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A N I N T E R N A T I O N A L J O U R N A L

Commercial Biological Control Agents Targeted Against Plant-Parasitic Root-knot Nematodes

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ABSTRACT

Root-knot nematodes are microscopic round worms, which cause severe agricultural losses. Their attacks affect the productivity by reducing the amount and the caliber of the fruits. Chemical control is widely used, but biological control appears to be a better solution, mainly using microorganisms to reduce the quantity of pests infecting crops. Biological control is developing gradually, and with time, more products are being marketed worldwide. They can be formulated with bacteria, viruses or with filamentous fungi, which can destroy and feed on phytoparasitic nematodes. To be used by the farmers, biopesticides must be legalized by the states, which has led to the establishment of a legal framework for their use, devised by various governmental organizations.

Key words: Biopesticides, *Meloidogyne* spp., Filamentous Fungi, Homologation, Production, Formulation

INTRODUCTION

Root-knot nematodes are disease-causative agents, well-known by farmers, for example, tomato producers in Morocco. Each year, these microscopic worms cause considerable losses. Biopesticides may be a good solution to reduce the damages, but they have to be legalized by the governments. Filamentous fungi may play a good role in the control of this pest, using different action pathways. They have to be formulated to preserve their viability and virulence against the nematodes, and then to be approved for use on the crops by farmers.

ROOT-KNOT NEMATODES (RKN)

Nematodes are classified under the *Nematoda* Phylum, which forms part of the *Ecdysozoa* superphylum, from the Greek ecdysis (moulting) and zoo (animal). They possess a cuticle and must moult to continue their development (Bélair 2005). Most of them are said “free” and feed essentially on bacteria, fungi, protozoa and other nematodes, and only a minority parasites animals and plants (Bélair 2005).

Meloidogyne spp. or RKN are round worms (Brand et al. 2009) belonging to *Tylenchida* family. They are cylindrical and thread-like triploblastic Metazoans that show bilateral symmetry, a thick cuticle, and non-ciliated, non-segmented teguments (Grassé et al. 1965). They were discovered during the 14^{th} century as phytoparasites of intertropical and warm climate cultures. The agricultural losses they cause, in particular in greenhouse cultures of tomatoes in the Souss-Maasra Drâa region in Morocco, are considerable. Worldwide, more than USD 125 billions/year are lost due to nematodes, most of

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them due to RKN (Koenning et al. 1999; Chitwood 2003; Collange et al. 2011; Bissadou et al. 2012). The degree of these damages can be even more severe as nematodes can increase susceptibility to other parasites (Bridge et al. 2005).

The *Meloidogyne* genus subdivides in different species, all phytophagous, with *M. arenaria*, *M. incognita*, *M. hapla* and *M. javanica* being commonly widespread (Netscher 1970; Sawadogo et al. 2000; Hunt and Handoo 2009). The number of plants susceptible to *Meloidogyne* spp. is very high (more than 2,000 species, a constantly increasing figure), and includes tobacco (*Nicotiana tabacum*), coffee (*Coffea arabica*), tomato (*Solanum lycopersicum),* Niebe (*Vigna sinensis*), Kenaf (*Hibiscus cannabinus L.)*, sugar cane (*Saccharum officinarum*), tea (*Camellia sinensi*), carrot (*Daucus carota*), melon (*Cucumis melo*), etc. *Meloidogyne* spp are round worms, 0.4 to 1.0 mm long and 0.25-0.75 µm in diameter for the females and 1.2-1.5 mm long and 30-36 µm in diameter for the males (Agrios 1997). These present a stylet, which is used to pierce the cell walls of the roots of the host plants (Davis et al. 2000). As they enter the root, the larvae will provoke a cortical cell hypertrophy due to the excretion of secretions through the stylet. Multi-nucleated "giant cells" are thereby formed (Bird 1962), which cause the formation of gall, which is characteristic of the attack by a *Meloidogyne* spp.

