Universidade de Évora - Instituto de Investigação e Formação Avançada

Programa de Doutoramento em Biologia

Tese de Doutoramento

GLOBOWARNING - Mitigation of Globodera spp. outbreaks in Portugal through an innovative early nano-detection system and biocontrol

Maria João Santiago Militão Camacho

Orientador(es) | Manuel Galvão de Melo Mota

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The best is yet to come!

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Djebroune A., Chakali *G.*, de Andrade E., **Camacho M.J.,** Rusinque L. and Inácio M.L. (2021). Integrative Morphometric and Molecular Approach to Update the Impact and Distribution of Potato Cyst Nematodes *Globodera rostochiensis* and *Globodera pallida* (Tylenchida: Heteroderidae) in Algeria. *Pathogens* 10, 216. https://doi.org/10.3390/pathogens10020216.

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Novakovic Z., Khalife M., Costache V., **Camacho M.J.**, Cardoso S., Martins V., Gadjanski I., Radovic M. and Vidic J. (2024). Rapid detection and identification of vancomycin-sensitive bacteria using an electrochemical apta-sensor. *ACS Omega* 9(2), 2841–2849. https://doi.org/10.1021/acsomega.3c08219

GLOBOWARNING - MITIGATION OF *GLOBODERA* Spp. OUTBREAKS IN PORTUGAL THROUGH AN INNOVATIVE EARLY NANO-DETECTION SYSTEM AND BIOCONTROL

ABSTRACT

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are quarantine organisms which are subjected to stringent regulatory measures when detected. The incidence of PCN in Portugal is quite high and both species are present in all regions. Detections were mostly due to *G. rostochiensis*, but recently the situation has reversed. The overuse of potato cultivars resistant to *G. rostochiensis* led to a decline of this species population densities, but had no effect on *G. pallida*, which continued its reproduction without restrictions, since it doesn't have effective resistant cultivars, being now the most detected species. Thus, breeding of commercially attractive cultivars targeting resistance to *G. pallida* (with different R-genes), should be a priority.

Aiming a rapid detection, a Loop-mediated-isothermal-amplification (LAMP) *G. pallida* specific assay was developed. The primers designed for LAMP amplification specifically detected *G. pallida*, even in samples mixed with other species. Because LAMP requires cheaper equipment due to its isothermal amplification and all steps are conducted within one reaction-tube, it clearly holds potential for on-site detections.

Modern agriculture uses sensor to provide accurate data on crop growth. Therefore, specific DNA-probe and PCR-primers were designed to be used in a magnetoresistive-biosensing-device developed at INESC-MN to detect *G. pallida*. The device receives the target PCR-products (further labelled with magnetic-nanoparticles), which hybridize with the probe immobilized on the sensor surface, being detected due to a variation in the electrical resistance of the sensor.

To improve the on-site detection, the combination of the magnetoresistive-biosensing-device with an easy DNA extraction method (FTA-cards) and a rapid DNA isothermal-amplification (LAMP) was tested. These three technologies allowed the detection of *G. pallida* with a detection limit of one juvenile, even when mixed with other species. FTA-LAMP based biosensors show great potential for rapid in-field detections or at border phytosanitary inspection. Thus, this work provides insights for a new strategy to construct advanced devices for in-field diagnostics of plant pests/pathogens.

Keywords: Potato Cyst nematodes, LAMP, Lab-on-chip

GLOBOWARNING - MITIGAÇÃO DE SURTOS DE *GLOBODERA* Spp. EM PORTUGAL ATRAVÉS DE UM SISTEMA INOVADOR DE NANO-DETEÇÃO PRECOCE E BIOCONTROLO

RESUMO

Os nemátodos-de-quisto da batateira (NQB), *Globodera rostochiensis* e *G. pallida*, são organismos de quarentena, sujeitos a medidas regulatórias quando são detetados. A incidência de NQB em Portugal é bastante elevada, estando já presentes em todas as regiões. As deteções eram principalmente de *G. rostochiensis*, mas recentemente a situação alterou-se. A utilização de cultivares de batata resistentes a *G. rostochiensis* levou ao seu controlo populacional, não afetando *G. pallida*, que continuou a reproduzir-se por não haver cultivares totalmente resistentes a esta espécie, sendo agora a espécie mais detetada. Assim, o melhoramento de cultivares comercialmente atraentes e resistentes a *G. pallida* (com diferentes R-genes) deve ser prioritário.

Ambicionando uma rápida deteção, foi desenvolvido um método de amplificação isotérmica de DNA (LAMP) para *G. pallida*. Os primers-LAMP desenhados detetaram especificamente *G. pallida*, mesmo em amostras com juvenis de outras espécies. Como o LAMP requer equipamentos menos dispendiosos, devido à amplificação isotérmica, e as etapas poderem ser conduzidas num único tubo, possui grande potencial para deteções *in loco*.

Na agricultura moderna já se utilizam sensores para obter dados precisos sobre o desenvolvimento das culturas. Assim, foram desenhados primers-PCR e uma sonda, específicos para *G. pallida*, para serem testados num protótipo com biossensores-magnetorresistivos, desenvolvido no INESC-MN. Neste dispositivo, os produtos-PCR (posteriormente marcados com nanopartículas-magnéticas), hibridam com a sonda imobilizada na superfície do sensor, sendo detetados devido a uma variação na resistência elétrica do sensor.

Para aperfeiçoar a deteção *in loco* de *G. pallida*, foi testada a combinação da utilização de cartões FTA para extração de DNA, com o método LAMP e o protótipo do INESC-MN. Estas tecnologias permitiram a deteção de *G. pallida* mesmo em amostras com um juvenil ou em misturas com outras espécies. Os biossensores associados com FTA-LAMP mostram-se promissores para deteções no campo ou nos postos de controlo de inspeção fitossanitária. Este trabalho fornece assim novas estratégias para diagnóstico *in loco* de pragas/doenças das plantas.

Palavras-Chave: Nemátodes-de-quisto da Batateira, LAMP, Lab-on-chip

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LIST OF ABBREVIATIONS

AIC	Akaike Information Criteria
BIP (B1c + B2)	Backward Inner Primer
BLAST	Basic local alignment search tool
bp	Base pair
B3	Reverse outer primer
CAOP	Carta Administrativa Official Portuguesa
CC domain	Coiled-Coil domain
cDNA	Complement DNA
CYP	Cytochrome
DGAV	Direção-Geral de Alimentação e Veterinária
DGT	Direção Geral do Território
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
dsDNA	Double-strand DNA
DV/V_{sensor}	Differential voltage values
EDTA	Ethylenediaminetetraacetic acid
EPPO	European and Mediterranean Plant Protection Organization
FIP (B1c + B2)	Forward Inner Primer
FTA	Flinders Technology Associates cellulose cards
Fw:Rv	Forward:Reverse
F3	Forward outer primer
ΔG	Change in free energy of hybridization
GC	Guanine and Cytosine content
GMO	Genetically Modified Organism
Gpa	*Globodera pallida*
Gro	*Globodera rostochiensis*
HKY+G	Hasegawa-Kishino-Yano with Gamma Distribution
H1	Histone - resistance gene from potato
INESC-ID	Inst. Eng. de Sistemas e Computadores - Investigação e Desenvolvimento
INESC-MN	Inst. Eng. de Sistemas e Computadores - Microssistemas e Nanotecnologias
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
INL	International Iberian Nanotechnology Laboratory
IPM	Integrated Pest Management
ITS	Internal Transcribed Spacer
J1	Juveniles of the first stage
J2	Second-stage juveniles
LAMP	Loop-mediated isothermal amplification
LoD	Limit of Detection
LRR genes	Leucine-Rich Repeat genes
LSU	Large subunit
miRNAs	Micro RNA
ML	Maximum Likelihood
MNP	Magnetic nanoparticles

MR	Magnetoresistive
NA	Negative Agreement
NB-ARC	Nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4
NCBI	National Centre for Biotechnology Information
NPPO	National Plant Protection Organization
NTC	No template control
NVWA	Netherlands Food and Consumer Product Safety Authority, Wageningen
PA	Positive Agreement
PA	Precision Agriculture
PB	Phosphate buffer
PCN	Potato cyst nematodes
PCR	Polymerase Chain Reaction
PD	Positive Deviation
PDMS	Polydimethylsiloxane
PM	Phytosanitary Measures
PPN	Plant Parasitic Nematodes
Primers B	Primers Backward
Primers F	Primers Forward
Primers R	Primers Reverse
QTL	Quantitative Trait Loci
R-genes	Resistance genes
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNAi	RNA interference
RNAseq	RNA sequencing
rRNA	Ribosomal RNA
rt-PCR	Real time PCR
RT-PCR	Reverse transcriptase-PCR
sp.	single species of a genus
spl	SQUAMOSA-promoter binding protein-like
spp.	several species of a genus
ssDNA	Single-strand DNA
ssRNA	Single-strand RNA
SV	Spin-valve
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TIR	Toll-Interleukin Receptor
Tmelting, Tm	Melting Temperature

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CHAPTER I

GENERAL INTRODUCTION

1. POTATO CYST NEMATODES

Nematodes are a group of vermiform invertebrates that can be found in almost all environments, but only 25% of presently known species are parasites, out of which 15% are animal parasites and 10% are plant parasites - there are nearly 4100 species of plant parasitic nematodes (PPN) reported to date (Nicol, 2011). PPN, mostly small in size, have a specialized feeding structure - the stylet, which allows them to perforate roots, introduce enzymes into plant cells and extract their contents, causing substantial damage to the host plant, with a consequent reduction in productivity of the infested crop (Eisenback and Rammah, 1987; Coyne *et al*., 2007). Damage depends on a wide variety of factors, such as population density, virulence of the species and host resistance (ability of the plant to reduce nematode population) or tolerance (ability of the plant to yield despite of the nematode attack) (Coyne *et al*., 2007).

Cyst nematodes are an economically important group of PPN, present throughout the world, affecting all major horticultural crops. Cyst nematodes comprise approximately 100 species belonging to six different genera. However, the most economically important species belong to the genera *Globodera* and *Heterodera*, since they represent a greater and significant threat to several agricultural crops worldwide (Lilley *et al*., 2005; Jones *et al*., 2013).

Within the genus *Globodera* two species stand out, the potato cysts nematodes (PCN) *G. rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 - the golden potato cyst nematode and *G. pallida* (Stone, 1973) Behrens, 1975 - the pale potato cyst nematode. PCN are sedentary endoparasites of the potato root system, highly devastating to potato fields and are considered harmful quarantine organisms. These phytoparasites are regulated by the Commission Implementing Regulation (EU) 2022/1192 of 11 July 2022 and are part of the European and Mediterranean Plant Protection Organization (EPPO) List A2 - quarantine organisms already present in the EPPO region - A2/125 and A2/124, respectively (EPPO, 2022). PCN are described in the EPPO PM 7/40 (5) diagnostic protocol and are subject to stringent regulatory measures when detected alone or in combination (EPPO 2022a), to prevent further spread. The success of these protocols depends on the efficient detection and monitoring of cysts in soils and other substrates (Kimpinski *et al*., 1993).

Globodera rostochiensis and *G. pallida* are two of the major species limiting potato yield, which can cause yield losses of up to 80% (Haukeland, 2016). Though, other *Globodera* species have been reported associated with potato. In 2012, a new species of potato-associated cyst nematode, *G. ellingtonae*, Handoo, Carta, Skantar, Chitwood (2012) was discovered in Oregon (USA), but its importance to potato plants is not yet established (Handoo *et al*., 2012; Lax *et al*., 2014; Zasada *et al*., 2013; 2015). In 1997, a different *Globodera* species was detected in Portugal by Reis on *Chamaemelum mixtum* rhizosphere (Reis, 1997; Sabo *et al*., 2002) and re-detected in potato fields by Camacho *et al*. (2020). This species has never been described and additional research is needed to determine its pathogenicity and impact on potato. In Mexico*, G. mexicana*, (Campos-Vela, 1967) Subbotin, Mundo-Ocampo and Baldwin (2010) was described in 1967, which despite being stimulated by potato root exudates, does not have the ability to establish and develop in potato plants. However, it can be considered as a subgroup of *G. pallida* populations, as it is capable of mating with *G. pallida* and shares a high degree of genomic similarity (Grenier *et al*., 2002; Sabeh *et al*., 2019). More recently, Xu *et al*. (2023) identified *G. vulgaris* on potato crops, in China. Despite being closely related to *G. rostochiensis* (from a molecular point of view), this species exhibits differences in morphology and host preference. Pathogenicity tests have shown that even though potatoes are infested by *G. vulgaris*, it does not affect the crop (Xu *et al*., 2023).

In addition to Globodera species, 70 other species, representing 24 genera have been reported associated with reduced potato yields or quality of the tubers (Holgado and Magnusson, 2012). Among them, *Meloidogyne* (Meloidogynidae), *Pratylenchus*/*Nacobbus* (Pratylenchidae), *Ditylenchus* (Anguinidae), and *Nanidorus*/*Paratrichodorus*/*Trichodorus* (Trichodoridae) are the most destructive genera (Abrantes *et al.*, 2023).

1.1. Taxonomy

Globodera rostochiensis was initially described by Wollenweber, in 1923, as *Heterodera rostochiensis*, in Tessin (Mecklenburg, Germany), and having *Solanum tuberosum* L. as host. In 1973, Stone described a second species of PCN, *H. pallida*. Originally, thought to be a pathotype of *H. rostochiensis* (of the same species, but with different pathogenic capacity), later it would be described as *Globodera pallida* (Stone, 1973) Behrens, 1975.

The common taxonomic classification for the genus *Globodera* is (Hodda, 2022):

- Kingdom: Animalia;
- Phylum: Nematoda (Cobb, 1932);
- Class: Chromadorea (Inglis, 1983);
- Order: Panagrolaimida (Hodda, 2007);
- Family: Heteroderidae (Filipjev, 1934 (Skarbilovich, 1947));
- Genus: *Globodera* (Skarbilovich, 1959).

1.2. Distribution and economical importance

Globodera rostochiensis and *G. pallida* have originated in the Andes region, in southern Peru and have spread as the result of anthropogenic activity into many regions of the world (Grenier *et al*., 2010). Although potatoes were brought to Europe in the 16-17th century, PCN came likely later. They are thought to have been introduced into Europe in the mid-19th century by means of potato tubers carrying infested soil (Evans *et al*., 1975), but nowadays, PCN have a worldwide distribution. PCN have been reported throughout Europe, South America and parts of Asia, North America, Oceania and Africa where potatoes are grown (EPPO, 2020). They are important invasive pests in all regions where they can negatively impact potato yield and the economy, representing a threat to global food security (Silvestre *et al*., 2021). However, new PCN detections continue to be reported (Hafez *et al*., 2007; Mburu *et al*., 2018; Niragire *et al*., 2019; Inácio *et al*., 2020; Mwangi *et al*., 2021). The knowledge on the geographical distribution, density and spatial dynamics of pest populations is indispensable in integrated pest management (IPM) systems, as it raises considerable interest among plant pathologists for the need to better understand the interaction between pest or pathogen and host, the *Globodera* spp. regional range of expansion since their first report, and the estimate the risk.

PCN high infection rates can lead to 80% loss in crop productivity (Haukeland, 2016). Yield losses due to the presence of PCN are estimated at €220 million/year in Europe (Viaene, 2016), $US80 billion/year in the United States (Jones *et al*., 2013) and £50 million/year in the United Kingdom (Wale *et al*., 2008; Orlando, 2022). Damage can vary from slight losses to complete crop failure depending on the infestation level (Lima *et al*., 2018). Besides the decrease on potato yield, nematodes deteriorate the quality and commercial value of tubers, and contribute to infestation of potatoes by other opportunistic plant pathogens, such as fungi (Back *et al*., 2006). As PCN can

be devastating to potato fields if they are not controlled in a timely manner, causing significant crop losses, it is extremely important to adopt preventive control measures.

1.3. Biology – life cycle

Globodera rostochiensis e *G. pallida* life cycle begins with an egg stage inside the cyst (Figure 1A), three juvenile stages separated by three molts and the adult stage. Under normal conditions, it is completed in five to eight weeks, which corresponds to one generation for each host culture. The first molt takes place, forming the juveniles of the first stage (J1). Juveniles, after moulting to stylet-bearing second-stage juveniles (J2) are stimulated by the exudates of the host plant roots and hatch from the eggs (Figure 1B) (Perry and Beane, 1988, Lavrova *et al*., 2017). Temperature is another factor that also influences the emergence of juveniles (Franco, 1979). In *G. pallida*, J2 hatch at around 10 °C or less and develop at low temperatures between 10 °C and 18 °C, while *G. rostochiensis* seems to be better adapted to a range of temperatures from 15 °C to 25 °C (Franco, 1979). Other factors that can affect juveniles hatching are soil moisture, air circulation and pH (Shepherd and Clarke, 1971).

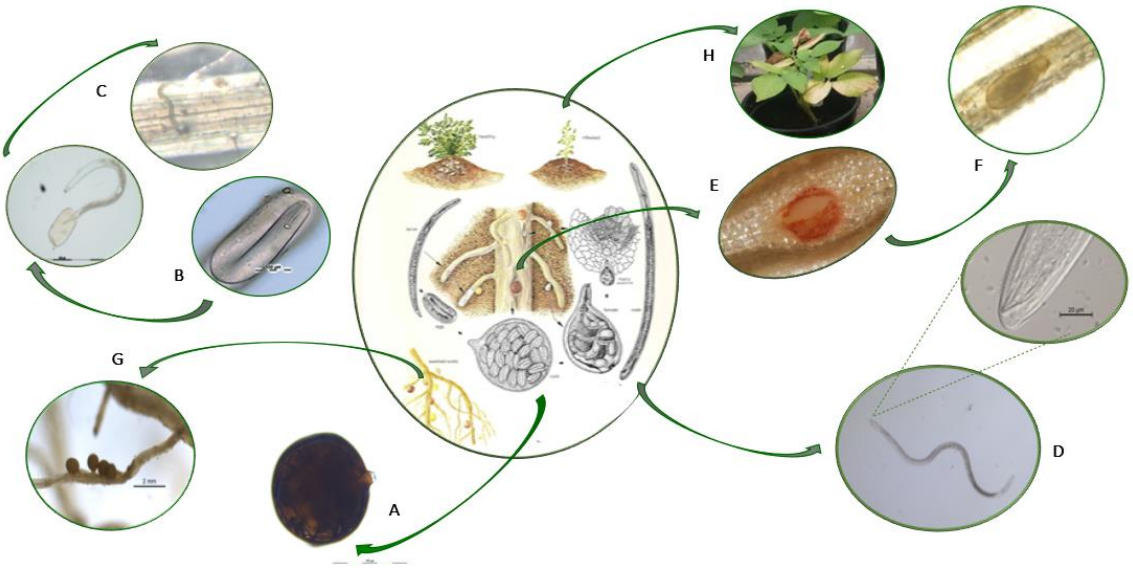

FIGURE 1 | Potato Cyst Nematodes life cycle (adapted from Papp, 2013) A) *Globodera* cyst, B) Juvenile inside the egg and juvenile hatching, C) Juvenile penetrating the root, D) Male and its spicule's position, E and F) Female breaking the root epidermis, G) Females with the anterior part of the body inside and the posterior part of the body outside the root, H) Symptoms are caused by PCN.

The freshly hatched juveniles are attracted to the host plant roots due to the presence of root exudates, which are released into the soil during root development (Perry, 1998). J2 penetrate the root (Figure 1C), while using the stylet and plant cell wall-degrading enzymes in stylet secretions, causing considerable damage to the plant cells along the migratory tracts (Lavrova *et al*., 2017). However, after being inside the cortex, J2 select a host cell to transform it into a metabolically active, multinucleate specialized feeding structure known as syncytium or syncytia (plural) (Sindhu, 2009), which will become their source of nutrients. At this point, the J2 become completely dependent on plant assimilates provided by the permanent feeding structure (Finkers-Tomczak, 2011). Later, the main stages develop into young males and females (EPPO, 2022a). After 4-6 weeks of feeding, males regain their mobility (Figure 1D) to inseminate the greatest number of females available, while in the meantime females secrete hormones to attract males (Green and Miller, 1969; Green and Plumb, 1970; Mugniery, 1979; Mugniery *et al*.,

1992). After fertilization, females increase in size due to the development of eggs and break through the epidermis of the root (Figures 1E and 1F), leaving the anterior part of the body inside the root and the posterior part of the body outside (Figure 1G). They are visible to the naked eye in the form of small globular structures - the cysts (Figure 1A). Females of *G. rostochiensis* are white followed by golden yellow while those of *G. pallida* are white or pearly white. When the eggs reach their complete development, females of both species die, and their body walls harden into brown and protective cysts that contain the fertilized eggs (Williamson and Hussey, 1996). Inside cysts, the embryos continue their development into first and second stage juveniles, which go into dormancy (Finkers-Tomczak, 2011). A cyst can contain between 100 and 500 eggs, and juveniles inside the eggs can hatch immediately or remain in a latent state and retain their reproductive capacity for many years. Some juveniles inside the eggs can survive within the cyst for 30 years, although at that time few may be viable (Perry and Beane, 1988). Ontogenesis of the nematode ends at the stage of cyst unrelated to plant roots, situated freely in the soil. These biological features of the nematode ensure its close interrelationship and synchronization of its lifecycle with the main developmental stages of the host plant (Lavrova *et al.,* 2017). When environmental conditions are favorable again, PCN begin a new cycle.

1.4. Host range and symptomatology

The host range of PCN is limited to plants of the Solanaceae family. More than 170 species of Solanaceae are potential hosts for PCN, however, the most economically significant hosts are potato (*Solanum tuberosum* L.), tomato (*S. lycopersicum* L.) and aubergine (*S. melongena* L.) (Sullivan *et al*., 2007). Additionally, numerous weeds are known to be hosts of these nematodes, such as bitter nightshade (*S. dulcamara* L.), black nightshade (*S. nigrum* L.), hairy nightshade (*S. sarrachoides* (L.) Sendtner), jamestown-weed (*Datura stramonium* L.) and silverleaf nightshade (*S. elaeagnifollum* Cav.) (Boydston *et al*., 2010).

The damage to potato production depends on the density of PCN present in the soil and the ability of the plant to tolerate the effects of the attack. During the growing season, the presence of PCN in the infested crops is reflected in the observation of declining patches, in which the plants have an atypical leaf discoloration and reduction in size, mainly during the hottest hours of the day. The leaves at the base of the stem become wilted, the upper leaves curl and show brown spots on the margins of the leaflets (Figure 1H). The roots may have brown lesions, irregular branching, a proliferation of lateral roots or evidence of advanced decay. A higher weed density can be observed, due to the plant infested by the nematode being less able to compete with the weeds. The tubers of attacked plants are smaller and fewer in number and on the surface small lesions can be seen, making them unmarketable and compromising the crop yield. Plants may also senesce prematurely and may have a higher incidence of other diseases, due to the suppressed resistance of plants infested by PCN (Coyne *et al*., 2007; EPPO, 2022a).

However, it is important to point out that these symptoms are not specific to nematode infection and may be mistaken with symptoms of other abiotic or biotic stresses. To confirm that these symptoms are caused by PCN, cysts must be observed on the host roots or must be detected in soil samples at nematology labs (Coyne *et al*., 2007).

1.5. Transmission

Cysts are highly resistant and an easily transportable stage in the life cycle of PCN, and can be found among soil particles, in the host roots and tubers. The microscopic size of the cyst makes

it difficult to detect in infested soil and facilitates its dissemination. The spread of these nematodes over great distances is ensured mainly through infested soil or tubers, plant roots, footwear, agricultural machinery and packaging. However, natural events such as wind, rain and runoff are also responsible for the dispersion of viable cysts (EPPO, 2022a). In addition, cyst nematodes are known to pass through the digestive tract of animals grazing in infested fields and are excreted intact in a viable condition ready to begin new infestations. Animal hooves, if carrying debris from infested soil, can also carry cysts from one area to another (Banks *et al*., 2012).

As human activity is the most probable mean of spreading PCN, there is a specific interest in the evaluation of the implemented control measures and their consequences to adopt more effective management practices.

2. CONTROL METHODS

Controlling PCN is a difficult task due to their high level of adaptation to new environments, the prolonged viability of juveniles inside the cysts in the absence of the host plant, either quiescent or in diapause in the form of encysted eggs (Christoforou *et al.,* 2014) or the risk of aggressive pathotypes appearance (a group of organisms of the same species that have the same degree of pathogenicity on a specified host) in the monoculture of nematode-tolerant potato cultivars (Matveeva, 2004). To assess the prevalence and distribution of PCN species across the territory, surveys were established in 2007, as set in the Council Directive 2007/33/EC on the control of potato cyst nematodes and repealing Directive 69/465/EEC, of 11 June 2007, outlining a new framework for phytosanitary protection measures against these harmful organisms to avoid dispersion in European Community territorie1Cs and to ensure potato production of a guaranteed quality for consumers. The main purpose of adopting preventive control measures is to maintain population densities below damage thresholds (Orlando and Boa, 2023).

In the case of a PCN positive detection, farmers must choose among different phytosanitary measures. An integrated approach to PCN management is usually based mainly on traditional methods (cultural practices, chemical treatments, solarization and biofumigation) and on the use of resistant cultivars.

Cultural practices include non-host crop rotation, uncultivated land for a quarantine period or trap-crops. Decline in *G. rostochiensis* and *G. pallida* populations usually ranges from 20 to 40% under non-host crops or fallow (La Mondia, 1986; Mugniery *et al*., 1984). On the other hand, trap crops can be used to promote hatching of juveniles without allowing the reproduction of the next generation, since the crop can be eliminated before nematode maturation. This technique has already been verified to reduce *Globodera* spp. by 80% (Dandurand *et al*., 2019), and has great potential for the biological market. However, if culture disposal is left too late, PCN density may increase.

Chemical treatments can be an effective method for PCN control and eradication. However, nematicides are normally not carried out because European legislation is very restrictive in the use of nematicides on European soil, and many of the chemicals previously used to control nematodes have already been banned or are in the process of being phased out, mostly due to environmental and health concerns (Desaeger et al., 2020). Additionally, G. pallida is more difficult to control through this method since it has a slower and longer hatching period, remaining in the soil after the degradation of the nematicide (Varandas *et al*., 2020).

