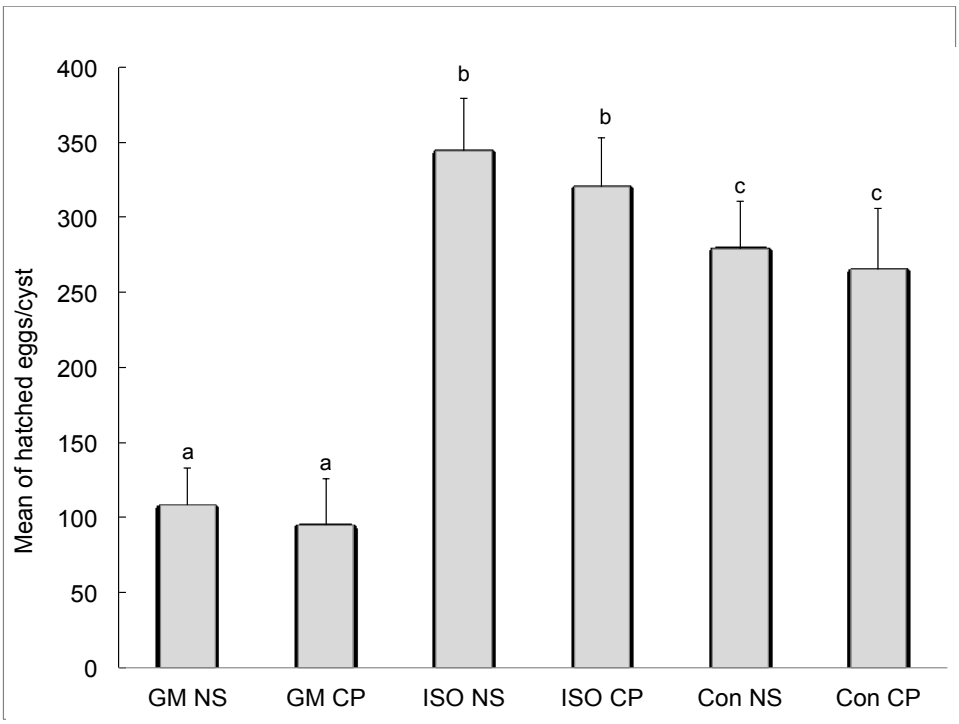

Fig. 2. Mean of hatched eggs for female of *Globodera pallida* collected from potato samples taken from Ireland (2013). Results are given as mean (n = 3 replicates of 5 cysts), and standard deviation. Bars with different letter (a, c) are statistically significant different at post hoc Tukey's t-test for multiple comparisons significant (P < 0.05). GM = GM cisgenic Desiree, ISO = Desirée, Con = Sarpo Mira, NS = no fungicide spray, CP = current fungicide practice

The morphological parameters showed no significant differences (P>0.05) as noted between the three varieties studied in the presence/absence of the fungicide treatments (Table 5).

Table 5. Average body measurements expressed in μm (lower - upper limits) taken from second larvae juveniles of *G. pallida* from potato varieties grown in Irish AMIGA plots in 2013. Results are given as mean (n = 3 replicates of 5 II J/replicate). No statistically significant differences were recorded as per post hoc Tukey's t-test for multiple comparisons. GM = GM cisgenic Desiree, ISO = Desirée, Con = Sarpo Mira, NS = no fungicide spray, CP = current fungicide practice

	GM NS	**GM CP**	**ISO NS**	**ISO CP**	**Con NS**	**Con CP**
Body length (μm)	477.4 (470-482)	476.8 (470-482)	480.2 (470-482)	478.8 (469-482)	476.6 (469-480)	476.2 (469-480)
Stylet length (μm)	23.2 (23-24)	23.2 (23-24)	23.6 (23-24)	23.6 (23-24)	23.4 (23-24)	23.2 (23-24)
Hyaline region (μm)	26.6 (26-27)	26.4 (26-27)	26.8 (26-27)	26.4 (26-27)	26.4 (26-27)	26.2 (26-27)
Tail length (μm)	51.6 (50-53)	51.4 (50-53)	51.6 (50-53)	51.2 (50-53)	51.6 (51-53)	51.4 (50-52)

Table 5 shows the morphometric parameters of the Juveniles (II J) hatched from the eggs collected form the different plots sown with the different potato varieties. The body length ranged from 470 to 482 μm and the length of the stylet was consistent among the specimens.

3.1.2. Laboratory incubations

The temporal development and the developmental stages from eggs to adults of *G. pallida* in 100 days are shown in Figure 3.

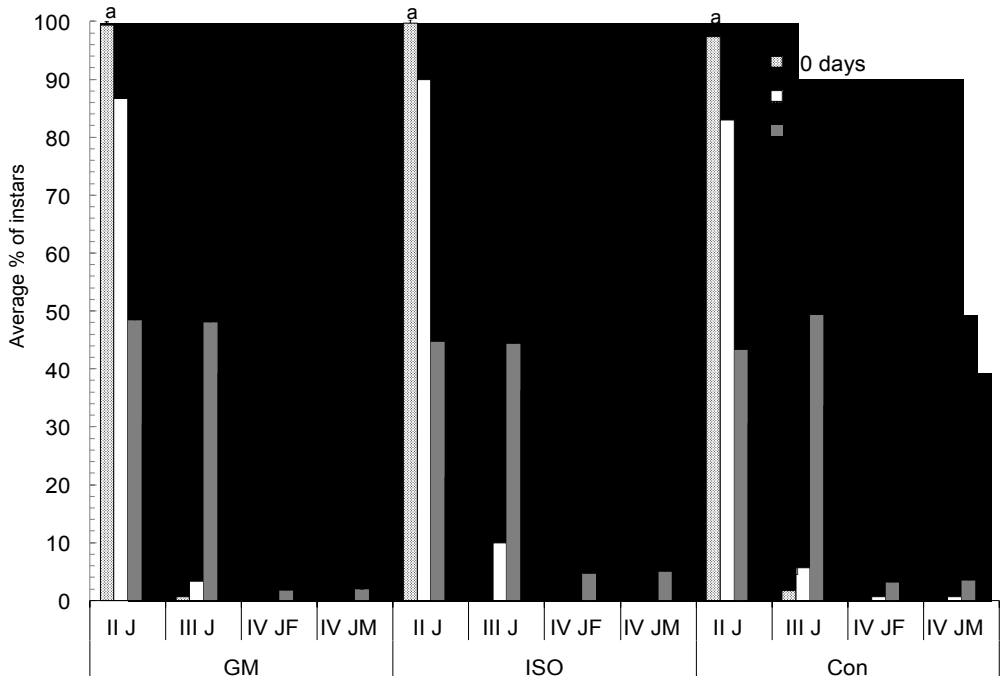

Fig. 3. Average percentage of stages found after inoculation of *G. pallida* [collected in Avezzano, Italy] on potato plants derived Ireland 2013. Results are given as mean (n = 3), and standard deviation. Bars with different letters (a-p) are statistically different (P<0.05) as per post hoc Tukey's t-test for multiple comparisons at the same days. II=stage larvae, III=stage larvae, J=juvenile, F= female, M= Male; GM = GM cisgenic Desiree, ISO = Desirée, Con = Sarpo Mira.

