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Collaborative Project

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

Work Package	**4**
WP Leader	**Christoph C. Tebbe, Thünen Institute of Biodiversity**
Deliverable 4.5	**Report on induced stress by GM plants on nematode focal species**
Task 4.3	**Impact on soil nematodes: Baselines of their diversity and physiological response to GM plants as potential stressors** **Task leader: Ewen Mullins, Teagasc**
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ABSTRACT

The goal of this study was to determine as an indicator for stress caused by genetically modified (GM) plants on soil-derived nematode populations the suitability of a novel approach based on the analysis of specific molecular stress markers (i.e., the abundance of heat shock proteins, HSPs). Employing GM maize and GM potato as the AMIGA model species, the study involved an examination of the abundance of HSPs in the whole nematode community collected from soils cultivated with (1) the GM cisgenic potato line (Desiree-vnt1), its isogenic equivalent (var. Desiree) and an additional conventional variety (var. Sarpo Mira) and (2), the maize MON810, expressing the insecticidal Cry1Ab protein (Bt-maize) and a relevant near-isogenic maize variety. For maize, soil samples were also included from the adjacent zones of the field in which no crop was cultivated. To detect specific HSPs (HSP27, HSP60, HSP70, HSP90), commercially synthesised monoclonal mammalian antibodies were applied, with HSP activity monitored in total nematode community as well as crop-specific nematode species, including *Globodera pallida* for potato, and *Rhabditis sp.* for maize. Based on the analysis conducted, no significant differences were noted in the amounts of HSP27, HSP60 and HSP70 across either the potato or maize genotypes. In the case of HSP90, the induction of this protein was at least six-fold more sensitive in the nematode community collected from the GM varieties of both maize and potato. Based on these results it is therefore suggested that HSP90 may potentially be a suitable biomarker to assess stress responses of whole soil nematode communities and, also, of the individual species *G. pallida*. In contrast, no differences in HSP90 expression were recorded with *Rhabditis* sp. In regard to maize, it was noted that the amount of HSP70 was lower in *Rhabditis* sp. extracted from soil cultivated with the transgenic maize variety compared to samples taken from near-isogenic variety. The reasons for these differences are unclear and further investigations are necessary to assess if they depend on the presence of the GM maize. The data cannot yet conclusively answer the question whether this stress response, in fact, is linked to the expression of the GM protein Cry1Ab itself or to other indirect effects. In conclusion, this preliminary study indicates the potential for utilizing stress proteins (particularly HSP90) to study effects of GM plants on soil nematode communities. The actual significance of the data presented here in regard to the particular risk assessment of GM and its variability across temporal (seasonal), and bio-geographical scales remains to be determined in more extended studies.

INTRODUCITON

Stress proteins (generally known as ‘heat shock proteins’, HSPs) are induced by multiple environmental stresses including exposure to trace metals, organic pollutants, pesticides, changes in temperature or osmolarity, hypoxia/anoxia, and exposure to ultraviolet radiation (Ritossa, 1962; 1963; Gupta et al., 2005a,b; Shivaiah Shashikumar & Rajini 2010). Indeed, almost any change in the cellular environment will induce the synthesis of HSPs (Csermely et al., 1998). Some HSPs are constitutively expressed while some are induced relative to specific stresses (Morimoto, 1998; Samali & Orrenius, 1998). Named according to their molecular weight (e.g. HSP100, HSP90, HSP70, HSP60, HSP40, and the smaller HSPs such

as HSP 27 and HSP10) HSPs are functionally related proteins involved in the folding and unfolding of proteins.

The value of HSPs as suitable biomarkers in ecotoxicology has already been described in regard to different species and/or stresses (Köhler et al., 1996; Bierkens, 2000; Nadeau et al., 2001, Skantar & Carta, 2004; Malaspina et al., 2006; Siamba et al. 2012). In particular for nematodes it has been shown that after a specific stress condition HSP 90 increased relative to the respective control (Birniby et al, 2000, Devaney et al., 2005). To our knowledge, only one study has yet explored the potential of utilizing HSPs for the identification of stress, as induced by genetically modified (GM) plants (Bondzio et al. 2013). In this report, the *in vitro* treatment of porcine cells with the Cry1Ab protein, as produced by maize MON810, resulted in an up-regulation of HSP70, which, however, was not matched with any detectable increase in cell toxicity.

The objective of this study was to analyze the effect of transgenic GM maize MON810 and a cisgenic GM potato on nematodes as non-target organisms (NTO). Due to the novelty of the approach proposed and explored in this study, the impact of the GM crop cultivations was analyzed for several HSPs, including HSP27, HSP60, HSP70, and HSP90, respectively. The selection of HSP60 and HSP70 as biomarkers was based on observations showing that the sensitivity of HSP60 to induction in certain organisms (e.g., the nematode *Plectus acuminatus* and the mussel *Mytilus edulis*) is significantly greater than the use of other comparable parameters (endpoints) such as quantifying adverse effects on biomass or reproduction (Kammenga et al., 1998; Hamer et al., 2004, Guo et al., 2013). Indeed, differences in the production of HSP60 would not only indicate a differential stress response but also a different ability for defending pathogen attacks (Vabulas et al., 2001).

HSP70 has already been identified and studied in the free-living nematode, *Caenorhabditis elegans*, (Heschl & Baillie, 1989) and it has also been characterised from other nematode species such as *Brugia malayi* (Selkirk et al., 1989) and *Parastrongyloides trichosuri* (Newton-Howes et al., 2006), respectively. HSP90 is an abundantly expressed molecular protection protein, called chaperone, which modulates the maturation and function of many key regulatory proteins within eukaryotic cells (Csermely et al., 1998). As a result of its unique biochemical function, although possessing no intrinsic signalling activity of its own, HSP90 plays a central role in regulating both normal developmental processes (Queitsch et al., 2002) and neoplastic transformation (Bagatell et al., 2001).