Solarization is a physical method for the control of nematode populations. The soil is covered with two layers of polyethylene, allowing the soil underneath to heat up quickly. Mani *et al*. (1993) found that 62 days of solarization reduced *G. rostochiensis* population density by 95%. Concerning biofumigation, plants from the *Brassicaceae* family have received much attention due to their suitable biofumigant effect caused by a series of compounds (isothiocyanates) released by these plants, which inhibit the motility of *G. pallida* and induced over 95% mortality of encysted eggs of *G. pallida* (Lord *et al*., 2011).

Other option available is biological control, which takes benefit of the use of fungal and bacteria biocontrol agents, that developed a wide range of strategies against plant parasitic nematodes. In terms of fungal biocontrol, *Pochonia chlamydosporia* has shown positive results against *G. pallida* eggs (Vieira dos Santos *et al*.,2019). On the other hand, several bacteria, such as Bacillus *cereus*, *B. pumilus*, *B. subtilis*, *Priestia flexa* (basionym: *B. flexus*), *P. megaterium* (basionym: *B. megaterium*), have a potential nematicide effect against *G. rostochiensis* (Widianto et al., 2021). The use of these biological agents is recommended as part of an IPM with other control methods (Varandas *et al*., 2020; Pires et al, 2022).

2.1. Resistant cultivars

Despite cultural control methods, there is an urgent need for novel approaches for controlling PCN since "traditional" methods were not, until now, full-effective solutions (Cotton *et al*., 2014). For instance, nematodes spend most of their life either inside plant roots or in the protective cyst, thus not accessible to pesticides. Additionally, dormant juveniles in the cysts persist for many years in the soil in the absence of a host plant, which makes crop rotation not so effective either (Finkers-Tomczak, 2011). Presently, the best control strategy for PCN is the use of resistant cultivars (Gartner *at al*.,2021), although there are insufficient potato cultivars available with full resistance to *G. pallida*. Most of the available cultivars are resistant to *G. rostochiensis* (Madani *et al*., 2010) and its intensive and generalized use may be responsible for the recent increase of *G. pallida* detections (Santos *et al*., 1995; Minnis *et al*., 2002; Hearne *et al*., 2017; Camacho *et al*., 2017; 2020). Classification of PCN pathotypes is based on the ability of nematodes to reproduce on resistant cultivars. Within a host species, there are distinct cultivars that differ in their susceptibility to diverse pathotypes of PCN (Kort *et al*., 1977). This differential susceptibility of host plant resistance is the basis for controlling the PCN.

PCN control will be significantly enhanced by a greater understanding of the interaction between those nematodes and the potato cultivars (Mantelin *et al*., 2017; Gartner *at al*., 2021). The interactions between plant-parasitic nematodes and their hosts are both complex and specific (Eves-van den Akker *et al*., 2016). Plant hosts are protected from nematodes by the activities of a complex and multi-level immune system and the immune response to infestation is a stage-by-stage activation of protective processes:

. The plant sensory system is the first to react to pathogen intrusion, initiating the plant's defensive response (innate immunity). Membrane receptors recognize the nematode molecular structures and participate in the development of nonspecific immunity. Nonetheless, the evolution of parasitic organisms is aimed at surmounting plant defences and establishing transport mechanisms for protein effectors (which are products of avirulence genes) to access plant cells (Lavrova *et al*., 2017);

. The second line of defence is an activation of the plants' signal system. Hosts have developed resistance genes (R-genes) encoding immune receptors that recognize nematode effectors

(Jones and Dangl, 2006; Sacco *et al*., 2009; Gartner *at al*., 2021) and lead to triggering a cascade of protective physiological reactions. The activation of nematode resistance genes results in hypersensitive reactions occurring in or near the feeding sites induced by nematodes, known as syncytium. These responses halt or reduce the nematodes' ability to feed, leading to either their death or transformation into males, which require less nourishment (Abramovitch *et al*., 2006; Jones and Dangl, 2006; Metraux *et al*., 2009) or do not require a sustained viable syncytium to reach maturity as they do not produce eggs (Rice *et al*., 1985; Sobczak *et al*., 2005; Gartner *at al*., 2021). This immune response depends greatly on the ability of a plant to modulate its transcriptome rapidly and specifically, for example to change the expression of genes related to the development of protective reactions in response to infestation (Katagiri and Tsuda, 2010; Zhang *et al*., 2013). These interactions are mediated by effector proteins responsible for a variety of processes, many of which have been acquired by horizontal gene transfer from other taxa (Smant *et al*., 1998; Wang *et al*.,2005; Danchin *et al*., 2010; Haegeman *et al*., 2011; Lee *et al*., 2011; Cotton *et al.,* 2014; Eves-van den Akker *et al*., 2014; 2016; Mei *et al*., 2015; Kikuchi *et al*., 2017; Gartner *at al*., 2021). Nematodes may interact with plant enzymes and transcription factors likely triggering gene expression alterations both at the level of protein-coding and protein non-coding genes as miRNAs (Baum, 2014).

Parasitism proteins, injected by nematodes into initial feeding cells, appear to provide many of the stimuli that result in the dramatically altered host gene expression observed in nematode-infected plant roots (Gheysen and Fenoll, 2002; Puthoff *et al.*, 2003; Jammes *et al.*, 2005; Ithal *et al.*, 2007*a*, *b;* Thorpe *et al*., 2014). Understanding this phenomenon has revealed RNA interference (RNAi) as a powerful tool to manipulate gene expression and to analyze gene function (Fire *et al.*, 1998).

To provide new methods for controlling PCN, a great deal of effort has been put into various approaches for effector identification due to the importance of effectors in plant-parasitic-nematode life cycle, including genomic and transcriptomic analyses (Thorpe *et al*., 2014), transcriptomic analyses of purified gland cells (Maier *et al*., 2013) and proteomic analyses (Bellafiore *et al*., 2008). However, the concerted action of many different effector functions in nematode parasitism is not well understood. Only a few of these effectors have been functionally characterized in sufficient detail (Gao *et al*., 2003). The great motivation for work on effectors is to provide new methods for controlling PCN (Mantelin *et al*., 2017).

Ultimately, understanding the mechanisms underlying the co-evolution between host plant resistance and nematode (a)virulence is essential to obtain durable resistance and even to genetically engineer new resistance specificities (Finkers-Tomczak, 2011) and develop durable crop protection strategies. However, exploiting natural resistance is complex and requires knowledge about their origin (taxonomy), the way they are inherited (genetics), the underlying genes (molecular biology), and their diversity and organization in the genome (genomics).

Fourteen PCN R-gene *loci* have been mapped on eight linkage groups in potato, on chromosomes III, IV, V, VII, IX, X, XI and XII (reviewed by Gebhardt and Valkonen, 2001; Caromel *et al.,* 2003; 2005). These resistances were originated from wild potato species like *S. tuberosum* ssp. *andigena* (*S. vernei* Bitter & Wittm*., S. spegazzinni* Bitter*), S. tuberosum* ssp. (*S. tuberosum* L.*, S. tarijense* Hawkes *and S. sparsipilum* (Bitter) Juz. & Bukasov) (reviewed by Finkers-Tomczak *et al.,* 2009).

These PCN R-gene *loci* grant varying levels of resistance, ranging from partial to nearly absolute, against different pathotypes or virulence groups of PCN populations (Finkers-Tomczak, 2011; Gartner *at al*., 2021):

. partial resistance:
Gro$^{1.2}$, Gro$^{1.3}$, Gro$^{1.4}$ (Kreike *et al*., 1993; 1996; Park *et al*., 2019); Gpa^{IV} (Bradshaw *et al*., 1998), Gpa$^{IV}_{adg}$ (Moloney *et al*., 2010); Gpa (Kreike *et al*., 1994; Caromel *et al*., 2005); Gpa^{V} (Rouppe van der Voort *et al*., 2000; van Eck *et al*., 2017); Gpa ^{VI} (Rouppe van der Voort *et al*., 2000; Bryan *et al*., 2002); Gpa^{4}, Gpa^{5}, Gpa^{6} (Rouppe van der Voort *et al*., 2000); Grp^{1} (Rouppe van Der Voort *et al*., 1998; Finkers-Tomczak *et al*., 2009); Gpa^{M1}, Gpa^{M2}, Gpa^{M3} (Caromel *et al*., 2003); Gpa$^{XII}_{tar}$ (Tan *et al*., 2009); Ro^{2_A}, Ro $^{2\_B}$, Pa$^{2/3_A}$, Pa $^{2/3\_B}$ (Park *et al*., 2019); H2 (Phillips *et al*., 1994; Strachan *et al*., 2019) and H3 (Bryan *et al*., 2004);

. nearly absolute resistance:
H1 (Gebhardt *et al*., 1993; Kreike *et al*., 1993; Pineda *et al*., 1993; Bakker *et al*., 2004; Finkers-Tomczak, 2011); Gro^{VI} (Jacobs *et al*., 1996); Gro$^{1-4}$ (Barone *et al*., 1990; Ballvora *et al*., 1995; Paal *et al*., 2004) and Gpa^{2} (Rouppe van der Voort *et al*., 1997; van der Vossen *et al*., 2000) and the combination of Gpa$^{V}_{spl}$ and Gpa$^{XI}_{spl}$ (Caromel *et al*., 2005).

Many of these PCN resistance *loci* are mapped in regions of the potato genome where clusters of resistance gene homologs are located. This is not only true for the single dominantly inherited PCN R-genes Gpa^{2} and Gro$^{1-4}$ (Barone *et al.,* 1990; Rouppe van der Voort *et al*., 1997), but also for quantitative trait loci (QTL) such as Grp^{1}, Gpa, Gpa$^{V}_{spl}$, Gpa^{M1} and Gpa^{5} (Kreike *et al*., 1994; Rouppe van Der Voort *et al*., 1998; Rouppe van der Voort *et al*., 2000; Caromel *et al*., 2003; Caromel *et al*., 2005). Some of nematode R-genes that have been characterized at the molecular level in potato are Gpa^{2} (Paal *et al*., 2004; Sacco *et al*., 2009) and Gro$^{1-4}$ (van der Vossen *et al*., 2000). These R-genes belong to the super family of nucleotide-binding – leucine-rich repeat (NB-LRR) genes (Ellis *et al*., 1999; Meyers *et al*., 1999, Gartner *at al*., 2021), representing the largest class of R-genes in plants. Based on the N-terminal part, NB-LRR proteins can be further subdivided into two classes containing either a coiled-coil (CC) domain (Gpa^{2} is a CC-NB-ARC-LRR gene) or a Toll-Interleukin receptor (TIR) homologue domain (Gro$^{1-4}$ belongs to the class of TIR-NB-ARC-LRR proteins) (Paal *et al*., 2004; Finkers-Tomczak, 2011). Gpa^{2} genes are able to recognize the *G. pallida* effector proteins and activate a hypersensitive response, inducing cell death through necrosis (Sacco et al., 2009) and Gro$^{1-4}$ genes confer resistance to all pathotypes of *G. rostochiensis* (Paal et al, 2004). None of the other important PCN resistances have yet been isolated, despite significant efforts to do so (Gartner *et al*., 2021).

Monogenic resistances are desirable for breeding purposes because of their simplicity in being introgressed. Unfortunately, in many plant–pathogen interactions, this monogenic resistance can be broken down relatively fast due to alterations in the co-evolving pathogen. Remarkably, in potato, QTL conferring resistance to the PCN often co-localize with hot spots for single dominant R-genes, suggesting that they may contribute to partial resistance to nematodes. Another view suggests that quantitative resistance is mediated by an R-gene but the PCN populations used to screen for resistance consist of a mixture of virulent and avirulent genotypes (Finkers-Tomczak, 2011),

While *G. rostochiensis* resistance is conferred by a single gene (H1) which is present in many new cultivars, *G. pallida* has no single gene conferring full resistance, so only partially resistant cultivars are available. H1 has been introgressed into many commercially available cultivars and

even today, after many decades of use, it is still effective against *G. rostochiensis*. It confers resistance by triggering a hypersensitive response in a layer of cells surrounding the feeding site, which leads to the degeneration of the syncytium (Rice *et al*., 1985). For *G. pallida*, the breeding process is more complex due to the greater genetic diversity of *G. pallida* populations and a high number of virulence groups (Gartner *at al*.,2021).

When assessing the practical utility of resistance genes, is crucial to determine their potential transferability to economically significant cultivars that lack comparable resistance. Currently, achieving successful transfers of functional R-genes to other species has been somewhat limited (Williamson and Kumar, 2006). For example, the tomato gene Hero (Ernst *et al*., 2002), which confers resistance to both PCN, was not effective in potato according to Sobczak *et al.* (2005). Understanding this phenomenon will be challenging, but it seems to be necessary for a successful transfer of nematode resistance to a different species (Finkers-Tomczak, 2011).

Determination of transcript profiles and patterns or identification of differentially regulated genes in plants can be performed by various procedures such as DNA microarrays, serial analysis of gene expression, cDNA fragment sizing combined with amplified fragment length polymorphism, differential display, differential screening of cDNA libraries, expressed sequence tag sequencing or massively parallel signature sequencing (Öktem *et al*., 2008).

Transcript profiling, in the area of plant-pathogen interactions, has provided insight to the mechanisms underlying specific gene resistance and basal defense, host/non-host resistance, biotrophy/necrotrophy, and pathogenicity of vascular/nonvascular pathogens, among many others. In this way, genomic technologies have facilitated a system-wide approach to unifying themes and unique features in the host-pathogen interactions (Wise *et al*. 2007). DNA microarrays have been intensely used to investigate plant transcriptomes to answer various biological questions involving tolerance to biotic diseases or abiotic stresses, germination, growth and development, fertilization, flowering and nutritional requirements, toxicity or deficiency (Öktem *et al.,* 2008). Further genomics, proteomics and metabolomics studies are necessary to enlighten whole cellular processes related with biotic and abiotic stress tolerance in plants and to understand how the components such as genes, proteins and metabolites work together to comprise functioning plant cells.

Until now, microarrays were frequently used to test large numbers of genes simultaneously for the selection of good reference genes. However, this technique requires previous knowledge of the nucleotide sequences of each candidate gene. With the advent of next-generation sequencing, especially, RNA sequencing (RNA-Seq), this problem has solved this issue, enabling the routine quantification of numerous gene transcripts even without prior knowledge of their gene sequences. The RNA-Seq method yields millions of reads that can be assembled to generate a transcript database, which contains the sequences and expression levels of all expressed genes at a given time. Because RNA-Seq is quantitative, it can be used to study DNA expression (Nagalakshmi *et al*., 2008; Wang *et al*., 2009). Therefore, analyzing RNA-Seq data from different potato cultivars under invasion of the roots with *G. rostochiensis* and *G. pallida* should allow the identification of R-genes for further plant breeding, a strategy to control potato cyst nematodes (Sabeh, 2018).

In a word, resistant cultivars raise considerable interest among plant breeders and plant pathologists for the need to better understand the interaction between PCN and their hosts and

to estimate the risk of crop damage (Camacho *et al*., 2020). Resistance ensures the reduction of nematode population levels and tolerance ensures reliable crop yields, even when the initial PCN levels are high (Blok *et al*., 2018; Gartner *et al*.,2021).

3. PCN ISOLATION AND IDENTIFICATION

Due to their huge economic and trade impact, it is crucial to timely and accurately distinguish the different PCN species to plan and implement strategies for an effective IPM (Subbotin *et al*., 2013; Camacho *et al*., 2017). Regular surveys should be performed in fields that had or will have potatoes. A visual analysis can be done to check for the presence of cysts on the roots, as young females and cysts are visible to the naked eye as tiny white, yellow or brownish dots on the surface of the root. However, detection by uprooting the plants is problematic, as the cysts can be easily missed during the survey operation. Soil analysis is therefore the best way to determine the presence of PCN (EPPO, 2022a).

3.1. Extraction methods

The different methods for PCN extraction take into account different aspects: size, shape, mobility and the specific density of nematodes, being most of them described in the EPPO diagnostic protocol PM 7/119 (1) (EPPO, 2013). In recent years, some studies have been carried out to test the efficiency of various methods of cyst nematode extraction, but few results have been published (Bellvert *et al*., 2008; Den Nijs and van den Berg, 2012; Kumar *et al*., 2012; Camacho *et al*., 2018).

Cyst extraction techniques rely on the principle that dry cysts contain air, causing them to float on water. For an efficient recovery of cysts, the soil must be completely dry and pass through a 4 mm mesh sieve to remove the coarser material. To obtain a representative sample, a dry soil amount ranging from 100 to 500 g is used (Marks and Brodie, 1998; van Bezooijen, 2006). Samples can undergo drying at room temperature or in a kiln, a crucial step for diagnostic precision. Inadequate drying can potentially result in false negative outcomes. Subjecting the soil to excessively high or abrupt temperatures during drying can harm the viability of the cyst contents. Therefore, it is important to avoid drying soil samples at temperatures exceeding 30 °C. Additionally, gentle air circulation also accelerates the drying process. Depending on conditions, the soil will dry within 2– 4 weeks. (EPPO, 2013, EPPO,2022a). The Fenwick method or its variations are widely used for the extraction of cysts from dried soil (Fenwick, 1940; Oostenbrink, 1950; EPPO, 2013). Unfortunately, young, full cysts do not float very well and can be lost; hence the total population can be underestimated (EPPO, 2013).

3.2. Morphological identification

The identification of *G. rostochiensis* and *G. pallida*, by morphological and molecular methods, is described in the EPPO protocols PM 7/40 (5) *Globodera rostochiensis* and *Globodera pallida* (EPPO, 2022a). Additionally, there is a lot of published literature that can be a good source of complementary information on EPPO protocols.

Morphological identification, based on a few characters of the J2 (Figures 2A and 2B) and of the perineal area of the cyst (Figure 2C), has been quite successful but always carries some uncertainty (Bačić *et al*., 2013; Seesao *et al*., 2016; Tirchi *et al*., 2016; EPPO, 2022a). Therefore, due to the variability of the main morphological features and the overlapping of standard

diagnostic parameters in these two species, confirmation through molecular methods is recommended (Subbotin *et al*., 2013; Camacho *et al*., 2017; EPPO, 2022a). The identification protocol should preferably combine morphological and molecular methods, especially when new introductions are suspected. Molecular methods have been successfully applied to differentiate *G. rostochiensis* from *G. pallida*.

Second-stage juveniles (J2) are vermiform and tapering at the ends (Figure 2A), the body cavity extends to the anus and ends in a conical tail. The head is offset and rounded. The stylet is strong (Figure 2B), and the basal bulbs are projected posteriorly in *G. rostochiensis* and projected anteriorly in *G. pallida*. The medium bulb (metacorpus) is well developed, has an elliptical shape and a large central valve. The nerve ring is located around the esophagus, the excretory pore is approximately 110 µm from the head and the hemizonoid (sensory organ) is located posterior to the excretory pore. The genital primordium is located 60% of the length of the body from the tip of the head (EPPO, 2022a). The common morphometric parameters used to differentiate the second-stage juveniles of these two species are shown in Table 1.

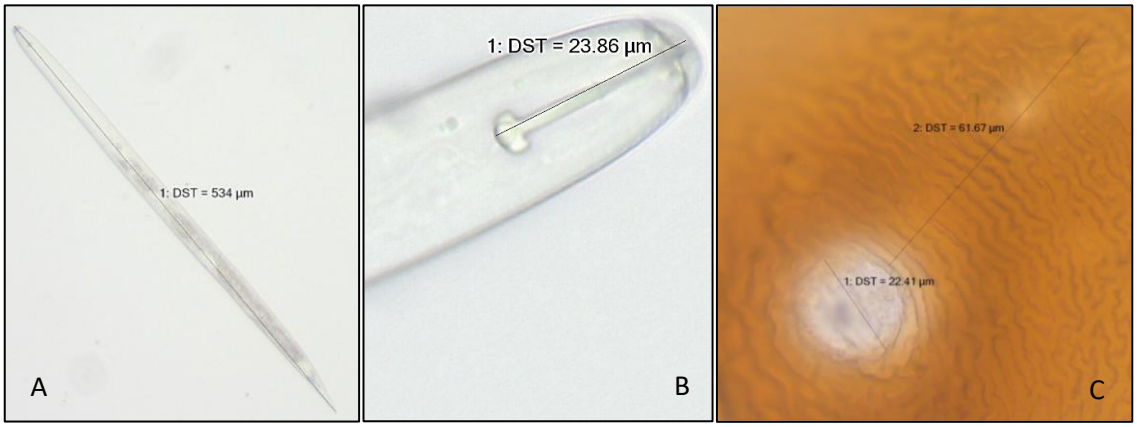

FIGURE 2 |Morphometric parameters for *Globodera rostochiensis* and *G. pallida* A) Body length, B) Stylet length, C) Vulva diameter and the vulva-anus distance.

Table 1 | Morphometric parameters for second-stage juveniles of *Globodera rostochiensis* and *Globodera pallida* (EPPO, 2022a)

Parameter	*G. rostochiensis*	*G. pallida*
Body length	468 (425– 505) µm	484 (440– 525) µm
Knob width	3 - 4 µm	4– 5 µm
Knob shape	Rounded to anteriorly flattened	Distinct forward projections
Stylet length	21.8 (19 – 23) µm	23.8 (22– 24) µm

The PCN adult female has an almost spherical body from which the neck and head emerge, without a terminal cone (Figure 1A). At the posterior pole, opposite to the neck, is the vulval–anal region (Figure 2C), a more translucent area of the cuticle, where the vulvar slit is located. As it has only one almost circular hole, it is characterized as 'circumfenestrated'. The anus is often observed at a point on the cuticle where the "V" shape decreases to a final point. The pattern and cuticular ridges found in the area between the anus and the tip of the vulva contribute to the identification of *Globodera* species (EPPO, 2022a).

The morphometric parameters commonly used to differentiate the cyst of *G. rostochiensis* and *G. pallida* are shown in Table 2.

Table 2 | Morphometric parameters of cysts of *Globodera rostochiensis* and *Globodera pallida* (EPPO, 2022a)

Parameter	*G. rostochiensis*	*G. pallida*
Number of cuticular ridges between anus and vulval basin	12 – 31b (usually >14)	8– 20 (usually <14)
Granek's ratio*	1.3 – 9.5 (>3)	1.2 – 3.5 (<3)

* The ratio between vulva-anus distance and diameter of vulval basin

3.3. Molecular identification

The most accurate and reliable identification of PCN is based on various DNA-based molecular approaches. These methods enable reliable and rapid identification of *G. rostochiensis* and *G. pallida* and their differentiation from each other and from closely related cyst nematode species. The available methods have already been validated through interlaboratory studies, meaning that there are performance criteria available and confirmation that the analytical procedure is suitable for its intended use (EPPO, 2022a).

Currently, PCN molecular identification is routinely performed through conventional PCR and real-time PCR (rt-PCR) based on protocols described in the EPPO protocols PM 7/40 - *Globodera* rostochiensis and *G. pallida* (EPPO, 2022a). The molecular tests recommended by the EPPO for the identification of isolated cysts or individuals of *G. rostochiensis* and *G. pallida* are (EPPO, 2022a):

. Multiplex rt-PCR test (Gamel *et al*., 2017);
. High-throughput diagnosis of PCN (*Globodera* spp.) in soil samples using rt-PCR (Reid *et al*., 2015);
. rt-PCR tests for species-specific identification as well as detection of *G. rostochiensis*, *G. pallida* and *G. tabacum* (based on LSU rDNA) available as an all-inclusive rt-PCR kit (www.clear detections.com);
. A multiplex PCR test using species-specific primers based on ribosomal 18S and ITS1 sequences (Bulman and Marshall, 1997);
. An internal transcribed spacer (ITS)- RFLP PCR test based on primers described by Vrain *et al*. (1992) (Thiéry and Mugniéry, 1996);
. A Taqman® rt-PCR targeting the internal transcribed spacer I (ITSI) gene (Fera);
. Identification of viable PCN (*Globodera* spp.) using RNA-specific RT-PCR (Beniers *et al*., 2014).

Although the sensitivity and specificity of these diagnostic assays are sufficiently high when they are properly applied, the procedures are time-consuming, require well-trained technicians and expensive laboratory equipment and cannot be performed in the field because of the lack of convenient portable instruments (Kogovšek *et al*., 2015). In consequence of the PCR-based protocols downsides, other methods have been developed.

4. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

Loop-mediated isothermal amplification (LAMP), developed by Notomi *et al*. in 2000, is a single tube technique for the amplification of nucleic acid, using four to six primers that target six to eight locations within a given DNA sequence, under isothermal conditions (60–65°C). It yields large amounts of products in a short time (30 to 60 minutes). LAMP amplified products can be visualized by gel electrophoresis, by a visible by-product (colorimetric detection) or by

measuring the fluorescence emitted by DNA intercalating dyes. Because LAMP does not require an expensive thermocycler (is used chemical denaturation of DNA instead of thermic denaturation at 95 ºC) and an optical detection equipment, is less sensitive to amplification inhibitors and all steps are conducted within one reaction tube, it clearly holds potential for testing in the field, allowing for precise, sensitive, specific and cost-effective early detections (Peng *et al*., 2012). Due to its speed, robustness and simplicity, the use of LAMP is gaining popularity for diagnostics in plant health (Tomlinson *et al*., 2010; Bekele *et al*., 2011; Hodgetts *et al*., 2011; Camacho *et al*., 2021).

LAMP assays have been developed to detect viruses (ssRNA and ssDNA), bacteria, fungi and nematodes, and some detection protocols are already validated, such as (EPPO, 2011):

. LAMP assay for detection of "flavescence dorée" phytoplasma;
. LAMP primer-sets for the detection of the whitefly transmitted Criniviruses, *Tomato chlorosis virus* (TOCV), *Tomato infectious chlorosis virus* (TICV), *Potato yellow vein virus* (PYVV) in white flies and the Begomovirus *Tomato yellow leaf curl virus* (TYLCV);
. LAMP procedures for the detection of the bacteria *Xanthomonas arboricola* pv. *pruni*, *Erwinia amylovora, Ralstonia solanacearum* and *Potato spindle tuber viroid* (PSTVd);
. LAMP assay for detection of *Bursaphelenchus xylophilus*;
. LAMP assay for the detection of *Xyllela fastidiosa*.

Concerning PCN detection, before this work started, LAMP assays were only available for the detection of *Globodera* sp. (Peng and Shiqi, 2016) and *G. rostochiensis* (Peng and Shiqi, 2014), but have not yet been tested in Portuguese isolates. Nowadays, there are more LAMP detection assays published (more information on Chapter III).