Differences were recorded between the potato varieties, including those between GM and isogenic controls for the average percentage of instars *G. pallida* in 100 days (almost the complete life cycle). For example, while no statistical differences (p>0.05) were recorded among the percentage of second stage larvae after 40 days of exposure across the varieties, the percentage of second stage juveniles at 60 days post-inoculation differed across the varieties (p<0.05).

At 80 days after inoculations, the percentage of juvenile females and males recorded in the GM variety was lower than the average percentage recorded in the isogenic variety but was comparable with the percentage of stages recorded in the conventional variety. By 100 days the percentage of females was significantly higher in the isogenic variety compared to the GM ($p<0.01$) and the conventional ($p<0.05$) varieties. For the number of cysts after 100 days of incubation, no significant differences were recorded across the three potato varieties ($P>0.05$) (Fig. 4).

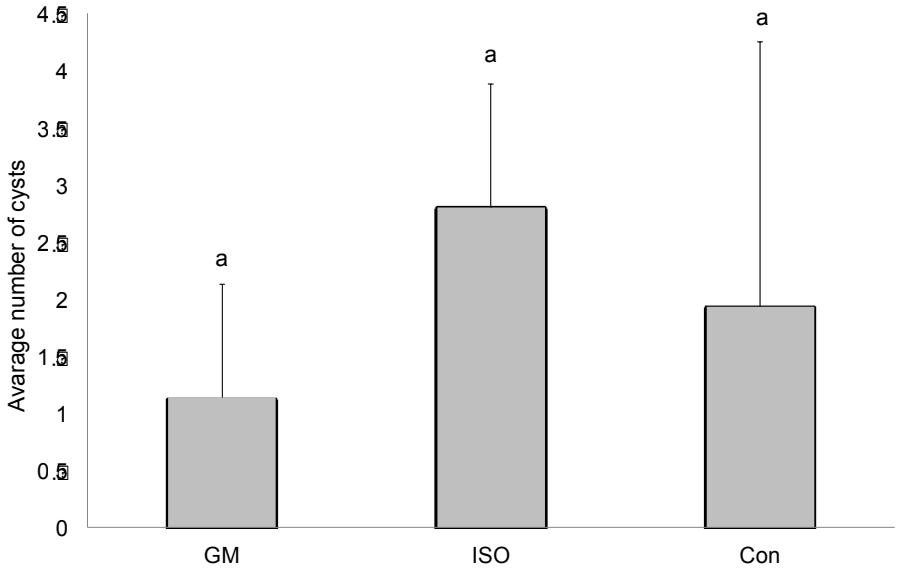

Fig. 4. Average number of *Globodera pallida* cysts taken from 10 grams of soil after 100 days incubation with potato varieties GM = GM cisgenic Desiree, ISO = Desirée and Con = Sarpo Mira. Results are given as mean (n = 3), and standard deviation. Bars with different letters (a) are statistically different at post hoc Tukey's t-test for multiple comparisons.

For viable eggs as an endpoint, the mean number of hatched eggs for female of *G. pallida* was lower in *G. pallida* extracted from GM potato plants ($P<0.05$) compared to the conventional Desiree plants and the Sarpo Mira conventional potato plants (Fig. 5).

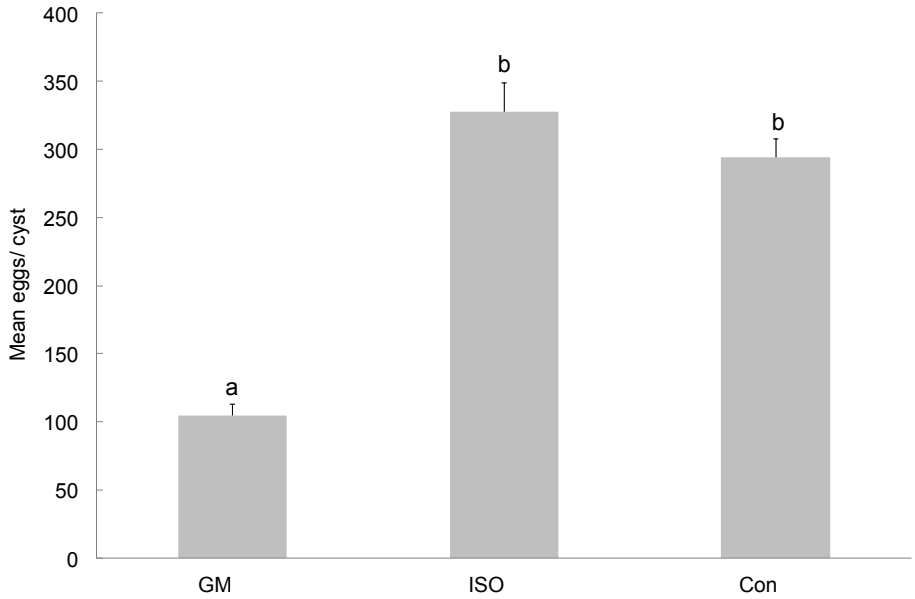

Fig. 5. Mean number of hatched eggs per cyst of *Globodera pallida* after 100 days incubation with potato varieties GM = GM cisgenic Desiree, ISO = Desirée and Con = Sarpo Mira. Results are given as mean (n = 5 x 3 replicates), and standard deviation. Bars with different letters (a,b) are statistically different at post hoc Tukey's t-test for multiple comparisons.

3.2. Meloidogyne sp.

3.2.1 Analysis of specimens collected from field plots

Females of *Meloidogyne sp.* exposed to GM variety showed no physical differences compared to the females collected in the other two varieties for all the nine morphometric parameters considered (Table 6).

Table 6. Morphological characters of mature female of *Meloidogyne sp.* from tubers of 3 potato varieties (Mean ± SD) collected in Netherlands in 2012. GM = GM cisgenic Desiree, ISO = Desirée and Con = Sarpo Mira. Data entries with different letter (a,i) are statistically different at post hoc Tukey's t-test for multiple comparisons within each character.

	GM	ISO	Con
Length (µm)	700.5 ± 50.3a	723.0 ± 55.4a	713.3 ±49.9a
Width (µm)	585.5 ±40.3b	593.6 ±42.2b	575.6 ±32.3b
Stylet length (µm)	17.7 ±5.5c	20.7 ±5.9c	18.7 ±6.0c
Neck length (µm)	245.3 ± 41.1d	248.5 ±35.8d	244.4 ±54.4d
Length of median bulb (µm)	31.7 ±8.8 e	29.7 ±7.9e	30.3 ± 9.0e
Width of median bulb(µm)	35.4 ±5.3f	37.7 ± 5.3f	35.2 ±5.5f
Anus to vulval slit(µm)	24.4 ± 7.3g	27.8 ± 5.3g	21.4 ± 9.6g
Length of vulval slit(µm)	21.1 ± 6.9h	23.3 ±4.5h	22.2. ± 6.6h
Anus to tail terminus area (µm)	13.4 ± 3.2i	14.3 ± 4.0i	14.0 ± 4.5i

The number of juveniles (II J) hatched from egg masses of the *Meloidogyne sp*. collected in The Netherlands (2012) and Ireland (2013) from the plots sown with the three potato varieties are shown in Table 7. With the exception of the number of *Meloidogyne sp*. juveniles recorded after three days co-cultivation with the respective potato varieties, which were lower from the egg masses recorded from the GM variety, no significant differences were noted in the number of juveniles hatching from *M. incognita* egg masses collected from the different potato varieties except. The viability of the hatched individuals showed no statistical differences among the varieties ($p>0.05$) for the mobility of juvenile up to 3 days after hatching (Table 8).