In this AMIGA deliverable baseline physiological responses of soil nematodes to GM-specific impacts were analyzed by employing antibody-based quantifications of the different HSPs mentioned above. The selected GM variants represent transgenic and cis-genic GM plants and include a genetic modification providing insect resistance for the maize and fungal resistance against the Oomycete and plant pathogen *Phytophtora infestans* (see Materials and Methods for more details) for the potato. The impact on the whole soil inhabiting nematode community as detected from soil samples collected from different cultivars of potato and maize, including GM variants, was investigated. Furthermore, additional analyses were conducted to gauge the impact of the GM on individual crop-specific nematode species including *G. pallida* (potato) and *Rhabditis* sp. (maize).

MATERIALS AND METHODS

To ensure nematode viability, soil samples (50g) were sent by express deliveries from AMIGA field locations across Europe directly to the laboratory of the University of Palermo and then, immediately, nematodes were extracted using the Bearmann funnel method (Bearmann, 1917). From each extracted sample, live nematodes were randomly picked and collected in a tube for HSP detection, or immediately frozen at -80°C. A series of preliminary assays were conducted to determine the best protocol for extraction and protein purification, as a requirement to perform the subsequent HSP analyses.

Maize-derived nematode community extraction and collection

Soil samples were received in August 2013, from AMIGA maize field sites located in Slovakia and Spain. The samples included the GM maize, its near-isogenic variety (ISO), and, in addition, also bulk soil samples originating from inter-field (IF) zones. Furthermore, as a positive control for stress treatment, a total of 250 nematodes extracted from soil samples of isogenic maize fields from both localities were treated under laboratory conditions with a biological nematocide (NemGuard, CBC Europe) at 10% of the recommended field dose. In general, nematodes were first extracted according to Bearmann (1917), with three sub-replicates, representing a total of 150 g of soil and then pooled to simplify the random nematode selection process and to ensure the correct number of individuals (n = 250) were obtained for each treatment. Three replicates for each type of variety were used for the HSPs analysis.

Potato-derived nematode community extraction and collection

Soil samples were collected at both AMIGA potato field sites in Ireland and The Netherlands, respectively. Only samples from 2013 were analyzed. The samples included the GM cisgenic potato line (GM), the respective near 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 (http://www.wageningenur.nl/en/Expertise-Services/Research-Institutes/plant-research-international/DuRPh.htm). It contains the cisgene *Rpi-vnt1*, which confers resistance to the potato late blight disease pathogen *P. infestans*. For each of the three potato genotypes, two treatments were applied *in situ*, which included current fungicide practice (CP) and a no spray control (NS). After Bearman (1917) extraction (see above), two sub-replicates representing a total of 100 g of soil (wet weight) were pooled to simplify nematode selection and to ensure the exact amount of nematodes (n = 250) for each experimental treatment. A total of three replicates for each potato variety and treatment (CP and NS) were included in the analyses for stress response measurements.

Globodera pallida (Stone) Behrens

Preliminary assessments were made on potato tubers and roots received from The Netherlands in 2012 in order to develop an optimal *G. pallida* extraction and adjust handling methods. *G. pallida* cysts, visible to the naked eye as pin-head white, yellow or brown coloured eggs, are generally more frequent on roots than on tubers (Greco et al., 2007). The analyses of these nematodes was performed on potato tubers and roots, shipped in sterile water, from both

potato AMIGA sites, and considered the following treatments: GM, ISO and Con (for abbreviations see above). For the Dutch samples, soils from field plots included those treated with fungicides (CP) or untreated (NS). For the Irish samples, soils from only unsprayed (NS) plots were analyzed. However, for the root samples, CP and NS were analyzed. Since the tubers from Ireland of 2013 showed only low infestation levels, the analysis was done using cysts collected from the roots sampled in both the CP and NS plots. In all cases, five mature *G. pallida* females were taken from each replicate with three replicates analysed per variety per treatment.

Rhabditis sp.

HSP production studies with *Rhabditis* sp. were conducted with soils of near-isogenic maize and GM maize collected from Swedish field site in the year 2012. As described above, the nematodes were extracted using the Bearman funnel method (1917). *Rhabditis* sp. was visually identified using inverted phase fluorescence microscope (Leitz Fluovert-FS) at 60x or 100x. Only alive individuals were manually selected and picked out for HSPs analysis . Three replicates per variety (GM and ISO) were performed.

Preparation of nematode homogenates

Extracted nematodes were centrifuged at 300 x *g* for 5 min and resulting pellets were sonicated in 0.5 ml of TNES lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 1% NP-40, 2 µg/µl antipain, leupeptin and bestatin, 1 µg/µl aprotinin and pepstatin, 1 mM benzamidine, and 0.1 mM AEBSF, pH 7.4) for a total of 60 min. Thereafter, samples were centrifuged at 15,000 x *g* for 30 min at 4°C, and subsequently the supernatants were collected and dialysed against 50 mM Tris-HCl (pH 7.5) overnight. The resulting protein content was estimated using a Qubit® 2.0 Fluorometer (Invitrogen by Life Technologies, Singapore).