5. LAB-ON-CHIP

Portable methods are being developed, aiming at less processing time, less hands-on work, easy portability for in-field analysis, higher sensitivity and the use of new and more affordable technological platforms. Portable lab-on-chip platforms (DNA or protein-based) have been developed at Instituto de Engenharia de Sistemas e Computadores – Microsistemas e Nanotecnologias (INESC-MN), which allow biomarker detection from a variety of matrices. Typically, the DNA platform will receive the amplified and labelled DNA targets - labelled with Magnetic NanoParticles (MNPs), which will hybridize with immobilized single-stranded DNA oligonucleotides or probes and are then detected by sensors on the detection chip (Martins *et al*., 2009; Dias *et al*., 2016; Freitas *et al*., 2016). A PCR module and DNA extraction kit need to be integrated in the device to allow full point of use utilization. INESC-MN is developing a microfluidic based LAMP module, that can further be automated in a miniaturized unit capable of field operation and included in the decision support system in potato production. Detailed information is described on Chapters VI and V.

6. OBJECTIVES AND THESIS OUTLINE

This thesis aims to contribute for the knowledge of potato cyst nematodes in Portuguese potato fields and understand the PCN evolution in the last decade; and develop innovative diagnostic methods for the detection of this species in routine analyses.

The current research comprises 5 chapters, with the results presented in the form of 4 scientific papers, either already published or submitted for publication to peer-reviewed journals. Each paper follows the journal's specific guidelines consisting of an introduction, material and methods, results, discussion of results and literature cited.

Chapter I reviews the state of the art on potato cyst nematodes, along with the presentation of the main objectives of this thesis.

Chapter II is focused on the assessment of *Globodera rostochiensis* and *G. pallida* dispersion and prevalence in Portugal through an epidemiological approach. The identification of regions at risk and the current distribution of the pest, combining the information from official services with the results of the PCN national plan obtained at INIAV's Nematology Laboratory. Such approach allows the formulation of hypotheses about the frequency of pest occurrence in risk regions and the impact of using potato resistant cultivars in disease epidemiology. A retrospective study was conducted taking the information available since 2013. The results were analysed to modulate the PCN epidemiologic situation in Portugal. The model enables the design of appropriate ways for sustainable pest control, which are subjacent to the development of diagnostic and control methods.

Chapter III presents the development, validation and implementation of a LAMP method regarding the identification of biomarkers for *Globodera* species identification in Portugal in order to accurately identify these nematodes.

On Chapters IV and V, the design of single-stranded DNA probes and PCR/LAMP primers adapted to the MNPs labelling system, to be used in the INESC-MN miniaturized and portable platform is discussed. DNA extraction and PCR/LAMP amplification were executed at INIAV facilities, whereas detection assays were performed at INESC facilities. The system was tested with bulk sample preparation and bench PCR/LAMP but, in the future, a PCR/LAMP module and on-site DNA extraction kit need to be automated in a miniaturized unit capable of field operation. Other possible application is the simultaneous multiplex detection of different pathogens based on universal primers to indiscriminately amplify any target *Globodera* sp. in conjunction with species-specific sensor-immobilized oligonucleotide probes.

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CHAPTER II

POTATO CYST NEMATODES: GEOGRAPHICAL DISTRIBUTION, PHYLOGENETIC RELATIONSHIPS AND INTEGRATED PEST MANAGEMENT OUTCOMES IN PORTUGAL

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SUMMARY

The identification and phylogenetic relationships of potato cyst nematodes (PCN) were studied to assess the potential value of geographical distribution information for integrated pest management of potato production in Portugal. This research focused on PCN species, *Globodera pallida* and *Globodera rostochiensis*. From 2013 until 2019, 748 soil samples from the rhizosphere of different potato cultivars were surveyed in the Portuguese mainland to detect and identify both species and track their location. PCN are widespread invasive species throughout Portugal. In fact, during the survey period an incidence of 22.5% was estimated for the tested samples. The patterns of infestation vary among regions, increasing from south to north, where PCN were first detected. Currently, both species are present in all potato producing regions of the country, with a greater incidence of *G. pallida*. Phytosanitary control measures are influencing to the observed results. The use of potato cultivars resistant to *G. rostochiensis* led to a decrease of this species but had no influence on *G. pallida* detections, which continues its reproduction freely since there are no effective resistant cultivars for this species. The relationship between the presence, infestation rate, spread and geographical distribution of PCN is discussed in terms of behavioral responses of the potato cultivars and the implications for developing new integrated crop protection measures.

INTRODUCTION

Potato crop (*Solanum tuberosum*) has great social and economic importance in Portugal since it is grown throughout the country. The most representative production regions are the North and West Regions (Figure 3), with a total potato growing area of approximately 20,000 hectares and a total production of 430,000 tons.

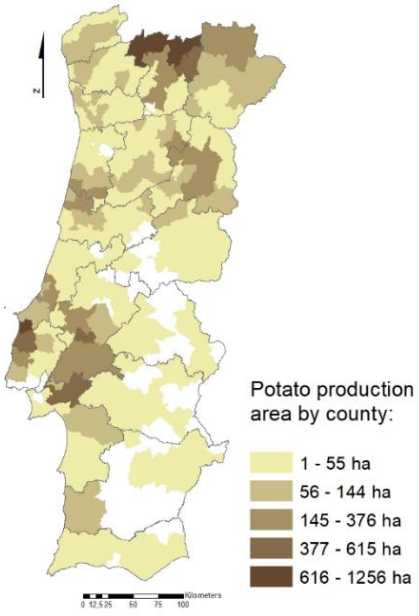

FIGURE 3 | Potato production areas (ha) in Portugal by county (data from INE, 2011).

Several nematode species have been reported associated with potato. Among those, the potato cyst nematodes (PCN), *Globodera rostochiensis* (Wollenweber, 1923; Skarbilovich, 1959) and *Globodera pallida* (Stone, 1973), are two of the major species limiting potato yield. These two

species are sedentary endoparasites of the potato root system, deteriorate the quality and commercial value of tubers and contribute to infection of potatoes by other opportunistic plant pathogens, such as fungi and bacteria (Lavrova *et al*., 2017).

Yield losses due to the presence of PCN, estimated at €220 million/year in Europe (Viaene, 2016), can vary from slight losses to crop failure depending on the infestation level (Lima *et al.,* 2018).

Both PCN species are considered harmful quarantine organisms and are subject to stringent regulatory measures when detected singly or in combination (EPPO, 2017).

The golden potato cyst nematode, *G. rostochiensis*, and the pale potato cyst nematode, *G. pallida*, originated from the Andes region in southern Peru and have spread as the result of anthropogenic activity into many regions of the world (Grenier *et al.,* 2010). They are thought to have been introduced to Europe in the 16–17th century by means of potato tubers carrying infested soil and nowadays have worldwide distribution. PCN have been reported throughout Europe, South America and parts of Asia, North America, Oceania and Africa where potatoes are grown (EPPO, 2020). However, new *Globodera* sp. detections continue to be reported (Hafez *et al.,* 2007; Mburu *et al.,* 2018; Niragire *et al*., 2019; Inácio *et al.,* 2020).

In Portugal, *G. rostochiensis* was first reported in 1956 (Macara, 1963) in a field of seed potatoes near Bragança (Trás-os-Montes district, North of Portugal) and is currently present in all potato producing regions of the country (DGAV, 2015; Camacho *et al*., 2017), including the Madeira and Azores islands (DGAV, 2015; Inácio *et al.,* 2020). *Globodera pallida* was first identified in 1988 (Santos and Fernandes, 1988), also in Trás-os-Montes, but its current national distribution has not yet been reported.

The knowledge on the geographical distribution, density and spatial dynamics of pest populations is indispensable in integrated pest management (IPM) systems, as it raises considerable interest among plant breeders and plant pathologists for the need to better understand the interaction between pest or pathogen and host and to estimate the risk of crop damage. Therefore, information of PCN distribution and potato cultivars used is essential to understand the *Globodera* spp. regional range of expansion since their first report. As human activity is the most probable means of spreading PCN, there is a specific interest in the evaluation of the implemented control measures and their consequences to adopt more effective management practices.

Controlling PCN is a difficult task due to their high level of adaptation to the environment, the prolonged viability of cysts in the absence of the host plant for more than 20 years, either quiescent or diapause in the form of encysted eggs (Christoforou *et al.,* 2014), and the risk of appearance of aggressive pathotypes in the monoculture of nematode-tolerant potato cultivars. To assess the prevalence and distribution of PCN species across the territory, a country-wide survey was established in 2010, outlining a new framework for phytosanitary protection measures against these harmful organisms to avoid dispersion in national and European Community territories and to ensure potato production of a guaranteed quality for consumers. The main potato growing regions of Portugal have been surveyed for the presence of *G. rostochiensis* and *G. pallida* since 2013.

Before the national survey started, infestations were almost entirely due to *G. rostochiensis* (Santos and Fernandes, 1988; Santos *et al*., 1995; Martins *et al*., 1996; Conceição *et al.,* 2003, Cunha *et al*., 2004, 2006, 2012). The few *G. pallida* population found in Portugal may suggest that it was introduced after *G. rostochiensis* or there were only few introductions that were kept

confined by their low natural mobility. Recently, the analysis of soils sampled in Portuguese potato fields revealed a spread of *G. pallida* (Camacho *et al*., 2017). In case of PCN positive detection, growers have to choose one of the following options as a phytosanitary measure: (a) culture with a PCN-resistant potato cultivar for a 3-year quarantine period, (b) culture with non-host species or (c) uncultivated land for a 6-year quarantine period. The use of resistant cultivars must be done carefully, in order to prevent the increase of *G. pallida* populations, which are more difficult to control as there are only a few available resistant cultivars.

Currently, in Portugal, there is a lack of detailed information on the geographical distribution of potato cyst nematodes, the correlation between their pattern, the potato cultivars and the near future implications for potato production. Therefore, this study aims to: (i) gather all PCN detections data in Portugal; (ii) carry out a molecular characterization of Portuguese *Globodera* isolates based on sequences of the ITS-rRNA region; (iii) study the phylogenetic relationships of *Globodera spp.* isolates from Portugal; and (iv) correlate cyst infestations with potato cultivars used.

The research reported herein includes PCN isolates collected from Portuguese potato fields for the national PCN surveys from 2013 to 2019, which made it possible to obtain an accurate assessment of the incidence and phylogenetic relationship of the two PCN species in the territory and their spread in different PCN-resistant cultivars fields.

EXPERIMENTAL PROCEDURE

1. Sampling

Soil was collected during the surveys between 2013 and 2019. Sampling was conducted by official inspectors of the National Plant Protection Organization (DGAV, Portugal). According to Annex II of DL 87/2010, sampling consists of a randomized collection of a soil volume with 1500 ml of soil/ha, harvested at least 100 subsamples/ha, preferably in a rectangular mesh, not less than 5 m wide and no more than 20 m long between sampling points, covering the entire field. Soil samples were stored in plastic bags and individually coded by the official services to ensure the anonymity of the samples during the analysis period. Potato field location at the county level and potato cultivars used in these fields were accessed only after analysis results.

The detection, identification and infestation rate of the PCN species were related to their sample location, given by DGAV, and species positive detection maps were made using the ArcMap 10.6 software (ESRI, United States), CAOP2017_PORTUGAL and CAOPP2017_DISTRITOS shapefiles (DGT, 2017).

2. *Globodera* spp. Molecular Identification

Cysts were extracted from soil samples using the Fenwick's can method (Fenwick, 1940), according to the EPPO PM7/40 (3) protocol, isolated and counted under a binocular microscope (Leica MZ6, Germany). Cysts (1 to 20 depending on the sample infestation) containing eggs and juveniles were used for DNA extraction by means of the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, United States) following the manufacturer's instructions. The internal transcribed spacer region (ITS) of the ribosomal DNA repeat unit was amplified by duplex PCR for species identification. PCR reactions were performed in a 25 µL final volume using the Promega GoTaq Flexi DNA Polymerase Kit (Promega, Madison, United States), containing 1 µL template DNA, 5

µL GoTaq Flexi PCR buffer (2x), 1.5 mM MgCl2, 0.20 mM each dNTPs, 1.25 U GoTaq Flexi DNA Polymerase (Promega, Madison, United States) and 0.4 mM of each primer in a Biometra TGradient thermocycler (Biometra, Gottingen, Germany). The set of primers was composed of the forward primer ITS_5 (50-GGA AGT AAA AGT CGT AAC AAG G-30) and the reverse $PITSr_3$ (50-AGC GCA GAC ATG CCG CAA-30) for *G. rostochiensis* and PITSp4 (50-ACA ACA GCA ATC GTC GAG-30) for *G. pallida* (Bulman and Marshall, 1997). The amplification profile for ITS-rDNA consisted of an initial denaturation of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension of 72 °C for 7 min (EPPO, 2017). The amplified products were loaded onto a 1.5% agarose gel containing 0.5 $mg.mL^{-1}$ ethidium bromide and 0.5xTris-borate-EDTA (TBE) running buffer and electrophoresed at 5 V/cm. Amplifications were visualized using the VersaDoc Gel Imaging System (Bio-Rad, United States). The expected length of the PCR products was 265 bp for *G. pallida* and 434 bp for *G. rostochiensis*. Possible contaminations were checked by including negative controls (no template control – NTC) in all amplifications.

3. *Globodera* spp. Phylogenetic Analysis

The ITS-rDNA region of 36 samples was amplified and sequenced using the primers 5'-CGT AAC AAG GTA GCT GTA G-30 and 50-TCC TCC GCT AAA TGA TAT G-3' (Ferris *et al*., 1993). The expected length of PCR fragments is 1040 bp and corresponds to the 3'end of 18S rDNA-ITS1-5.8S-ITS2-5' of 28S rDNA. The thermal cycling conditions performed consisted of an initial denaturation of 95 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 55 C for 30 s, and 72 °C for 33 s and a final extension of 72 °C for 7 min. Nucleotide sequences were edited and analyzed using BioEdit v7.2.0 (Hall, 2007). The resulting ITS-rDNA sequences were used as query at BLAST from NCBI GenBank to retrieve the most similar sequences within *Globodera* species for phylogenetic reconstruction, and they were deposited in the GenBank database (NCBI). Sequences from *Globodera artemisiae*, *Globodera tabacum*, and *Globodera hypolysi* were selected as outgroup taxa. All sequences were aligned by CLUSTAW (Thompson *et al*., 1994) with default parameters, trimmed manually and evaluated by Maximum Likelihood phylogeny using the best AIC (Akaike Information Criteria) nucleotide substitution model determined, namely Hasegawa-Kishino-Yano with Gamma Distribution (HKY+G). A bootstrap analysis with 1000 replications was also conducted to infer robustness of the phylogenetic tree. The CLC Main Workbench software package 8.1 (https://www.qiagenbioinformatics.com) was used for phylogenetic analysis.

4. Statistical Analysis

The differences obtained in the detection of the two PCN species in Portugal were achieved through a Z-test for the equality of two proportions using the software R (https://www.r-project.org). Only soil samples with one or more cysts were used. The hypothesis tests were performed with a significance level $\alpha = 0.05$.

Subsequently, the same test was used, with the same level of significance, to infer differences between PCN detections in north, center and south producing regions and between *G. pallida* and *G. rostochiensis* detections in fields with PCN susceptible and *G. rostochiensis* resistant potato cultivars.

RESULTS AND DISCUSSION

During the survey period (2013–2019), 748 soil samples were collected throughout the country by the official services and tested in the plant health national reference laboratory (INIAV).

Potato cyst nematodes were identified in 168 samples, representing 22.5% of the tested samples. Forty-eight samples tested positive for *G. rostochiensis* populations alone (28.6%) and 83 for *G. pallida* populations alone (49.4%). Mixed populations were found in 37 samples (22%) (Table 3). Statistics revealed that two species detections are significantly different (p-value = 0.00014, $\alpha = 0.05$), *G. pallida* detection being greater than *G. rostochiensis* detection (p-value = 0.999, $\alpha = 0.05$, which allows us to accept the null hypothesis that *G. pallida* detections are significantly greater to *G. rostochiensis* detections) between 2013 and 2019. These results contrast with those reported by Cunha *et al*. (2004) in which out of 423 tested populations (samples collected from various districts of continental Portugal), 83% were *G. rostochiensis* populations alone, 8% were *G. pallida* populations alone and 9% consisted of a mixture of the two species. This reverse situation can be explained due to the use of *G. rostochiensis* resistant potato cultivars (Figure 4), which has been considered the most widespread PCN species in Portugal.

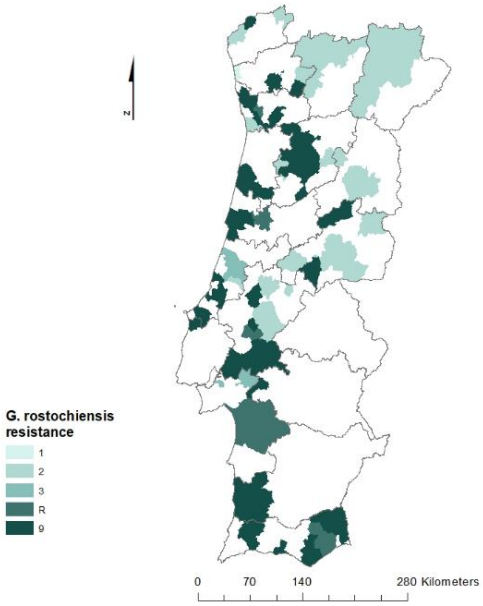

FIGURE 4 - Location and level of resistance to *Globodera rostochiensis* of potato cultivars installed in the fields on the date soil was harvested for laboratory analysis.

Table 3 | Samples tested for *Globodera rostochiensis* and *Globodera pallida* in Portuguese regions between 2013 and 2019 (absolute values and %)

Region	Positive detections								Negative detections		Total
	G. rostochiensis		*G. pallida*		*Gr + Gp*		Total				
	Value	%	Value	%	Value	%	Value	%	Value	%	
North	30	40.5	39	52.7	5	6.8	74	42.5	100	57.5	174
Centre	11	18.0	32	52.5	18	29.5	61	25.5	178	74.5	239
South	7	21.2	12	36.4	14	42.4	33	9.9	302	90.1	335
Total	48	28.6	83	49.4	37	22.0	168	22.5	580	77.5	748

The use of *G. rostochiensis* resistant potato cultivars (Table 4), effective only against certain races of G. rostochiensis and with no resistance to *G. pallida*, is leading to the predominance in Portugal of the more difficult species to control, *G. pallida*. The obtained p-value (p-value = 0.996, $\alpha = 0.05$) supported the null hypothesis, confirming that *G. rostochiensis* detection in potato fields with *G. rostochiensis* resistant cultivars is significantly smaller than *G. rostochiensis* detection in potato fields with PCN susceptible cultivars. With this result it is possible to infer that resistant cultivars are more efficient in reducing cyst infestations in potato production fields compared with susceptible cultivars fields. However, *G. pallida* detection in potato fields with *G.* rostochiensis resistant cultivars is not different to *G. pallida* detection (p-value = 0.2048, $\alpha = 0.05$, which allows us to accept the null hypothesis that *G. pallida* detections in *G. rostochiensis* resistant cultivars are significantly similar to *G. pallida* detections in PCN susceptible cultivars) and *G. rostochiensis* detection in PCN susceptible potato cultivars fields (p-value = 0.5415, $\alpha =$ 0.05, which allows us to accept the null hypothesis that *G. pallida* detections in *G. rostochiensis* resistant cultivars are significantly similar to *G. rostochiensis* detections in PCN susceptible cultivars). With this result it is possible to infer that resistant cultivars used in Portugal allow us to reduce *G. rostochiensis* cysts infestation but has no influence on *G. pallida* cysts infestations in potato production fields. Therefore, the use of *G. rostochiensis* resistant potato cultivars has led to a decrease in *G. rostochiensis* detection but has no influence on *G. pallida* detection. These results agree with the published literature (Minnis *et al*., 2002; Pickup *et al*., 2019).

Table 4 | Potato cultivars grown in Portuguese sampled fields (2013-2019) and their resistance status towards *Globodera rostochiensis* and *Globodera pallida*

Cultivar	Resistance status		Cultivar	Resistance status	
	G. rostochiensis	***G. pallida***		***G. rostochiensis***	***G. pallida***
Agria	R	S	Jelly	R	S
Alcander	R	R	Kenebeck	S	S
Allison	R	R	Lady roseta	R	S
Asterix	R	S	Manitou	R	S
Aurea	R	S	Monalisa	S	S
Baraka	R	S	Montecarlo	R	R
Bellarosa	R	ND	Olho de perdiz	R	ND
Camberra	R	ND	Picasso	R	ND
Carlita	R	ND	Red Lady	R	ND
Colomba	R	S	Red scarlet	R	ND
Daifla	R	S	Romano	S	S
Delila	S	S	Rudolph	S	S
Désirée	S	S	Soleny	S	S
Evolution	R	S	Stemster	R	ND
Evora	S	S	Taurus	R	S
Hermes	S	S	Yona	R	S

* R = resistant; S= susceptible; ND= No data available

There is no available data to infer about the use of *G. pallida* resistant potato cultivars. This raises the question of whether phytosanitary measures are effective or whether they are contributing to the increase of *G. pallida*, as also reported in the United Kingdom (Minnis *et al*., 2002). On the other hand, the market has caused potato growers to predominantly use *G. rostochiensis* resistant potato cultivars (i.e., Aurea, Agria, Lady rosetta, Taurus), and this is the main cause of *G. pallida* detections increase.

The geographical distribution of PCN infestations in Portugal is illustrated in Figures 5 and 6, which present the infestation rate in counties with positive detections of *G. rostochiensis* and *G. pallida* between 2013 and 2019. This information completes a picture of the PCN situation in Portugal to date.

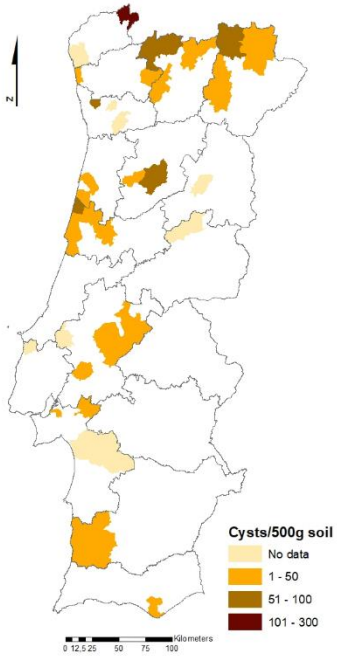

FIGURE 5 | Counties with positive detections of *Globodera rostochiensis* in Portugal between 2013 and 2019.

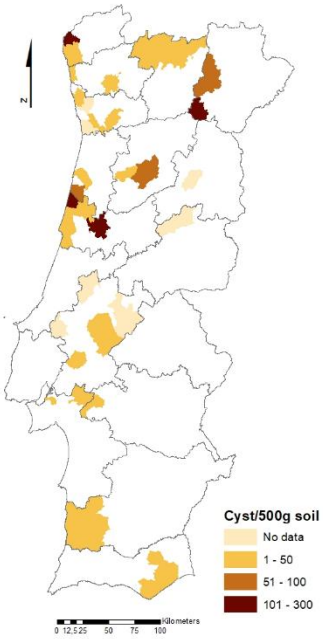

FIGURE 6 | Counties with positive detections of *Globodera pallida* in Portugal between 2013 and 2019.

According to these results, the incidence of PCN in Portugal is quite high, and both species are currently present in all potato producing regions of the country. PCN detections in the different regions are significantly different. Statistics revealed that the Northern PCN detection is greater than the Center PCN detection (p-value = 0.998, $\alpha = 0.05$, which allows us to accept the null hypothesis that PCN detection in northern fields is significantly greater than PCN detection in central fields) and the Center PCN detection is greater than the Southern (Lisbon and Tagus Valley, Alentejo and Algarve regions) PCN detection (p-value = 1, $\alpha = 0.05$, which allows us to accept the null hypothesis that PCN detection in central fields is significantly greater than the PCN detection in southern fields), meaning that PCN detection increases from south to north (see Figures 5 and 6), where PCN were first detected and nematode reproduction are happening for a longer period. These results are also in line with previous reports, which state that the cysts are adapted to higher altitudes (Jones *et al*., 2017) since the altitude grows from south to northern regions in Portugal.

To infer the phylogenetic relationship of *Globodera* isolates, ML analyses were performed (Figure 7). Two major clades, highly supported, can be observed: clade (I) with sub-clades *G. rostochiensis* and *G. pallida* and clade (II) with the sub-clades *Globodera* sp. recently re-detected. Within the first clade, two sub-clades were formed with *G. rostochiensis* and the related species *G. tabacum* and *G. pallida*. The second clade groups a Portuguese *Globodera* sp., discovered in 1997 (Reis, 1997; Sabo *et al*., 2002) and not re-detected until recently (data not shown), and their most closely related *Globodera* species, *G. hypolysi* and *G. artemisiae*. As can be clearly

seen, no spatial-temporal relation can be redrawn evidencing the coexistence between the two major species of *Globodera* in Portugal. These results are in accordance with those reported by Cunha *et al*. (2012), who reported that no relationship could be found between the two-dimensional electrophoresis protein patterns or virulence behavior of the isolates and their geographic origin within Portugal.