Table 7. Effect of different potato varieties on the average number of juveniles hatching from *M. incognita* egg masses collected from GM = GM cisgenic Desiree, ISO = Desirée and Con = Sarpo Mira varieties. Results are given as mean (n = 3 replicates of 5 egg masses each) with standard deviation. Data entries with different letter are statistically different at post hoc Tukey's t-test for multiple comparisons within each character ($P < 0.05$).

TUBERS (NETHERLANDS) 2012			
	GM	**ISO NS**	**Con**
3 days	55.4 ±5.3b	67.4 ± 5.3a	68.2 ±5.3a
5 days	74.4 ± 4.3a	85.1 ± 2.0a	86.4 ± 4.6a
7 days	88.8±4.6a	89.6 ± 1.9a	88.8±5.7a
SOIL/ROOTS (IRELAND) 2013			
	GM	**ISO**	**Con**
3 days	35.7 ±15.8a	47.5 ± 5.3a	48.7 ±11.3a
5 days	44.4 ± 9.4a	58.1 ± 7.5a	66.5 ± 7.8a
7 days	67.8±5.5a	49.7 ± 11.2a	51.8±7.5a

Table 8. Mobility (expressed as mean of mobile individuals) of second-stage juveniles (J2) of *M. incognita* recorded after 3 days from hatching and a 24 h water rinse (24 WR). GM = GM cisgenic Desiree, ISO = Desirée and Con = Sarpo Mira varieties. Results are given as mean (n = 3 replicates of 5 egg masses each) with standard deviation. Data entries with different letter are statistically different at post hoc Tukey's t-test for multiple comparisons within each character ($P < 0.05$).

TUBERS (THE NETHERLANDS) 2012			
	GM	**ISO**	**Con**
3 Days	16.4 ± 0.8 a	15.1 ± 2.0a	16.0 ± 3.6a
24 WR	15.8± 2.6a	16.3 ± 1.7a	15.8±2.5a
SOIL/ROOTS (IRELAND) 2013			
	GM	**ISO**	**Con**
3 Days	9.8 ± 3.2 a	11.9 ± 3.1a	12.8 ± 4.5a
24 WR	6.7± 2.8a	9.3 ± 3.4a	9.8±3.3a

Second stage juveniles, showed no significant differences in response to the presence of GM (Fig. 6): The body length of J2 individuals was 288.3 µm (SD= ± 54.4) with a maximum recorded in *Meloidogyne sp.* derived from isogenic potato plots (350.0 µm) and the minimum for individuals collected from the GM plots (245.3 µm) from Ireland. However, no statistical differences were recorded for the parameters evaluated ($p<0.05$) in respect to the variety or sampling area (Figure 6).

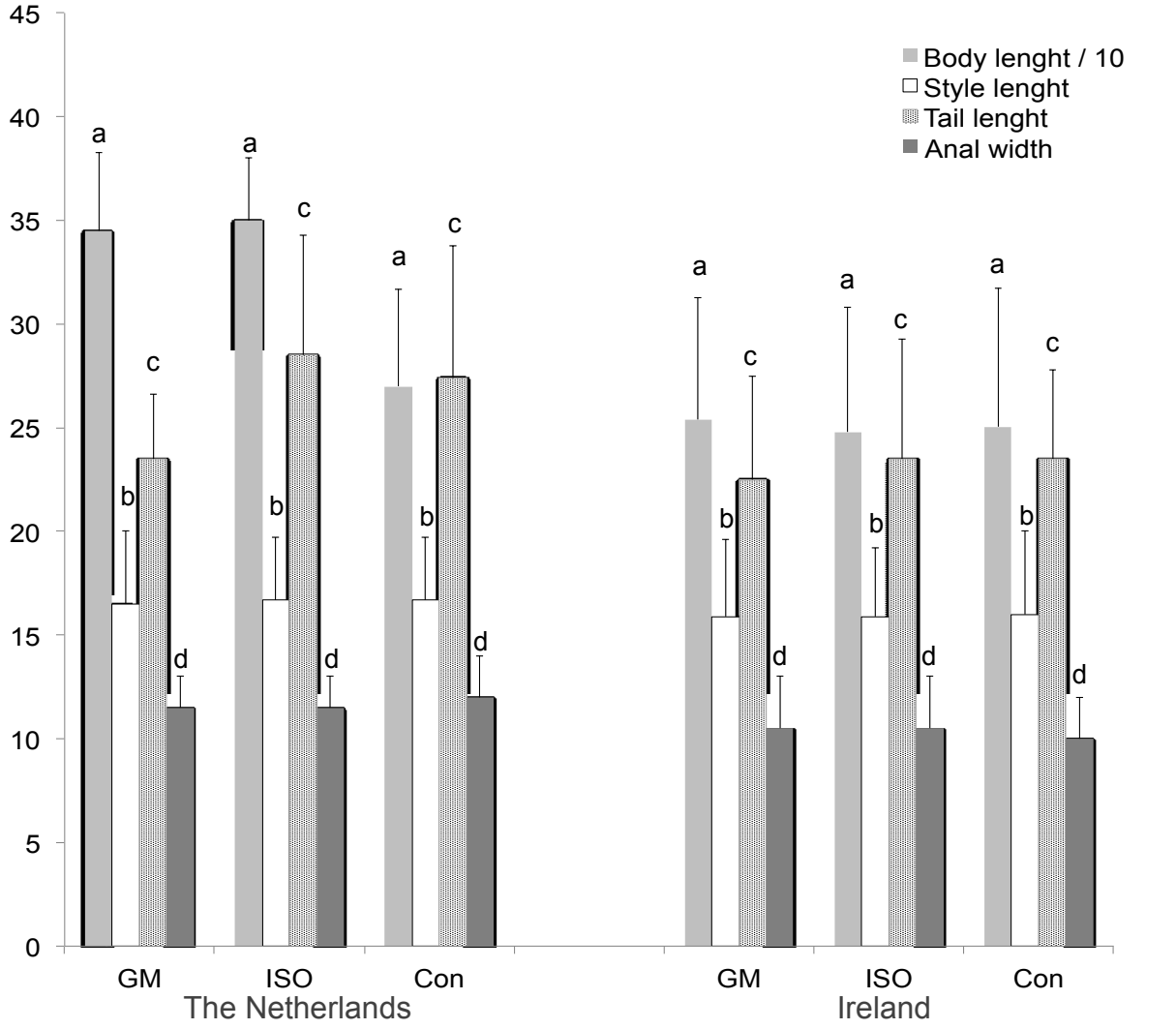

Fig. 6. Average of morphological characters (in µm) of second-stage juveniles (J2) hatched form *Meloidogyne* sp. collected in Netherlands (2012) and Ireland (2013) from the three potato varieties GM = GM cisgenic Desiree, ISO = Desirée and Con = Sarpo Mira. Bars with different letters are statistically different at post hoc Tukey's t-test for multiple comparisons.