Detection and quantification of HSP proteins

Preliminary studies were carried out to ascertain the specificity of mammalian antibodies (primary and secondary, Sigma Aldrich, St. Louis, MO USA) with the specific HSP proteins with molecular weights of 27 KDa, 60 KDa, 70 KDa and 90KDa, respectively. Antibody selection was based on information collected from a review of available literature (Matranga et al., 2006; Celi et al., 2012; Celi et al., 2015). Varying concentrations of nematode extracted protein were first tested against increasing concentrations of the primary and secondary antibody and, to standardize the Western Blot protocol, β-actin was included as an internal positive control. After the antibody specificity was confirmed, analyses were conducted with the AMIGA samples. Equal amounts of cellular lysate samples (15 µg) after incubation at 100°C for 5 minutes (reducing conditions), per treatment/replicate were analysed on 10% acrylamide gels for SDS-PAGE. SDS-polyacrylamide mini-gels were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Trans-Blot SD, BioRad, Hercules, CA) and blocked with 5 % bovine serum albumin in TBS-T (20 mM Trizma base, pH 7.5, 300 mM NaCl, 0.1% (v/v), Tween-20 with 0.02% sodium azide) for 60 min at room temperature (RT). The membranes were incubated overnight at 4 °C with gentle stirring, in a solution of various anti-HSP antibodies: anti-HSP90 monoclonal antibody produced in mice (H1775 Sigma Aldrich) 1:1000 in 3% BSA in TBS-T; anti- HSP70 monoclonal antibody

produced in mice (H-5147, Sigma Aldrich) 1:2500 in 3% BSA in TBS-T; anti- HSP60 monoclonal antibody produced in mice (H3524, Sigma Aldrich) 1:500 in 3% BSA in TBS-T and anti- HSP27 polyclonal antibody produced in rabbits (SAB4501457, Sigma Aldrich) 1:1000 in 3% BSA in TBS-T as the primary antibody. After incubation, the membranes were incubated for 2.0 h at 20 °C with a diluted solution of the secondary antibody anti-mouse IgG (A5324, Sigma Aldrich, 1:10000 anti-mouse IgG in TBS-T) conjugated with alkaline phosphatase to highlight the presence of HSP70, HSP90 and with a diluted solution of the secondary antibody anti-rabbit IgG (A3937, Sigma Aldrich, 1:7500 anti-rabbit IgG in TBST) conjugated with alkaline phosphatase to mark HSP27. Immunoreactivity was then detected using a mixture of BCPI-NBT (Sigma Aldrich). The analysis of the molecular weights and the densitometric analysis of the band intensities were performed using AlphaEaseFC software after image acquisition using a flatbed scanner (EPSON PERFECTION V700 PHOTO, Dual Lens System). The data were evaluated as integrated optical density value (IDV). For each biological replicate three technical replicates were performed according to Celi et al. (2013).

Statistical analysis

All analyses were performed for each treatment/variety with a minimum of three independent biological replicates and 3 technical replicates for each. Statistical significance was assessed for comparisons either by Student's t-test ($p < 0.05$) or, for multiple comparisons by post-hoc Tukey test using Statistica 6.0 (StatSoft, Tulsa, OK, USA).

RESULTS

Production of HSP by nematodes in response to maize cultivation

Compared to the near-isogenic variety, the cultivation of GM maize in Spain and Slovakia did not exhibit a significant difference on the amount of HSP27 in the tested nematodes (Fig. 1A). For HSP27, Western Blotting (Fig. 1A) indicated a uniformity of values across all treatments and locations. This was confirmed by the IDV analysis (Fig. 1B), which recorded the absence of significant variations between extracts from ISO-, GM cultivated plots and from the uncultivated IF samples, and no difference caused by the two geographically distinct locations. For the nematocide treatment, there was a reduction of about 40% in HSP expression in the Slovakia samples, but this was not significant due to the high biological variation recorded (Fig. 1B). For the site in Spain, no significant increase/decrease in HSP27 production was recorded in the nematode communities treated with the biological nematocide compared to the HSP27 recorded for the nematode communities from the other plots.