Once hatched, J2 free larvae become obligatory parasites that have to continue their cycle into adult stage in host-plant roots only (Sharon et al. 2001). For that purpose, they swim into the water pellicle surrounding the soil particles. Once they penetrate the root thanks to the stylet, the larvae move both in and between the cells. They move to the central cylinder along which they immobilize (Fig. 1A). At this time, L3 and L4 larvae turn into sexual adults, presenting strong dimorphism (Fig. 1B): males stay vermiform whereas females become pyriform and sedentary, their heads lodged in giant cells. Once fecundated, females lay eggs (around 500/mass) agglomerated at all the stages of development in a gelatinous substance, from the unicellular stage to the ready-to-hatch stage. Egg development between these two stages could take from seven to nine days at 28°C. During this time, nematodes undergo a first molting and the larvae that hatch are J2 second-stage larvae (De Guiran and Netscher 1970).

A higher temperature may accelerate the cycle; yet temperatures above 40°C are lethal. Eggs are the nematodes' resistant form, so they survive unfavorable conditions of life. In the absence of males, females are also able to produce fertile eggs in a parthenogenetic way (Agrios 1997). Typical symptoms of damage caused by the nematodes are a reduction of the root system, root structure distortion or root diameter increase, and gall presence. Plants turn yellow and wither and a production decrease is observed (Agrios 1997).

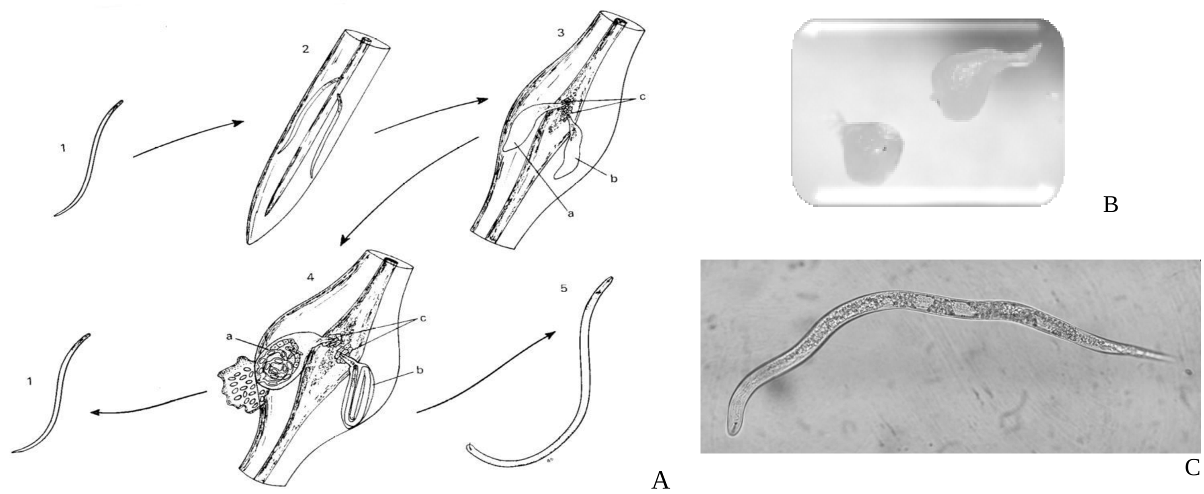

Figure 1 - A. Schematized cycle of *Meloidogyne* spp. - 1. Free second stage larva; 2. Second stage larva penetrating a growing root; 3. Root in formation : a-b : Swolled second stage larva ; c : Giant cells ; 4. Gall containing : a : adult female which had laid her eggs in the gelatinous substance ; b : adult male curled up in the larval envelopes ; c : Giant cells; 5. Free adult male (By De Guiran G. and Netscher C. 1970) ; B. Photo of females of *M. javanica* (M. Tranier) and C. J2 larvae of *M. javanica* (M. Tranier).

BIOLOGICAL CONTROL

General Definition

Founded in 1956, the International Organization of Biological Control (Iobc 2014) is set to promote environmentally-, socially- and-economically-efficient methods to eliminate the diseases and pests from agriculture and forests. It defines biological control as « the use of living organisms or their products to eliminate or reduce the damages or losses due to pests ». Biological control involves the use of beneficial organisms, of their genes and/or of products such as metabolites that reduce the negative effects of plant pathogens and promote positive responses from the plant (Junaid et al. 2013). More precisely, it uses living populations of parasitoid, predatory, pathogenic, antagonist or competing organisms to reduce a pest population, making it less abundant and less harmful than it would otherwise have been (De Bach 1964; Stirling 1991; Siddiqui and Mahmood 1996). The Biological Control Agent (BCA) is then the organism or its product which combats the pest. It is usually called a « biopesticide ».