3.2.2. Laboratory incubations

Thirty days after inoculation, most individuals of *M. incognita* (collected in Chieri, Ragusa, Italy) found in the roots were egg-laying females, and a proportion of swollen juveniles were still present (Fig. 7). More females were found in the isogenic variety compared to the GM variety ($p<0.05$). While the isogenic variety recorded more egg masses and eggs this difference was not statistically significant ($p>0.05$) (Fig. 6). The mean root galling index (RI) of *M. incognita* estimated for each potato variety after 30 days indicated no significant difference between the potato genotypes: ISO = 3.66 ±0.57, GM = 3.16 ±0.28, Con = 3.33 ±0.57.

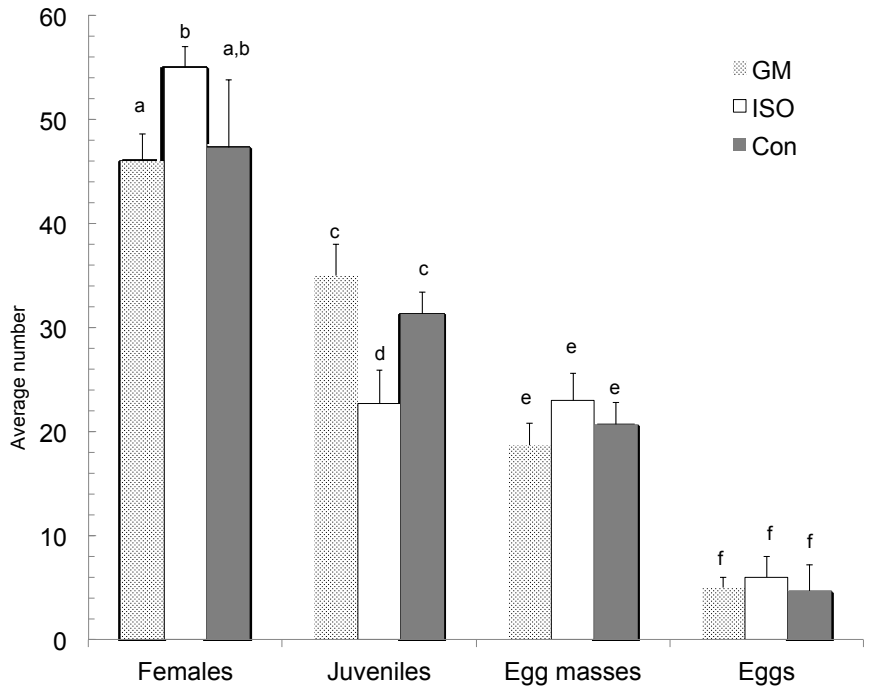

Fig. 7. Average number of females, shallow juveniles, egg masses and eggs produced *M. incognita* on the different potato varieties by 30 days after inoculation. GM = the genetically modified cisgenic Vnt1-gene potato, ISO = is the respective isogenic cultivar Desirée. Con= conventional cultivar. Bars with different letter are statistically different at post hoc Tukey's t-test for multiple comparisons within each character ($P < 0.05$).

3.3 *Aphelenchus avenae* analysis

3.3.1 Analysis of specimens collected from field plots

The morphological assessment of individuals of *A. avenae* taken from the different maize varieties from Sweden and Denmark did not show a statistically significant difference across all the parameters evaluated (Table 10).

Table 10. Morphological characteristic of females of *Aphelenchus avenae* collected in 2013 from GM and non-GM maize varieties cultivated in Denmark and Sweden. Bt = the genetically modified cry1Ab-gene maize, ISO = is the respective isogenic cultivar. Results are given as mean ± standard deviation (n = 5 for 3 replicates). Datasets with alternative letters are significantly different at post hoc Tukey's t-test for multiple comparisons significant (P < 0.05). Note, a= body length/greatest body width, b=body length/distance from the anterior end to base of median oesophageal bulb, c=body length/tail length, stylet length (µm), tail length (µm), V= (distance of vulva from anterior end) x 100/ body length.

	DENMARK		SWEDEN	
	Bt	**ISO**	**Bt**	**ISO**
Length (µm)	607 ± 52.3a	651.0 ± 58.a	615.3 ±49.9a	610.0 ± 55.4a
Width at lip region (µm)	5.5 ±0.6b	5.6 ±0.4b	5.6 ±0.3b	5.6 ±0.4b
Width at anus (µm)	12.4 ± 3.2c	12.0 ± 3.0c	12.4 ± 2.5c	12.3 ± 3.1c
With at mid body (µm)	20.1 ± 3.9d	22.3 ±4.5d	20.2. ± 3.6d	21.2 ±3.5d
a (µm)	28.7 ±3.7 e	31.7 ±4.8e	29.3 ± 3.9e	31.5 ±3.9e
b (µm)	4.4 ±0.7f	4.7 ± 0.8f	4.5 ±0.5f	4.7 ± 0.6f
c (µm)	1.75 ± 0.7g	1.78 ± 0.5g	1.78 ± 0.6g	1.77 ± 0.5g
Stylet length (µm)	16.0 ±.0.5h	15.3 ±0.9h	14.9 ±0.6h	15.2 ±0.7h
Tail length (µm)	20.3 ± 4.1i	20.5 ±5.3i	20.4 ±4.4i	20.5 ±5.8i
V (%)	75.20 ± 4.9l	76.3 ± 4.3l	76.62 ± 5.1l	76.77 ± 4.9l

3.3.2. Laboratory incubations

The potato-based bioassay results were based on the examination of 10 females, 30 days after exposure to the respective potato varieties (Fig. 8). A higher level of reproduction was recorded in the agar control compared to the reproduction of *A. avenae* on the isogenic potato material ($p<0.05$) and the cisgenic GM potatoes ($p<0.01$).