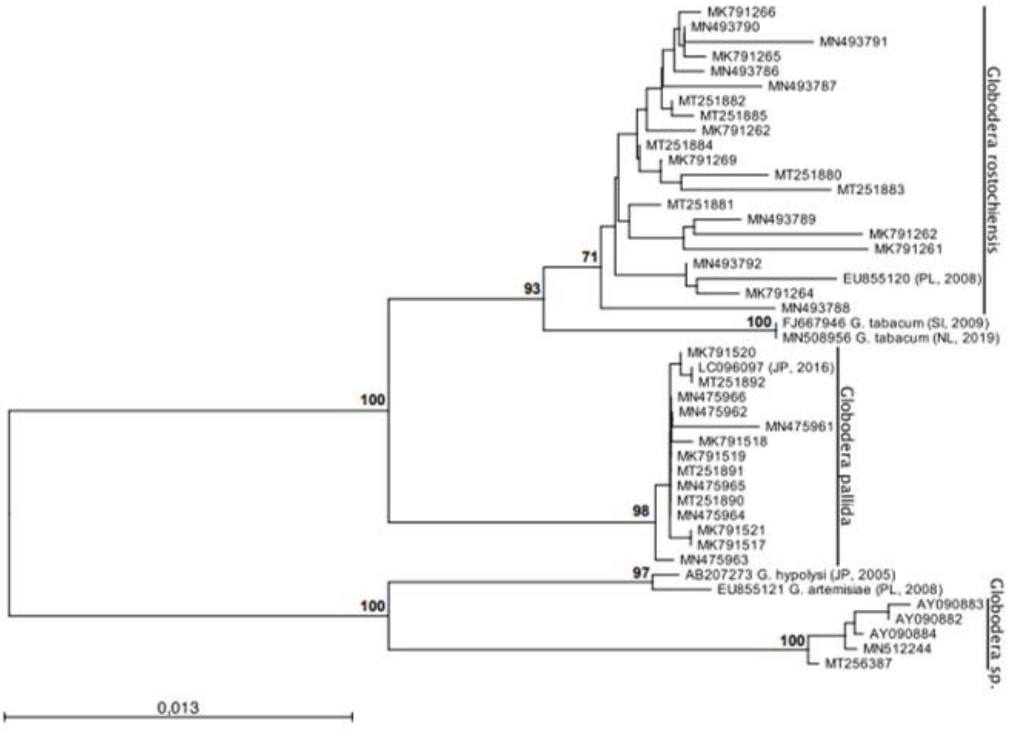

FIGURE 7 | Phylogenetic relationships of *Globodera* sp. isolates collected from Portugal based on the sequence alignment of the ITS-rDNA loci. The condensed phylogenetic tree was generated using the Maximum Likelihood method based on the HKY+G model with 1,000 bootstrap replications. Bootstrap values are indicated at the nodes (bootstrap higher than 70%). The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. *Globodera tabacum*, *G. hipolysi* and *G. artemisiae* were used as outgroup.

It is also worth noting that the topology differs between *G. rostochiensis* and *G. pallida* sub-clades. The first is more branched, with 96–100% of similarity, showing more genetic variability due to being present for a longer period in Portugal, while the second is flatter, with 99–100% of similarity, showing more identical sequences (Supplementary Table 1).

Concerning the new species *Globodera* sp. (Reis, 1997; Sabo *et al*., 2002), re-detected recently in Portugal, it is out of the scope of this work, but additional research is being carried out to determine its pathogenicity and impact on potato.

The nucleotide sequences obtained in this study were deposited in the GenBank database (NCBI) under the accession numbers given in Table 5.

Table 5 | *Globodera* spp. isolates sequenced in the present study (E-value =0.0).

***Globodera* species**	**GenBank accession number**	**Locality**	**Collection code/year**		**Sequence length (bp)**	**NCBI BLAST homology (%)**
Globodera rostochiensis	EU855120	Poland	*	2008	4064	100.00
	MK791260	Coimbra	650P	2014	893	100.00
	MK791261	Montalegre	5244	2015	888	100.00
	MK791262	Montalegre	5245	2015	909	100.00
	MK791263	Viseu	9996	2018	871	98.62
	MK791264	Mirandela	14598	2018	969	99.79
	MK791265	Mirandela	14600	2018	871	99.89
	MK791266	Bragança	14601	2018	909	99.89
	MN493786	Montalegre	13486	2017	937	99.25
	MN493787	Chaves	8850	2016	937	98.50
	MN493788	Viseu	9610	2017	920	98.58
	MN493789	Viseu	5967	2016	936	98.82
	MN493790	Viseu	7047	2017	973	100.00
	MN493791	Odemira	3663	2018	915	99.13
	MN493792	Aveiro	7913	2018	897	99.78
	MT251880	Coimbra	1252	2019	929	99.14
	MT251881	Montalegre	1681-2	2019	909	99.34
	MT251882	Montalegre	1681-6	2019	924	99.89
	MT251883	Chaves	1681-7	2019	933	98.71
	MT251884	Mirandela	1681-10	2019	928	99.35
	MT251885	Melgaço	1249-1	2019	946	98.94
Globodera tabacum	FJ667946	Slovenia	*	2009	923	99.46
	MN508956	Netherlands	NL:c6876	2018	953	99.89
	LC096097	Japan	*	2016	964	100.00
Globodera pallida	MN475961	Viseu	3876	2014	898	99.33
	MN475962	S. Magos	4261	2016	970	99.90
	MN475963	S. Magos	15731	2018	933	99.03
	MN475964	Vagos	9993	2018	977	98.89
	MN475965	Montalegre	14002	2017	914	99.89
	MN475966	Esposende	5087	2016	926	99.56
	MK791517	Penafiel	4694	2015	873	100.00
	MK791518	Viseu	5961	2016	890	99.22
	MK791519	Guimarães	11309	2018	901	99.78
	MK791520	Mirandela	14593	2018	878	100.00
	MK791521	Mirandela	14599	2018	873	100.00
	MT251890	Vagos	1223-7	2019	938	100.00
	MT251891	Aveiro	1223-8	2019	915	99.89
	MT251892	Mira	1086-3	2019	913	99.67
Globodera sp.	AY090883	Bouro	*	2002	908	99.89
	AY090882	Canha	*	2002	908	99.89
	AY090884	Ladoeiro	*	2002	908	99.78
	MN512244	Montijo	12031	2018	953	99.45
	MT256387	Lagameças	1479-2	2019	913	99.67
Globodera artemisiae	EU855121	Poland	*	2008	4092	100.00
Globodera hypolysi	AB207273	Japan	*	2005	909	99.45

* Sequences available from GenBank, NCBI.

Phytosanitary measures have been taken to prevent further spread of *Globodera* spp. in recent years. In the case of *G. rostochiensis*, up until now the dominant species, measures include non-host crop rotation (for 6 years), fallow (for 6 years) or growing of resistant potato cultivars (for 3 years). The use of resistant cultivars containing the H1 gene (single dominant resistance gene for *G. rostochiensis*) (Gebhardt *et al*., 1993), as already shown, is effective against many populations of *G. rostochiensis* and is likely to be an advantageous management tactic to reduce population densities and thereby yield losses. However, the deployment of resistance in such cultivars may have caused the predominance of *G. pallida* in Portugal, as already predicted by Cunha *et al*. (2004) and statistically verified in this study.

Therefore, it is urgent to follow a new approach for the management of PCN, mainly *G. pallida*. Non-infested areas need to be managed to minimize the opportunities for the introduction of *Globodera* species. On the other hand, and in infested soils, a greater use of integrated control strategies (such as crop rotation, solarization, trap cropping, biofumigation and selected nematicides) (Evans and Haydock, 2000; Alptekin, 2011; Davie *et al.,* 2019), in addition to PCN-resistant potato cultivars, should be a priority. These interactions require careful research into the effects of one or another strategy under a specific set of environmental conditions and a specific nematode infestation level. The efficacy of the integrated program will be determined by the interaction, overlap and complementarity of the various components. Despite the difficulties associated with *G. pallida* resistance being quantitatively inherited, the breeding of more resistance with different R-genes to avoid PCN capacity to overcome the plant resistance and commercially attractive cultivars is highly important. As *G. pallida* field populations tend to show increased virulence toward a particular partially resistant cultivar each time that it is grown (Trudgill et al., 2003; Pickup *et al.,* 2019), potato growers would need a choice of different cultivars to allow effectiveness to be maintained. Currently, there are insufficient alternatives to partially resistant cultivars for growers to meet the requirements of markets.

SUPPORTING INFORMATION

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.606178/full#supplementary-material.

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CHAPTER III

DEVELOPMENT AND VALIDATION OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION DIAGNOSTIC METHOD TO DETECT THE QUARANTINE POTATO PALE CYST NEMATODE, *GLOBODERA PALLIDA*

Data published in:

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SUMMARY

The potato cyst nematode (PCN) *Globodera pallida* has acquired significant importance throughout Europe due to its nefarious effects on potato production. Rapid and reliable diagnosis of PCN is critical during the surveillance programs and for the implementation of control measures. Molecular DNA-based methods are available, but they require expensive laboratory facilities, equipment and trained technicians. Moreover, there is an additional need of time for sample shipment and testing. In this work, we have developed a new and simple assay which reliably discriminates *G. pallida* from other cyst nematodes in less than 40 min. This assay may be applied either on cysts or juveniles with the ability to detect a single juvenile of *G. pallida* in a sample of at least 40 juveniles of the non-target species *G. rostochiensis*. This test should be a tool to improve the performance of the laboratory and has the potential to be performed on-site.

INTRODUCTION

The potato cyst nematodes (PCN), *Globodera rostochiensis* (Wollenweber, 1923; Skarbilovich, 1959) and *Globodera pallida* (Stone, 1973) constitute one of the greatest threats to potato crops. These plant parasitic nematodes originated from the Andes region in southern Peru and have spread as the result of anthropogenic activity into many regions of the world (Grenier E., *et al*., 2010). They are thought to have been introduced into Europe in the 16–17th century by means of potato tubers carrying infested soil. Beyond Europe, PCN have been reported throughout South America and parts of Asia, North America, Oceania and Africa where potatoes are grown (EPPO, 2020). The golden potato cyst nematode, *G. rostochiensis*, and the pale potato cyst nematode, *G. pallida*, are sedentary endoparasites of the potato root system that deteriorate the quality and commercial value of tubers and contribute to infection of potatoes by other opportunistic pathogens, such as fungi and bacteria (Lavrova *et al.,* 2017). Therefore, PCN are considered harmful quarantine organisms and are subject to strict quarantine regulations in many countries (EPPO, 2022a).

Owing to their huge economic and trade impacts, it is crucial to distinguish these species using diagnostic tools in order to plan and implement strategies for an effective integrated pest management. Since the identification of these *Globodera* species based on morphology may be ambiguous due to the variability of the main morphological features and the overlapping of morphometrics in these two species, confirmation via molecular methods is recommended (Camacho *et al.,* 2017).

PCN molecular identification is routinely performed through multiplex conventional PCR (Bulman and Marshall, 1997) and real-time PCR based on LSU rDNA protocols described in the European and Mediterranean Plant Protection Organization (EPPO) protocols PM 7/40—*Globodera rostochiensis* and *Globodera pallida* (EPPO,2022a). Although the sensitivity and specificity of these diagnostic assays are sufficiently high when properly applied, the procedures are time consuming, require well-trained technicians and expensive laboratory equipment and cannot be performed in the field due to the lack of convenient portable instruments (Kogovšek *et al.,* 2015).

As a result of the PCR-based protocols limitations, other methods have been developed, aiming at less processing time, less hands-on work, easy portability for in-field analysis, higher sensitivity and the use of new and more affordable technological platforms. Overall, they aim at lower costs

for laboratories and for the inspection services when applicable. As result, a loop-mediated isothermal amplification method (LAMP) has been developed (Notomi *et al*., 2000).

LAMP is a single tube technique for the amplification of nucleic acid, using four to six primers that target 6 to 8 locations within a given DNA sequence under isothermal conditions (60–65 °C), yielding large amounts of products in a short time (30 to 60 min). Amplified products can be visualized by gel electrophoresis, by a visible by-product (colorimetric detection) or by measuring the fluorescence emitted by DNA intercalating dyes such as SYBRGreen (Subbotin *et al*., 2013). It does not require expensive thermocycle (chemical denaturation of DNA instead of thermic at 95 °C) and optical detection equipment and is less sensitive to amplification inhibitors, allowing for precise, sensitive, specific and cost-effective early detections (Subbotin *et al*., 2013).

LAMP clearly holds potential for in-field testing. Portable lab-on-a-chip platforms (based on DNA or proteins) have already been developed which allow biomarker detection from a variety of matrices. The DNA platform receives the amplified and labelled DNA targets (labelled with MNPs), that hybridize with immobilized probes and are then detected by sensors on the detection chip (Freitas *et al*., 2016; Martins *et al*., 2009; Dias *et al*., 2015). Because of its speed, robustness and simplicity, the use of LAMP is gaining popularity for diagnostics in plant health. LAMP-based assays have been developed for the detection of plant pathogenic viruses and phytoplasmas (Sarkes *et al*., 2020;.Panno *et al*., 2020; Dickinson, 2015), insects (Blaser *et al*., 2018), fungi (Duan *et al*., 2014; Tian *et al*., 2016; Khan *et al*., 2018; Aglietti *et al*., 2019; King *et al*., 2019; Li *et al*., 2019; Zhang 2019) and bacteria (Aglietti *et al*., 2019; Chen *et al*., 2020).

In addition, LAMP-based assays have been developed for the detection of several plant parasitic nematodes (PPN) (Ahuja and Somvanshi, 2020). The first LAMP assay for any PPN was developed for the pinewood nematode *Bursaphelenchus xylophilus*, along with an easy method to extract nematode DNA directly from wood samples (Kikuchi, *et al*., 2009). More highly specific LAMP-based assays for *B. xylophilus* have also been developed (Kanetani *et al*., 2010; Kang *et al*., 2014; Leal *et al*., 2015; Meng *et al*., 2018; Nakajima *et al*., 2019). A LAMP assay is also available for detection of *Bursaphelenchus cocophilus* (Ide *et al*., 2017).

For detection of different species of root-knot nematodes, several LAMP assays have been developed, such as for *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, *M. javanica* (Niu *et al*., 2011), *M. enterolobii* (Niu *et al*., 2012), *M. hapla* (Peng *et al*., 2017), *M. mali* (Zhou *et al*., 2017), *M. chitwoodi* and *M. fallax* (Zhang and Gleason, 2019). Recently, a LAMP-based diagnostic assay was published for the pecan root-knot nematode, *M. partityla* (Waliullah *et al*., 2020). In a variant assay to detect *M. hapla*, DNA from the root galls was directly crushed onto Flinders Technology Associates (FTA) cellulose cards and stored at room temperature for years and directly used as a template in LAMP reactions (Kanetani *et al*., 2010; Peng *et al*., 2017).

Many other LAMP assays have been developed to detect PPN, such as Aphelenchoides besseyi (Yang and Yu, 2019), *A. ritzemabosi* (Wang *et al*., 2019), *Anguina wevelli* (Yu *et al*., 2020) and *A. agrostis* (Yu *et al*., 2018), *Radopholus similis*, directly from infected plant tissues (Peng *et al*., 2012), *Ditylenchus destructor* from complex plant/nematode DNA mixtures (Deng *et al*., 2019) and *Tylenchulus semipenetrans* in soil samples (Lin *et al*., 2016; Song *et al*., 2017).

To detect *Globodera* spp., LAMP assays are only available for the detection of *Globodera* sp. and *G. rostochiensis*, based on sequences of Belgian and Netherlands populations (Peng *et al*., 2014; Peng *et al*., 2016). The objective of this work is to develop a LAMP assay for *G. pallida*, to be used in routine analyses, since the analysis of soils sampled in Portuguese potato fields has revealed an increased spread of *G. pallida* in the country (Camacho *et al*., 2020). The rapid identification

of the two species is essential to detect their presence in potato fields, to re-evaluate the control measures implemented so far and adopt more effective practices. Our LAMP assay reliably allows for the differentiation of species of *Globodera* within less than 40 min and 3 h if including DNA extraction.

RESULTS

1. Sequencing and Primer Design

The sequence alignment of the "3'end 18S-ITS1-5.8S-ITS2-5'end 28S" region of Portuguese isolates (*G. pallida, G. rostochiensis* and *Globodera* n. sp., the three predominant species in Portugal (Camacho *et al*., 2020) and several from the GeneBank database for *G. pallida*, *G. rostochiensis*, *Globodera* n. sp., *G. mexicana* and *G. ellingtonae* (Supplementary Table S1) was performed. There is a considerable amount of genetic information publicly available from GenBank, which reflects unbiased diversity of full sequences of the "18S-ITS1-5.8S-ITS2-28S" fragment with low sequence error rate. Some shorter sequences were brought into the analysis when they were necessary to ensure worldwide coverage. Therefore, only the fragments with no undetermined nucleotides among all the *G. pallida* accessions were taken to create the consensus sequence of 221 bp in the ITS1 ribosomal spacer region. This fragment covers the most conserved part of the "3'end 18S-ITS1-5.8S-ITS2-5'end 28S" sequence among *G. pallida* accessions (inclusivity) while demonstrating sufficient sequence variation among those species that can be found in Europe when exclusively using environmental samples. This guaranteed coverage of a wide range of genetic variability and robustness of the study.

A total of 100 primer sets (Supplementary Figure S1) was the outcome of the online LAMP designer tool Primer Explorer V5 (Eiken Chemical Co. LTD, Tokyo, Japan) when applied on this fragment of the *G. pallida* consensus sequence. This enabled the design of *G. pallida*-species-specific primers, as demonstrated by in silico analysis (Figure 8).

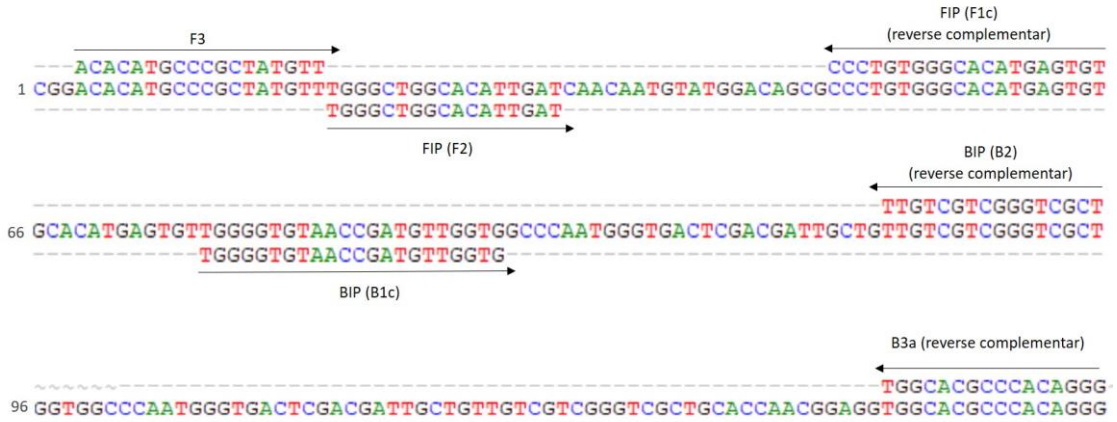

FIGURE 8 | Partial ITS consensus sequence created after the alignment of all *Globodera pallida* selected accessions and localization of target sequences used for LAMP primers. Arrows indicate the direction and location of the primers. Numbers at the left side indicate solely the position in this fragment.

Although the 221 pb fragment was conserved among *G. pallida* accessions, the specificity of all primers designed by the online tool was also manually checked. The aim was to have two out of the three primers F and two out of the three primers B having the nucleotide of the last position at the 3'end mismatching in all of the non-target species (vertical boxes in Figure 9).

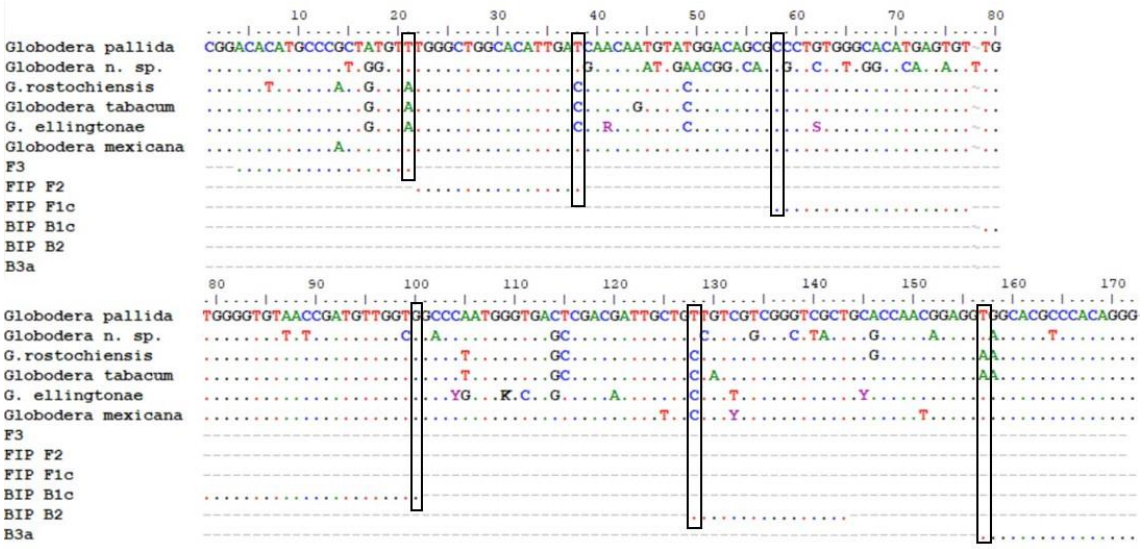

FIGURE 9 | Alignment of partial ITS sequences of *G. pallida*, *Globodera* n. sp., *G. rostochiensis*, *G. tabacum*, *G. ellingtonae*, *G. mexicana* and set2a LAMP primers.

For all non-target species but *G. mexicana*, mismatches were found in the F2, F3, B2 and B3 primers (Figure 9). *G. mexicana* sequences are very close to *G. pallida* sequences. Only one primer (B2) will not amplify as the 30 nucleotide is different (Figure 9). This fact may alter the amplification time (more delayed) and eventually the melting temperature (Tmelting). However, we think that this species will not be a problem for PCN surveys in potato fields and for the specificity of the method because although *G. mexicana* is stimulated by potato root exudates, it is unable to establish and develop on potato crops (Grenier *et al*., 2002; Sabeh *et al*., 2019). This species seems to be present in a restricted area of Mexico (not widely spread) and only in wild *Solanancearum* species (Subbotin *et al*., 2020).

Due to some similarity of the sequences among species, only two sets of primers (Table 6) were selected for further analysis, but just one was kept for use in the subsequent validation studies (Figure 8). All primers but B3a were kept as designed in order to have the best thermodynamic conditions, considering the formation of secondary structures and unwanted hybridizations. The primer B3a was manually designed to improve specificity which gave rise to set2a and to the amplification product of 171 bp.

Table 6 | Sets of primers tested for *Globodera pallida* LAMP assays.

Primers	**Set 1**
FIP (F1c + F2)	CAC GGC CAC GGA CGT AGC ACA TGT CGT ACG TGC CGT ACC C
BIP (B1c + B2)	GAG ACG ACG TGT TAG GAC CCA CTC ATC AAG TCT TAA ACC G
F3	CAT GGA GTG TAG GCT GCT AT
B3	TTA TAA AAA TGA GAA AAA G
Primers	**Set 2a**
FIP (F1c + F2)	ACA CTC ATG TGC CCA CAG GGT GGG CTG GCA CAT TGA T
BIP (B1c + B2)	TGG GGT GTA ACC GAT GTT GGT GAG CGA CCC GAC GAC AA
F3	ACA CAT GCC CGC TAT GTT
B3(a)	CCC TGT GGG CGT GCC A

2. Optimization of the LAMP Assay Protocol and Specificity

In the first preliminary analytical study to evaluate the two primer sets, a total of eight isolates from five species (*G. pallida, G. rostochiensis, Globodera* n. sp., *G tabacum* and *Heterodera* sp.—Table 7-I) were used. The reaction conditions were those of protocols A and C (Table 8). Set 1 of primers identified *G. pallida* within 20 min but it cross-reacted with all the other species (Supplementary Table S2).

Table 7 | Samples from Portugal, Netherlands and other European isolates used for LAMP specificity assay. Spectrophotometric estimates for the concentration and quality of DNA extracts.

	Species	Isolate	Origin	ng/µL
I	*G. pallida*	MK791521	Portugal	5.2
	G. pallida	NPPO-NL Pa3 HLB	Netherlands	1.4
	G. rostochiensis	MK791264	Portugal	28.2
	G. rostochiensis	NPPO-NL Ro1 HLB	Netherlands	2.9
	G. tabacum	NPPO-NL C6876	Netherlands	39.4
	Globodera n. sp.	MT256387	Portugal	11.6
	Heterodera sp.	SV-18-10003 *	Portugal	18.1
	G. rostochiensis	058	Samples from an interlaboratory test(European origin)	16.8
II	*G. pallida*	094	Samples from an interlaboratory test (European origin)	1.5
	G. pallida	138		13.1
	G. tabacum	185		3.1
	Heterodera sp.	414		2.3
	G. tabacum	447		1.4
	G. rostochiensis	471		3.1
	G. pallida	546		2.2
	G. pallida	580		2.5
	G. rostochiensis	629		3.0

* Not deposited at the NCBI GeneBank database. INIAV internal reference number.

Table 8 |Protocols tested for *Globodera pallida* LAMP optimization.

Protocol	Master Mix	Primer Volume (µL)		Amplification Temp. (°C), Time (s)	$T_{Melting}$ Heat-Cooling (°C)
		F3, B3 (Initial Conc. 50 µM)	FIP, BIP (Initial Conc. 50 µM)		
A	ISO-004 ([Mg^{2+}] = 5 mM)	**0.10**	0.80	65 °C, 60 min	95 °C–75 °C
B			0.60	65 °C, 20 min	
C		0.15	0.80		
D			0.40		
E			0.60		
F			0.80	64 °C, 20 min	
G		0.12	0.70	66 °C, 20 min	
H		0.15	0.90	64 °C, 20 min	
I		0.12			
J	ISO-001 ([Mg^{2+}] = 3 mM)	0.15	0.80	64 °C, 30 min	
K				64 °C, 60 min	
L					95 °C–85 °C
M	ISO-004			64 °C, 20 min	

Set2a of primers identified *G. pallida* and has no homology with other cyst nematodes (Figures 10 and 11). It showed more than 99% perfect matching for inclusivity in more than 88% of the replicates of *G. pallida*. Exclusivity showed less than 94% homologies with the other *Globodera* species. No match was found for *Heterodera* sp.. Therefore, these primers are not expected to react and yield false positive results (Supplementary Table S2). To further test specificity, genomic DNA from other nematode species and genera were tested. No match was found with *Pratylenchus penetrans, Xiphinema* sp., *Helicotylenchus* sp., *Bursaphelencus xylophilus* and *B. mucronatus* (Figure 11).