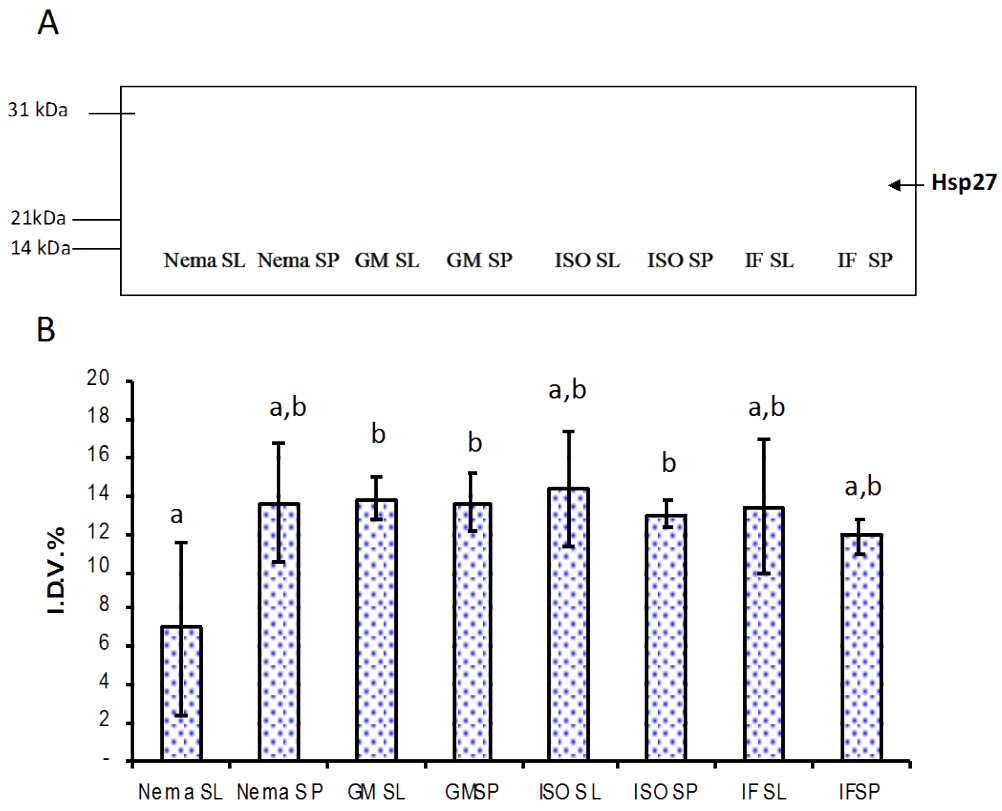

Fig. 1. Impact of cultivating GM and isogenic maize on production levels of HSP27 by the soil nematodes collected from experimental plots in Slovakia (SL) and Spain (SP). A) Representative Western blot with HSP27 levels from 250 individual nematodes combined for each variety. (B) Histogram of Integrated optical Density Value (IDV) of the HSP27 bands. Results are given as mean of three replicate and error bars indicate standard deviations. GM = genetically modified maize, ISO = near-isogenic cultivar; IF= inter-field zones. Nema = nematocide applied in laboratory on the nematodes collected from the plot ISO. Different letters (a-b) are assigned to statistically different results (ANOVA with post-hoc Tukey's tests, $p<0.05$).

Irrespective of the location or varieties grown there was no production of the HSP60 and HSP70 proteins by the nematode communities tested (data not shown). The production of HSP90 was greater in the cultivation of maize in samples from both Spain (Fig. 2A) and Slovakia (Fig. 2B). The densitometric analysis of HSP90 protein levels (Fig. 2C) revealed significantly higher amounts produced by nematodes collected from soils cultivated with GM maize ($p<0.05$) compared to plots grown with the near-isogenic variety. Significantly smaller amounts of HSP90 were also recorded for the plots with near-isogenic maize compared to those originating from the uncultivated inter-field zones (IF). Also, nematodes subjected to the nematocidal treatment showed a higher production of HSP90 compared those from the near-isogenic cultivated plots. The amount of HSP90 was apparently neither influenced by the location of the field site for the same variety, nor by the treatment ($p<0.05$).

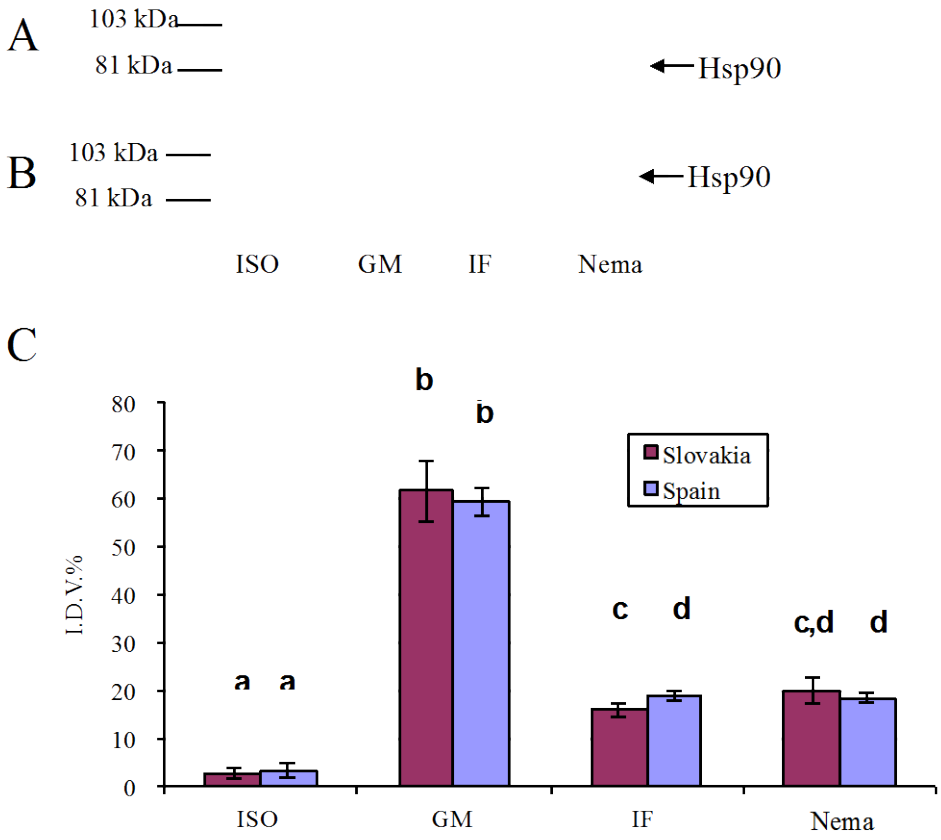

Fig. 2 Impact of cultivating GM and near-isogenic (ISO) maize on the amount of HSP90 in soil nematode populations collected from experimental field plots in Slovakia (SL) and Spain (SP) and in inter-field zones (IF) Representative Western blot of HSP90 levels in soil taken from Spain (A) and from Slovakia (B) for both GM and ISO cultivated plots. (C) Histogram of integrated optical density (IDV) of the HSP90 protein bands. Results are given as means (n = 3), and standard deviations. Nema = nematocide applied in laboratory on the nematodes collected from the plot ISO. Bars with different letters (a-d) indicate statistical differences ($P<0.05$) as indicated by post-hoc Tukey's t-test for multiple comparisons.