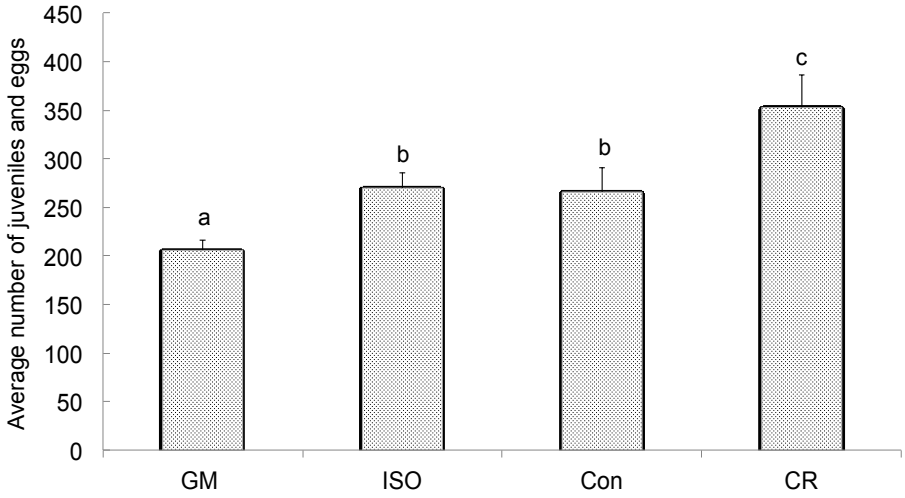

Fig. 8. Reproduction, expressed as the number of juveniles and eggs, of *Aphelenchus avenae* on potato varieties collected from Ireland in 2013, GM: = the genetically modified cisgenic Vnt1-gene potato, ISO = is the respective isogenic cultivar Desirée, Con = conventional cultivar, CR = control. Results are given as mean (n = 3), and standard deviation. Bars with different letter (a, c) are statistically significant different at post hoc Tukey's t-test for multiple comparisons significant ($P < 0.05$).

The percentage of mortality expressed as non-mobile individuals indicated no significant difference between the GM and isogenic potato varieties and similarly, no significant difference was noted between the isogenic and the other conventional potato variety (Fig. 9).

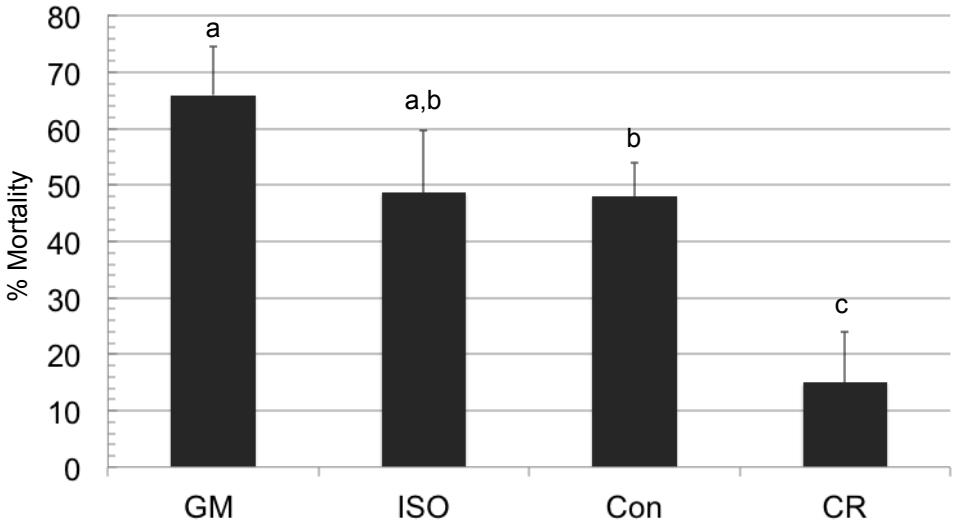

Fig. 9. Average percentage mortality of *Aphelenchus avenae* on potatoes varieties collected in Ireland in 2013, estimated 30 days after inoculation of gravid females GM: = the genetically modified cisgenic Vnt1-gene potato, ISO = is the respective isogenic cultivar Desirée, Con = conventional cultivar, CR = control, Difco PDA + *R. solani* . Results are given as mean (n = 3), and standard deviation. Bars with different letter (a, c) are statistically significant different at post hoc Tukey's t-test for multiple comparisons significant ($P < 0.05$).

All three potato varieties recorded significantly higher mortality than the media control (Fig. 9). A morphological analysis of *A. avenae* females reared on each of the sampled potato varieties indicated no significant differences between GM, isogenic or conventional (Table 8). This was the case across all parameters studied with the data collated comparable to the datasets recorded for *A. avenae* exposed to the GM and non-GM maize material (Table 11).

Table 11. Average morphometric measurements of females of *Aphelenchus avenae* reared in laboratory on different potatoes varieties and feed with *Rhizoctonia solani*. GM: = the genetically modified cisgenic Vnt1-gene potato, ISO = is the respective isogenic cultivar Desirée, Con = conventional cultivar, CR = control Difco PDA + *R. solani* . Results are given as mean ± standard deviation (n = 5 for 3 replicates). Different letter (a, l) are statistically significant different at post hoc Tukey's t-test for multiple comparisons significant (P < 0.05). Comparison was made only among the same morphometric characters. a= body length/greatest body width, b=body length/distance from the anterior end to base of median oesophageal bulb, c=-body length/tail length, stylet length (µm), tail length (µm), V= (distance of vulva from anterior end) x 100/ body length.

	GM	ISO	Con	CR
Length (µm)	600 ± 54.3a	649.0 ± 56.0a	625.3 ±50.0a	650.0 ± 35.4a
Width at lip region (µm)	5.5 ±0.5b	5.6 ±0.5b	5.6 ±0.5b	5.6 ±0.4b
Width at anus (µm)	11.5 ± 3.0c	12.0 ± 3.0c	11.8 ± 2.8	12.3 ± 4.0c
With at mid body (µm)	20.1 ± 4.0d	21.3 ±4.4d	20.7 ± 3.4d	21.0 ±3.0d
a (µm)	29.7 ±3.5e	31.7 ±4.5e	30.3 ± 2.9e	31.5 ±4.0e
b (µm)	4.5 ±0.5f	4.7 ± 0.8f	4.5 ±0.5f	4.6 ± 0.7f
c (µm)	1.75 ± 0.5g	1.80 ± 0.5g	1.79 ± 0.6g	1.78 ± 0.7g
Stylet length (µm)	15.5 ±.0.5h	15.5 ±0.5h	15.5 ±0.5h	15.2 ±0.7h
Tail length (µm)	14.6 ± 1.1i	20.5 ±4.9i	20.4 ±4.4i	21.5 ±4.5i
V (%)	72.00 ± 4.8l	75.6 ± 4.8l	76.00 ± 5.0l	76.50 ± 4.5l

3.4 Summarizing overview of the effects detected with the focal species in this study

Summarizing the results, it becomes evident that the morphometric parameters neither of females or juveniles are affected by the different varieties. However, more juvenile stages are collected in the GM potato variety compared to the non GM potato variety after the same time of exposure, thus GM potato negatively affected the development time of the specimens. *G. pallida* was more susceptible than the other two species because also the vitality of juveniles decreased in GM plots.

Table 12. Parameters analysed for the selected focal species and differences among the varieties. GM = Genetically modified variety, ISO = respective isogenic variety, Con = conventional variety.

