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Management of white grubs (Coleoptera: Scarabeidae) with entomopathogenic nematodes in Rwanda

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Abstract

White grubs are largely unsolved problems in vegetable and tuber production in East Africa. Novel Rwandan as well as international entomopathogenic nematodes (EPNs) were screened in nine laboratory bioassays and two small-scale field trials in 2014 and 2015. Soil-based laboratory bioassays revealed that all EPNs were able to infect *Anomala graueri* larvae (Coleoptera: Scarabeidae), although a relatively high number of infective juveniles were needed. Rwandan EPNs were as infectious as their corresponding international strains. At 100 infective juveniles per larva, the *Heterorhabditis bacteriophora* strains (Rwanda14-N-C4a and international H06) as well as the international *Steinernema carpocapsae* All caused 18 to 22% grub mortality within 7 days compared to the control. At 1000 infective juveniles, both *H. bacteriophora* strains as well as the Rwandan *S. carpocapsae* RW14-G-R3a-2 killed 34 up to 58%. The Rwandan *Steinernema* RW14-M-C2a-3 least performed in the bioassays (2 to 6%). In two Irish potato fields, the into-soil-applied 1×10^9 infective juvenile *Steinernema* RW14-M-C2b-1 per hectare reduced 29 ± 33% and 96 ± 3% of grubs within 30 and 60 days, respectively. About 1 up to 2.5×10^9 infective juvenile *Steinernema longicaudum* X7 per hectare reduced 77 up to 85% of grubs until day 30, respectively, and 82 up to 95% until day 60. Avermectin + Chlorpyrifos tuber coatings reduced 39% of grubs, and Fipronil + Chlorpyrifos reduced 27%, whereas handpicking did not help much. In conclusion, at least *Steinernema* RW14-M-C2b-1 and *S. longicaudum* X7 are promising for managing white grubs in tuber production, this is at a rate of at least 1.5×10^9 EPNs per hectare, but *H. bacteriophora* RW14-N-C4a needs further field research. Findings will support the biocontrol product development in Rwanda, including registration if any would be needed for native macrobial biocontrol agents.

Keywords: *Anomala graueri*, Augmentation biological control, Entomoparasitic nematodes, Rwanda

Background

Similar to many agricultural regions across the globe, East African agriculture suffers considerable losses due to soil-inhabiting insects (Trutman and Graf, 1993; Cock and Allard, 2013; Nyamwasa et al., 2017). Those pests usually damage early developmental stages of horticultural crops, but can also attack mature below-ground plant parts, such as tubers. Damaged tubers are prone to secondary infections, thus reducing their shelf-life and marketable value (Toepfer et al., 2016).

At least 40 soil insect species impact agriculture in Rwanda. The most troublesome are scarabeid white grubs in the genera *Anomala* and *Hoplochelus* or in the tribe *Melolonthini*, followed by cutworms (*Agrotis* species), bean flies (*Ophiomyia* species) and tuber-attacking weevils (*Cylas* species) (Nyamwasa et al., 2017). This pattern is found across East Africa, but also in other world regions, such as in East Asia (Guo et al., 2013; 2015; Toepfer et al., 2016).

Since about 2011, white grub outbreaks have been more and more frequently occurring across Rwanda,

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inflicting heavy damage in many cultivated vegetable and tuber crops. In some cases, up to 60 grubs were found per square metre (Nyamwasa et al., 2017). Those included *Copris, Oniticellus, Onitis, Onthophagus, Pachysoma, Anomala, Lepadoretus, Adoretus, Cyclocephala, Pycnoschema, Hoplochelus, Maladera, Trochalus*, *Lepiserica* species and *Melolonthini* genera from Rwanda, but probably there are even more. However, the pest status of some of those genera is unclear, and for some pest species, it is not clear why their populations recently increased. It is hypothesised that the continuous strip cropping of the same combination of vegetables or vegetables with field crops favours, as does monoculture, the survival and build-up of certain soil pest populations because many of them are restricted to certain plants or have long life cycles over several cropping seasons (Chen et al., 2004; Toepfer et al., 2014; Li et al., 2016).

Unfortunately, white grub pests are difficult to control because of their hidden nature, sometimes combined with insufficient knowledge on their life cycles and below-ground ecology. It is difficult to effectively use synthetic insecticides against soil pests due to problems of leaching, adsorption or rapid break down, and the impracticality of applying contact pesticides (Mayo and Peters, 1978; Furlan et al., 2006). Soil pesticides are highly concentrated formulations and can endanger the handling farmer. They are often also highly toxic, with many being restricted or indeed banned (WHO, 2009). As for Rwanda, the availability of soil pesticides is limited. They are often too expensive or impractical for small-holder growers (Toepfer et al., 2014). Socioeconomic constraints have kept pesticide use in Africa the lowest among all the world regions (Musebe et al., 2017), a fact also valid for Rwanda. These factors combined present a barrier to the effective control of soil pests in Rwanda.

As a consequence, alternative strategies such as entomopathogenic nematodes (EPNs; syn. Entomoparasitic nematodes) could be the solution, but research from East Africa is scarce. Therefore, EPNs are being investigated in an Agricultural Technology Transfer project funded by the UK Department for International Development (DfID) (Li et al., 2016; Toepfer et al., 2016). For example, EPNs of the genus *Heterorhabditis* and *Steinernema* are found naturally in soils throughout the world and possess a number of attributes that facilitate their use as biological control agents. They have the ability to search for hosts and, due to their high reproductive potential, can react to changing pest densities. They are non-toxic to humans, considered safe to the environment, and can often be mass cultured, formulated and applied with common agricultural practises. Globally, mainly *H. bacteriophora*, *H. marelatus*, *S. glaseri* and *S. scarabaei* are known to be virulent to white grubs (Klein and Georgis, 1992; Selvan et al., 1993; Ansari et al., 2003; Koppenhöfer and Fuzy, 2003 and 2009; Grewal et al., 2005; Bal et al., 2014; Guo et al., 2015), but some, such as *S. scarabaei*, are difficult to mass-rear (Koppenhöfer and Fuzy, 2003 and 2009). Prior to the here-reported study, locally adapted EPNs were searched for in semi-natural and small-holder farming habitats across Rwanda (Yan et al., 2016). The found isolates (species/strains) were described as *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, *S. carpocapsae* RW14-G-R3a-2, *H. bacteriophora* RW14-N-C4a and *Heterorhabditis* sp. RW14-K-Ca. They are currently maintained at the biocontrol agent factory at the Rubona Research Centre of the Rwanda Agriculture Board and needed investigation on their potential in killing soil insect pests, such as the white grub pests of Rwanda.

We therefore implemented bioassay-based laboratory screenings and field efficacy trials in Irish potatoes. Not only were the Rwandan nematodes assessed, but they were also compared with three international EPNs commonly used for soil pest control. As for the field trials, EPNs were also compared to insecticide tuber coatings and to the locally practised handpicking of grubs during soil preparations and mechanical weed control. This study will serve as a baseline for future work on using these EPN species/strains for white grub management in Rwanda or neighbouring countries. Results will particularly help to decide which EPN species should be mass-produced and applied to effectively control soil-inhabiting insect pests in vegetable and tuber production in a safe and environmentally friendly way.