Impact of potato cultivation on the production of HSP by nematodes

No quantifiable production of HSP27 and HSP60 was detected for any of the soil samples analyzed; neither from Ireland nor from The Netherlands. In case of HSP70, the amounts produced were at the detection threshold (Fig. 3), as seen for both locations, with no significant difference observed across treatments ($p>0.05$). In 2013, intervention in the Dutch potato trials by activists led to an unintentional admixture of GM and non-GM tubers. As this action affected the genetic identity of the designated field plots, the respective data were not further considered for this report.

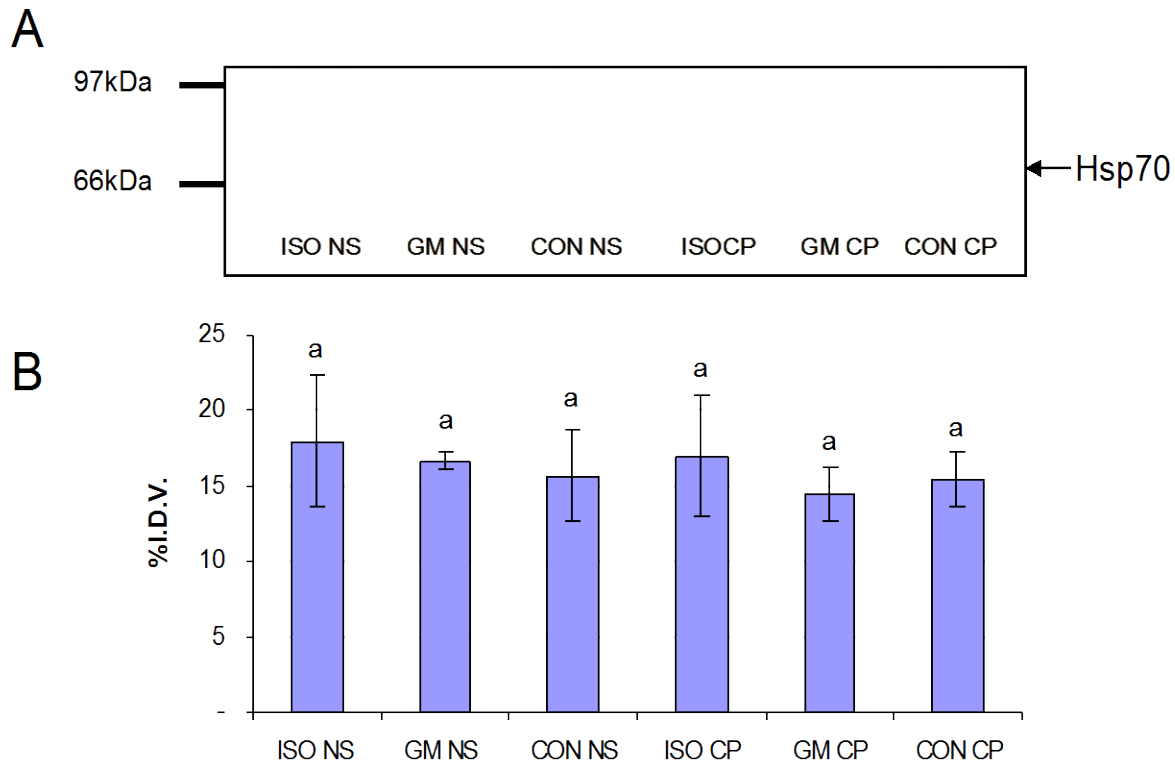

Fig. 3 A) Representative Western blot of HSP70 levels in soil samples taken from AMIGA plots grown in Ireland in 2013. B) Histogram of integrated optical density (IDV) of the HSP70 proteins bands. Results are given as means (n = 3), and standard deviations. GM = GM cisgenic Desiree, ISO = Desirée, Con = Sarpo Mira, NS = no fungicide spray, CP = current fungicide practice. Bars with different letters indicate statistical differences ($P<0.05$) as elaborated by post-hoc Tukey's t-test for multiple comparisons.

For samples taken from the field site in Ireland in 2013, the HSP90 was significantly more abundant ($p<0.05$) in whole nematode community collected from GM potato plots (Fig. 4) compared to the near-isogenic and conventional varieties, irrespective of whether the plots received fungicide treatments or not.