	Endpoint	**Differences between cultivars**	**Differences between GM and non GM**	**Comment**
Globodera pallida	Female morphometry	No	No	…
	Juvenile morphometry	No	No	
	Fertility	No	No	
	Vitality of offspring	Decreased in Con	Decreased in GM	
	Life cycle in 100 days (% of instars)	Longer development time the first 60 days of juveniles in GM	Longer development time of juveniles in GM	Significant only for certain stage at certain period
	Number of female after 100 days exposure	No	No	
	Vitality of offspring after 1 generation	No	Lower in GM	
Meloidogyne sp.	Female morphometry	No	No	
	Juvenile morphometry	No	No	
	Vitality of offspring	No	No	
	Time of development	Shorter in ISO	Shorter in ISO	As stages developed after 30 days exposure
	Fertility of female after 30 days exposure	No	No	
A. avenae	Female morphometry	No	No	
	Fertility	No	Lower in GM	
	Mortality	Increased GM vs. conventional	No	

4. DISCUSSION

The objective of this study was to analyse the effect of GM plant material on three selected focal nematode species and develop a laboratory test systems that could be used for testing of the impact of GM crops (here mainly potatoes and maize) from different biogeographical regions in Europe for NTO risk assessment, as also considered in the EFSA ERA guidance document (EFSA, 2010).

To develop the standardized laboratory test system, specimens collected from AMIGA field sites were directly analysed for their morphometric parameters and fecundity (only for the plant feeding). In addition, microcosm feeding experiments were carried out with plantlet of GM and non-GM potatoes from Ireland and The Netherlands.

Considering current literature data on culture techniques for the three focal nematode species (e.g. Greco et al, 2005; Barker et al., 1985; Li et al., 2005; Wesemael et al., 2011; Niere and Unger, 2012; d'Errico et al., 2014), appropriate laboratory conditions and a reproducible experimental design were developed.

Specifically, the AMIGA task 4.3, to which this deliverable contributes, is concerned with the development of assays to establish biological responses, e.g., growth, fertility, vitality of off-springs, by nematodes to GM plants, as potential stressors for the soil fauna. Although it is a serious economic pest of commercial potato production, *G. pallida* was included in this study because of its intimate association with the root system of its potato host. Regrettably, the datasets presented here were based on samples only received from Ireland, due to vandalism and loss of study material from the Dutch AMIGA site. However, the analyses completed for Ireland indicates that exposure to GM cisgenic potato material induced a reduction in the mean number of hatched eggs/cyst; a phenomenon that, if confirmed with additional analyses, should be considered as an agronomic advantage. As another important result, an examination of the life stages of *G. pallida* identified significant variation across the physiological stages relative to each potato variety tested. It is therefore clear that further in depth analysis is required to determine the reproducibility of the results seen here for the potential physiological response of GM potato to *G. pallida*.

Comparable to *G. pallida, M. incognita* is a serious plant parasite of potato production systems. As for the impact of exposure to GM potato; although some

influence was noted in the number of females and juveniles, the presence of GM potatoes did not negatively affect the vitality or the life cycle of *Meloidogyne* sp.

In contrast to both *G. pallida* and *M. incognita*, the fungal feeding *A. avenae* is non-pathogenic on potato and for this study was extracted from maize soil samples. No significant differences were recorded for morphometric parameters due to the genetic modification (expression of the Cry1Ab protein in plant material). While *A. avenae* is partially affected by exposure to GM potatoes, it could not be determined if this was a direct or indirect effect, since the rate of colonisation by the substrate fungus, *R. solani* on each of the three potato varieties was not quantified here. However, it was confirmed that exposure to GM potato did not affect significantly the percentage mortality of *A. avenae*.

In conclusion, this study highlights the potential of comprehensive morphological analyses of select nematode species as a means to analyse the NTO effects of a GM variety. Considering the outstanding importance of nematodes for soil health and ecosystem functions, the methodological approach and the preliminary data reported in this deliverable call for additional and complementary investigations for future case-by-case novel GMO considered for the cultivation in Europe.

5. REFERENCES

Baermann G. (1917) Eine einfache methode zue affindung von *Ankylostomum* (Nematoden) larven in Erdproben. *Geneeskundig Tijdschrift voor Nederlandsch-Indië*, **57**, 131 – 137.

Barker K.K., Carter C.C., Sasser J.N. (1985). An advanced treatise on *Meloidogyne*. Volume II: Methodology. Eds. Barker K.K., Carter C.C., Sasser J.N. A cooperative publication of the Department of Plant Pathology and US States Agency for International Development. North Caroline. pp. 223.

Barker K.R., Darling H.M. (1965) Reproduction of *Aphelenchus avenae* on plant tissues in culture. *Nematologica*, **11**, 162 – 166.

Bongers T. (1990) The maturity index: an ecological measure of environmental disturbance based on nematode species composition. *Oecologia* **83**, 14 – 19.

Bongers T. and Ferris H. (1999) Nematode community structure as a bioindicator in environmental monitoring. *Tree*, **14**, 224 – 228.

Bonkowski M., Villenave C., Griffiths B. (2009). Rhizosphere fauna: the functional and structural diversity of intimate interactions of soil fauna with plant roots. *Plant Soil*, **321**, 213 – 33.

Buecher EJ., Yarwood EA., Hansen EL., (1974). A screen to separate young larvae of *Aphelenchus avenae* from adults and eggs. *Nematologica*, **194**, 565 – 566.

Byrd D.W., Ferris H., Nusbaum C.J. (1972) A method for estimating numbers of eggs of *Meloidogyne* spp. in Soil. *Journal of Nematology*, **4**, 266 – 269.

Byrd D.W., Kirkpatrick Jr T., Barker K.R. (1983) An improved technique for clearing and staining plant tissues for detection of nematodes. *Journal of Nematology,* **15**, 142 – 143.

Calder WA (1984) Size, function, and life history. Harvard University Press, Cambridge, MA. UK. pp 328.

Celis C., Scurrah M., Cowgill S., Chumbiauca S., Green J., Franco J., Main G., Kiezebrink D., Visser R.G.F., Atkinson H.J. (2004) Environmental biosafety and transgenic potato in a centre of diversity for this crop. *Nature* **432**, 222 – 225.

Colombo A., Cataldi S., Marano G., Genna G. (2011) Control of the root-knot nematode, *Meloidogyne incognita,* in organic protected crops in Sicily. *Redia*, **94**, 127 – 131.

Colyer P.D., Kirkpatrick T.L., Caldwell W.D., Vernon P.R. (2008) Root-knot nematode reproduction and root galling severity on related conventional and transgenic cotton cultivars. *Journal of Cotton Science*, **4**, 232 – 236.

Coolen W.A. (1979) Methods for the extraction of *Meloidogyne* spp. and other nematodes form roots and soil. In Root-knot Nematodes (*Meloidogyne* species) Systematics, Biology and Control (eds Lamberti F & Taylor CE), pp. 317–329. Academic Press, London & New York. pp. 215.

Cowgill S.E., Danks C., Atkinson H.J. (2004) Multitrophic interactions involving genetically modified potatoes, nontarget aphids, natural enemies and hyperparasitoids. *Molecular Ecology*, **13**(3), 639 – 647.