Methods

Target insect pests

The target insect pests were larvae of scarabeid beetles, i.e. white grubs known to damage vegetables and tubers in Rwanda (Nyamwasa et al., 2017). These included *Anomala* as well as *Hoplochelus* species (Coleoptera: Scarabeidae).

As for laboratory screenings, *Anomala graueri* grubs were field-collected. They were identified using morphological and molecular DNA comparison methods (Nyamwasa et al., 2017). They were collected from ploughed fields after sweet or Irish potatoes in Akanyandoli in Nyamagabe district in September 2014 as well as in February and March 2015. More larvae were collected between rows of sweet potatoes and from grassy fallows after sweet potatoes near Mujuga and Kitabi in Nyamagabe district in October and November 2015. White grubs were visually searched for through digging with a spate or hook about 30 cm deep through the soil. Each grub was individually placed into a soil-filled small plastic container with holes in the lid. They were transported to the laboratory and held at 24 ± 2 °C for no more than 1 day until testing. Tested larvae were mainly of third instar (mean 2.6 ± 0.2 SD cm long).

As for field trials, sites were chosen wherefrom farmers had reported serious crop damage by white grubs the previous season. Population levels of white grubs were assessed through soil samplings prior to planting and again during weeding 15 days after crop emergence and during earthing up of Irish potatoes. Natural white grub populations were between 1 and 4 grubs per square metre. The dominant white grub genera were *Anomala* and *Hoplochelus* species as per morphological and DNA comparisons. But identification to species level was not possible in most cases due to lack of comparative information in DNA databases and difficulties to morphologically discriminate larval instars (Nyamwasa et al., 2017).

Source and handling of EPNs

The Rwandan EPN species/isolates, *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, *S. carpocapsae* RW14-G-R3a-2 and *H. bacteriophora* RW14-N-C4a originated from surveys in several provinces of Rwanda in 2014 (Yan et al., 2016) (Table 1). *Steinernema longicaudum* X7, *H. bacteriophora* H06 and *S. carpocapsae* All are commonly used biocontrol agents of soil insect pests in many world regions (Poinar Jr., 1990; Shen and Wang, 1991; Yan et al., 2013) and served as positive controls. They were provided by Lvbenyan Biotech Ltd., a spin-off of the Guangdong Institute of Applied Biological Resources (GIABR) in China.

All EPNs were reared at the biocontrol agent facility of RAB Rubona (Holmes et al., 2015) using the in vitro semi-solid, sometimes called the solid system as per Shapiro-Ilan and Gaugler (2002) and Kaya et al. (2006). The harvested infective juvenile (IJ) EPNs were acclimated to room temperature for 1 h. Their viability was assessed on the basis of movements checked under a stereomicroscope and considered alive when actively moving or showing response after probing with a needle. EPN suspensions were used for the experiments when more than 90% of IJs were viable.

All EPNs were assessed in laboratory bioassays, but only the Rwandan *Steinernema* sp. RW14-M-C2b-1 and the international *S. longicaudum* X7 in field efficacy trials, as those were the easiest to mass-rear.

Laboratory bioassays

Bioassay-based laboratory screenings of EPNs were carried out on *A. graueri* larvae under controlled conditions to compare four Rwandan EPN species/isolates versus three international EPNs commonly used in biological control (Table 1). This is a pre-requisite of deciding for which EPN a mass production technique should be established.

Screening for EPN infectiousness

In total, nine experiments were implemented to screen seven different EPNs at five different concentrations for their infectiousness on third instar *Anomala graueri* larvae (Table 2). The infectiousness was here defined as the insect mortality caused by EPNs that include the processes of host finding, host recognition, penetration, release of bacteria, bacteria proliferation, overcoming insect immune response, nematode proliferation and insect death as per Peters and Smits (2000), in other words the situation in a bioassay arena.

In each of at least three experimental replicates per EPN and concentration, three sets of ten small bioassay arenas were set up containing one white grub each (Table 2), as bioassay arenas served toothpick boxes (3 cm diameter × 7 cm high, 3 holes of 2 mm in the lid). The arenas contained 25 g sandy loam soil (78% sand, 14% loam, 5% clay, 0.4% organic matter; pH 6.8). The

Table 1 Origin of Rwandan and comparative international entomopathogenic nematodes

EPN	Origin	Provider	Reference
Rwandan			
H. bacteriophora RW14-N-C4a	Ploughed cropland on terraces, South province [Nyamagabe district], Rwanda	RAB Rubona, Rwanda	Yan et al., 2016
S. carpocapsae RW14-G-R3a-2	River bench; North province [Gakenke district], Rwanda	RAB Rubona, Rwanda	Yan et al., 2016
*Steinernema sp.*RW14-M-C2a-3 *feltiae*-group	Banana- pumpkin- sorghum intercrop field; North province [Musanze district], Rwanda	RAB Rubona, Rwanda	Yan et al., 2016
Steinernema sp. RW14-M-C2b-1, *feltiae*-group	Banana- pumpkin- sorghum intercrop field, North province [Musanze district], Rwanda	RAB Rubona, Rwanda	Yan et al., 2016
International			
H. bacteriophora H06	Sandy soil peanut field; Shandong, China	GIABR, Guangzhou, China	Yan et al., 2013
S. carpocapsae All	Vineyards, East coast, USA	Biosys, Columbia, MD, USA via GIABR	All et al., 1981; PoinaJr, 1990
S. longicaudum X7	Shandong, China	GIABR, Guangzhou, China	Shen and Wang, 1991

All EPNs tested in soil-based small arena laboratory bioassays; *Steinernema* sp. RW14-M-C2b-1 and *S. longicaudum* X7 also tested in small-scale field efficacy trials
GIABR Guangdong Institute of Applied Biological Resources, China, *RAB* Rwanda Agriculture Board

Table 2 Experimental replicates, sets of bioassay arenas, and total sample sizes of arenas per tested entomopathogenic nematode and applied concentration in soil-based laboratory bioassays (concentration = 100, 200, 300, 400 and/or 1000 infective juveniles (IJs) per grub equalling 4, 8, 12, 16 and 40 IJs per gram soil, 2.4, 4.8, 7.1 and 9.5 IJs per cm^3 soil and 14, 28, 42, 57, and 140 IJs per cm^2 soil surface). An experiment consisted of at least two sets of 10 bioassay arenas each, and each arena containing one Anomala graueri grub

Treatment	Experiments/(sets of 10 arenas across experiments)/sample size of arenas across experiments																	
	Concentration (IJ per larva)																	
	100			200			300			400			1000			Total		
H. bacteriophora RW14-N-C4a	3	(9)	90	2	(6)	60	2	(6	60	3	(9	90	2	(6	60	12	(36)	360
H. bacteriophora H06	5	(15)	150	5	(15)	150	4	(12)	120	5	(15)	150	2	(6)	60	21	(63)	630
S. carpocapsae All	3	(9)	90	2	(6	60	1	(3)	30	1	(3)	30	2	(6)	60	9	(27)	270
S. carpocapsae RW14-G-R3a-2	3	(9)	90	3	(9)	90	2	(6)	60	3	(9)	90	4	(12)	120	15	(45)	450
Steinernema sp. RW14-M-C2a-3	3	(9)	90	2	(6)	60	2	(6)	60	3	(9)	90	2	(6)	60	12	(36)	360
Steinernema sp. RW14-M-C2b-1	3	(9)	90	3	(9)	90	2	(6)	60	3	(9)	90	4	(12)	120	15	(45)	450
S. longicaudum X7	3	(9)	90	3	(9)	90	2	(6)	60	3	(9)	90	2	(6)	60	13	(39)	390
Untreated control	Similar numbers for each experiment																	

soil originated from an experimental field at RAB Rubona station north of Huye town in the southern province of Rwanda. The soil had been sieved at 2 mm mesh size, and autoclaved at 121 °C for at least 15 min to destroy any natural enemy of grubs or EPNs as well as any potential natural population of EPNs. The soil was then dried at 100 °C for 24 h and then adjusted to 15% *w*/*w* moisture. An about 1-cm^3 piece of fresh Irish potato was added in each arena as food for the white grubs. The arenas were kept at room temperature of 24 ± 2 °C for 2 h, and then only arenas with moving grubs, e.g. which had entered into the soil, were selected for experiments.