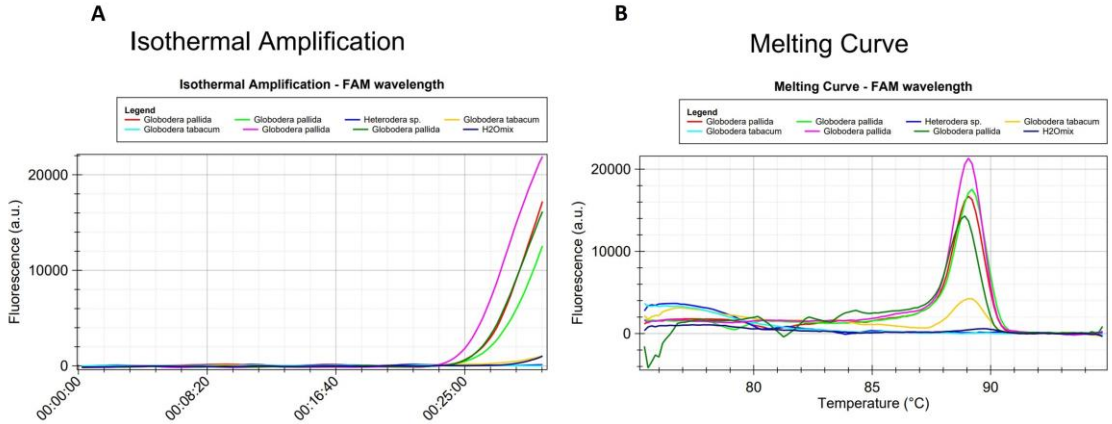

FIGURE 10 |Specificity test of the LAMP using genomic DNA from *Globodera pallida*, *G. tabacum* and *Heterodera* sp.: (A) amplification curves and (B) derivative of the melting temperature curve.

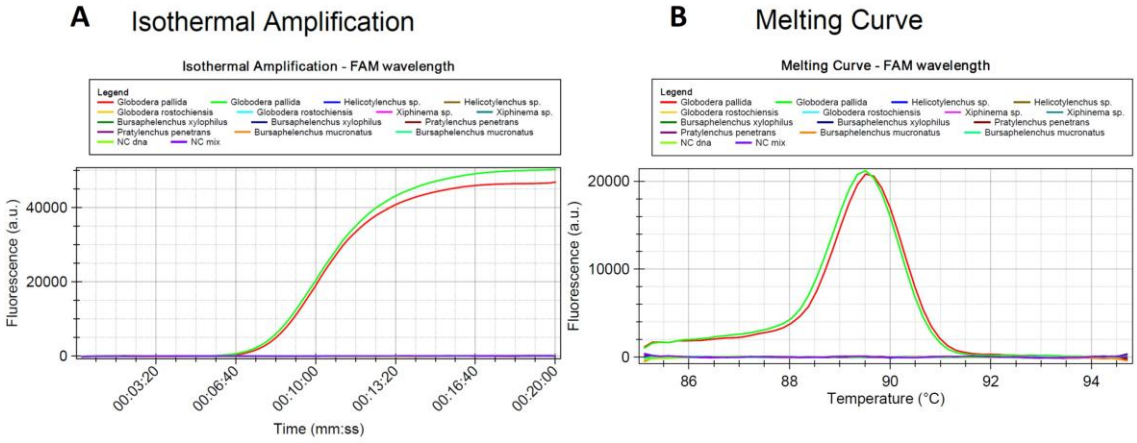

FIGURE 11 | Specificity test of the LAMP assay using genomic DNA from *Globodera pallida*, *Pratylenchus penetrans, Xiphinema* sp., *Helicotylenchus* sp., *Bursaphelencus* (*B. xylophilus* and *B. mucronatus*): (A) amplification curves and (B) derivative of the melting temperature curve.

The set2a of primers which provided the expected results (i.e., correct species identification within 40 min) was then tested under several master mix compositions to determine the optimal primer concentration, temperature and time for each of the two master mixes tested (ISO-001 and ISO-004) differing in the concentration of MgSO4 (Table 8). DNA from the cyst nematode isolates referred in Table 7-I were used as template for different lengths of time. Of all protocols provided in Table 8, the L protocol with master mix ISO-001 and the M protocol with master mix ISO-004 were the ones which obtained the best results (Supplementary Table S2). LAMP assay for *G. pallida* detection should be performed according to the protocols summarized in Table 9. The reaction mixtures prepared with master mix ISO-004 should be incubated at 64 °C, for 20 min and terminated by incubation at 95–85 °C, 0.05 °C/s or for 60 min if the isothermal master mix ISO-001 (OptiGene, Horsham, UK) is used.

Table 9 | Preparation of LAMP reaction master mix for *Globodera pallida* positive amplification control.

Component	Initial Concentration	Vol/Reaction (µL)
ISO-004 (or 001) master mix	-	15
Primers FIP and BIP	50 µM	0.80
F3 and B3a	50 µM	0.15
Molecular grade water	-	3.1
DNA template	≥5 pg	5

In all LAMP reactions, the acceptance criterion for a positive result combines a sigmoid amplification curve within 40 min with the expected Tmelting of the amplified products. Tmelting was set at 89.66 °C (0.61 °C) and 89.87 °C (0.61 °C) for mastermix ISO-004 and ISO-001, respectively. With the 2a primer set, no positive signal could be generated from non-target cyst nematode species (Table 7). Positive signals were only generated from *G. pallida* DNA (Figures 10 and 11).

3. LAMP Sensitivity Assay for *Globodera pallida*

3.1. Analytical Sensitivity

To determine the level of analytical sensitivity of the LAMP assay, serial dilutions of *G. pallida* total DNA were used as template for the reactions. Each dilution from the series was analyzed in triplicate in the Molecular Biology Laboratory at INIAV. Amplifications were detected in all replicates from all dilutions from 5 ng/ µL to 5 pg/µL of *G. pallida* DNA (Figure 12A). In contrast, only two replicates out of the three from the dilution at the concentration of 5 pg/ µL have amplified.

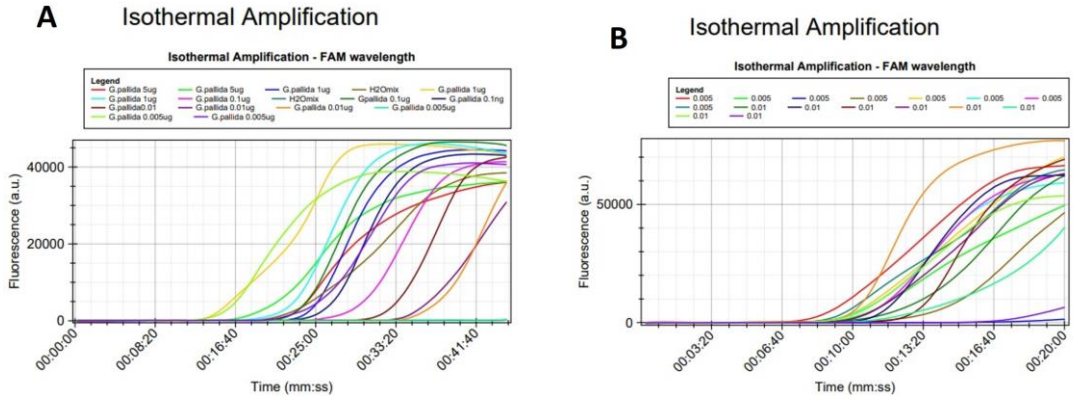

FIGURE 12 | Analytical sensitivity test of the LAMP assay performed in two different times and facilities: (A) Laboratory of Molecular Biology at INIAV; (B) NemaLab-Laboratory of Nematology in Évora.

The experiment was repeated in NemaLab (Évora University). Only the two lower concentrations (0.01 ng/µL and of 5 pg/µL) were tested (Figure 12B), as the failure in the amplification was observed at 5 pg/µL. To ensure a higher level of confidence, octuplicates were performed. Again, this LAMP assay produced positive results down to 5 pg of DNA (25 pg/25 L reaction volume), however, the sensitivity decreased from 100% at 0.01 ng/µL to 87.5% at 5 pg/µL (7 PA out 8 reactions). For DNA extracts with concentrations lower than 10 ng/µL, the variation between replicates was high and, therefore, the accuracy of the measurement could be low. Further evaluation of the sensitivity of the assay was done by using DNA extracted from a single juvenile. The LAMP assay was able to detect/identify *G. pallida* even when the DNA was diluted 102-fold without knowing the initial concentration. In routine work, DNA is extracted from cysts having an unknown number of juveniles rather than from individual juveniles. As a consequence, DNA concentration estimate is not a key performance parameter. Therefore, we can establish as a rule of thumb that DNA extracts should be diluted at least 100 times.

3.2. Diagnostic Sensitivity

The detection of the target species within pools of non-target species was attempted because it was previously demonstrated that *G. pallida* and *G. rostochiensis* cohabit in mixed populations in potato fields (Camacho *et al*., 2020, 56. Djebroune *et al*., 2021). Samples of pure *G. pallida* and pure *G. rostochiensis* were not used since the specificity had been previously demonstrated. The assay was able to identify *G. pallida* in all combinations (Table 10). Amplifications were detected in all DNA extracts obtained from pools containing different proportions of *G. rostochiensis*/*G. pallida* J2 (Figure 13), even when one *G. pallida* J2 was mixed with 40 *G.*

rostochiensis J2. The average time for detection did not change much, but the 40:1 was the latest (15 min).

Table 10 | Samples with different proportions of *G. rostochiensis* and *G. pallida* second stage juveniles (J2).

Samples	Samples Ratio (J2 *G. rostochiensis*/ J2 *G. pallida*)	ng/µL
1	1:5	3.2
2	1:9	1.7
3	1:19	2.3
4	1:40	2.6
5	5:1	2.4
6	9:1	2.0
7	19:1	4.3
8	40:1	2.0

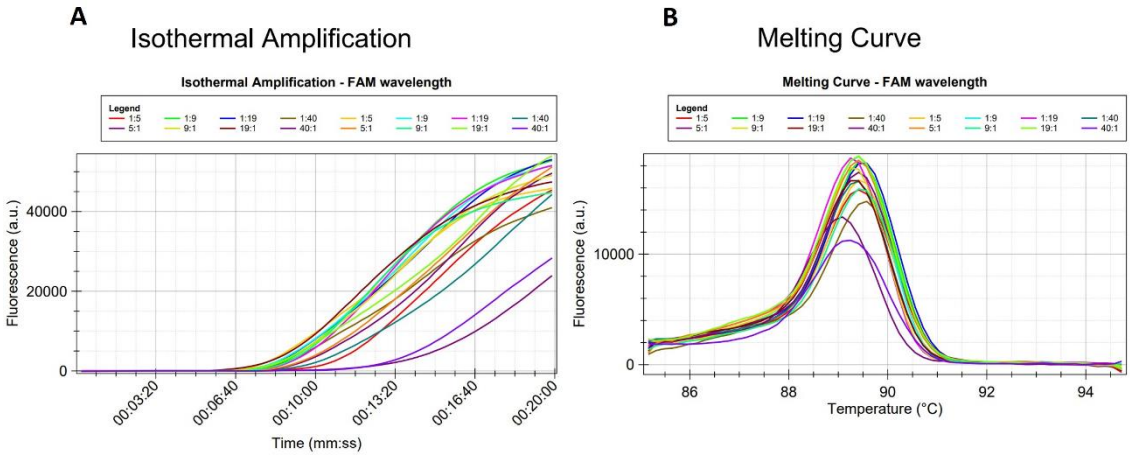

FIGURE 13 | Diagnostic sensitivity test of the LAMP assay performed in the Laboratory of Nematology in Évora. (A) Isothermal amplification and (B) Melting curve. Amplification of DNA extracts from pools having different proportions of *G. rostochiensis*/*G. pallida* J2.

3.3. LAMP Reproducibility

Reproducibility was assessed by analyzing DNA extracts of very low concentration (0.01 ng/L and 5 pg/L) in triplicates and octuplicates in two different laboratories. Consistent results were obtained between the two laboratories (Figure 12).

An additional evaluation of the LAMP assay was done by a comparative test using the same samples and a rt-PCR instrument. Amplifications were detected in all *G. pallida* samples and in *Heterodera* sp. sample (Figure 14A), however the derivative of the melting curve of the later indicated a different value than that determined for *G. pallida* (Figure 14B). In contrast, no amplification was observed from other nematode species samples including the closely related species, *G. rostochiensis, G. tabacum* and *Globodera* n. sp., which are difficult to distinguish from

G. pallida by its morphological characteristics (Camacho *et al*., 2017). There was concordance between the identified species and the expected.

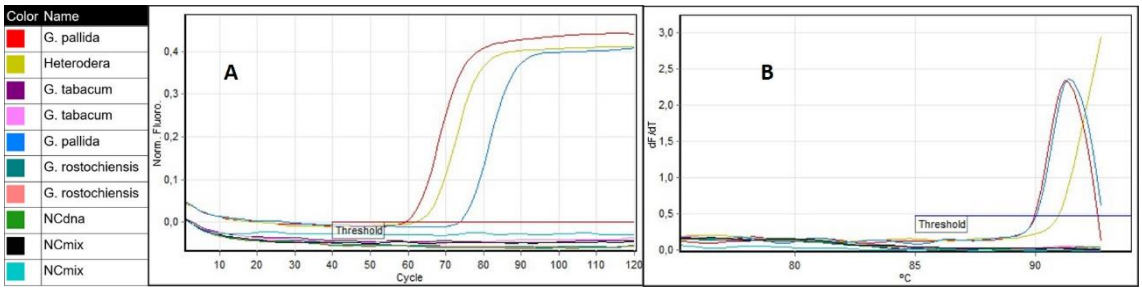

FIGURE 14 | LAMP assay run on a rt-PCR instrument using genomic DNA from *Globodera pallida* (MK791521; NPPO NL Pa3 HLB), *G. rostochiensis* (MK791264; NPPO NL Ro1 HLB) *G. tabacum* (NPPO NL C6876) *Globodera* n. sp (MT256387) and *Heterodera* sp. (SV-18-10003): (A) amplification curves of *G. pallida* and *Heterodera* sp. *G. rostochiensis*, *Globodera* n. sp., *G. tabacum* and negative controls did not amplify and are represented by the horizontal lines and (B) derivative of the melting temperature curve.

DISCUSSION

In recent years, we have seen an increasing need for early detection methods, mainly for emerging and invasive organisms and plant pathogens, either regulated or nonregulated, in all areas of diagnostics (Huang *et al.,* 2020). Among many new methods and technologies, LAMP is one of the most explored techniques to detect invasive and quarantine species both at the laboratory level and on site (farms, water resources, border inspection points) (Blaser *et al.,* 2018; Martinez *et al.,* 2019; Tomlinson, 2008).

Cost-effectiveness is an important parameter of phytosanitary analysis (Tomlinson, 2008). Moreover, costs associated with the damage caused by new pests in the invaded areas as a result of decreases in production, market value and pest management, surveillance and inspection may benefit from an early detection.

Currently, *G. pallida* represents a real threat to production in all potato-producing countries. Its control is affected by the lack of attractive potato resistant/tolerant cultivars and by the existence of cultivars with high tolerance to *G. rostochiensis* which create a pressure on the selection of *G. pallida*. There is substantial evidence suggesting that European countries bear an increasing burden with this nematode due to the high circulation of people and goods.

Therefore, in this report, we describe the development of a LAMP-based assay for the specific identification of *G. pallida* by targeting the ITS1 sequence. We present a more rapid and precise, simpler and more affordable diagnostic method than the traditional diagnostic methods (Deng, *et al*., 2019). Indeed, a demand for simpler and low-cost detection methods that retain the sensitivity of PCR but avoid the costly rt-PCR equipment and laborious practices was the motivation for the development of this assay (Tomlinson and Boonham, 2008). Additionally, it does not require specific knowledge or experience by the operator. Thus, our LAMP assay can be considered essential for surveillance and disease control purposes.

The primers used for the LAMP amplification specifically detected *G. pallida* in DNA extracts with concentrations, at least, equal or above 5 pg/L. No false positives were observed either with other closely related species or non-related species. In a single situation, the DNA of one

Heterodera sp. amplified but the melting temperature of the product was different from the expected for *G. pallida*. Since either DNA or cysts from *G. mexicana* were not available, the specificity of our LAMP assay could not be tested against this species. However, knowing that *G. mexicana* is present in a restricted area of Mexico, is not a potato cyst nematode and the spread of these pests happened mainly through potato seed, the risk of false positives is very low when performing potato field surveillances. False positives due to cross-reaction with non-related species were also analytically not observed. This was expected from both the in-silico analysis of DNA sequences and the nematode extraction process from soil samples.

In this work, LAMP assays optimized for a portable instrument in real time allowed for a complete analysis in less than 40 min even when using pooled samples with one *G. pallida* J2 mixed with 40 *G. rostochiensis* J2. Positive amplifications started from ca. 9 min (Figure 13) the average time being ca. 10.5 min when the DNA was extracted from 1 single juvenile of *G. pallida* (mixed with up to 19 juveniles of *G. rostochiensis*) by the DNeasy Blood and Tissue Kit. In all cases, the DNA concentration of the extracts was in the range of 2 to 4 ng/L, what is not sufficiently variable to yield significant differences in the amplification time, besides the fact that this assay was not designed to be quantitative. The relatively low amount of DNA that originated from one *G. pallida* juvenile combined with the used primer concentration was not the limiting factor for obtaining a positive signal when the DNA was extracted by the DNeasy Blood and Tissue kit. Similar results were observed when DNA was extracted from 5, 9, 19 and 40 juveniles of *G. pallida* that were always mixed with one single juvenile of *G. rostochiensis*.

A higher number of juveniles did not improve the final concentration of DNA in the extracts obtained from the samples with more specimens combined with one juvenile of *G. rostochiensis*. The most evident difference can be seen in the sample having the ratio 40:1 or the lowest representativeness of *G. pallida*.

These observations show that the established LAMP is highly specific for detecting *G. pallida* even in samples infested with cysts of other *Globodera species*. For specificity checks, DNA from several European isolates from three non-target species of the *Globodera* genus and isolates from other cyst nematodes were examined. We focused on those species present in Europe and in potato fields where they may co-habit (Khan *et al*., 2018; Li *et al*., 2019; Kang, *et al*., 2014; Niu *et al*., 2011). Different Portuguese populations (unknown pathotypes), a population from the Netherlands (pathotype Pa3) and four isolates from a European interlaboratory study (from different origins and probably of different pathotypes) tested systematically positive. As the number of isolates from other origins was limited, interlaboratory performance studies are needed to confirm the specificity and to determine the repeatability and reproducibility of this method in order to be standardized and validated. In the Molecular Biology Laboratory at INIAV and in the independent laboratory of the University of Évora, we obtained 100% matches. Further improvement of this LAMP assay will include the use of DNA extracted on-site from the potato rhizosphere by the rapid method and optimization for the potential use under field conditions at the point-of-care in the farms.

To our knowledge, this is the first reported LAMP method for differentiating *G. pallida* from both other cyst nematodes (*G. rostochiensis*, *G. tabacum* and *Heterodera* sp.) and motile nematodes.

EXPERIMENTAL PROCEDURE

1. Samples, Chemicals and Standard Techniques

An initial assay development was undertaken using either cysts or second stage juveniles (J2) from all isolates which had originated from different potato growing regions in Portugal (Camacho *et al*., 2020). This material was obtained at the Nematology lab of INIAV (NemaINIAV).

Later, for the specificity characterization of the assay and to estimate the risk of future false negatives, nematode populations from The Netherlands, kindly provided by NVWA–The Netherlands Food and Consumer Product Safety Authority, Wageningen, composed of three different nematode species (*G. pallida*, *G. rostochiensis* and *G. tabacum*) were analyzed as well as DNA extracts obtained from the European isolates provided for an interlaboratory study. The identities of the former were known whereas the identities of the latter were not (blind samples). The second set of samples also allowed evaluation of the practical application of the LAMP assay. The extraction of total DNA was always conducted using the DNeasy Blood and Tissue Kit (Qiagen) and following the manufacturer's instructions. DNA extracts were used directly for the LAMP reactions without any additional purification step.

2. *Globodera* sp. Sequences and Primer Design

Nucleotide sequences of the "3'end 18S-ITS1-5.8S-ITS2-5'end 28S" rDNA region from 14 *Globodera pallida* isolates collected from Portuguese potato fields (Camacho *et al*., 2020) were chosen as the candidate targets for primer design. To ensure the specificity of this new assay, sequences from the closely related non-target species *G. rostochiensis*, *G. tabacum* and *Globodera* n. sp. (only detected in Portugal (Camacho *et al*., 2020; Reis, 1997) and *G. mexicana* and *G. ellingtonae* were also included in the primer design and in the in-silico verification of the specificity of the primers (Supplementary Table S1). A total of 89 sequences retrieved from the National Centre for Biotechnology Information (NCBI), a quality curated sequence database, covering regions from all potato production regions were grouped using BioEdit v7.2.0 (Hall, 2007) and aligned by means of ClustalW Multiple Alignment tool (Higgins *et al*., 1996). Based on the alignment of the *G. pallida* accession sequences, a consensus sequence was created and used to design sets of LAMP primers (Supplementary Figure S2) by the online LAMP designer tool Primer Explorer V5 (Eiken Chemical Co. LTD, Tokyo, Japan). Two sets of four primers were selected for the LAMP development each set composed of two outer primers (F3 and B3), one forward inner primer (FIP) and one backward inner primer (BIP) (Table 6).

3. LAMP Assay

All LAMP reactions were conducted in the B-cube device (Hyris, London, UK) in 16-well cartridges. Each reaction was 25 L final volume comprising 15 L of the isothermal master mix ISO-004 or ISO-001 (OptiGene, Horsham, UK), which vary in the MgSO4 concentration, and 5 L of the template DNA. In this step only DNA from *G. pallida* was used. For all primers (Table 6), five different concentrations were tested in different combinations during the optimization process. For the FIP and BIP primers (50 M) the volume varied from 0.4 to 0.9 L and was combined with different volumes of the F3 and B3 outer primers (50 M), which varied from 0.10 to 0.15 L each. For the optimization of the temperature and time, the reaction mixtures were incubated at 65 to 63 C, for 60 to 20 min. To determine the product melting temperature, the generated products were heated from 75 to 95 °C at a rate of 0.05 °C/s. In all LAMP assays, as

a negative amplification controls (NAC), 5 L of water was added to the reaction instead of DNA extract. The LAMP products were detected by the SybrGreen fluorescence.

The protocols in Table 8 were tested during the optimization of the LAMP protocol for *G. pallida* identification.

Briefly, at the end, the LAMP reactions should be performed as described in Table 9.

4. LAMP Specificity

Analytical specificity inclusivity was assessed by in silico analysis taking sequences from specimens from all regions reported as having *Globodera* sp., therefore, covering a wide range of genetic diversity and geographic origins. In order to assess the analytical specificity exclusivity of the LAMP assay, genomic DNA extracted from cysts of non-target species from different origins (Table 7) were used as template.

A second LAMP experiment was performed according to Table 9 with blind samples from an interlaboratory study (Table 7-II). A negative control sample was also prepared using PCR grade-H_2O instead of a DNA template. LAMP results were visualized by measuring the fluorescence emitted by the DNA intercalating dye SYBRGreen. All experiments were done twice, within two weeks by the same operator, and the samples were analyzed in triplicate to ensure repeatability.

Specificity or true-negative rate was calculated as: Specificity = [NA/(NA + PD)] 100. Where NA is the number of true negative results (negative agreement) and PD is the number of false positive results (positive deviation) (EPPO, 2019).

An extra LAMP experiment was performed with Portuguese genomic DNA (Table 11) from *Globodera pallida*, *Globodera rostochiensis*, *Pratylenchus penetrans*, *Xiphinema* sp., Helicotylenchus sp., *Bursaphelencus xylophilus* and *B. mucronatus* provided by the Nematology lab of INIAV (NemaINIAV). Negative control samples were also prepared using PCR grade-H_2O instead of a DNA template. LAMP results were visualized by measuring the fluorescence emitted by the DNA intercalating dye SYBRGreen.

Table 11 | No cyst nematode samples from Portugal used for LAMP specificity assay.

Species	Isolate	Origin
Pratylenchus penetrans	A44L4 *	Portugal
Xiphinema sp.	SV-21-00826 *	Portugal
Helicotylenchus sp.	SV-20-0967-01 *	Portugal
Bursaphelencus xylophilus	SV-21-0502-02 *	Portugal
Bursaphelencus mucronatus	BmCh3 *	Portugal

* Not deposited at the NCBI GeneBank database. INIAV internal reference number.

5. LAMP Sensitivity

Sensitivity was estimated at two different levels, analytical and diagnostic. To assess analytical sensitivity, the ability to detect low concentrations of DNA was studied. Different serial dilutions of *G. pallida* DNA (5 ng/µL, 1 ng/µL, 0,1 ng/µL, 0,01 ng/µL and 5 pg/µL) were separately subjected to the optimized LAMP protocol (Table 9) in triplicate. This can be referred as the Limit of Detection (LoD) as it represents the number of DNA copies that can be consistently detected in more than 95% of the times.

A second LAMP assay was performed with eight replicates of two DNA extracts from *G. pallida* at two low concentrations (0,01 ng/µL and 5 pg/µL) to confirm the assay detection limit. LAMP results were visualized by measuring the fluorescence emitted by the DNA intercalating dye SYBRGreen.

Diagnostic sensitivity was assessed by preparing mixtures with different proportions of *G. rostochiensis*/*G. pallida* J2. Cysts from both species were cut and J2 were picked up according to Table 11 composition. Two independent samples for each ratio of *G. rostochiensis*/*G. pallida* were prepared and analyzed.

Sensitivity or true-positive rate was calculated by means of the following formula: Sensitivity = PA/(PA + ND). Where, PA is the number of true positives (positive agreement) and ND is the number of false negatives or positive deviations.

6. LAMP Reproducibility

The reproducibility was tested performing analyses on two different devices: B-cube (Hyris, UK) and rt-PCR (RotorGene Q, Qiagen, Hilden, Germany) and in two different laboratory facilities (GMO and Molecular Biology Laboratory at INIAV and NemaLab in Évora University).

A LAMP experiment was performed in the RotorGene Q instrument under the same reaction conditions. In order to determine the time, the thermal cycling profile consisted of 120 cycles at 64 ^{o}C for 1 + 29 s (totalizing 60 min) and a final step of 93 ^{o}C and cooling to 75 ^{o}C, 0.05 ^{o}C/s to determine the Tmelting. Two isolates of *G. pallida* and *G. tabacum* were tested whereas the *Heterodera* sp. sample was loaded alone (Table 6). LAMP results were visualized by measuring the fluorescence emitted by the DNA intercalating dye SYBRGreen.