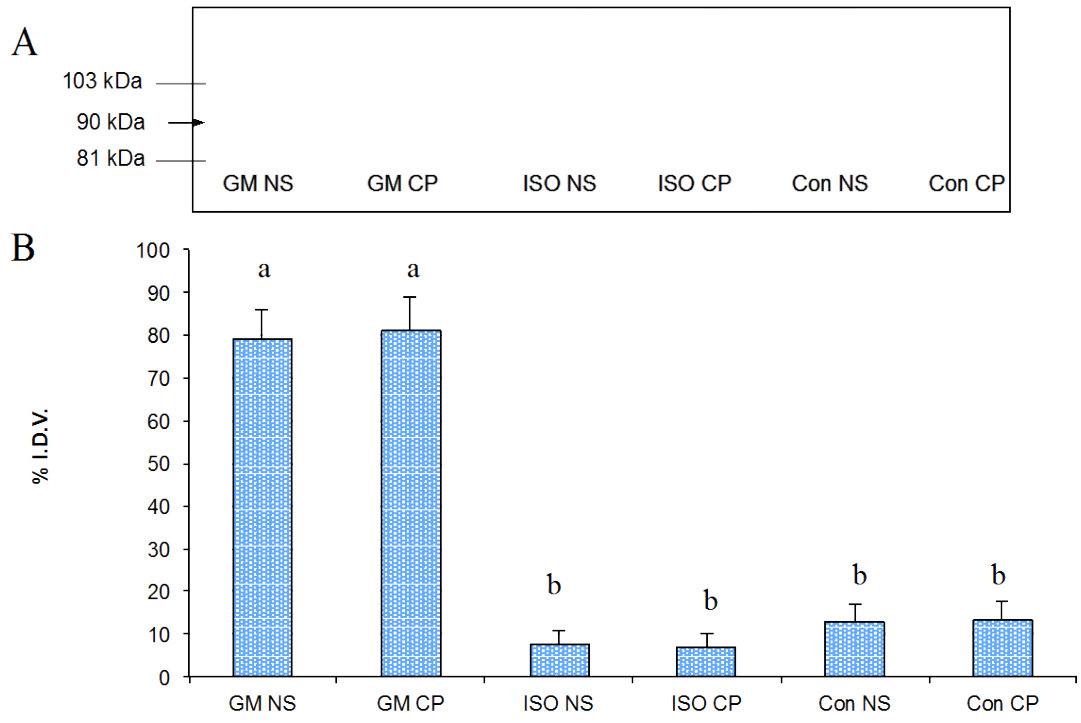

Fig. 4. Impact of cultivating GM potato on levels of the HSP90 protein in nematode populations collected from the soil of potato crops in Ireland in 2013. (A) Representative Western blot of HSP90 levels across nematode community samples taken from plots cultivated with GM = GM cisgenic Desiree, ISO = Desirée, Con = Sarpo Mira varieties with NS = no fungicide spray, CP = current fungicide practice treatments. Results are given as means (n = 3), and standard deviations. (B) Histogram of integrated optical density (IDV) of the HSP90 proteins bands. Bars with different letters indicate statistical differences as revealed by post-hoc Tukey's t-test for multiple comparisons ($P < 0.05$).

Impact of potato cultivation on *Globodera pallida* HSP expression

In accordance to the results described above for the whole nematode populations, only results from Ireland are presented. The amount of HSP27 and HSP60 was below the threshold of detection (data not shown). HSP70 of *Globodera pallida* was detected, but differences between cultivars and treatments were not significant (Fig. 5).

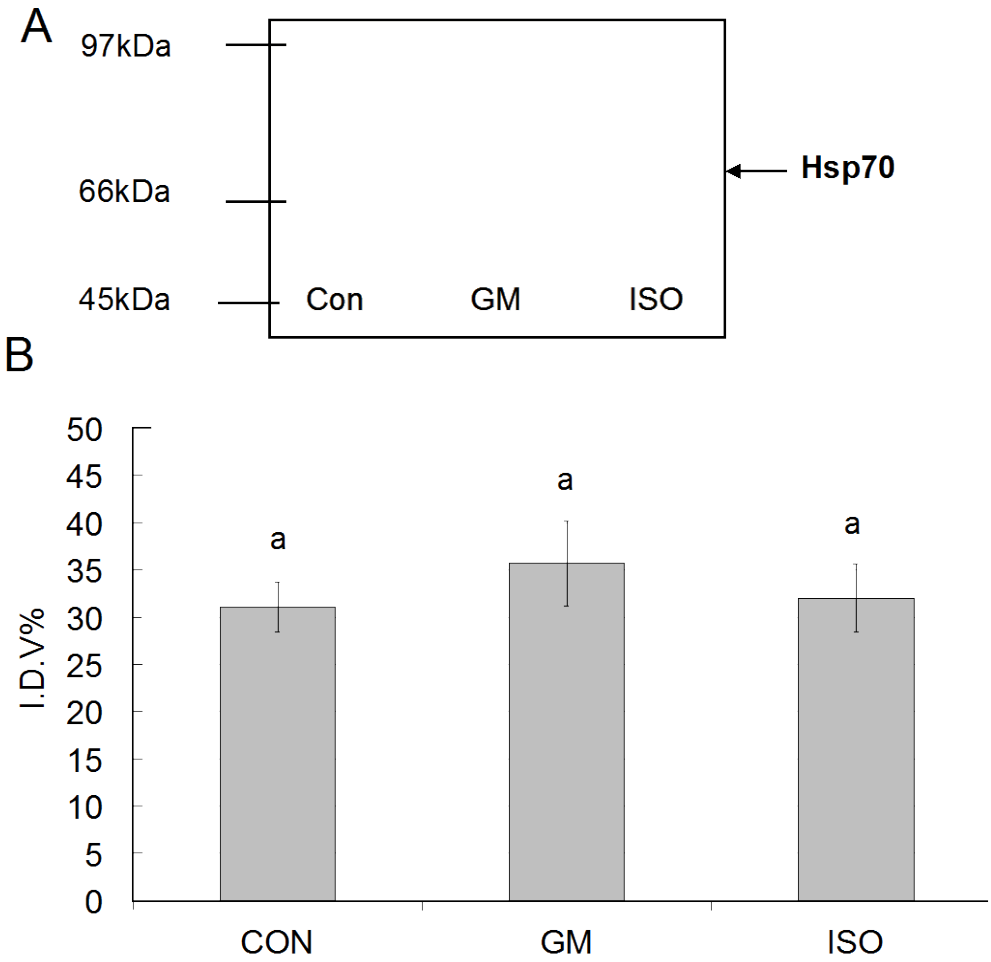

Fig. 5 Effect of GM potato on levels of the HSP70 protein in *Globodera pallida* (Stone) Behrens collected in Ireland in 2013 from infested tubers and/or roots. GM = GM cisgenic Desiree, ISO = Desirée, Con = conventional variety. (A) Representative Western blot of HSP70. (B) Histogram of integrated optical density (IDV) of the HSP70 proteins bands. Results are given as means (n = 3) and standard deviations. For more details see previous figure legends.