Up to five different concentrations were tested; that is, 100, 200, 300, 400 and 1000 IJs per white grub, thus per 28 m^2 soil surface in a bioassay arena (Table 2). The 100 to 1000 IJ step in concentrations reflects a $\log_{10}$ step, as commonly used in dose trials allowing a more linear response analyses. One-millilitre suspensions containing the required concentration of IJs were distributed across the soil surface of each arena using a precision pipette. No direct application onto an insect body was carried out. Water without nematodes was used on the untreated controls. The arenas were placed in the dark at 24 ± 2 °C for 14 days.

Grub mortality was assessed after 4, 7 and 14 days. In addition, mortality likely caused by EPNs and not by other mortality factors was assessed as follows: In case a dead grub was found, it was washed in 1% NaOCl (syn. bleach, jijk) or in 70% ethanol, and then washed in clean water. Individual cadavers were then placed onto a slightly moist filter paper in a 9-cm Petri dish for another 4 days and then dissected to confirm adult or young EPNs under a stereomicroscope. The infectiousness was calculated as the corrected (=relative) mortality of grubs in each treatment minus the natural mortality found in the untreated controls (Figs. 1 and 2). Distributions of data were investigated using histograms and the one-sample Kolmogorov-Smirnov test against normal distribution (Kinnear and Gray, 2000). For non-normal distributed data, $\log_{10}(x+1)$, $\mathrm{sqrt}(x+1)$, or $\arcsin(x)$ transformations were used to achieve better data distribution. The influences of the EPN species/strains/isolates, the concentration of EPNs and the assessment period of 4, 7 and 14 days after treatment were examined on grub infectiousness through GLM univariate analyses (binomial with logit link function). Due to the large number of concentrations and time steps assessed, treatments and time steps, the p values obtained from the post hoc tests were adjusted to q values to reduce the probability of type I errors of p values using the false discovery rate-correction of Benjamini & Hochberg through the package Q-VALUE in R (R Development Core Team, 2009).

Field efficacy trials

To evaluate EPNs' potential under field conditions, field efficacy trials were implemented for controlling *Anomala* and *Hoplochelus* grubs. Soil applications of the Rwandan *Steinernema* sp. RW14-M-C2b-1 and the international *S. longicaudum* X7 were compared with insecticide-tuber coatings, handpicking and untreated controls.

Field sites

Two experimental field sites of 0.13 ha were established in 2015 for assessing the control effect of EPNs against natural populations of white grubs. The fields are referred to as field M in Kinigi in Musanze district, northern zone of Rwanda (2285 m, S01° 2′ 11.1″, E29° 35′ 50.8″) and field

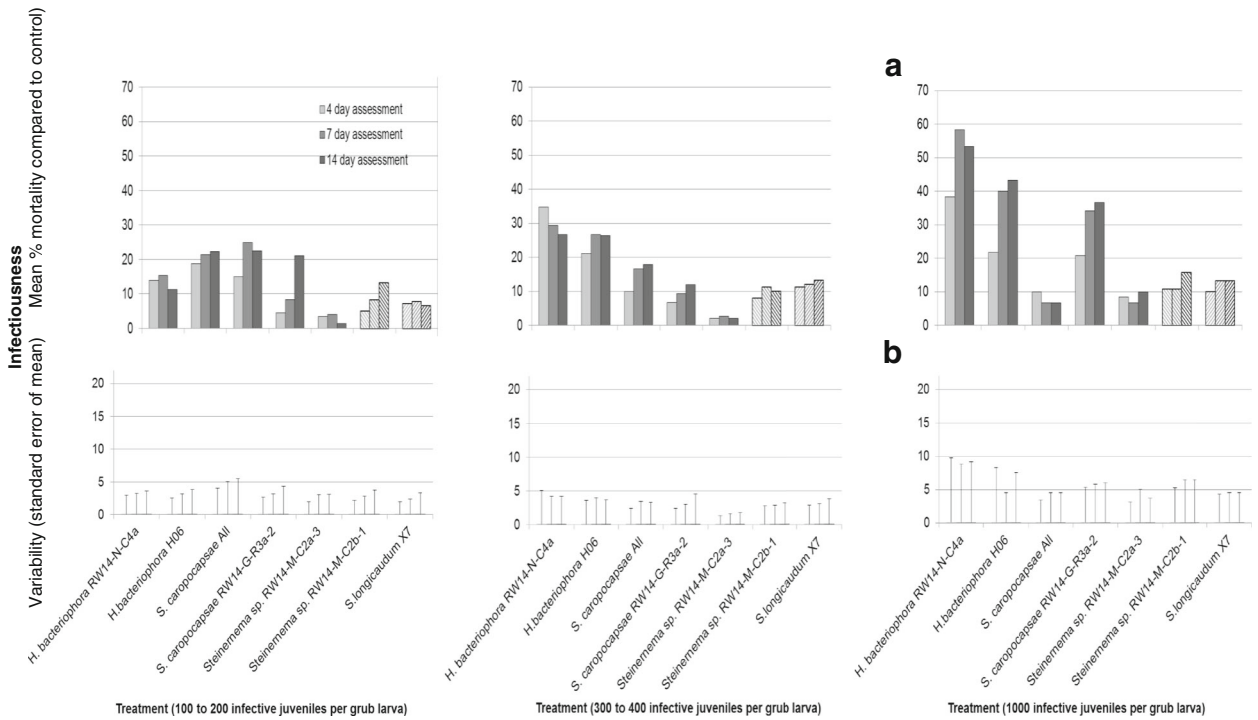

Fig. 1 Assessing which exposure period of *Anomala graueri* grubs to entomopathogenic nematodes leads to most distinct differences between treatments in laboratory bioassays. Mean value and variability separately presented to better assess (**a**) differences between percent-corrected mortality compared to control (mortality in EPN treatments minus natural mortality in untreated controls), and (**b**) smallest data variability. $n = 6$ to 15 grubs assessed per EPN species/strain and per concentration in single-grub arenas with soil

N in Akanyandoli in Nyamagabe district, southern zone (2153 m; S20° 30′ 43.2″, E29° 30′ 007″).