Cunha A. (2012)**.** Innate immunity: Pathogen and xenobiotic sensing - back to basics. *Nature reviews. Immunology* **12**, 400.

d'Errico G., Crescenzi A., Landi S., (2014) First Report of the Southern Root-Knot Nematode *Meloidogyne incognita* on the invasive weed *Araujia sericifera* in Italy. *Plant Disease*, **98**, 1593.

Di Vito M., Greco N., Carputo D., Frusciante L. (2003) Response of wild and cultivated potato clones to Italian populations of root-knot nematodes *Meloidogyne* spp. *Nematropica,* **33**, 65 – 72.

EFSA (European Food Safety Authority) Panel on Genetically Modified Organisms (GMO) (2010). Guidance on the environmental risk assessment of genetically modified plants. *EFSA Journal* **8** (11): 1879, 111p.

Fiscus D.A., Neher D.A. (2002) Distinguishing nematode genera based on relative sensitivity to physical and chemical disturbances. *Ecol. Appl*., **12**, 565 – 575.

Fisher J.M., Davies K.A. (1990) On size and moulting of *Aphelenchus Avenae* (Nematoda: Aphelenchida). *International Journal of Parasitology*, **20**, 175 – 182.

Fonderie P., Steel H., Moens T., Bert W. (2013) Experimental induction of intraspecific morphometric variability in a single population of *Halicephalobus* cf. *gingivalis* may surpass total interspecific variability. Nematology, 15, 529 – 544.

Fontanetti C.S., Nogarol L.R., de Souza R.B., Perez D.G., Maziviero G.T. (2011) Bioindicators and biomarkers in the assessment of soil toxicity. In *Soil Contamination*, pp. 143 – 168. Ed S. Pascucci, InTech, ISBN: 978-953-307-647-8.

Giacometti R., d'Errico G., d'Errico F.P. (2010) *In vitro* Nematocidal activity of the experimental formulation Tequil against *Meloidogyne incognita* and *Heterodera daverti*. *Nematropica*, **40**, 263 – 268.

Greco N., Brandonisio A., De Cosmis P. (2007a) Pathotypes and heterogeneity of Italian populations of *Globodera rostochiensis* and *G. pallida*. *Nematologia Mediterranea,* **35**, 137 – 142.

Greco N., Carputo D., Frusciante L., Russo G., Brandonisio A. (2005) Screening of clones of *Solanum* spp. for resistance to potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*. *Nematologia Mediterrranea,* **33**, 163 – 170.

Greco N., Di Vito M., Parisi B., Ranalli P., Brandonisio A., Catalano F. (2007b) Resistance of new Italian potato breeding clones to cyst and root-knot nematodes. *Nematologia Mediterranea,* **35**, 227 – 235.

Greco N., Frusciante L., Carputo D., Della Rocca F., Brandonisio A. (2002) Resistance to potato cyst nematode (*Globodera rostochiensis*) of tetraploid potato clones with different genetic backgrounds. *Nematologia Mediterranea*, **30**, 111 – 114.

Griffiths B.S., Caul S., Thompson J., Birch A.N.E., Scrimgeour C., Andersen M.N., Cortet J., Messéan A., Sausse C., Lacroix B., Krogh P.H. (2005) A comparison of soil microbial community structure, protozoa and nematodes in field plots of conventional and genetically modified maize expressing the *Bacillus thuringiensis* CryIAb toxin. *Plant Soil*, **275**, 135 – 146.

Griffiths B.S., Caul S., Thompson J., Birch A.N.E., Scrimgeour C., Cortet J., Foggo A., Hackett C.A., Krogh P.H. (2006) Soil microbial and faunal community responses to *Bt* maize and insecticide in two soils. *Journal of Environmental Quality*, **35**(3), 734 – 741.

Griffiths B.S., Geoghegan I.E., Robertson W.M. (2000). Testing genetically engineered potato, producing the lectins GNA and Con A, on non-target soil organisms and processes. *Journal of Applied Ecology* **37**, 159–170.

Hansene L., Buechere J., Yarwoode A. (1972) Sex differentiation of *Aphelenchus avenue* in axenic culture. *Nematologica*, **18**, 253 – 260.

Hechlerh C. (1962) The development *of Aphelenchus avenae* Bastian, 1865. *Proceedings of the Helminthological Society of Washington*, **29***,*162 – 167.

Hilbeck, A.; Arpaia, S.; Birch, A. N. E.; Chen, Y.; Fontes, E. M. G.; Lang, A.; Le Thi Thu Hong; Lovei, G. L.; Manachini, B.; Nguyen Thi Thu Cuc; Nguyen Van Huynh; Nguyen Van Tuat; Pham Van Lam; Pham Van Toan; Pires, C. S. S.; Sujii, E. R.; Trac Khu'o'ng Lai; Underwood, E.; Wheatley, R. E.; Wilson, L. J.; Zwahlen, C.; Andow, D. A. (2008) Non-target and biological diversity risk assessment. In Environmental risk assessment of genetically modified organisms: challenges and opportunities with Bt cotton in Vietnam, *Volume 4: Challenges and Opportunities with Bt Cotton in Vietnam*. 115-137. Edited by Andow DA, Hilbeck A, Nguyen Van Tuat. Wallingford, UK, CABI Publishing.

Höss S., Menze R., Gessle F., Nguyen H.T., Jehle J.A., Traunspurg W. (2013) Effects of insecticidal crystal proteins (Cry proteins) produced by genetically modified maize (Bt maize) on the nematode *Caenorhabditis elegans. Environmental Pollution*, **178**, 147 – 151.

Höss S., Nguyen H.T., Menzel R., Pagel-Wieder S., Miethling-Graf R., Tebbe C.C., Jehl E.J.A., Traunspurger W. (2011) Assessing the risk posed to free-living soil nematodes by a genetically modified maize expressing the insecticidal Cry3Bb1 protein. *Science of the Total Environment*, **409**, 2674 – 2684.

Hunt D.J., Handoo Z.A. (2009) Taxonomy, identification and principal species. In *Root-knot nematodes*, pp. 55 – 97. Eds R.N. Perry, M. Moens. Wallingford, UK, CABI Publishing.

Ishibashi N., Takayama S and Kondo E. (2005) Propagation and feeding behavior of the mycetophagous nematode, *Aphelenchus avenae*, on four species of soil fungi. Japanese Journal of Nematology **35**:1 – 6.

Jepson S.B. (1987) Identification of root-knot nematodes (*Meloidogyne* species). Wallingford, UK, CABI Publishing, 265 pp.

Karuri H.W., Amata R., Amugune N., Waturu C. (2013) Reproduction of root knot nematode (*Meloidogyne incognita*) on Bt cotton expressing Cry1Ac and Cry2Ab2 protein. *Journal of Applied Biosciences*, **69**, 5487 – 5495.

Kline P.J. (1976) Morphometric variation in *Aphelenchus avenae* Bastian, 1865 with varied nutrition and time. *Nematologica*, **22**, 94 – 102.

Korthals G.W., Popovici I., Iliev I., Lexmond T.M. (1998) Influence of perennial ryegrass on a copper and zinc affected terrestrial nematode community. *Applied Soil Ecology*, **10**, 73 – 85.