For HSP90, the amount of protein was significantly higher ($P<0.05$) in *G. pallida* collected from the GM potato variety (Fig. 6). Statistical differences were recorded also in for HSP90 of *G. pallida* when samples taken from Sarpo Mira treated with fungicides were compared to those without fungicide treatments (Fig. 6).

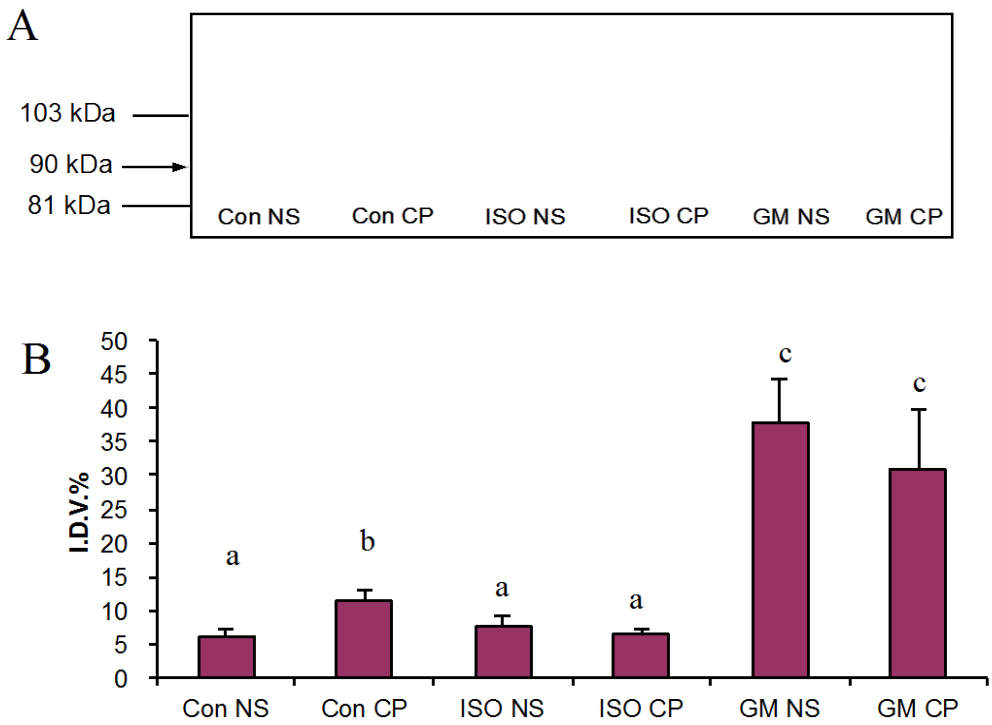

Fig. 6 Effect of GM potato on levels of the protein HSP 90 in *Globodera pallida* (Stone) Behrens, collected from infested tubers and/or roots of *Solanum tuberosum L.* sampled in Ireland in 2013 (A) Representative Western blot of HSP90 levels in cysts for each different potatos variety (B) Histogram of integrated optical density (IDV) of the HSP90 proteins bands. For more details on abbreviations and statistical analyses see legends above

Impact of maize cultivation on the production of HSP by the nematode *Rhabditis* sp.

It was not possible to detect significant amounts of either HSP27, HSP60, or HSP90 proteins from *Rhabditis* sp. for the different varieties of maize cultivated in Sweden, 2012. In contrast, for HSP70 a significant increase in its abundance in *Rhabditis* sp. was detected in samples taken from the near-isogenic maize cultivated plots (Fig. 7). The densitometric analysis of HSP70 protein levels (Fig. 7A) confirmed a significant decrease of HSP70 of this indicator nematode when incubated with soil exctracts from plots cultivated with GM maize ($p<0.05$) compared to plots grown with the near-isogenic maize (Fig. 7B).

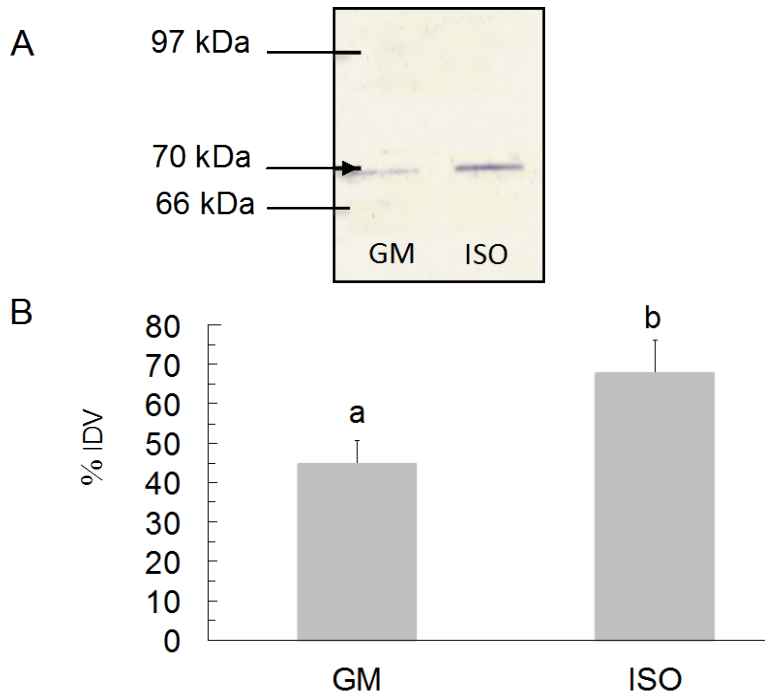

Fig. 7 Impact of GM maize cultivation on levels of the HSP70 protein in *Rhabditis sp.* collected from soils of different maize plots in Sweden in 2012. A) Representative Western blot of HSP70 levels in each different soil samples taken from AMIGA plots cultivated with GM maize and its isogenic equivalent (ISO). B) Histogram of integrated optical density (IDV) of the HSP70 proteins bands.