Both fields were ploughed twice prior planting for weed control as well as for soil preparation. Irish potatoes (Var. Cruiser, seed program at Rwanda Agriculture Board) were planted in field N on 5 May 2015 and in field M on 15 May 2015. At the day of planting, well-composted farm cow manure (20 t per ha) and mineral fertiliser (NPK 17-17-17 at 300 kg per ha) were applied into the planting holes just before planting. Irish potatoes were planted into 8- to 10-cm deep holes at 30 cm distance between plants and 80 cm between rows, equalling 41,000 plants per hectare. About 1-kg seed tubers were used per 4 m^2, equalling 2.5 tons per hectare. About 60 cm buffer spaces were kept between treatment plots. Mechanical weeding was implemented 15 days after potato germination, and soil was earthed up along potato rows at potato flowering stage. Natural white grub populations were up to one grub per square metre in field M and up to four grubs per square metre in field N (Fig. 3). Field M was harvested on 24 September 2015 and field N on 22 September 2015.

Experimental setup and treatments

In total, eight different treatments were implemented against white grubs, consisting of two different EPN species/strains (one tested in three concentrations), two insecticide tuber coatings, handpicking of grubs and an untreated control.

In detail, IJs of the Rwandan *Steinernema* sp. RW14-M-C2b-1 (60,000 per row-metre, equalling 0.75×10^9 IJs/ha) and the international *S. longicaudum* X7 (60,000/row-metre, equalling 0.75×10^9 IJs/ha; 120,000/row-metre, equalling 1.5×10^9 IJs/ha; 200,000/row-metre, equalling 2.5×10^9 IJs/ha) were applied with water (2000 l/ha) into the soil along the plant rows. For that, an about 5-cm-deep furrow had been made at about 10 to 15 cm distance to the plants. EPNs formulated in sponge (Yan et al., 2016) were diluted in water and transferred through a sieve into an 8-l knapsack sprayer. Nozzles were removed and EPNs applied into the soil. EPNs were applied about 30 days after planting (4 June 2015) in field N, and about 55 days after planting in field M (10 July 2015).

Liquid microcapsule formulations of 0.2% Avermectin + 14.8% Chlorpyrifos (Pesticide factory at IPP, CAAS, Beijing, China) or 3% Fipronil + 15% Chlorpyrifos (Yoloo Pesticide Co., Ltd., Beijing, China) were coated onto the to-be planted tubers. Briefly, 2.5 ml of the liquid microcapsule formulation was diluted in 10 ml water, then pulled over around 1 kg of tubers and manually mixed to achieve surface coating. This equals a 1: 400 ratio of the products

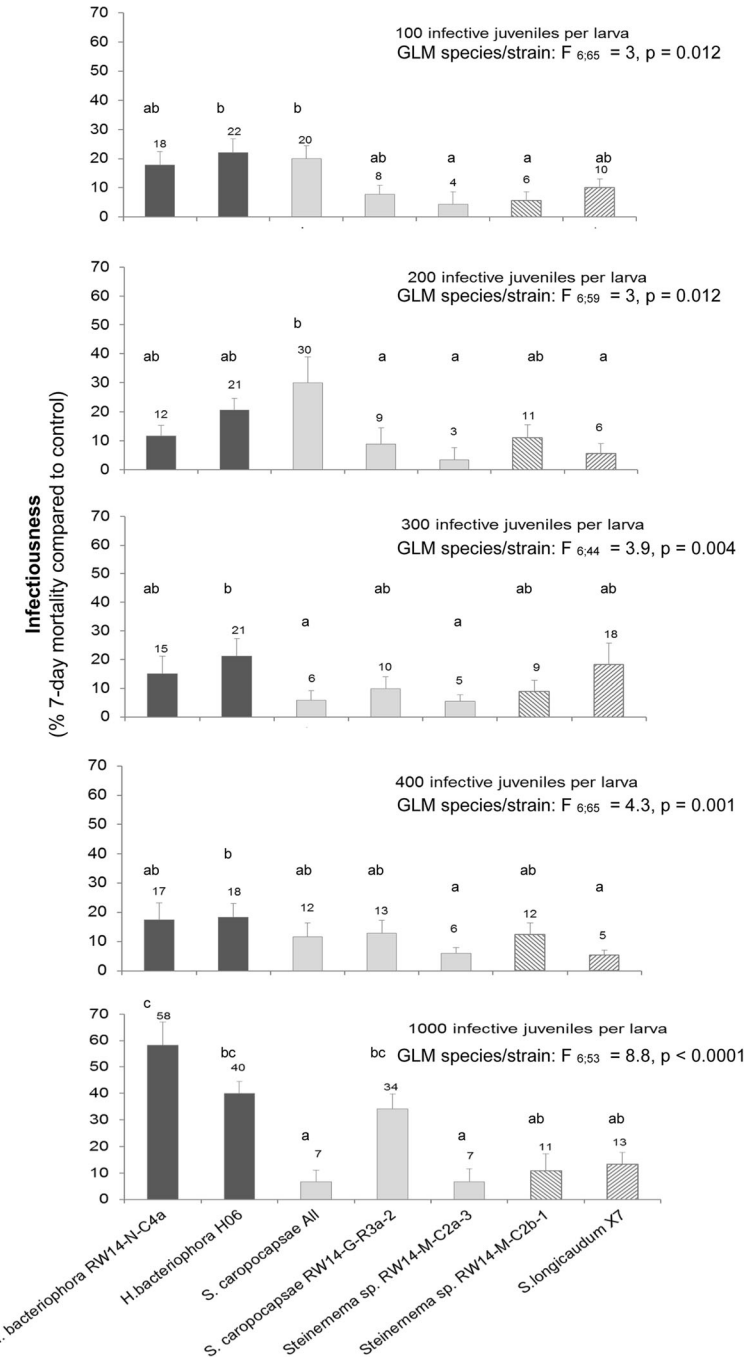

Fig. 2 Comparing the infectiousness of Rwandan (RW) and international entomopathogenic nematodes on *Anomala graueri* grubs in soil-based laboratory bioassays, depending on species/strain and on applied concentrations of 100, 200, 300, 400 and 1000 infective juveniles per grub equalling 14, 28, 42, 57 and 140 IJs per square centimetre soil. Dark grey bars = heterorhabdits, light grey = steinernematids, lined = verified in field trials (Figs. 4 and 5.); 7-day-corrected morality shown, this is mortality in EPN treatments minus natural mortality in untreated controls. Three sets of 10 bioassay arenas with single grubs in soil used per each of 3 to 15 experimental replicates; letters on bars indicate difference according to multiple comparison Tukey post hoc test at $p < 0.05$, additionally fdr-corrected among concentration experiments; error bars = SEM

versus tuber weight. After coating, potatoes were dried in the sun for 5 to 10 min. One kilogram of potato seed tubers was used per 4 m^2, equalling 2.5 tons per hectare (totalling 6.25 l product in 25 l water/ha). Grubs were handpicked from the freshly opened furrows during planting and again during at first mechanical weeding 15 days after germination, and finally during earthing up at plant flowering stage. Larvae were visually searched for and picked up.