SUPPORTING INFORMATION

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10060744/s1, Supplementary Table S1: Geographical origin, accession reference number and year of collection of sequences from *Globodera* species used in either in silico or in the laboratory evaluation to verify the specificity of the primers. Supplementary Table S2: Results of protocols for *Globodera pallida* LAMP optimization assays. Supplementary Figure S1: LAMP designer tool Primer Explorer V5 (Eiken Chemical Co. LTD, Tokyo, Japan) outcome primers sets.

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CHAPTER IV

A LAB-ON-A-CHIP APPROACH FOR THE DETECTION OF THE QUARANTINE POTATO CYST NEMATODE *GLOBODERA PALLIDA*

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SUMMARY

The potato cyst nematode (PCN), *Globodera pallida*, has acquired significant importance throughout Europe due to its widespread prevalence and negative effects on potato production. Thus, rapid and reliable diagnosis of PCN is critical during surveillance programs and for the implementation of control measures. The development of innovative technologies to overcome the limitations of current methodologies in achieving early detection is needed. Lab-on-a-chip devices can swiftly and accurately detect the presence of certain nucleotide sequences with high sensitivity and convert the presence of biological components into an understandable electrical signal by combining biosensors with microfluidics-based biochemical analysis. In this study, a specific DNA-probe sequence and PCR primers were designed to be used in a magnetoresistive biosensing platform to amplify the internal transcribed spacer region of the ribosomal DNA of *G. pallida*. Magnetic nanoparticles were used as the labelling agents of asymmetric PCR product through biotin–streptavidin interaction. Upon target hybridization to sensor immobilized oligo probes, the fringe field created by the magnetic nanoparticles produces a variation in the sensor's electrical resistance. The detection signal corresponds to the concentration of target molecules present in the sample. The results demonstrate the suitability of the magnetic biosensor to detect PCR target product and the specificity of the probe, which consistently distinguishes *G. pallida* (DV/V > 1%) from other cyst nematodes (DV/V < 1%), even when DNA mixtures were tested at different concentrations. This shows the magnetic biosensor's potential as a bioanalytical device for field applications and border phytosanitary inspections.

INTRODUCTION

Globodera rostochiensis (Wollenweber, 1923: Skarbilovich, 1959) and *Globodera pallida* (Stone, 1972), known as potato cyst nematodes (PCN), are one of the greatest threats to potato crops. These plant parasitic nematodes are originated from the Andes region in southern Peru and spread worldwide due to human activities (Grenier *et al*., 2010) and lack of phytosanitary measures as they exist nowadays. In Europe, PCN were introduced in the 16–17th century, by means of infested potato tubers, and were also reported throughout North and South America, parts of Asia, Africa and Oceania where potatoes are grown (EPPO, 2020). The golden PCN, *G. rostochiensis*, and the pale PCN, *G. pallida*, are worm-like microscopic endoparasites which feed on potato roots, deteriorating the quality of tubers and reducing their commercial value. In addition, PCN may facilitate the infection of potatoes by opportunistic pathogens, like bacteria and fungi (Lavrova *et al*., 2017), significantly reducing yield, increasing the overall costs of production and imposing trade restrictions. Therefore, upon PCN detection, crop fields are subjected to severe quarantine in many countries, where these nematodes are considered harmful quarantine organisms (EPPO, 2022).

Owing to their commercial and environmental impacts, it is essential to detect these species early. A promising tool relies on the use of diagnostic devices in order to implement strategies for an effective integrated pest management. As the morphological identification of these *Globodera* species may be uncertain due to the overlapping morphometric values between both species, molecular confirmation is recommended (Camacho *et al*., 2017).

PCN molecular identification, described in the European and Mediterranean Plant Protection Organization (EPPO) protocol PM 7/40 (EPPO, 2022), is performed through duplex conventional and/or real-time PCR based on the nuclear large subunit ribosomal DNA (rDNA) sequences. Despite the high sensitivity and specificity of these diagnostic methods, the procedures require

highly trained staff, are time consuming, the laboratory equipment is expensive and cannot be used in agricultural fields due to the lack of portable devices.

Therefore, due to PCR-based protocol constraints, other methods should be developed, aiming at less practical and technical expertise and at the use of new portable and affordable technological devices for in-field analyses. As a result, different prototypes have been developed concerning the miniaturization of biomolecular methodologies. Microfluidic systems have been used for the automation of experiments and minimization of user intervention (Llandro *et al*., 2010; Romão *et al*., 2017), allowing completely integrated systems, including all steps from sample preparation until DNA amplification (Martins *et al*., 2019).

Biosensors, in combination with microfluidics-based biochemical analysis, in a miniaturized device, can rapidly detect the presence of specific nucleotide sequences with high sensitivity and convert the presence of biological compounds into an easy-to-read electrical signal. The detection of DNA amplicons (fragments amplified either by PCR or isothermal reactions) is based on specific target DNA sequence hybridization with a complementary immobilized oligo probe, that can be spotted on chip surfaces in a microarray format (Romão *et al*., 2017).

An existing portable electronic reader and magnetoresistive (MR) biochips developed in a collaboration between INESC MN and INESC ID (Lisbon, Portugal) (Martins *et al*., 2009; Germano *et al*., 2015) were used to discriminate the internal transcribed spacer (ITS) region rDNA of *Globodera pallida* (tested as a model organism) from other related species.

The MR biochip is comprised of an array of 30 spin-valve (SV) sensors which offer particular advantages in terms of reduced size, low limit of detection, analytical sensitivity, high signal-to-noise ratio and integration capability (Li *et al*., 2006; Lin *et al*., 2019). The target molecules are marked with magnetic nanoparticles (MNPs) through biotin–streptavidin interaction, generating a fringe magnetic field when an external magnetic field is applied, proportionally changing the electrical resistance of the sensors (Graham *et al*., 2004; Freitas *et al*., 2012). Asymmetric PCR products, after amplification, go through a microfluidic system to the probes immobilized on the sensors, allowing their hybridization. The probe sequence can be manually or robotically spotted over the sensing sites and when complimentary target amplicons specifically hybridize, a signal is generated in the transducer (Martins *et al*., 2019).

Oligonucleotide probe and specific PCR primers were designed at GMO and molecular biology lab of INIAV (Oeiras, Portugal) to specifically target *G. pallida* based on the ITS rDNA while avoiding the detection of *G. rostochiensis*, *G. tabacum* and *Heterodera* sp., which can be found in the same fields as *G. pallida*. The analytical specificity and sensitivity of this system was evaluated using detection assays with target DNA amplified by asymmetric PCR using one pair of specific primers and various ratios of template DNA in mixtures of the closely related non-target species *G. rostochiensis*.

Biosensors are growing at a fast pace in human diagnostics, while applications for agriculture remain limited. This work intends to demonstrate the applicability with economic viability of the use of biosensors in agricultural fields for soil pest management or at border phytosanitary inspections facilities.

EXPERIMENTAL PROCEDURES

1. Sensors

MR sensor microfabrication is described in the work of Martins *et al.* (2009). Briefly, the biochip consists of an array of 30 SV sensors passivated with an oxide layer, arranged in six sensing regions, each one containing five active sensors covered with a gold layer, and surrounded by a gold frame for the discrete spotting of the probes. The SV stack consists of the following magnetic thin layers: Ta 2.0 nm/NiFe 2.5 nm/CoFe 2.8 nm/Cu 2.6 nm/CoFe 2.4 nm/MnIr 7.0 nm/Ta 5.0 nm. The sensors are arranged in series of two SVs (active area of 80 × 2.6 μm2) electrically contacted by aluminum leads. The sensors' magnetic response was characterized, obtaining an average MR of 6.0% and sensitivity of 1.3%/mT.

2. Biochemical Reagents

TE buffer was supplemented with KH2PO4 (0.1 mM), Tris (10 mM), EDTA (1 mM) and pH was adjusted using HCl (1 M) to 7.4. Phosphate buffer (PB) was prepared from stock solutions of Na2HPO4 and NaH2PO4 at 0.2 M and pH 7.2. PB-Tween20 consisted on PB buffer with 0.02% (v/v) of Tween® 20 from Promega (Madison, WI, USA). All solutions were prepared with ultra-pure grade water.

The customized primers and probes were synthesized by Eurogentec (Seraing, Belgium).

The Magnetic Nanoparticles were nanomag®-D from Micromod (Rostock, Germany), with a diameter of 250 nm and 75–80% (w/w) magnetite in a matrix of dextran (40 kDa), and streptavidin coated. The particles had a magnetic moment of ~1.6 × 10−16 Am2 for a 1.2 kA/m magnetizing field and a susceptibility of χ~4.

3. Nematode Samples

Nematode isolates of *G. pallida*, *G. rostochiensis*, *G. tabacum*, *Heterodera* sp. and different mixtures of *G. pallida*/*G. rostochiensis* (Table 12) were obtained at the Nematology lab of INIAV (NemaINIAV, Oeiras, Portugal).

Table 12 | Samples from Portugal and The Netherlands used for on-chip assays.

Species	Isolate	Origin	ng/μL
G. pallida	MK791521	Portugal	5.2
G. pallida	NPPO-NL Pa3 HLB	Netherlands	1.4
1 Gp/5 Gr	MK791521/ MK791264	Portugal	4
1 Gp/19 Gr	MK791521/ MK791264	Portugal	2.2
1 Gp/40 Gr	MK791521/ MK791264	Portugal	5.4
G. rostochiensis	MK791264	Portugal	28.2
G. rostochiensis	NPPO-NL Ro1 HLB	Netherlands	2.9
G. tabacum	NPPO-NL C6876	Netherlands	39.4
Heterodera sp.	SV-18-10003	Portugal	18.1

The extraction of total DNA was always conducted using the Qiagen DNeasy Blood and Tissue kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. Genomic DNA was quantified using the thermo-NANODROP 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 ◦C until further use. DNA extracts were used directly for the PCR reactions without any additional purification step. Total DNA extraction, purification and conservation was performed as described in Camacho *et al*. (2020).

4. *Globodera pallida* Probe and Primer Sequence Design

The nucleotide sequences of the "3'end 18S-ITS1-5.8S-ITS2-5'end 28S" rDNA region used to design the probe specific for the detection of *G. pallida* were acquired during a previous study to develop a new LAMP assay (Camacho *et al*., 2020; 2021). The primers B2 and F3 were selected as the forward and the reverse primers, respectively, to amplify a 141 bp biotinylated product. B2 was biotinylated on the 5'end. The detection of this product, by immobilization, needs a probe which was labelled with a thiol group and a 15-mer poli-T sequence at the 5'end for immobilization purposes.

Primers and probe's sequences and characteristics are summarized in Table 13. Additionally, a probe sequence not related with any target sequence was used as the negative control. The primer properties (were indicated by the manufacturer—Eurogentec (Seraing, Belgium). The probe properties (Table 13), including guanine and cytosine (GC) content, melting temperature (Tm) and change in free energy of hybridization (ΔG), were calculated using the IDT Oligo Analyzer tool.

Table 13 | Sequence, size, GC content, and melting temperature (Tm) of a universal pair of primers designed based on the ITS-rDNA of *Globodera pallida* and the change in free energy of hybridization (ΔG) of the oligonucleotide probe specifically designed to target *Globodera pallida*, and of the negative control probe.

Primers	Sequence (5′-3′)	Size (bp)	GC%	Tm (^{0}C)	ΔG (kcal/mol)
F3 - Reverse primer (Rv) (camacho *et al*., 2021)	ACA CAT GCC CGC TAT GTT	18	50	54	
b-B2 - Forward primer (Fw)	Biotin-AG CGA CCC GAC GAC AA	16	62.5	52	
G. pallida	Thiol 15T GTG TAA CCG ATG TTG GTG GCC CAA TG	26	53.8	62.1	-51.85
Chikungunya	Thiol 15T CGC ATA GCA CCA CGA TTA G	19	52.6	53.4	-36.7

5. Asymmetric PCR Amplification

The ITS-rDNA was amplified by an asymmetric PCR, with a primer ratio of 10:1 (Fw:Rv). PCR reactions were performed in a 25 µL final volume containing 5 µL template DNA, 5 µL GoTaq Flexi PCR buffer (2×), 5 µL MgCl2 (25 mM), 0.4 µL dNTPs (10 mM), 0.5 µL GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 0.375 µL of F3 primer (10 µM), 3.75 µL of b-B2 primer (10 µM) and 4.975 µL of DNA-free water. The amplification profile for ITS-rDNA consisted of an initial

denaturation of 94 ◦C for 2 min followed by 35 cycles at 94 ◦C for 30 s, 55 ◦C for 30 s, and 72 ◦C for 15 s and a final extension of 72 ◦C for 7 min. The amplified products were visualized using the VersaDoc Gel Imaging System (Bio-Rad, Hercules, CA, USA) after being electrophoresed at 5 V/cm in 0.5× SGTB buffer (GRISP, Porto, Portugal) and in a 1.5% agarose gel stained with ethidium bromide (0.5 µg.mL^{-1}). Possible contaminations were checked by including negative controls (no template control—NTC) in all amplifications.

6. Detection Assays in the Biochip Platform

Prior to probe immobilization, biochips underwent a cleaning procedure described in Viveiros *et al*. (2020). The probes designed for *G. pallida* detection were diluted in the TRIS-EDTA buffer to a concentration of 5 µM and immobilized by manual spotting on the biochip surface (Figure 15—Probe immobilization). Each spot consisted of a drop volume of 1 µL. After spotting, the probes were left to immobilize for 1 h in a humid chamber at room temperature.

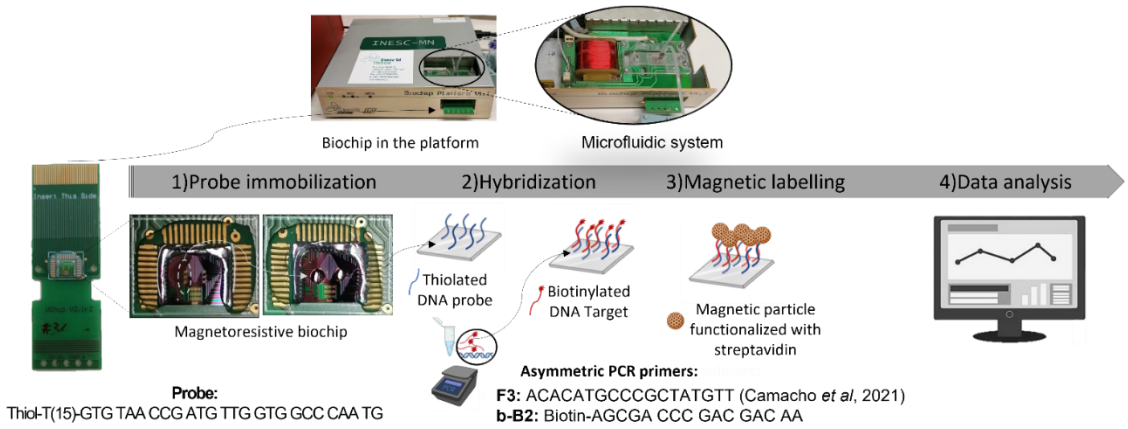

FIGURE 15 | Schematic representation of the main steps involved in a measurement.

The biochip platform was fabricated by INESC ID and INESC MN (Lisbon, Portugal) as described by Germano *et al*. (2009). The sensor functionalized with the *G. pallida* probe was inserted in the platform and an U-shaped PDMS microfluidic system was placed over the sensor to transport the reagents, in sequential order, over the sensing area (Figure 15) (Martins and Germano, 2010), All reagents were loaded at a flow rate of 50 µL/min, with the help of a syringe pump (NE-300, NEW ERA, Buffalo, NY, USA). First, sensors were washed with PB buffer to remove unbound probes, followed by the loading of 10 µL of target asymmetric PCR product to cover the sensing sites (Figure 15—Hybridization). The hybridization was left to occur for 30 min, after which, unbound target molecules were washed off with PB buffer. Next, the MR measurement was initiated by first acquiring the baseline voltage of the sensors for 5 min, followed by the injection of the MNPs (10× diluted from stock) into the PDMS channel which were then left to incubate over the sensors for 20 min (Figure 15). After the resistance signal of the sensors saturated, the unbound particles were washed off for 5 min at continuous flow, or until signal stabilization. In total, data acquisition took about 30 min. The main steps of the measurement are represented in Figure 15.

The sensors were biased with 1 mA DC current, and the MNPs magnetized with an external AC magnetic field of 13.5 Oe at 211 Hz and a DC field of 35 Oe. A voltage signal was acquired for each sensor and the data was recorded (Figure 16).

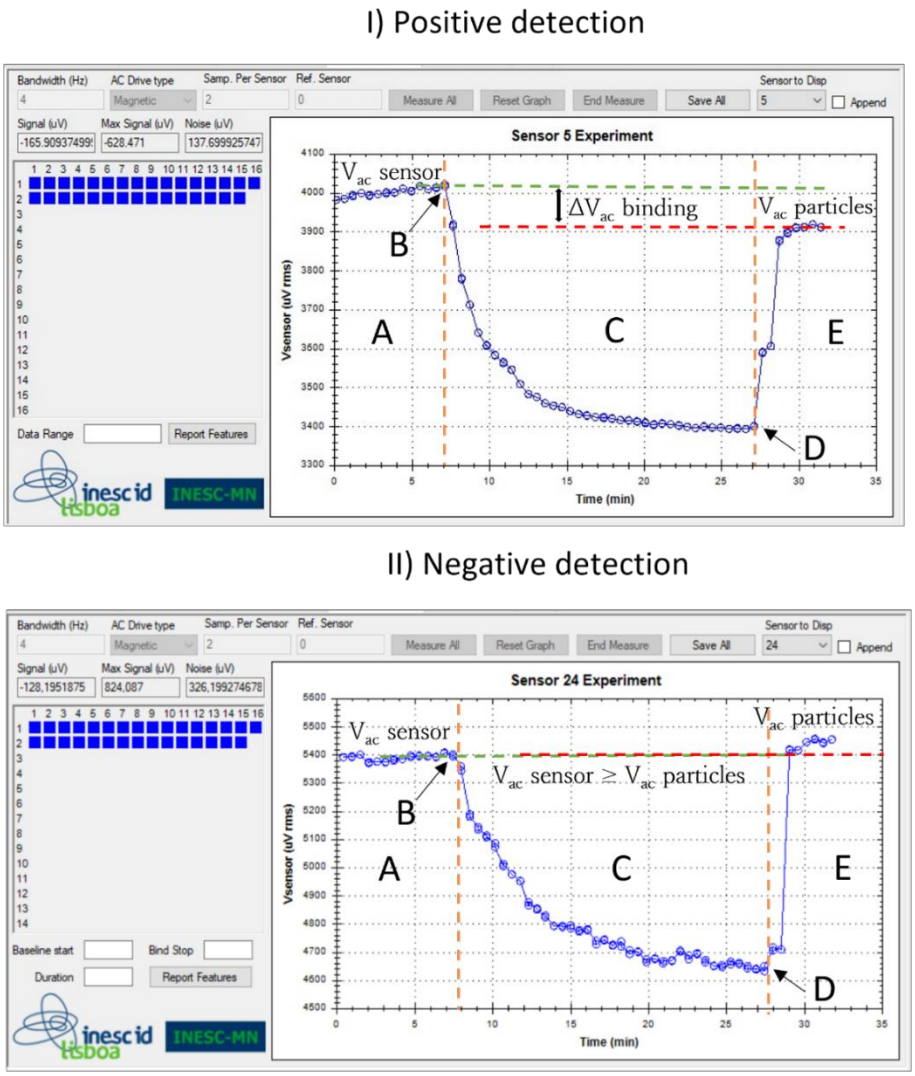

FIGURE 16 | Voltage signal acquired from two sensors. Both measurements occur through five phases: (A) acquisition of the sensor baseline signal (Vac sensor); (B) magnetic particle addition; (C) decreasing signal due to the magnetic particles settling down over the sensor; (D) saturation signal; and (E) washing step and final signal corresponding to the presence of target bound magnetic particles over the sensor (Vac particles): (I) positive detection event: hybridization with a complementary target DNA (*Globodera pallida*) labeled with 250 nm magnetic particles ending at a lower voltage and (II) negative detection event: non-hybridization with a non-target DNA (*G. rostochiensis*) ending at an equal voltage value.

7. Data Analysis

The binding signals are differential voltage values identified as ΔVac binding, calculated from the difference between the sensor baseline (Vac sensor) and the signal originating from the specifically bound MNPs over the sensor (Vac particles). The ΔVac binding signal is then normalized by the sensors' baseline and taken as the final output read-out signal (ΔVac binding/Vac sensor). Additionally, in each substrate, a reference spot with an unspecific probe (whose target is Chikungunya—Table 13) was performed to remove the influence of unspecific binding. The measurement curves on Figure 16 correspond to the sensors used to detect (I) target DNA and (II) a non-complementary target.

RESULTS AND DISCUSSION

1. Asymmetric PCR

DNA samples of *Globodera pallida*, *G. tabacum*, *G. rostochiensis* and *Heterodera* sp. were amplified by asymmetric PCR using the pair of primers indicated in table 13, designed on a region of the ITS-rDNA conserved among different isolates of *G. pallida* and variable among other species. Figure 17 presents the agarose gel of asymmetric PCR amplification products.

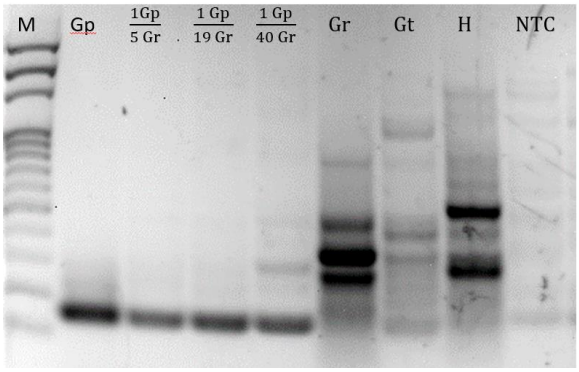

FIGURE 17 | Agarose gel of the amplified products obtained with asymmetric PCR using F3 and b-B2 primers. M = 100 bp DNA Ladder (Thermo Scientific); Gp = *Globodera pallida*.; Gp/Gr = ratios of *G. pallida* and *G. rostochiensis*; Gr = *G. rostochiensis*; Gt = *G. tabacum*; H = *Heterodera* sp.; NTC = negative control (no DNA template).

For all targets, more than one band was observed. These bands correspond to both double strand (dsDNA) and single strand DNA (ssDNA) products from the asymmetric PCR. The limiting primer was involved in the production of dsDNA since the first reaction cycle until it was fully consumed, when the ssDNA production started, supported by the forward primer in excess.

2. Detection Assays in the Biochip Platform

Detection assays were performed in the magnetoresistive biochip device with the target amplified by asymmetric PCR of genomic DNA samples. The data acquired from each sensor was analyzed as previously described. Different samples were tested against the specific probe for *G. pallida* and a negative control probe was used as a reference signal. At least three replicated measurements were performed for each sample, corresponding to the detection signal of at least 12 sensors in each measurement. The results obtained are summarized in Figure 18. Each bar of the graphic represents the normalized signal acquired from the probe against the *G. pallida*, mixed samples and non-target species PCR products. The threshold value (dashed line) was obtained from the value between the highest non-specific signal achieved against a non-complementary target and the lower specific signal obtained against a complementary target (standard deviation was taken into consideration). Above the threshold value, the detection signal was considered positive.

The tested probe showed specific signals against its complementary target (*G. pallida*) without significant cross reactivity, even when using pooled samples with *G. pallida* mixed with *G. rostochiensis* (ratios of 1/5, 1/19 and 1/40), corresponding to a diagnostic sensitivity of one (1) juvenile. All samples with *G. pallida* DNA obtained detection signals higher than 1% (1.9 ± 0.77%) and all samples with non-target DNA obtained detection signals lower than 1% (−0.04 ± 0.44%). *Globodera rostochiensis* samples, as expected due to PCR product amplification be the closest

related to *G. pallida*, obtained higher detection signals than the others non-target species (*G. tabacum* and *Heterodera* sp.), even so lower than 1%. These data are in line with previous studies, whose reports show a positive detection signal of 1.8 ± 0.7% and a negative control of 0.4 ± 0.3% (Viveiros *et al.,* 2020)

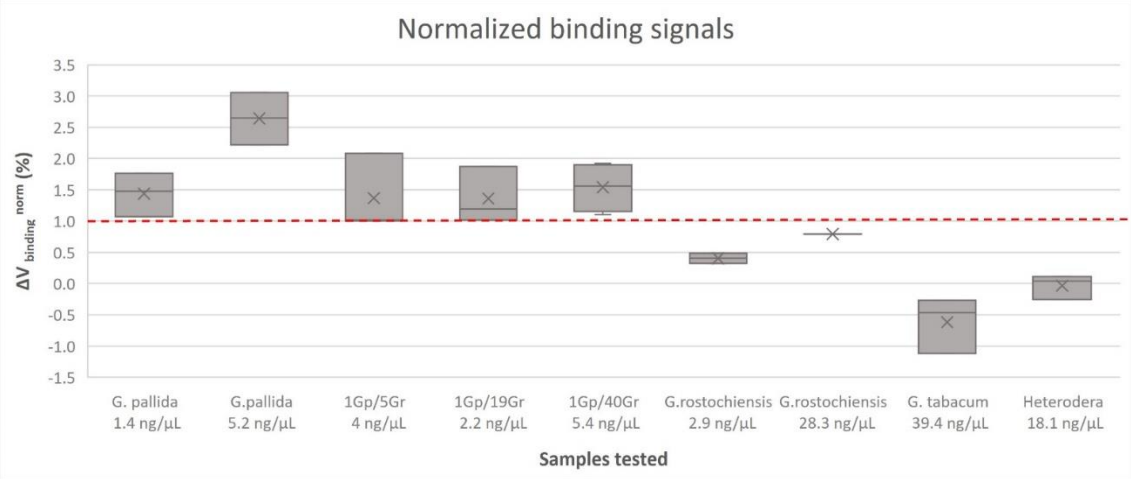

FIGURE 18 | Normalized binding signals obtained for each sample group, obtained from asymmetric PCR against the specific probe for *Globodera pallida* detection. The error bars are standard deviations coming from at least 12 sensors acquired from three measures for each sample. The dashed line represents the threshold, a minimum value above which a detection signal is considered positive. DNA of pure samples (*Globodera pallida*, *G. rostochiensis*, *G. tabacum* and *Heterodera* sp.) was extracted from cysts and DNA extracted from pooled samples was extracted from mixed juveniles.