Biopesticides are generally defined as the “products aimed at protecting the plants, made from living organisms or natural substances from species co-evolution, not produced by chemistry, and the use of which is recommended for the control of pests or bio-aggressors for a better respect of the biocenosis and environment”. According to this definition, Genetically Modified Organisms (GMO) that are not issued from the co-evolution of species and which have known too little anteriority in terms of impact on biological communities and the environment should so far be excluded from the field of biopesticides.

If the antagonist is a microorganism, microbiological control could be considered. In general, the microorganism infects the host by ingestion and has a resistant form that permits it to pass through – and stay in – the medium (soil, foliage, litter). The pathogen multiplies in the host and causes its death by tissues destruction, septicemia, and sometimes the emission of a toxic substance (case of bacteria). Host cadavers release the pathogenic agents in the medium. When the antagonists have to be inoculated in large numbers to effectively reduce the pest, the method is called inundative biological control; it is frequently used, but poses the problem of the future of the auxiliary populations introduced in the field.

Biological control appears to be an excellent alternative to the chemical one, which is the major method used to fight the pests. It consists in a general disinfection of fields (before each plantation) with fumigants or precursors, but presents a real danger for the environment and human health. These chemical products indeed present serious drawbacks (wide action spectra, disturbed ecological balances, pollution of the environment and food-chain, insufficient results, long-term resistance of pests). This method of control also poses the problem of the necessary delay between the product’s application and harvest. Moreover, widely-used chemical products (like methyl bromide) are increasingly banned, which poses the problem of the existence of alternatives, if possible within the legal framework of organic farming. However some alternatives to chemical products do exist, which are used but present limited effects: inundation and summer solarization are two examples (Van der Putten et al. 2006; Djian-Caporalino et al. 2009; Collange et al. 2011). Inundation causes anaerobic conditions that reduce the occurrence of RKN, but it requires a long flooding period (eight weeks) to be efficient, and causes water consumption, lack of oxygen and degradation of the soil’s structure, which is not suitable to agronomic practices (Collange et al. 2011). Summer solarization, in the intercropping period, consists in covering the soil with a plastic film during the hottest months of the year to increase the soil’s temperature. It helps neutralize thermosensible pathogens at depths up to 20 cm, like root-knot nematodes. However, the heat can’t penetrate into the deeper layers of the soil where nematodes can also shelter. Soil amendments with composts or manures can stimulate natural microbial soil flora and have a repressive effect on phytoparasitic nematode populations (Bélair 2005). Over the last few years, biological control has, thus, been developing. This article presents a review on nematode-antagonistic fungi and some of the commercial products associated.

FUNGI AS BCA AGAINST *MELOIDOGYNE* SPP

Fungi naturally exist that have an antagonistic action against the nematodes in general (Sarhy-Bagnon et al. 2000, Brand et al. 2004), more precisely *Meloidogyne* spp. (Viaene and Abawi, 1998a; Stirling et al. 1998; Stirling and Smith

1998; Duponnois et al. 1998; 2001; Kumar and Singh 2006; Thakur and Devi 2007; Liu et al. 2008; Collange et al. 2011). The infection process is generally the same: cuticle penetration, immobilization, invasion and digestion of the nematode (Huang et al. 2004). The nematodes' cuticles and egg walls play an important role in the infestation by fungi. The cuticle is essentially made of proteins (chitin, collagen, fibers) and can play the role of a precursor in the invasion of the nematodes by nematophagous fungi (Huang et al. 2004). Whatever the mode of action of the fungus, predation or parasitism, the interaction between a fungus and a host organism or prey requires penetration of the outer shell of the body. This penetration occurs before the colonization of the internal structures of the body through digestion, allowing the fungus to meet its nutritional needs (Gaspard et al. 1990; Hajieghrari et al. 2008). Cayrol et al. (1992) classified the nematophagous fungi according to their mode of action against the nematodes.