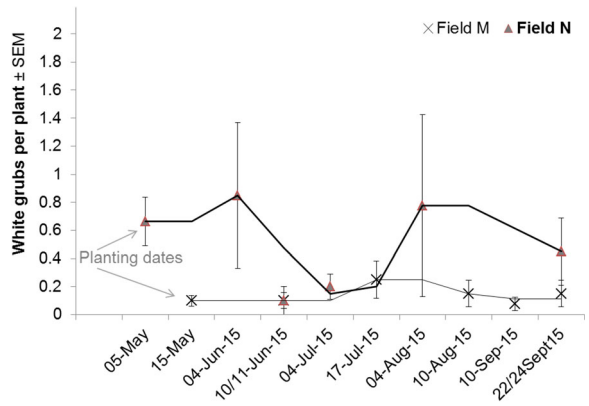

Fig. 3 Population dynamics of *Anomala* and *Hoplochelus* white grubs in Irish potato fields during experimental periods in field M in Musanze and field N in Nyamagabe in Rwanda in 2015. Assessed from the soil at 0 to 15 cm below and around 10 plants in 4 untreated control plots in each field per date. Error bars = SD. Trendline = moving average

The control plots were only treated with water, that is, at a rate equalling 2000 l of water per hectare.

The study was conducted according to the efficacy evaluation standards PP 1/212 and PP 1/152 of EPPO (Anonymous, 2007). Trial permits were obtained from the Rwanda Agriculture Board Authorities. The experimental fields were divided into plots of 4 × 10 m. Each treatment was applied on four such plots per field, totalling 32 plots per field. A randomised block design was used for the placement of plots. Thus, the within-field replicate number per treatment was four totalling eight for the entire study.

Assessment of EPN efficacy at reducing white grubs

In each of the four plots per each of the treatments, soil beneath 10 randomly pulled-out plants were assessed for white grub population levels, and this in both fields at six time steps from planting until harvest (Fig. 3). Grubs were visually searched for by crumbling an about 40 × 40 × 20 cm deep area of soil per plant hole. Only for the assessment before planting, samples were taken from five random spots of 1 × 1 × 0.25 m soil per treatment per plot, and data converted to per-plant-data depending on the expected plant density.

In field M, this was done at the date of planting (15 May 2015), 57 days later (10 July 2015, date of EPN treatment), then 64, 94 and 124 days later (= 7, 30, 60 days after EPN treatment) and 133 days later, i.e. at harvest (24 September 2015, 77 days after EPN treatment).

In field N, this was done at the date of planting (5 May 2015), 31 days later (4 June 2015, date of EPN treatment), then 38, 68 and 98 days later (= 7, 30, 60 days after EPN treatment) and 141 days later, i.e. at harvest (22 September 2015, 111 days after EPN treatment).

The mean efficacy of each treatment was calculated as the reduction of grubs relative to the untreated control at each time step and as a seasonal average., i.e. the corrected % efficacy = [100 – (insects in treated plots * 100/ insects in the untreated control)].

Distributions of data were investigated using histograms and one-sample Kolmogorov-Smirnov test against normal distribution (Kinnear and Gray, 2000). As for distribution of average grub data per plot along treatments, all data appeared normally distributed; except the 7-day data needing sqrt($x + 1$) transformation. As for the distribution of raw grub data, only the data at harvest time were normal distributed and, after log10($x + 1$) and sqrt(x+) transformation, also the data from day 7—after EPN treatment. The 30- and 60-day raw data remained non-normal distributed, regardless of transformation.

The influences of treatments on white grub populations were examined through GLM analyses or through independent samples Kruskal-Wallis H test (nonparametric analogue of GLM analyses). Once a significant factorial effect was detected by those models, the averages of grubs per plot were compared between treatments at each date. For this, Tukey post hoc range test was used in case of normally distributed data and equal homogeneity of variances (Kinnear and Gray, 2000).

Assessment of EPN efficacy at preventing yield losses

In each of the four plots per each of the treatments, 10 plants were harvested and their tubers weighted immediately. Differences among different treatments were analysed at $p < 0.05$ via multiple comparison Tukey post hoc range test after GLM.

Assessment of EPN persistence

The nematode-baiting method with alternative host insects as baits was employed as per Bedding and Akhurst (1975) and Yan et al. (2016) to assess whether the applied EPNs had established and persisted in the field soil. Around 400 to 600 g of soil was taken from the holes remaining from each of the 10 sampled plants for white grub assessments (see above). Soil samples were taken at a depth of 10 to 20 cm. The 10 soil samples per plot were then mixed, and a 500-g mixed subsample was taken. The mixed subsamples were placed into plastic containers and returned to the laboratory. Then, five late instar *Galleria mellonella* (Lepidoptera: Pyralidae) were added to each mixed sample to bait for potential EPNs (container 20 × 12 × 6 cm) (Caroli et al., 1996). The baited samples were then incubated in the dark at room temperature (24 ± 2 °C). The *Galleria* larvae were recovered about 5 days later, and dead *Galleria* counted. Rotting larvae, those with a bad (viz. bacterial) odour, or

larvae with multiple colours (EPN-killed larvae show one colour) were discarded, as they had unlikely been killed by EPNs (Grewal, 1992). The cadavers of potential interest were surface-sterilised in 0.5 to 1% NaOCl for 3–5 s, washed in water, then individually placed onto a nematode trap modified as per White (1927). Briefly, the trap consisted of cellulose tissue on an inverted Petri dish lid onto which the cadaver was placed. The dish was placed into a larger Petri dish. The dish was left open for 1–2 days to desiccate and kill saprophytes (EPNs inside cadavers are protected from desiccation). Then, a bit of sterilised tap water was added to the tissue. Between 4 to 7 days later, the traps were checked daily for IJ emergence from the cadavers. Emerging nematodes were visually assessed under a stereomicroscope (×10 magnification) for saprophytic nematodes and EPNs. A mixed soil sample was considered EPN positive in case that at least one of the five *Galleria* larvae was infected by EPNs per sample (Kurtz et al. 2007). Proportions of positive samples, thus persistence, were assessed over time using Pearson correlations, and between treatment using Tukey multiple comparisons after GLM.

Results and discussion

To evaluate the potential of newly described Rwandan EPNs (Yan et al., 2016) for the development of biological control products against soil insect pests, bioassay-based laboratory screenings and small-scale field efficacy trials had been implemented and the results, as presented here, are promising.

Laboratory bioassays

The small arena bioassays revealed that all the four tested Rwandan EPN species/strains were able to infect and kill white grubs, although it took quite a high number of infective juveniles per grub larva. Nevertheless, this is considered a good basis for further research, as it is often not too easy to find EPNs being infectious to grubs as they are often able to defend themselves to EPNs or other ground-living natural enemies (Ansari et al., 2003; Guo et al., 2013; Laznik and Trdan, 2015). And other EPNs, such as *S. scarabaei*, are not easily mass-produced. Consequently, there are only few EPN products on the market against white grubs, globally. However, as the tested Rwandan EPNs originated from small-holder farming habitats of Rwanda (Yan et al., 2016), they are, potentially, more adapted than EPNs from other habitats, such as sea shores. Moreover, the tested Rwandan EPNs seem to be as good as international species/strains commonly used for soil pest control. This is a hint that the tested EPNs may have evolved in areas of white grub prevalence in Rwanda.