The results demonstrate the specificity of the probe which reliably discriminates *G. pallida* from other cyst nematodes. The MR biosensor shows specific signals for qualitative *G. pallida* detection through a double specific control—PCR and probe hybridization efficiency—avoiding false positives for non-targets samples, such as *G. rostochiensis*, *G. tabacum* and *Heterodera* sp. This approach shows great promise for field application in the early detection and surveillance of plant soil pests and in assisting the implementation of management practices to reduce the risk of infestations. Another possible application is at border phytosanitary inspections. New technologies are in high demand in the agricultural market to address the problem of plant pest detection and there is a clear opportunity for new developments in portable devices for agriculture applications. Further improvement of this technique will include an isothermal amplification of DNA (e.g., LAMP—Loop Isothermal AMPlification) (Notomi *et al*., 2020)] to avoid the need for high temperatures which is the major impediment for its application in-field, and the use of Flinders Technology Associates (FTA) card protocol for DNA extraction on-site (Marek *et al*., 2014).

Despite not being the goal of this work, whose purpose was to qualitatively detect *G. pallida* (tested as a model organism), other works have achieved the simultaneous multiplex detection of different pathogens based on an asymmetric PCR protocol coupled with a magnetic array biochip functionalized with species-specific probes (Viveiros *et al.,* 2020; Miguéis *et al*., 2021). In the future, a multiplex detection protocol can be designed for the detection of different *Globodera* species using a single pair of primers in asymmetric PCR to indiscriminately amplify any target *Globodera* sp. in conjunction with species-specific sensor-immobilized oligonucleotide probes.

CONCLUSIONS

Recently, we have seen an increasing need for new detection methods, mainly for plant pests and diseases. An essential consideration in phytosanitary study is the cost–benefit ratio. Although the use of biosensors in human diagnosis is expanding quickly, there are still few applications in agriculture. With this work, we tried to manage plant pests in agricultural fields by integrating the use of biosensors. This activity is in line with the European Green Deal, which acknowledges digitization as a tool to enhance output by lowering the impact of pests and diseases, improving productivity and enabling an ecological transition (reduction in pesticide applications).

Nowadays, *G. pallida* constitutes a big threat to all potato-producing regions. Its management is being affected by the few attractive *G. pallida* resistant/tolerant potato cultivars, compared to several cultivars with a high tolerance to *G. rostochiensis*, which is leading to *G. pallida* selection. Therefore, for field detection, we used a magnetoresistive biochip device for the specific identification of *G. pallida* by targeting the ITS-rDNA sequence. The primers designed for the PCR amplification in combination with the probe specifically detected *G. pallida* in DNA extracts. No false positives were observed with other closely related species. These observations show that the tested biosensors are highly specific for detecting *G. pallida* even in samples infested with cysts of other *Globodera* species.

It is possible to investigate this technology for the detection of other organisms and plant pests and pathogens. It does not require specific knowledge or experience from the operator. Thus, this method can be considered very beneficial for the surveillance and disease plant control purposes.

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CHAPTER V

FTA-LAMP BASED BIOSENSOR FOR A RAPID IN-FIELD DETECTION OF *GLOBODERA PALLIDA* - THE PALE POTATO CYST NEMATODE

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SUMMARY

The combination of a sensitive and specific magnetoresistive sensing device with an easy DNA extraction method and a rapid isothermal amplification is presented here targeting the on-site detection of *Globodera pallida*, a potato endoparasitic nematode. FTA-cards were used for DNA extraction, LAMP was the method developed for DNA amplification and a nanoparticle functionalized magnetic-biosensor was used for the detection. The combinatorial effect of these three emerging technologies has the capacity to detect *G. pallida* with a detection limit of one juvenile, even when mixed with other related species. This combined system is far more interesting than what a single technology can provide. Magnetic biosensors can be combined with any DNA extraction protocol and LAMP forming a new solution to target *G. pallida*. The probe designed in this study consistently distinguished *G. pallida* (($\Delta V_{ac}^{binding}/V_{ac}^{sensor}$ above 1%) from other cyst nematodes (($\Delta V_{ac}^{binding}/V_{ac}^{sensor}$ below 1%). It was confirmed that DNA either extracted with FTA-cards or Lab extraction Kit was of enough quantity and quality to detect *G. pallida* whenever present (alone or in mixed samples), ensuring probe specificity and sensitivity. This work provides insights for a new strategy to construct advanced devices for pathogens in-field diagnostics. LAMP runs separately but can be easily integrated into a single device.

INTRODUCTION

Sensors are instruments capable of gauging a physical signal and converting it into an easy-to-read electrical signal. Within the realm of agricultural industry, different types of sensors are used as part of crop management to foster sustainability and enhance crop productivity – a concept referred to as precision agriculture (PA).

These agricultural sensors play a central role in collecting data during crop growth cycle, from seed-plot to harvest, providing farmers a large amount of information to optimize their decision-making process. They have the capability to measure a wide range of parameters, including but not limited to air temperature, atmospheric pressure, rainfall, wind direction, solar radiation, soil moisture, temperature, nutrient content, electric conductivity and pH at different depths, light reflectance frequencies, carbon dioxide concentrations and other volatile substance. These measurements are used to monitoring crops health (Bogue, 2017; MacDougall *et al.,* 2022).

Sensors are designed to detect physicochemical parameters. For crop pests/diseases, are necessary biosensors, which combine a transducer with a biological receptor to achieve sensitive and selective detection of a range of analytes. When it comes to detecting crop pests and diseases detection, there are an increasing number of biosensors available (Mahlein, 2016). Advances in biosensing, information technologies, and nanotechnologies are opening up new opportunities for PA. Optical and thermal sensors are well-suited for detecting patches in the field afflicted by soilborne pathogens during crop production. However, these technologies encounter challenges in accurately differentiating between symptoms caused by different plant pests or diseases and damages resulting from abiotic stresses (Mahlein, 2016; Shao *et al.*, 2023). PA relies on specialized equipment and software to capture data and to infer the expertise of various scientific domains such as, plant health and informatics. There is still room for improvement in all these scientific areas, but a critical aspect in enhancing plant health management is the integration of the different technologies, allowing farmers to swiftly and precisely detect crop pests and diseases. This is where microfluidic sensing devices come into play. Loop Isothermal Amplification (LAMP)-based microdevices have been developed for plant pathogens detection (Fu *et al.*, 2021, Sivakumar *et al*, 2021) and fully integrated microfluidic

devices, comprising DNA extraction, amplification, and the detection of different plant pathogens, were already developed by Loo *et al*. (2017), Wu *et al.* (2021) and Das *et al.* (2022).

In this work, was adapted a microfluidic-based portable magnetoresistive (MR) device, that has been previously developed for detecting different pathogens affecting human and animal health (Martins *et al*., 2009, Viveiros *et al*., 2020, Albuquerque *et al*. 2022), to plant parasitic nematodes detection, namely, potato cyst nematodes (PCN).

The device was specifically modified to detect the internal transcribed spacer (ITS) rDNA region of *Globodera pallida*, which was used as a model organism and is distinct from other related species. The portable analytical platform (Germano and Martins, 2009), comprising the electronic reader and biochips, was a collaborative effort between INESC-MN (Instituto de Engenharia de Sistemas e Computadores – Microsistemas e Nanotecnologias, Lisbon, Portugal) and INESC-ID (Investigação e Desenvolvimento, Lisbon, Portugal). The biochips microfabrication, characterization, and encapsulation on chip carriers was performed in the clean room at INESC-MN. The MR biochip includes six discrete sensing areas, framed by gold squares, with each area containing five MR-based sensors (Spin valves - SV), resulting in a total of 30 active sensors per biochip (Martins *et al.*, 2009). Sensor functionalization involves the immobilization of an oligoprobe with a thiol group, facilitating strong chemisorption onto the gold-pads of the sensing sites (Martins *et al.*, 2009). This biologically active layer on the top of each individual sensor enables the hybridization of the biotinylated target sequence, through specific interactions between complementary sequences of the probe and the target LAMP amplified sequence. Magnetic labelling is achieved by flowing the streptavidin-modified magnetic nanoparticles (MNPs) over the biotinylated target molecules that have been previously immobilized on the sensors, within an U-shaped microfluidic PDMS-channel. Following the removal of unbound entities, the observed variation in the sensor's electrical resistance (baseline signal - MNPs signal = V_{ac}^{sensor} - $V_{ac}^{particles}$ = $\Delta V_{ac}^{binding}$ signal) corresponds to the amount of hybridized target molecules.

Previously, this technique has been successfully demonstrated for the detection of *Globodera pallida* using a laboratory DNA extraction kit and asymmetric PCR, as documented by Camacho *et al.*, (2023). However, in order to enhance field-testing conditions and streamline the process, reducing the time and sample preparation requirements, in this study was opted to use the Whatman Flinder Technology Associates (FTA) cards (Whatman, Maidstone, United Kingdom) rather than the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) to extract DNA . FTA cards are composed of cellulose and include patented chemicals that burst cells, denature proteins, and adsorb nucleic acids, allowing for long-term preservation at room temperature while maintaining quality needed for molecular assays; and have previously been demonstrated to be suitable and effective for plant parasitic nematodes DNA extraction (Marek *et al.*, 2014).

Additionally, a LAMP assay was developed to amplify a DNA fragment of *G. pallida* with the appropriate length to hybridize with newly designed probe.

LAMP was developed by Notomi (2000) as an alternative method for rapid and accurate nucleic acid amplification. It is advantageous because it operates at a constant temperature (typically between 60–65 ºC), yielding large amounts of LAMP products within a short timeframe (from 20 to 60 min). Therefore, LAMP only requires a straightforward heating device keeping a stable temperature, which is readily compatible with a microfluidic system (Wong *et al*., 2017; Das *et al.*, 2022). Previous studies have confirmed the suitability of LAMP assays for detecting *Globodera pallida* (Camacho *et al.*, 2021; Bairwa *et al*., 2023), the chosen model organism for evaluation in this work.

Globodera pallida and *G. rostochiensis*, commonly referred to as potato cyst nematodes (PCN), are prevalent nematode species in potato crops. These microscopic, worm-like endoparasites feed on potato roots, deteriorating the quality of tubers, causing a significant reduction in yield, increasing the overall costs of production, and imposing trade restrictions. Both species are worldwide distributed, with *G. rostochiensis* historically having a wider range compared to the more limited distribution of *G. pallida*. Managing *G. pallida* is particularly challenging due to the limited availability of attractive potato cultivars resistant/tolerant to this nematode, whereas several cultivars exhibit high tolerance to *G. rostochiensis*. However, there is a shifting balance between both species driven by the pressure of selection resulting from current nematode management practices, leading to the dominance of *G. pallida* in some countries (Minnis *et al.*, 2002; Camacho *et al*., 2020).

Owing the detrimental effects of *G. pallida* on potato crops and the difficulties associated with its management, the development of a portable device for a field-specific early detection is crucial to prevent its dispersion. Microfluidics biochips combined with FTA cards and LAMP are promising solution for on-site detection, enabling the implementation of effective integrated pest management strategies. This work represents a step towards a fully integrated device for rapid in-field crop pest and disease detections.

MATERAIL AND METHODS

1. LAMP primers and probe design

The ITS rDNA region used to design the specific probe and LAMP primers for detecting *G. pallida* were acquired from a previous study envisaging to develop a LAMP assay (Camacho *et al.*, 2020; Camacho *et al.*, 2021). The primers, including F3 and FIP as forward primers and b-B3 and b-BIP (biotinylated on the 5'end) as reverse primers, were designed to amplify a 172 bp biotinylated LAMP product. The detection of this product involved hybridization with a specific probe that has been previously immobilized on the chip. The design of the DNA probe followed the criteria outlined in Table 14.

Table 14 | List of criteria for probe design (Viveiros *et al*., 2020).

Criteria	Functional requirements
Size	≈20 and ≈ 45 bases
Position (PPS)	100 - 75 and 75 - 50
Modification	Thiol group in the 5´end
Spacer	15-T bases in the 5´end
G-C content	40-60% (recommended)
Max. surface-proximal tails	200 bases (shorter possible)
Max overhanging end	Depends on the amplicon size
Starting/Ending bases	G or C (recommended)
Stretches of a single base	No more than 4 in a row
ΔG value of any self-dimers, hairpins and heterodimers	> -9.0 kcal/mol
Heterodimers	Less than 5 bases in a row
Homologies to non-target	Less than 50%

Key characteristics of the primers and probes, such as guanine and cytosine (GC) content, melting temperature (Tm) and change in free energy of hybridization (ΔG) were computed using the

Integrated DNA Technologies Oligo Analyzer (RRID:SCR_001363). Furthermore, a probe sequence unrelated with any target sequence was used as a negative internal control to correct the positive signals. The primers and probes properties are resumed in s Table 15.

Table 15 | Sequence, size, GC content, and melting temperature (Tm) and change in free energy of hybridization (ΔG) of LAMP primers designed based on the ITS-rDNA of *Globodera pallida*, the probe specifically designed to target *Globodera pallida* and the negative control probe.

Primers/Probes	Sequence (5'-3')	Size (bp)	GC%	Tm (ºC)	ΔG (kcal/mol)
F3 - Forward outer primer*	ACA CAT GCC CGC TAT GTT	18	50.0	54.7	-35.32
FIP - Forward inner primer*	ACA CTC ATG TGC CCA CAG GGT GGG CTG GCA CAT TGA T	37	56.8	71.0	-74.76
b-B3 – Reverse outer primer*	Biotin-CCC TGT GGG CGT GCC A	16	75.0	62.0	-37.33
b-BIP - Reverse inner primer*	Biotin-TGG GGT GTA ACC GAT GTT GGT GAG CGA CCC GAC GAC AA	38	57.9	70.8	-79.27
G. pallida probe	Thiol-15T-CAC ATT GAT CAA CAA TGT ATG GAC AG	26	53.8	62.1	-51.85
Chikungunya probe (negative control probe)	Thiol-15T-CGC ATA GCA CCA CGA TTA G	19	52.6	53.4	-36.7

* Camacho *et al.*, 2021

The design of these probes and LAMP primers were carried out at the GMO and Molecular Biology lab of INIAV (Oeiras, Portugal) and synthesis was performed by Eurogentec (Seraing, Belgium).

2. Biochemical Reagents

All the solutions for the assay were meticulously prepared with ultra-pure grade water, and the specificities of this preparation is resumed in Table 16.

Table 16 | Biochemical reagents preparation conditions.

Reagent	Prepared with	pH
TE buffer	KH_2PO_4 (0.1 M) Tris (10 mM) EDTA (1 mM)	7.4. adjusted with HCl (1 M)
Phosphate buffer (PB)	Na_2HPO_4 (0.2 M) NaH_2PO_4 (0.2 M)	7.2
PB-Tween20	PB buffer with 0.02% (v/v) of Tween® 20 from Promega (Madison, WI, USA)	

The Magnetic Nanoparticles (MNP) were nanomag®-D from Micromod (Rostock, Germany), with 75–80% (w/w) magnetite in a matrix of dextran (40 kDa), a diameter of 250 nm and streptavidin coated. The particles had a magnetic moment of ~1.6 10^{-16} Am^2 for a 1.2 kA/m magnetizing field and a susceptibility of χ~4. To be used, MNPs needed to be 10 times diluted from stock solution.

3. DNA Nematode Samples

The samples used by Camacho et al. (2023), which included nematode isolates of *G. pallida*, *G. rostochiensis*, various mixtures of *G. pallida* and *G. rostochiensis*, *G. tabacum*, and *Heterodera*

sp. (as indicated in Table 17) were obtained from the nematode collection of INIAV Nematology lab (NemaINIAV, Oeiras, Portugal).

Table 17 | Samples from Portugal and Netherlands used for FTA-LAMP based biochip assays.

Species	Isolate	Origin	ng/µL
G. pallida	SV-18-14599	*Portugal*	*5.2*
G. pallida	NPPO-NL Pa3 HLB	Netherlands	1.4
G. pallida (FTA)	SV-20-1451-8	Portugal	143
1 *Gp*/5 *Gr*	SV-18-14599/ SV-18- 14598	Portugal	4
1 *Gp*/19 *Gr*	SV-18-14599/ SV-18- 14598	Portugal	2.2
1 *Gp*/40 *Gr*	SV-18-14599/ SV-18- 14598	Portugal	5.4
G. rostochiensis	SV-18- 14598	Portugal	28.2
G. rostochiensis	NPPO-NL Ro1 HLB	Netherlands	2.9
G. tabacum	NPPO-NL C6876	Netherlands	39.4
Heterodera sp.	SV-18-10003	Portugal	18.1

4. DNA extraction

Each quadrant of the FTA card was allocated to a single sample. The available cysts were smashed onto the FTA card and let to air-dry for 20 minutes. At the place where each cyst was smashed (Figure 19), a small disk was punched out and placed in a 1.5 mL tube containing 150 µL of water (DNase and RNase free). These disks were then subjected to incubation in a thermomixer at 70 °C for 25 minutes and subsequently stored at -20 °C until needed for further analysis. The total DNA content was quantified using the thermo-NANODROP 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To compare the efficacy of the DNA extraction method at the lab with FTA cards, the extraction of the other samples DNA was conducted using the DNeasy Blood and Tissue Kit (Qiagen) and following the manufacturer's instructions. The DNA extracts (Table 17) were directly used for the LAMP reactions without the need for any additional purification step.

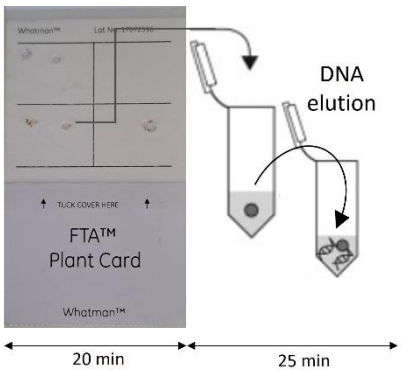

FIGURE 19 | FTA card used for DNA extraction.

5. DNA amplification

All LAMP reactions were conducted in the B-cube device (Hyris, London, UK) in 16-well cartridges. Each reaction was 25 µL final volume comprising 15 µL of the isothermal master mix

ISO-004 (OptiGene, Horsham, UK), 0.8 µL of FIP and b-BIP primers (50 µM), 0.15 µL of F3 and b-B3 primers and 5 µL of the template DNA. The isothermal conditions are resumed in Table 18.

Table 18 | LAMP Isothermal conditions.

Amplification Temperature, Time	Temperature of melting (Heat-Cooling)
65 °C, 60 min	95 °C – 75 °C

6. Detection Assays in the Biochip Platform

MR sensor microfabrication is described in the work of Martins *et al*. (2009) and Viveiros *et al*. (2020). The schematic representation of the main steps involved in a positive or negative detection is represented in Figure 20.

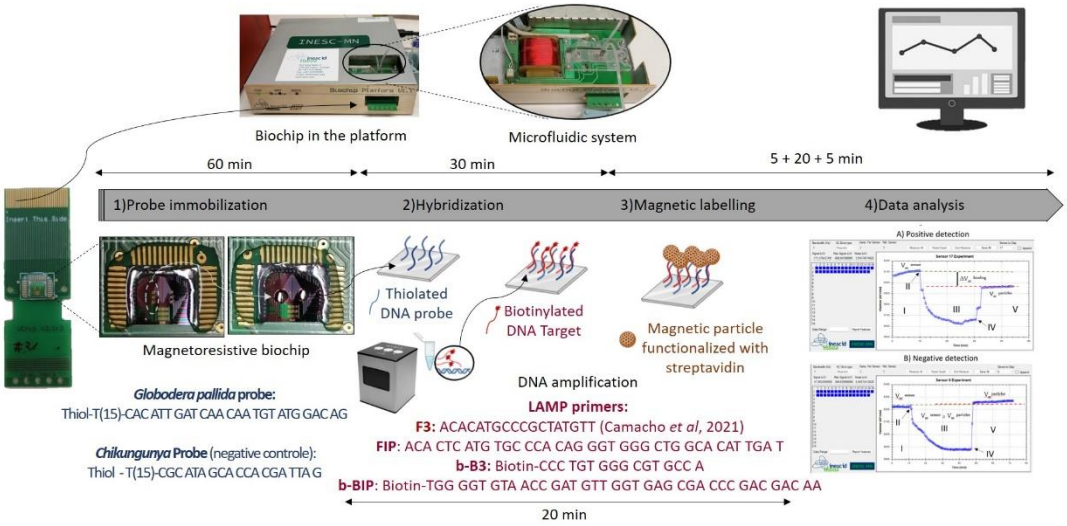

FIGURE 20 | Schematic representation of the main steps involved in a measurement.Step 1) Probe immobilization – Probes are immobilized over the sensing areas (positive probe at the left side, corresponding to sensors 1 to 15 – Circled area, and negative probe at the right side, corresponding to sensors 16 to 30), Step 2) LAMP products hybridization, Step 3) Magnetic labelling of Magnetic nanoparticles through a streptavidine-biotine interaction, and Step 4) Measurement and data analysis, above is a positive measurement and below is a negative measurement.

7. Chip functionalization

Before probe immobilization, the biochips underwent a cleaning procedure as described in Viveiros *et al.* (2020).

For *G. pallida* detection, the probe was diluted to a concentration of 5 µM in the TE buffer (Table 16), and 1 µL of this solution was spotted on the left side of the sensing area of biochip surface – sensors 1 to 15 (area encircled and zoomed in Figure 20 - Probe immobilization). The same procedure was followed for the negative control probe (the negative probe was used for Shikungunya detections and was spotted on the right side of the sensing area of biochip surface – sensors 16 to 30). After a 1-hour incubation period, the chip was rinsed with PB buffer and

then inserted in the platform. The U-shaped PDMS microfluidic system was placed over the sensor to transport the reagents (Figure 21).

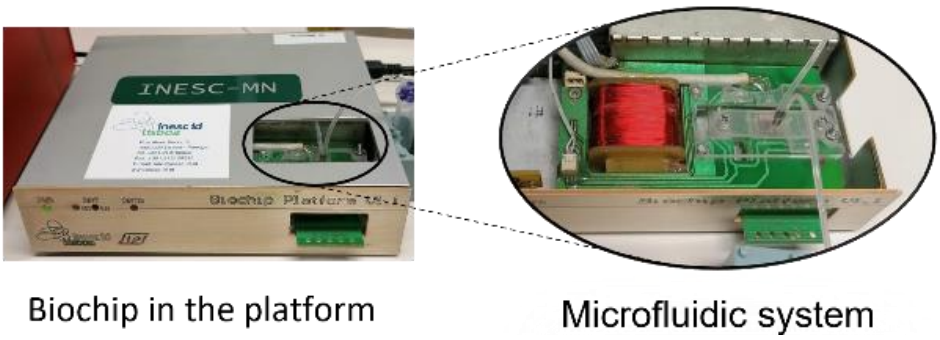

FIGURE 21 | Platform and microfluidic system

8. Biochip Platform measurement

Initially, the sensors were washed with PB buffer to remove weakly bound probes and establish equilibrium within the system. Subsequently, 10 µL of target LAMP product (previously melted at 90 °C for 5 min to denature the DNA) was applied over the sensing sites and allowed to incubate for 30 min. After target-probe hybridization, any unbound target molecules were washed off by rinsing with PB buffer. The measurement with MR sensors started by acquiring the baseline voltage for 5 min (Step I in Figure 22). Then, the magnetic nanoparticles were introduced into the microfluidic system (Step II in Figure 22) and left to incubate over the sensing area for 20 min (Step III in Figure 22). Once the resistance signal of the sensors saturates (Step IV in Figure 22), any unbound particles were washed away within a 5 minutes timeframe at continuous flow. If the signal stabilizes before 5 minutes, the wash can be stopped since all the unbound particles were totally washed (Step V in Figure 22). All reagents were loaded at a flow rate of 50 µL/min with the help of a syringe pump (NE-300, NEW ERA, NY, USA). In total, the data acquisition process took approximately 30 min.

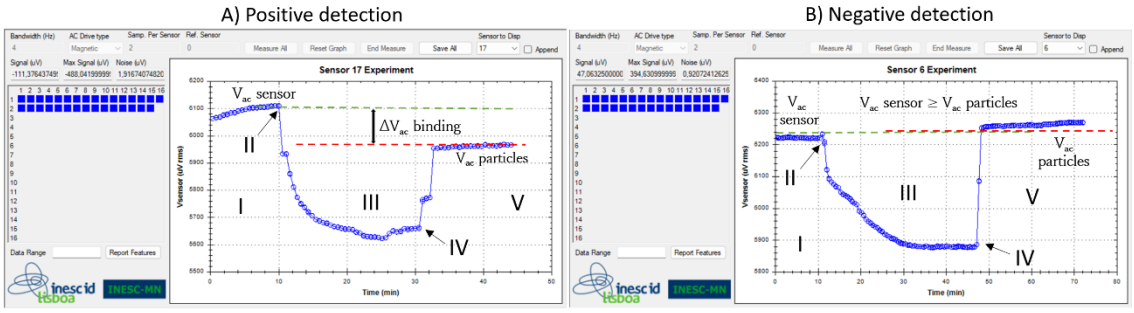

FIGURE 22 | Steps of voltage signal measurements, obtained simultaneously from two different sensors: Step I: Base line signal acquisition (V_{ac}^{sensor}); Step II: Injection of magnetic particles; Step III: Signal changes due to the presence of magnetic particles over the sensor; Step IV: saturation signal and washing step; Step V: final signal due to the presence of target bound magnetic particles over the sensor ($V_{ac}^{particles}$). A) positive detection event: hybridization with a complementary target DNA (*Globodera pallida*) - ending at a lower voltage and B) negative detection event: non-hybridization with a non-target DNA (*G. rostochiensis*) - ending at a higher voltage value.

9. Data Analysis

The binding signals were determined by calculating the difference between sensor baseline (V_{ac}^{sensor}) and the signal originated from the MNPs specifically bound to the sensor ($V_{ac}^{particles}$). Then, the voltage differential values ($\Delta V_{ac}^{binding}$ signal) were normalized based on the sensor's baseline and taken as the final output readout signal ($\Delta V_{ac}^{binding}$ / V_{ac}^{sensor})x100. At the same time, a reference spot (negative control - spotted on the right side of the sensing area of biochip surface), as shown in Figure 20 - Probe immobilization, was established using an unspecific probe (whose target is *Chikungunya* - Table 16). The final calculated output signal was the percentage difference of the sensor signals average obtained from positive and negative sensors. This was done to remove the influence of unspecific binding and any potential signal drift. The measurement curves in Figure 22 correspond to the sensors used to detect A) target and B) non-complementary target DNA.