DISCUSSION

Previous studies have supported the use of HSPs as eco-toxicological soil biomarkers in nematodes (Arts et al., 2004; Sanchez-Moreno et al., 2006). So far, only single species had been studied, including *Plectus acuminatus* (a nematode), *Lumbricus terrestris* (earthworm, annelids) and some gastropods (molluscs). Emphasis was mainly on HSP70 and/or HSP60 as important biomarkers of eco-toxicity (Köhler et al., 1992, 1996; Nadeau et al., 2001). In this study, the production of HSP60 by soil nematodes or selected nematode species incubated with soil extracts was never detectable. In contrast, HSP70 production was generally detected by both soil nematode communities and the two selected species, except for certain instances. However, except for *Rhabditis* sp., the amount of HSP70 did not respond to field site location, crop variety or treatment, as applied at the AMIGA field sites. For *Rhabidtis* sp., HSP70 levels were responsive only in the case of maize but not of potatoes. It is important to note that the procedure to manually select *Rhabditis* sp. under the inverted stereomicroscope was time consuming and as a result the number of individuals per treatment/plot was limited. For that reason it is not a suitable procedure to recommend as a routine tool to investigate the potential effect of GM plants. Moreover the reasons for these differences are unclear and further investigations are necessary to assess if they depend on the presence of GM maize. Whether this stress response is, in fact, linked to the presence of the GM proteins i.e. Cry1Ab, or other indirect effects remains to be investigated. The role of HSP70/HSP60 as a biomarker is highly topical. Certain studies have produced contradictory results (Luo et al., 2014); some indicate a high HSP70 sensitivity to pollutants, while others suggest the opposite (Khalid

Mahmood et al., 2014). Moreover the type of potential stress due to the presence of Bt maize is rather different from that of the xenobiotics, as chemical pesticide and heavy metals ,tested. Until now, no data are available to compare the results of the present research with other similar findings.

In the case of HSP90, expression levels, as indicated by temporal abundance of the protein, fluctuated in whole soil nematode community collected from the GM plots of maize and potato compared to the respective plots with near-isogenic cultivars, and also in the case of *G. pallida* collected from GM potatoes. The HSP90 protein is a chaperone which ensures correct folding patterns and at the same time preventing possible non-specific interactions with other proteins. Because of its role in cellular operation, HSP90 has been well studied, but much of the available information comes from studies on mammalian cell lines and yeast (Devaney et al. 2005; Skanta & Carta 2004). For nematodes, most investigations have been made with the model organism *Caenorhabditis elegans* (Birnby et al., 2000). In addition to that, Skanta & Carta (2004) reported that nematodes with resistance to certain chemical pesticides were found to have a lower expression of HSP90 compared to nematocide sensitive populations. In contrast, Khalid Mahmood et al. (2014) reported that HSP90 did not link to the nature of a specific stressor as the protein showed a marked variation in concentrations in response to a variety of stress conditions. In the case of whole soil nematode communities it appears not yet feasible to ascertain which species, if one or more than one, produce a higher level of HSP90. However such an analysis would potentially provide new insights into the exhibition of direct/indirect environmental stresses on the soil microfauna.

The fundamental goal of this study was to develop and validate methods for the analysis of markers of stress in nematodes and evaluate whether these could become useful tools for the analyses of non-target effects on the soil microfauna, especially on soil nematodes. The study considered both, whole nematode communities as extracted from soils and two single species extracted directly from soils or plant tissues. The experimental design was done to assess if *in situ* exposures will provide some insights into understanding and predicting effects of genetically modified plants on natural communities under realistic exposure conditions. The results of this study indicate that from the selected HSP proteins, HSP90 was the most suitable candidate as an indicator for stress, while the other HSPs were hardly detectable or did not show any differences. The responses of HSP90 were consistent and indicated potential stress induced by the cultivation of GM plants with an increment of specific stress protein production. However, as HSPs are induced by several stresses, it is impossible to establish the entity of the stress, for how long it could persist, and if it was due to a direct or indirect effect. It is known that certain xenobiotics interfere with organism integrity at the biochemical level, with changes in the HSPs production, and with consequent adverse effects at the individual level such as growth, reproduction and survival. However, these biochemical parameters have a reduced long-term ecological relevance at the population and community levels. Indeed, such early warning biomarkers would be useful for detecting sub-lethal effects before changes in community structure or species composition occur (Hyne, & Maher, 2003; De Jong, 2006). However, it should be noted that a single biomarker cannot reflect the overall physiological state of an organism.

In summary, this is the first report to show the response of HSP90 in nematode communities and in *G. pallida* due to the presence of a GM plant. These data may also be

valuable for studying the potential biological traits related to this answer. In conclusion, this study indicates some potential of using HSP90 for analysing NTO effects on GM, but the inconsistent responses recorded so far clearly call for more fundamental work on the selection and quantification of HSP to become applicable as a biomarker in the context of GMO environmental risk assessments.

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