EPN infectiousness against grubs

The overall accumulated mortality of *A. graueri* white grubs due to EPNs, as verified by dissections, was 13 ± 15 SD % until day 4; 17 ± 17% until day 7; and 20 ± 18% until day 14 ($n = 291$). Comparable results were obtained when correcting the total accumulated mortality of grubs in the treatments to the natural mortality in the untreated controls. That is, 13 ± 15% more grubs died in the EPN treatments than in the control until day 4; 16 ± 18% until day 7; and 17 ± 19% until day 14. Without any treatment, 6 ± 7% of grubs naturally died until day 4; 10 ± 13% until day 7; and 14 ± 18% until day 14.

Infectiousness depending on EPN species/strain

Overall, tested EPNs appeared to have medium infectiousness against white grubs in the laboratory bioassays. Nevertheless, all tested EPNs, regardless if Rwandan or international, were able to infect and kill *A. graueri* grubs (Figs. 1 and 2). However, species/strains differed in their infectiousness (GLM: "species/strain" for 7 day corrected mortality: $F_{6;290} = 16$, $p < 0.0001$; "species × concentration": $F_{24;290} = 2.5$, $p < 0.0003$, adjusted $R^2 = 0.33$; Fig. 2). The Rwandan *H. bacteriophora* RW14-N-C4a and *S. carpocapsae* RW14-G-R3a-2 reached the efficacies of their corresponding international strains, indicating that local EPNs could be used as biocontrol agents against *Anomala* grubs.

The heterorhabdits slightly more consistently and usually more effectively killed the grubs than did most steinernematids (Fig. 2); except for the Rwandan and international *S. carpocapsae* which reached the infectiousness level of the heterorhabdits in some of the assays.

On average across concentrations, the Rwandan *H. bacteriophora* RW14-N-C4a killed 29 ± 18 SD % of grubs, the international *H. bacteriophora* H06 27 ± 9%, the Rwandan *S. carpocapsae* RW14-G-R3a-2 14 ± 11% and the international *S. carpocapsae* All 18 ± 9%. *Steinernema* sp. RW14-M-C2a3 was, overall, the least performing (4 ± 2% of grubs killed across concentrations, Figs. 1 and 2). The Rwandan *Steinernema* sp. R14-M-C2b1 (10 ± 3%) and the international *S. longicaudum* X7 (11 ± 3%) were both of comparable medium, but relatively constant infectiousness (5 to 18% mortality across different concentrations), as well as easily mass-reared (data not shown), and therefore suggested for field trials.

Field assessments are the logical next step, because the infectiousness data from EPNs in the laboratory can only partly be transferred to the field situation (Peters et al., 1996). This is, for example, because the foraging behaviour of EPNs is less important for the attack of host insects in small arenas of bioassays, than under field conditions. Field conditions also require a longer survival of EPNs without the host, i.e. before finding the host, than in small arena bioassays.

Infectiousness depending on EPN concentration

The concentration of applied EPN species/strains influenced their infectiousness on *A. graueri* grubs (GLM "concentration" for 7 day corrected mortality: $F_{4;290} = 5.5$, $p < 0.0003$; "concentration × species/strain" $F_{24;290} = 2.5$, $p < 0.0003$, adjusted $R^2 = 0.33$) (Fig. 2). This effect was due to an increasing infectiousness at high concentration of 1000 IJs per larva, whereas concentration between 100 and 400 IJs did not matter much (GLM $F_{3;236} = 0.8$, $p = 0.49$). When analysing concentration effects separately per EPN species, often no dose efficacy-response was detected, that is, for *S. carpocapsae* All, *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1 and *S. longicaudum* X7 (GLM $F_{3;44\ to\ 65)} < 3$, $p > 0.05$). Only the following species/strains showed a positive dose-infectiousness response, i.e. *H. bacteriophora* RW14-N-C4a and H06 and *S. carpocapsae* RW14-G-R3a-2. In other words, at low concentrations of 100 to 200 IJs per larva, both the Rwandan and international *H. bacteriophora* strains as well as the international *S. carpocapsae* All performed best (12 to 30% additional mortality compared to untreated control) (GLM in Fig. 1). At high concentrations of 1000 IJs per larvae, again both *H. bacteriophora* strains were the best as well as the Rwandan *S. carpocapsae* RW14-G-R3a-2 (34 to 58% mortality compared to control).

In many cases, results were variable, and therefore a clear dose-response can hardly be concluded. This is typical for natural strains of EPNs that have not yet gone through many cycles of mass production and therefore through a selection for constant traits. It also shows that higher concentrations than 1000 IJs per grub should probably have been included in the experiments to get better dose-response trendlines. Nevertheless, we conclude that all Rwandan EPNs are worth to be further investigated on a number of target soil insect pests of Rwanda.

Infectiousness depending on exposure period to EPNs

Overall, the 7-day assessment appeared to be promising to detect differences in the infectiousness of the different EPN species/strains as well as concentrations. Differences among EPN species/strains in their infectiousness on *A. graueri* grubs over time of exposure were more obvious at low EPN concentrations than at medium or high concentrations (Fig. 1a). Variability of data depended more on the EPN species/strain or concentration (see large SEMs for 1000 IJs per grub in Fig. 1b), than on exposure period. In other words, increasing exposure period from 4 up to 14 days did not consistently increase or decrease variability of data, except at low concentrations (see below).

As for low EPN concentrations of 100 to 200 IJs per larva, differences between EPN species/strains seemed most obvious for the 7-day assessment as data later became disturbed by increasing natural mortality. Infectiousness usually only little increased with time (Fig. 1a), except from day 4 to day 7 for *S. carpocapsae* All and *Steinernema* sp. RW14M-C2b-1, and up to day 14 for *Steinernema* sp. RW14M-C2a-3 and *Steinernema* sp. RW14M-C2b-1 (Kruskal-Wallis H tests at $p < 0.05$, d.f. 2; 40 to 60). For all tested EPNs, the variability of relative infectiousness increased over time due to an increase in natural mortality (Fig. 1b). In conclusion, the 7-day assessment should provide reliable information from the bioassay on differences between EPNs at an acceptable variability.

As for medium EPN concentrations of 300 to 400 IJs per larva, infectiousness did not or only little increase with time (Fig. 1). The 14-day assessment added little additional information and/or was disturbed by natural mortality, as seen in the decreasing values of *H. bacteriophora* RW14-N-C4a with time. The variability of relative infectiousness data was comparable among time periods, but increased in few cases for the 14-day assessment (Fig. 1). In conclusion, the 4- and 7-day assessment should provide reliable information from the bioassays.

As for high EPN concentrations of 1000 IJs per larva, infectiousness increased particularly after day 4 up to day 7, but only in few cases or not at all after day 7 (Fig. 1). This indicates that the high concentration led to maximum infection already at day 7 and no additional information could be obtained running the assays until day 14.

Field efficacy trials

White grub infestation

The natural population levels of grubs ranged from 0.1 to 0.25 larvae per plant in field M and from 0.3 to 0.9 larvae per plant in field N in 2015 (average across fields and cropping season 0.3 ± 0.29 SD larvae per plant). This equals about 4000 to 10,000 white grubs per hectare at site M and 4000 to 35,000 at site N, a pest level that can lead to significant yield loss as a single grub usually destroys at least one planted tuber.