Lagerlöf J., Insunza V. Lundegårdh B., Rämert B. (2011) Interaction between a fungal plant disease, fungivorous nematodes and compost suppressiveness, *Plant Soil Science*, **61**, 372 – 377.

Li F., Neher D., Darby B.J., Weicht T.R. (2005) Observed differences in life history characteristics of nematodes *Aphelenchus* and *Acrobeloides* upon exposure to Copper and Benzo(a)pyrene. *Ecotoxicology* **14**, 419 – 429.

Liu Q.L., Williamson V.M. (2006) Host-Specific Pathogenicity and Genome Differences between Inbred Strains of *Meloidogyne hapla. Journal of Nematology*, **38**, 158 – 164.

Manachini B., Landi S., Agosti M. (2003) Attacchi di *Meloidogyne hapla* Chitwood su *Lactuca sativa* L. e *Spinacia oleracea* L. nell'Italia Settentrionale. *Informatore Fitopatologico*, **4**, 53 – 54.

Manachini B., Landi S., Fiore M. C., Festa M., Arpaia S. (2004) First investigations on the effects of transgenic *Brassica napus* L. on the trophic structure of the nematofauna. *IOBC/wprs Bulletin* **27**, 103 – 108.

Manachini B., Lozzia G.C. (2002) First investigations into the effects of *Bt* corn crop on Nematofauna. *Bollettino di Zoologia Agraria e Bachicoltura*, **34**, 85 – 96.

Melo J. A., Ruvkun G. (2012). Inactivation of conserved *C. elegans* genes engages pathogen- and xenobiotic-associated defenses. *Cell*, **149**, 452 – 466

Nascimento I.A., Pereira S.A., Leite M.B.N.L. (2008) Biomarcadores como instrumento spreventivos de poluição. In *Ecotoxicologia: Princípios e Aplicações*, pp. 413 – 432. Eds P.A. Zagatto, E. Betoletti. RiMa, ISBN 9788576560906, São Carlos.

Ngo Xuan Quang, Nguyen Ngoc Chau, Vanreusel Ann (2014) Nematode morphometry and biomass patterns in relation to community characteristics and environmental variables in the Mekong Delta, Vietnam. *Raffles Bulletin of Zoology* **62**: 501 – 512.

Niere B., Unger J.-G. (2012) Extensive literature search on the PCN and PCN-host interaction. Supporting Publications of European Food Safety Authority (EFSA) EN-272, 224 pp.

O'Callaghana M., Emily M. Gerarda E.M., Bellb N.L., Waiparac N.W., Aaldersb L.T., Bairda D.B., Conner A.J. (2008) Microbial and nematode communities associated with potatoes genetically modified to express the antimicrobial peptide magainin and unmodified potato cultivars. *Soil Biology & Biochemistry*, **40**, 1446 – 1459.

Perez D.G., Fontanetti C.S. (2011) Hemocitical responses to environmental stress in invertebrates: a review. *Environmental Monitoring and Assessment,* **177**, 437 – 447.

Pey B., Nahmani J., Auclerc A., Capowiez Y., Cluzeau D., Cortet J., Decaëns T., Deharveng L., Dubs F., Joimel S., Briard C., Grumiaux F., Laporte M.A., Pasquet A., Pelosi C., Pernin C., Ponge J.F., Salmon S., Santorufo L., Hedde M. (2014). Current use of future needs for soil invertebrate functional traits in community ecology. *Basic and Applied Ecology* **15**, 194-206.

Pham Van Toan, Hoang Ngoc Binh, Anyango, B., Zwahlen, C., Manachini, B., Andow, D. A., Wheatley, R. E. (2008). Potential effects of transgenic cotton on soil ecosystem processes in Vietnam. In Environmental risk assessment of genetically modified organisms: challenges and opportunities with Bt cotton in Vietnam, *Volume 4: Challenges and Opportunities with Bt Cotton in Vietnam*. 258 – 273. Edited by Andow DA, Hilbeck A, Nguyen Van Tuat. Wallingford, UK, CABI Publishing.

Pillai J.K., Taylor D.P. (1976) Influence of fungi on host preference, host suitability, and morphometrics of five mycophagous nematodes. *Nematologica*, **13**, 529 – 540.

Russo G., Greco N., (2006) Response of Italian populations of *Globodera rostochiensis* and *G. pallida* to hatching agents. *Nematologia Mediterranea,* **34**, 93 – 94.

Russo G., Greco N., d'Errico F.P., Brandonisio A. (2007) Impact of the root-knot nematode, *Meloidogyne incognita*, on potato during two different growing seasons. *Nematologia Mediterranea*, **35**, 29 – 34.

Sasser J.N., Carter C.C. (1985). An advanced treatise on *Meloidogyne*. Volume I. Eds. Carter C.C., Sasser J.N. A cooperative publication of the Department of Plant Pathology and US States Agency for International Development. Raleigh: North Carolina State University Graphics. North Caroline. pp. 235.

Seinhorst J.W., Den Ouden H. (1966). An improvement of Bijloo's method for determining the egg content of *Heterodera* cysts. *Nematologica*, **12**, 170-171.

Sheila Shahab (2014). Effects of sowing dates on variability of perineal-pattern of *Meloidogyne incognita*. International *Journal of Science and Research,* **3**, 269 – 271.

Souza T.S., Angelis D.F., Fontanetti C.S. (2011) Histological and histochemical analysis of the fat body of *Rhinocricus padbergi* (Diplopoda) exposed to contaminated industrial soil. *Water, Air & Soil Pollution.* DOI 10.1007/s11270-011-0786-9.

Souza T.S., Fontanetti C.S. (2011) Morphological biomarkers in the *Rhinocricus padbergi* midgut exposed to contaminated soil. *Ecotoxicology and Environmental Safety*, **74**, 10 – 18.

Tita G., Vincx M., Desrosier G. (1999) Size spectra, body width and morphotypes of intertidal nematodes: an ecological interpretation. *Journal of Marine Biological Association* UK **79**, 1007 – 1015.

Townshend J.L., Blackith R.E. (1995) Fungal diet and the morphometric relationships in *Aphelenchus avenae*. *Nematologica*, **21**, 19 – 25.

Vanaverbeke J., Soetaert K.E.R., Vincx M. (2004) Changes in morphometric characteristics of nematode communities during a spring phytoplankton bloom deposition. *Marine Ecology Progress Series*, **273**, 139 – 146.

Vauramo S., Pasonen H.L., Pappinen A., Setala H. (2006). Decomposition of leaf litter from chitinase transgenic silver birch (*Betula pendula*) and effects on decomposer populations in a field trial. *Applied Soil Ecology* **32**, 338–349.

Wesemael W.M.L., Viaene N., Moens M. (2011) Root-knot nematodes (*Meloidogyne* spp.) in Europe. *Nematology*, **1**, 3 – 16.

Wyss U., Grundler F.M.W., Munch A. (1992) The parasitic behaviour of second stage juveniles of *Meloidogyne incognita* in root of *Arabidopsis thaliana. Nematologica*, **38**, 98 – 111.