RESULTS AND DISCUSSION

1. LAMP

The DNA samples listed in Table 17 were amplified through LAMP, using the primers indicated in Table 15. These primers were designed based on a region within the ITS-rDNA that remains conserved across various isolates of *G. pallida*, but displays variability among other species. The LAMP amplification products are visually illustrated in Figures 23, 24 and 25. In all LAMP reactions, the acceptance criterion for a positive result combines a sigmoid amplification curve within 40 min (Figures 23A, 24A and 25A) with a clear pick at the expected temperature on the derivative of the melting temperature curve (Figures 23B, 24B and 25B). In Figure 23, the DNA extraction efficacy with FTA cards was tested. Positive signals were generated after 10 minutes (Figure 23A) from *G. pallida* DNA extracted with both protocols - FTA cards and Qiagen Kit. No difference between both extractions was observed, demonstrating the efficacy of the FTA cards to field DNA extractions, which has an easier and faster procedure than the Qiagen kit protocol, which is mainly used for laboratory DNA extraction.

Amplification was detected for all targets, even when the DNA was originated from species other than *G. pallida* (Figure 24). This held true whether the DNA was a mix of different ratios of two species (Figure 25). Despite of having different amplification products, only samples containing DNA of *G. pallida* exhibited successful hybridization with the immobilized probe.

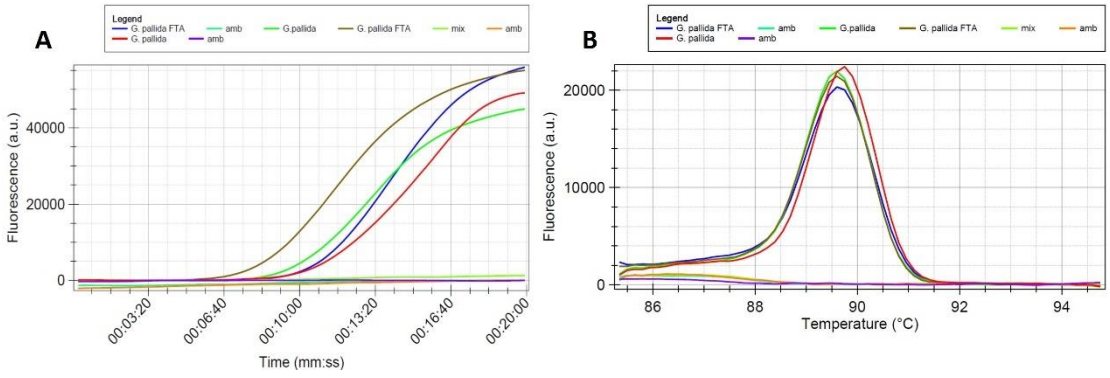

FIGURE 23 | LAMP sensitivity assay using *Globodera pallida* DNA extracted with FTA Cards and with Qiagen kit. Mix and amb samples are non-template control prepared in different laboratories areas (A) Isothermal amplification curves and (B) derivative of the melting temperature curve.

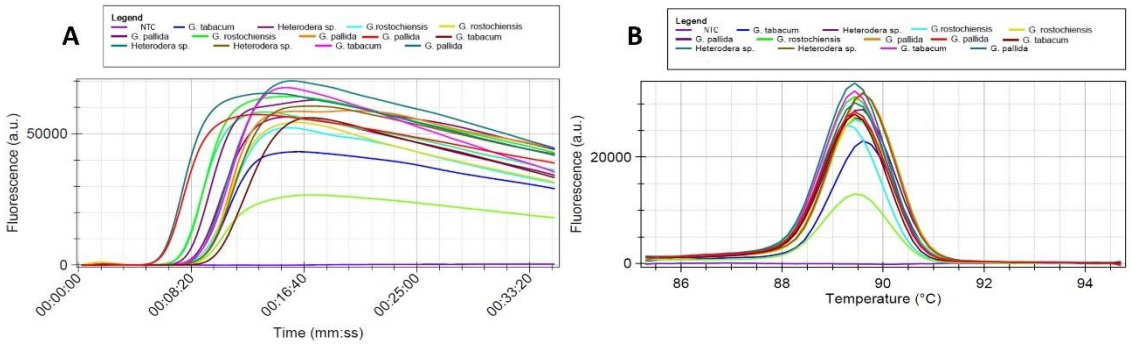

FIGURE 24 | LAMP assay using total DNA of *Globodera pallida, G. rostochiensis, G. tabacum* and *Heterodera* sp. NTC is a non-template control sample: (A) Isothermal amplification curves and (B) derivative of the melting temperature curves.

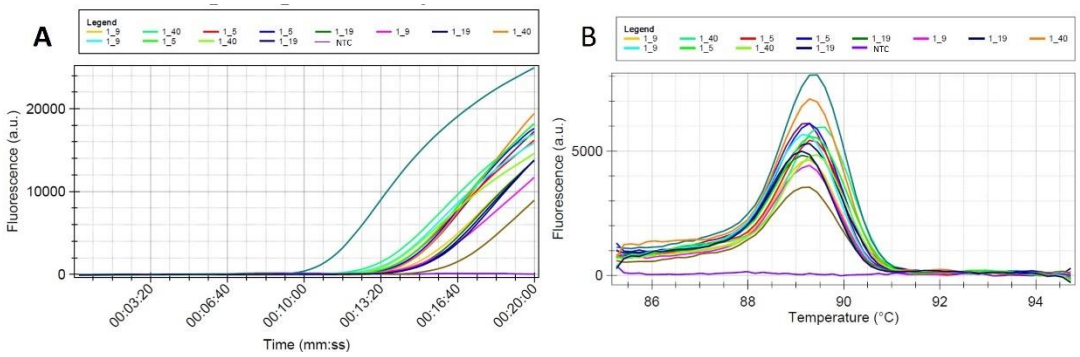

FIGURE 25 | LAMP sensitivity assay using total DNA of pools having different ratios of *Globodera pallida/G. rostochiensis* second stage juveniles (1-5, 1-9, 1-19 and 1-40 represents 1 *G. pallida* juvenile mixed with 5, 9, 19 or 40 *G. rostochiensis* juveniles, NTC is a non-template control sample): (A) Isothermal amplification curves and (B) derivative of the melting temperature curve.

2. Detection Assays in the Biochip Platform

The detection assays were performed in the MR biochip device with target DNA amplified by means of LAMP (Figures 23, 24, 25). All samples were tested with the specific probe for *G. pallida* in the same assay as the negative control probe, which was tested with the specific probe for Chikungunya virus and used as reference signal. Each sample underwent three or more measurements, corresponding to the detection signal from a range between 12 to 15 sensors in each measurement.

At the end of the experiments, the normalized signals acquired from the LAMP products from active and control sensors were compared. The normalized average signals (ΔV/Vsensor) acquired for positive sensors covered with *G. pallida* probe or with negative sensors covered Chikungunya probe, after the washing of the unbound MNP on the chip surface at a flow rate of 50 µL/min, presented a clear difference (as shown in Figure 26). In Figure 26, each bar in the graph represents the normalized signal acquired from the LAMP products of *G. pallida*, *G. pallida* extracted from FTA cards, mixed samples, and non-target species (*G. rostochiensis*, *G. tabacum* and *Heterodera* sp.). The threshold value (dashed line) is set at 1%, this value was established as the average ΔV/V from the non-specific signal obtained against a non-complementary target plus

its standard deviation of each signal (Albuquerque et al., 2022; Viveiros et al., 2020). It results from the physical behavior of the sensors and represents the minimal difference in the magnetic field that raises an electrical signal. Signals above this threshold were considered positive detection, signifying a successful match with a complementary target. Conversely, signals below the threshold value were set as negative detection, demonstrating de specificity of the designed probe for *G. pallida*.

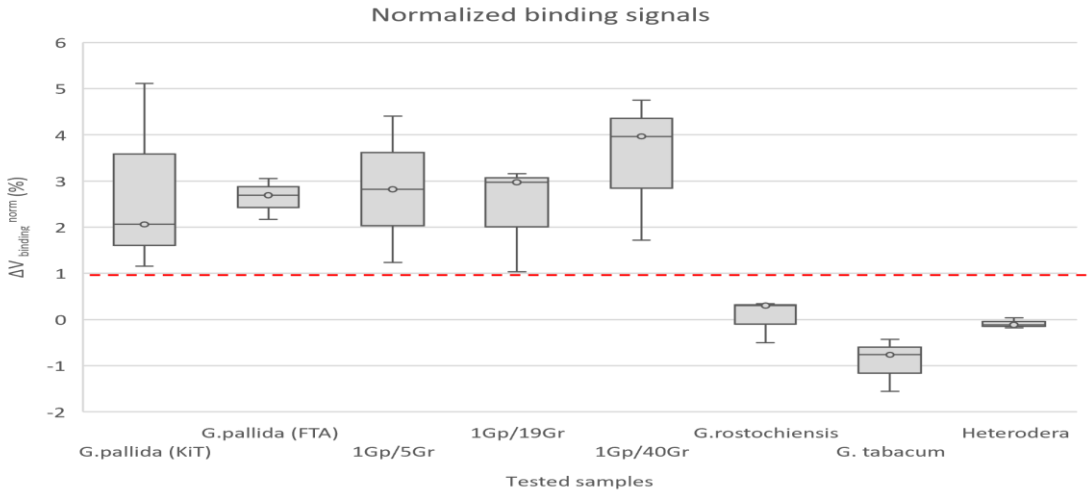

FIGURE 26 | Normalized binding signals obtained from LAMP products of *Globodera pallida*, *G. pallida* extracted with FTA cards, mixed samples of *G. pallida/G. rostochiensis* (1Gp/5Gr, 1Gp/19Gr and 1Gp/40Gr represents 1 *G. pallida* juvenile mixed with 5, 19 and 40 *G. rostochiensis* juveniles - diagnostic sensitivity), *G. rostochiensis*, *G. tabacum* and *Heterodera* sp. (analytical specificity) against the specific probe for *G. pallida* detection. The error bars are standard deviations coming from at least 12 sensors acquired from three measures for each sample. The dashed line represents the threshold. 1%is the value above which a detection signal is considered positive.

The FTA-LAMP-based MR biosensor functionalized with a specific oligoprobe (Table 15) showed a remarkable degree of specificity when detecting *G. pallida* LAMP products. This was concluded from the notably free of significant cross-reactivity, enabling the reliable discrimination of this species from other cyst nematodes, including *G. rostochiensis*, *G. tabacum* and *Heterodera* sp. In this analysis, all samples with the target sequence generated detection signals exceeding the 1% (with the average of 2.8 ± 1.3%), while those with non-target sequence produced detection signals below this threshold (with an average of -0.3 ± 0.6%). These findings are aligned with preceding works, where positive detection signals of 1.9 ± 0.8% and 1.8 ± 0.7% were reported, along with negative control results of -0.04 ± 0.4% and 0.4 ± 0.3% (Camacho *et al.*, 2023; Viveiros *et al.*, 2020). The capability to detect *G. pallida* persisted even when working with DNA extracted via FTA Cards or mixed samples containing *G. pallida* and *G. rostochiensis* (with ratios of 1 *G. pallida* juvenile mixed with 5, 19 or 40 *G. rostochiensis* juveniles: 1/5, 1/19, and 1/40). This underscores a diagnostic sensitivity equivalent to one second stage juvenile (Figure 26).

Developing a mobile biosensor with FTA-LAMP technology application may result in a significant improvement, as it can detect the presence of pathogens directly from environmental samples and has high specificity. FTA-LAMP based microfluidic devices also comes with many advantages, such as easy to operate, expedited and advanced method, palm-sized, high output applicability, and can be applied in the early detection of crop pest and diseases.

CONCLUSIONS

Modern agriculture uses sensor technology to provide accurate and timely data on crop growth, benefiting crop management and yields. The integration of sensors into agriculture systems aligns with the objectives of the European Green Deal, since it offers notable environmental benefits and acknowledges digitization as a tool to enhance productivity by lowering the impact of crop pests and diseases, and enabling an ecological transition, which includes the reduction of pesticides applications. The device used in this work has already been validated for individual detection of bacteria (Barroso et al., 2015; Fernandes et al., 2014, Viveiros *et al*., 2020), proteins (Albuquerque et al., 2019; Fernandes et al., 2020), nucleic acids (Dias et al., 2016; Martins et al., 2009) and virus (Albuquerque *et al*. 2022) and can be adapted to field detection of several crop pest and diseases. The FTA-LAMP-based biosensor here demonstrated, is specific for *G. pallida* detection, but can be simultaneously functionalized with species-specific probes for the related species *G. rostochiensis*, *G. tabacum* and *Heterodera* sp., hold significant potential for multiplex detection and for rapid in-field detection or at border phytosanitary inspections.

Although there is still a journey ahead before sensors become commonplace tools in agriculture, the technology underpinning sensors continues to advance, and their use is expected to increase, playing an important role in the future of sustainable agriculture. In this work, a magnetoresistive biochip device was used for the specific detection of *G. pallida* by targeting the ITS-rDNA sequence, and no false positives were observed with closely related species. Our results show that the tested LAMP-FTA based biosensors is exceptionally specific in detecting *G. pallida* even in samples infested with cysts of other *Globodera* species. Thus, the device adapted in this work has proven to simplify and reduce testing time to a complete field detection.

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CHAPTER VI

GENERAL DISCUSSION

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are an economically important group of plant-parasitic nematodes, affecting potato crops worldwide. PCN are sedentary endoparasites of the potato root system, being devastating to potato fields and affecting economic trade when serious losses occur. Studying PCN offers the opportunity to comprehensively explore various aspects of their biology, including reproduction, interactions with host plants, breeding habits, dissemination, and how they react to control measures. This research allowed us to gain a holistic understanding of PCN and its effects on potato crops.

Epidemiological situation

The knowledge on the geographical range, population density and spatial dynamics of pest populations is essential for effective IPM systems. In this study, was adopted an epidemiological approach to investigate the presence of PCN in Portugal. During the survey period, 748 soil samples were collected throughout the country by official services of the national plant protection organization (DGAV). These samples were subsequently analyzed in the national reference laboratory for plant health (INIAV). It is important to note that PCN are classified as quarantine organisms, and the relevant official entities were duly notified about this study.

PCN were detected in 22.5% of the tested samples, being 49.4% for *G. pallida* populations alone, 28.6% positive for *G. rostochiensis* populations alone and 22% for mixed populations. According to these results, the incidence of PCN in Portugal is quite high, and both species are currently present in all potato producing regions of the country, representing a real threat to potato crops. PCN detections within the Portuguese regions are significantly different, increasing from south to north, where PCN were first detected, and consequently, nematode reproduction is happening for a longer time. These data are aligned with Jones *et al*. (2017), who stated that cysts are adapted to higher altitudes, and there is a noticeable increase in altitude from southern to northern regions in Portugal.

The results presented here contrast with those previously reported by Cunha *et al*. (2004) in which 83% detections were *G. rostochiensis* populations alone, 8% were *G. pallida* populations alone and 9% consisted of a mixture of the two species. Statistical analysis revealed that this reverse situation is explained as a lateral consequence of the use of *G. rostochiensis* resistant potato cultivars in the last decades.

Phylogenetic relationship between different Portuguese isolates

The phylogenetic relationship of Portuguese *Globodera* isolates was analyzed and two main clades stand out. Within the first clade (with *Globodera* species parasites of solanaceous plants), two sub-clades were formed, one with *G. rostochiensis* and the related species *G. tabacum* and another with *G. pallida*. The second clade includes a group with the Portuguese *Globodera* n sp., discovered in 1997 (Reis, 1997; Sabo *et al*., 2002) and re-detected throughout this research, and their most closely related *Globodera* species, *G. hypolysi* and *G. artemisiae* (*Globodera* species parasites of non-solanaceous plants). The results show that no spatial-temporal relation can be drawn, evidencing the coexistence between the two major species of *Globodera* in Portugal, as Cunha *et al*. also reported in 2012. These authors concluded that no relationship could be found between the two-dimensional electrophoresis protein patterns or virulence behavior of the isolates and their geographic origin across Portugal. It is also worth noting that the clade topology differs between *G. rostochiensis* clade, which is more branched, with 96–100%

similarity, showing more genetic variability due to being present for a longer period in Portugal; and *G. pallida* clade, which is flatter, with 99–100% similarity, due to more identical sequences.

Concerning the new *Globodera* species (Reis, 1997; Sabo *et al*., 2002), its detection reveals once more the need of this kind of studies to have an up-to-date overview of PCN in Portugal. For the moment, it is out of the scope of this study, but additional research will be carried out to determine its pathogenicity and impact on potato.

Control measures - Resistant cultivars

Phytosanitary measures have been taken to prevent further spread of *Globodera* spp. in recent years. In the case of *G. rostochiensis*, until recently the dominant species, measures included non-host crop rotation (for 6 years), fallow (for 6 years) or growing *G. rostochiensis* resistant potato cultivars (for 3 years).

The use of *G. rostochiensis* resistant potato cultivars, containing genes effective only against certain races/pathotypes of *G. rostochiensis* and with no resistance to *G. pallida*, has led to a predominance in Portugal of the more difficult species to control, *G. pallida*. The results from this study confirmed that the detection of G. *rostochiensis* in potato fields with G. *rostochiensis* resistant cultivars is significantly lower when compared to its detection in fields with susceptible cultivars. Besides*, G. pallida* detections in sampled fields with *G. rostochiensis* resistant cultivars are similar to those in fields with *G. rostochiensis* susceptible cultivars. This suggests that *G. rostochiensis* resistant potato cultivars have contributed to a reduction in *G. rostochiensis* detections in Portugal, while they do not appear to affect the detections of *G. pallida.* These results agree with the published literature (Minnis *et al*., 2002; Pickup *et al*., 2019). Thus, it is possible to conclude that resistant cultivars are an efficient option to reduce cyst infestations in potato fields, as already predicted by Cunha *et al*. (2004) and statistically verified in this study.

Currently, *G. pallida* poses a substantial threat to production in all potato-producing countries. Its control is affected by the lack of attractive *G. pallida* resistant/tolerant potato cultivars and by the existence of cultivars with high tolerance to *G. rostochiensis*. Additionally, the influence of markets has led farmers to primarily adopt *G. rostochiensis* resistant cultivars like Aurea, Agria, Lady rosetta and Taurus. This market-driven trend has placed pressure on the selection for *G. pallida*. This raises the need for breeding new and effective *G. pallida* resistant potato cultivars, which can serve as a beneficial management strategy to reduce *G. pallida* population densities and, consequently, mitigate yield losses.

Therefore, it is imperative to follow a new approach for the management of PCN, with a greater use of integrated control strategies (such as crop rotation, solarization, trap cropping, biofumigation and selected nematicides) (Evans and Haydock, 2000; Alptekin, 2011; Davie *et al.,* 2019), to complement PCN resistant potato cultivars. These interactions require careful research concerning the effects of one or another strategy under a specific set of environmental conditions and a specific nematode infestation level. The efficacy of the integrated program will be determined by the interaction, overlap and complementarity of the various control methods.

Despite the difficulties associated with *G. pallida* resistance being quantitatively inherited, the breeding of more resistance with different R-genes, to avoid PCN capacity to overcome the plant resistance and commercially attractive cultivars, should be a priority. As *G. pallida* field populations tend to show increased virulence toward a particular partially resistant cultivar each time that it is grown (Trudgill *et al*., 2003; Pickup *et al.,* 2019), potato growers would need a

choice of different PCN resistant cultivars to maintain their effectiveness. Currently, there are insufficient alternatives to partially resistant cultivars for growers to meet the requirements of markets. There is substantial evidence suggesting that European countries bear an increasing burden with this nematode due to the high circulation of people and goods.

Loop-mediated isothermal amplification (LAMP)

PCN molecular identification is routinely performed through PCR-based methods. Although the sensitivity and specificity of these diagnostic assays are sufficiently high when properly applied, the procedures are time-consuming, require well-trained technicians, expensive laboratory equipment and cannot be performed in the field due to the lack of convenient portable instruments. Given the substantial economic and trade impact associated with PCN, it is crucial to timely and accurately differentiate among the various species in order to plan and implement strategies for an effective IPM (Subbotin *et al*., 2013; Camacho *et al*., 2017).

As a result of the PCR-based protocols limitations, other methods have been developed, with the objective of reducing processing time, minimizing hands-on work, enabling portability for in-field analysis, enhancing sensitivity, and making use of newer and more affordable technological platforms. Among many new methods and technologies developed, Loop-mediated isothermal amplification (LAMP) is gaining popularity for phytosanitary diagnostics and is one of the most explored techniques to detect invasive and quarantine species both at the plant health laboratories and on site (farms, water resources or border inspection points).

Prior to this work, detecting *Globodera* spp. through LAMP assays was only possible for *Globodera* sp. and *G. rostochiensis* detections, based on sequences of Belgian and Netherlands populations. Thus, as there was a lack in *G. pallida* LAMP detection methods, one of the goals of this research was to develop a LAMP assay for *G. pallida* identification, to be used in routine analyses. Consequently, this study successfully developed a LAMP-based assay for the specific identification of *G. pallida* by targeting the ITS1 sequence. The primers designed for the LAMP amplification allow the specific *G. pallida* identification within less than 40 minutes, even when using pooled samples with one *G. pallida* J2 mixed with 40 *G. rostochiensis* J2. A higher number of juveniles did not improve the final concentration of DNA in the extracts. Positive detections were made with DNA extracts concentrations at least, equal or above 5 pg/L and no false positives were observed either with other closely related species or non-related species. As the number of isolates from other origins was limited, interlaboratory performance studies are needed to confirm the specificity and to determine the repeatability and reproducibility of this method in order to be standardized and validated. Thus, tests carried out in the Molecular Biology Laboratory at INIAV and in the independent laboratory of the University of Évora, yielded 100% positive and negative agreement detections.

As far as is known, this is the first reported LAMP method for differentiating *G. pallida* from both other cyst nematodes (*G. rostochiensis*, *G. tabacum* and *Heterodera* sp.) and motile nematodes (*Pratylenchus penetrans, Xiphinema* sp., *Helicotylenchus* sp., *Bursaphelencus xylophilus* and *B. mucronatus*) and can be considered essential for future surveillance and disease control purposes.

LAMP clearly holds potential for in-field testing and can be automated in a miniaturized unit capable of field operation to be included in the decision support system in potato production.

Lab-on-a-Chip for detection of Globodera pallida

Modern agriculture uses sensor technology to provide accurate and timely crop growth data for crop management. The use of sensors in agriculture is in line with the European Green Deal, since it comes with significant environmental benefits and acknowledges digitization as a tool to enhance output by lowering the impact of pests and diseases, improving productivity, and enabling an ecological transition (reduction of pesticides applications). Although the use of biosensors in health diagnosis is expanding quickly, there are still few applications in agriculture.

The aim of this work was to integrate the use of biosensors in plant pest management. Thus, a magnetoresistive biochip device, developed at INESC facilities, was used for a specific asymmetric PCR-based identification of *G. pallida* by targeting the same ITS-rDNA sequence as the LAMP assay. The primers designed for the asymmetric PCR amplification in combination with the oligonucleotide probe specifically detected *G. pallida* in DNA extracts. In this assay, there are two steps ensuring the specificity - the annealing of the primers and hybridization of probe. No false positives were observed with other closely related species, such as *G. rostochiensis*, *G. tabacum* and *Heterodera* sp.. These observations reveal that the tested biosensors are highly specific for detecting *G. pallida* even in samples infested with cysts of other *Globodera* species.

Despite not being a goal of this work, this technology can be optimized for multiplexing allowing the detection of more than one plant pest and/or pathogen at once. Researchers in other fields have already achieved the simultaneous multiplex detection of different pathogens based on an asymmetric PCR protocol coupled with a magnetic array biochip functionalized with species-specific oligonucleotide probes (Viveiros *et al.,* 2020; Miguéis *et al*., 2021). In the future, a multiplex detection protocol can be designed targeting different *Globodera* species. This approach will use a single pair of primers in asymmetric PCR to indiscriminately amplify any *Globodera* sp. along with species-specific sensor-immobilized probes.

FTA-LAMP based Biosensor for a rapid in-field detection of Globodera pallida

With the increasing demand for advanced technologies in the agricultural sector to tackle plant pest detection, there is a clear opportunity for the creation of innovative portable devices. To improve the practicability of biosensors techniques in field conditions, the developed LAMP assay, which, already avoided the need of temperature cycles including high temperatures, was combined with a DNA extraction procedure based on Flinders Technology Associates cellulose cards (FTA cards). This innovation removes the requirement for centrifugations, which are a significant obstacle for DNA extraction in field applications.

In this work, DNA from cysts were directly crushed onto FTA cards and stored at room temperature until used as a template in LAMP reactions. Furthermore, the magnetoresistive biochip device was used for the specific detection of *G. pallida* by targeting the ITS-rDNA sequence. No false positives were observed with other closely related species and results show that the tested LAMP-FTA based biosensors are highly specific for detecting *G. pallida*, even in samples infested with cysts of other *Globodera* species.

Although there is still a long way to go before sensors are common tools in agriculture, the technology behind sensors is being improved and their use will increase and play an important role in the future of sustainable agriculture. FTA-LAMP based biosensors, functionalized with species-specific oligonucleotide probes, can be considered very beneficial for the surveillance and disease plant control purposes. This approach shows great promise for field application in

the early detection and surveillance of plant pests and in assisting the implementation of management practices to reduce the risk of infestations. Other important possible application is at inspections points (BIPs) for phytosanitary controls.

PCN are challenging and amazing organisms to be studied. The goals achieved in this work allowed us to have a complete picture of the current epidemiological situation in Portugal. National incidence, prevalence and distribution of PCN were investigated, the best approach for future PCN control was discussed and new strategies for new/early detection were developed, providing new data for complementary studies.

This research will proceed by employing gene editing technology CRISPR/Cas to modify non-functional resistance genes in potato cultivars, making them genes variants naturally occurring in wild potato species that exhibit resistance to *G. pallida*. A preliminary study involving the existing potato cultivars revealed varying levels of susceptibility to *G. pallida*. The data obtained from this research, together with the outcomes of RNA-seq will enable us to identify new candidate genes involved in the activation of the immune system.

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