At day of planting Irish potatoes, 0.1 ± 0.03 grubs were found per plant in field M and 0.7 ± 0.17 in field N. Before EPN treatment about 30 to 55 days after planting, 0.1 ± 0.1 grubs were found per plant in field M and 0.9 ± 0.52 in field N. Subsequently, the population remained relatively stable until harvest in field M (see untreated controls), but it decreased within 30 days after planting in field N until it remained relatively stable until harvest M (Fig. 3). This means, during the period of agents' efficacy assessments, a relatively constant natural pest population was present. The overall average pest populations across time periods was not different between the two fields (independent samples t test, $t_{78} = -1.2$, $p = 0.26$).

EPN efficacy at reducing grubs

Overall, treatments reduced *Anomala* and *Hoplochelus* white grub numbers (GLM, averages pooled over season; $F_{7;79} = 2.4$; $p = 0.03$; adjusted $R^2 = 0.1$; Fig. 4). Variability of grub data between plots was high as well as between time steps (Fig. 4). Therefore, it was difficult to come to quantitative conclusions particularly when separately looking at grub values of each field and time step.

When standardising grub data to their respective controls and combining data of both fields, the control effects of treatments became obvious (Fig. 5). Biological and insecticidal treatments were effective in reducing grubs, sometimes at highly efficient rates. Biological and insecticidal treatments were comparably effective when considering overall effects along the entire season (multiple Tukey post hoc comparisons, all $p > 0.05$). Control effects (compared to untreated controls) were highest about 60 to 90 days after planting, but less at earlier stages as well as towards harvest time. Only the handpicking method did not significantly reduce white grubs (Fig. 5).

As for EPNs, effects were highest about 30 to 60 days after their application when they reached up to 87 and 96% control on average across fields (Fig. 4). In contrast, 7 days after treatment, hardly any effects of EPNs were observed. This is because EPNs needed time to find and kill the hosts. However, the 7-day assessment as a first sample time is a common practice to evaluate dynamics of EPN efficacy after application (Yan et al., 2013). But, it is not surprising that shortly after the EPN treatment, still no effects were found.

The Rwandan isolate *Steinernema* sp. RW14-M-C2b-1 reached 29 ± 33% efficacies after 30 days and 96 ± 3% after 60 days, an increase likely due to EPN propagation in the soil. The Rwandan isolate was at least as good as the international strain *S. longicaudum* X7 or even better (e.g. see at concentration of 0.75×10^9 IJs/ha in Fig. 4).

The international *S. longicaudum* X7 reached, at least at medium and high application concentrations, high control efficacies (77 ± 16 up to 85 ± 9% respectively about 30 days after application, and 95 ± 3% and 82 ± 17% after about 60 days). Also, the low concentration reduced grubs, but it took longer to reach effects (Fig. 5). As for other studies, *S. longicaudum* X7 is known to attack white grubs, such as *Holotrichia parallela* or *H. oblita* in peanut fields in China, and is used by growers for biological control purposes (Guo et al., 2013, 2015).

However, more field trials with more Rwandan EPN species and strains will be needed once the EPN-based biocontrol factory at RAB Rubona will run at full capacity. Particularly, both heterorhabditids, i.e. the Rwandan *H. bacteriophora* RW14-N-C4a and the international *H. bacteriophora* H06, need further investigation under field conditions. Both revealed promising infectiousness levels in the laboratory screenings. Moreover, *H. bacteriophora* is known to attack white grubs, such as *Popillia japonica* in turf grass (Klein and Georgis, 1992; Selven et al., 1993; Downing, 1994), and *H. parallela* and *H. oblita* in peanut fields and grasslands in China (Guo et al., 2013, 2015).

As for the insecticides, both were comparably effective on average over the season, this is 39 ± 35% for Avermectin + Chlorpyrifos and 27 ± 40% for Fipronil + Chlorpyrifos (independent samples t test, $t_{18} = 0.7$, $p = 0.49$). However, 37 to 60 days after transplanting, Avermectin + Chlorpyrifos reached efficacies of 58 up to 87%, whereas Fipronil + Chlorpyrifos only reached 4 to 44%. However, the pesticides are difficult to be compared to the EPN treatments due to different application types

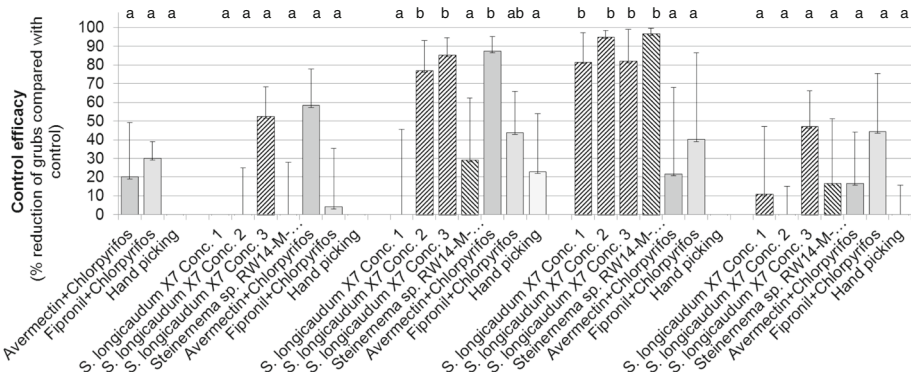

Fig. 4 Efficacy of entomopathogenic nematodes, insecticide tuber coatings and handpicking at reducing Anomala and Hoplochelus white grub numbers depending on time periods. EPN concentration 1 = 0.75×10^9 infective juveniles (IJs)/ha; concentration 2 = 1.5×10^9 IJs/ha; concentration 3 = 2.5×10^9 IJs/ha. Field M in Musanze and field N in Nyamagabe districts in Rwanda in 2015. Four plots per treatment per field. Different letters on bars indicate differences among different treatments at $p < 0.05$ according to multiple comparison Tukey post hoc range test after GLM. Error bars = SEM

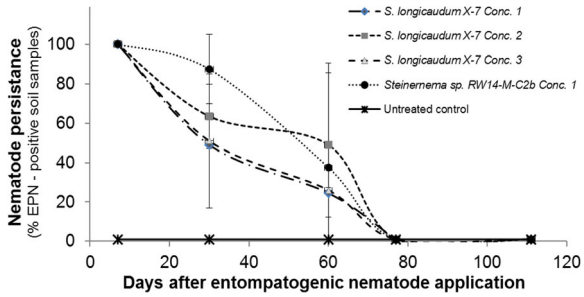

Fig. 5 Percentage of entomopathogenic nematode-positive soil samples collected from two experimental Irish potato fields in Rwanda treated with entomopathogenic nematodes. Nematode concentration 1 = 0.75×10^9 infective juveniles (IJs)/ha; concentration 2 = 1.5×10^9 IJs/ha; concentration 3 = 2.5×10^9 IJs/ha. Field M in Musanze and field N in Nyamagabe districts in Rwanda in 2015. Four plots assessed per treatment per field per time step. GLM revealed treatment effects, but Tukey multiple comparison did not detect differences between each treatment combination. Error bars = SD

(coating versus into-soil stream spray), application periods and water amounts. Thus, it is likely that the insecticides have caused effects earlier in the season, i.e. soon after they had been applied at the moment of planting. It needs to be, however, mentioned that Rwandan smallholder farmers do not have or have only limited personal protective clothing. Therefore, there is an acute risk that farmers are intoxicated (WHO, 2009) when coating tubers with pesticides, and therefore this is not advised. Chlorpyrifos is of WHO acute toxicity class II, thus moderately hazardous and Fipronil and Avermectin are slightly hazardous (WHO, 2009).

As mentioned above, the local common practice of handpicking did not significantly reduce white grub populations. This might be due to the fact that the grubs were collected only from a small proportional area of the field, such as from the opened furrow for planting. At that time period, grubs are still not aggregated around crop roots or tubers as there is still no crop. And later, during mechanical weeding and during earthing up of potatoes, only between-row soil was searched. But at this time, grubs are expected feeding on the tubers and roots. Consequently, handpicking likely misses a large part of the pest population; thus, the effect of this method is limited.

Considering factor "time" after planting and/or after EPN treatment the following effects on white grubs were found. Around 30 days after planting, the insecticide and handpicking treatment had no detectable effect on grub numbers (Kruskal-Wallis H test, chi square = 2, p = 0.37; Fig. 4). At 37 days after planting (7 days after EPN treatment), the factor "treatment" had no detectable effect on grub numbers (sqrt-transformed data, Univariate GLM, $F_{7;16} = 0.8$, $p = 0.6$; Fig. 4).

Around 60 days after planting (30 days after EPN treatment), treatments had reduced grub numbers (sqrt-transformed, Univariate GLM, $F_{7;16} = 3.6$, p = 0.045) as well as around 90 days after planting at day 60 after EPN treatment; GLM, $F_{7;16} = 4$, $p = 0.034$; Fig. 4).

At harvest, 128 days after planting (day 71 to 108 after EPN treatment), no effects of EPNs on grub numbers were detected anymore (sqrt-transformed, Univariate GLM, $F_{7;16} = 0.7$, $p = 0.67$) (Fig. 4).

EPN efficacy at preventing yield loss

The average yield was 0.3 ± 0.14 kg Irish potato tubers per plant, equalling about 9 to 10 tons per hectare. The yield was double in field M as in field N, that is, 0.4 ± 0.03 kg versus 0.2 ± 0.02 kg per plant, respectively (independent sample t test, $t_{62} = 11.8$, $p < 0.0001$).

However, treatments had no detectable effect towards an increased yield (GLM of yields compared to control, $F_{7;63} = 1.6$; $p = 0.15$).

EPN persistence

The Rwandan or international EPNs were recovered from the soil of the treated experimental plots, but no natural EPN population was detected in the untreated plots.

Both applied EPN species well-established in the soil, because soil samples from the treated plots were all found EPN-positive 7 days later (Fig. 5). Later on, EPN persistence decreased with time (Pearson correlation $r = -0.67$ with time, $p < 0.001$, $n = 40$). Multiple comparison tests at each time step did not reveal differences between the persistence of the two EPNs and their concentrations (too few data), but a slight overall treatment effect remained (e.g. GLM at 30 days, $F_{4;9} = 5.8$, $p = 0.041$; Fig. 5). *S. longicaudum* X7 persisted in the soil for at least 60 days after treatment in both fields. The Rwandan *Steinernema* sp. RW14-M-C2b1 persisted in the soil for at least 60 days in field N, whereas it was only detected until day 30 in field M. No EPNs were anymore detected towards time of harvest in both fields.

In summary, baited soil samples in our study proved that both the Rwandan and international EPN can establish in the soil after treatment. This indicates that the applied EPNs were of good quality as they need energy to survive some time in the soil until finding and propagating in the host insects. It moreover showed that the application method of EPNs into the moist just-opened soil furrow is a method appropriate for EPNs. Applications into the soil are known to be a good practice for EPN use as they prefer permanent moist conditions (Dutky, 1969). Also against other soil pests at a field scale, such as against the chrysomelid larvae of *Diabrotica* rootworms in maize, the fluid application into the soil had proven most suitable for EPNs compared with

onto-soil fluid row-applications or into-soil granule applications (Toepfer et al., 2010). Our results also showed that the applied EPNs persisted in the fields of Rwanda for at least 2 months, which indicates that they were able to propagate. After more than 3 months, i.e. during harvest time, no EPNs were detected anymore. This is a typical situation for crops with large surfaces of bare soil such as maize (Kurtz et al., 2007), or, as here, in wide-spaced senescent Irish potatoes. One season persistence is advantageous in case of commercialisation of the EPNs, as they would need to be more frequently applied. Short persistence is however less typical for vegetables or berries where EPNs persist longer (Burlando and Kaya, 1992), or grasslands or orchards where they can persist for years (Belair et al., 1994).

Conclusions

Most data of this study showed that there was variability within a field, between fields and between times steps. This is common for field trials, particularly when working with natural insect pest populations that are often aggregated. Moreover, the diversity of white grub species as well as their life cycles in Rwandan habitats is largely unclear, leading to different instars of different grub species in the soil. Due to the variability of data, it might be advised to field test the EPNs with higher plot numbers (at least six instead of four) at more locations in different Rwandan provinces and under different farming conditions as well as under different levels of natural white grub populations. This may lead to a broader view on the potential of the new Rwandan *Steinernema* sp. RW14-M-C2b-1 and the other isolates.

In conclusion, The Rwandan EPN *Steinernema* sp. RW14-M-C2b-1 is suggested to be used, in the first place, for white grub control in tubers and vegetables of Rwanda. As for the other Rwandan nematodes, more field trials are needed particularly with the heterorhabditids such as *H. bacteriophora* Rwanda14-N-C4a. It is expected that also these will work, or even better, if applied in the same way as presented here.

What remains is to consider whether indigenous nematodes need to be registered in Rwanda, and whether the EPNs should be mass produced either subsidised through governmental programs or through commercialization. Registration processes for macrobial biocontrol agents, such as EPNs, are currently not in place in Rwanda. The country may either follow the numerous countries that do not require any registration for indigenous EPNs as biocontrol agents, such as Germany, UK, or even not at the European Union level (Akhurst and Smith, 2002), or may require field efficacy trials and a formal registration approval by an expert committee, such as in Tanzania (J. Mwangi, Ministry of Agriculture, Food Security and Cooperatives MAFSC, 2015, pers. comm.) or in Kenya (KEPHIS, 2014). Discussion on setting up a Rwanda expert group for biocontrol agents is currently ongoing (B. Uwumukiza, MINAGRI Rwanda, pers. comm.).

Ultimately, we believe that EPNs, as here investigated, can deliver effective, safe and environmentally benign pest controls for soil-inhabiting pests to farmers.

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Authors' contributions

The whole team jointly planned the experiments. JK and AH contributed equally to this work. JK and HL played an additional role in coordinating the study. AH, BW and CU were particularly important for data collections. KL, DK and JY supported the field experiments. LC and XY particularly supported the nematode production, handling and application. BW and ST analysed the data. XY and ST wrote the manuscript with help of co-authors and are both corresponding authors. All authors read and approved the final manuscript.

Competing interests

Joelle Kajuga, Athanase Hategekimana, Bancy Waweru, Christine Umulisa from the Rwanda Agriculture Board and their farmer groups are representatives of the beneficiaries. The authors from the Rwanda Agriculture Board (RAB) agreed that the Rwandan EPN isolates can be maintained and researched outside Rwanda. The authors declare that they have no competing interests.

Publisher's Note

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