Predatory fungi (Cayrol et al. 1992)

Some fungi have the ability to trap the nematodes they feed on. They differ by their trapping mode: network traps (*Arthrobrotrys oligospora*, *A. superba*), constrictive rings (*A. anchonia*, *A. dactyloides*, *Dactylaria brochopaga*, *etc*), or adhesive knobs (*Monacrosporium cionopagum*, *Dactylella lobata*). Some fungi, such as *Dactylaria candida* (Hyphomycete) present two types of trapping mechanism: i) adhesive knobs, and ii) constrictive but non-adhesive rings (B'Chir 1984). These fungi are saprophytes, but they can trap the nematodes in a larval or free adult stage in the soil. It has been proven that the production of peptides by extracellular proteases hydrolyzing the nematodes' cuticles may induce trap formation (Huang et al. 2004).

Egg-parasitic fungi (Cayrol et al. 1992)

These fungi have the ability to infect and destroy nematode's eggs. Most of the fungi that have this mode of action are saprophytes, and can secondarily invade already-dead eggs. Egg-parasitic fungi include *Paecilomyces*, *Pochonia* and *Verticilium* genera. *Paecilomyces lilacinus* and *Pochonia chlamydosporia* are probably the most effective egg-parasites. *P. lilacinus* has been proven to successfully control root-knot nematodes, *M. javanica* and *M. incognita* on tomato, egg-plant and other vegetable crops (Cayrol et al. 1989; Verdejo-Lucas et al. 2003; Goswami and Mittal 2004; Van Damme et al. 2005; Goswami et al. 2006; Haseeb and Kumar 2006).

Nematophagous fungi with adhesive spores (Cayrol et al. 1992)

Nematodes can be parasited by the fungi with adhesive spores belonging to several classes: Oomycetes (*Catenaria anguillulae*, *Myzocytium lenticulare*, *M. anomalum* with biflagellated zoospores able to encyst in the nematode's cuticle), Zygomycetes (*Meristracum asterospermum*, with spherical conidia, which produce a germinative filament after adhesion that germinates and creates new conidiospores), Deuteromycetes (*Meria coniospora* with club-like spores, which fix on the host via their anterior part), and Basidiomycetes (*Nematoctonus leiosporus*, with adhesive spores) and Hyphomycetes (*Hirsutella sp*). Exclusively endoparasitic fungi must live their entire cycle in the host nematodes.

Endomycorhizes with arbuscular vesicles (Cayrol 1992)

Mycorhizes are fungi associated with the plant roots in a symbiotic way, presenting a double mycelial network: one external, in the soil, and one internal, in the tissues of the host-plant. The mycelial network expands widely in the rhizosphera and exploits a large volume of soil, which ensures abundant nutrition for host-roots, increasing the assimilative potential of the plant. Nematodes living in these roots compete in intra-root nutrition and face a plant becoming more resistant, thanks to the nutritional amelioration due to mycorhizes.

Microbial toxins

Some substances are naturally nematifuge (repulsives), nematostatic (perturbating host-plant reconnaissance by the nematode; blocking the development of the egg or the larva; or temporarily paralyzing the animal) or nematicide (*sensus stricto*: ovicide, larvicide or lethal for all stages). Some studies have been conducted, but it seemed that some enzymes produced by the fungi, or volatile organic compounds (e.g., 6-pentyl-α-pyrone, produced by *Trichoderma harzianum*), could have some antagonistic effects on nematodes (Sarhy-Bagnon et al. 2000). It has been proven that the toxin produced by *Bacillus thuringiensis* is efficient *in vitro* (Cayrol et al. 1992)

Actinomycetes against *Meloidogyne*

Gram-positive parasite bacteria such as actinomycetes (e.g. *Pasteuria penetrans)* possess BCA qualities against phytoparasitic nematodes (Mateille 1993). Spores staying in soil adhere to the J2 when they pass near the colonies. Adhesion is very specific of the *Meloidogyne* genera and of its larval stage. Spore germination occurs eight days after the J2 penetrates the root and starts feeding on it. This Actinomycete is perfectly synchronized with the development and the physiology of *Meloidogyne*. Then, a germinative tube is formed by the cuticle and infestation occurs. At the end, *Pasteuria penetrans* forms new hypha and spores inside the females, which become sterile as their reproductive system is destroyed.

Other organisms

There are carnivorous predatory nematodes belonging essentially to the order of *Mononchida*, to the *Diplogasteridae* suckers family, and to the *Dorylaimidae* omnivorous family, and others with toxic effects (Aphelenchidae). Some insects can also be nematophagous such as *Entomobryoides dissimilis* or nematodes' predatory mites (*Alliphis* spp., *Alicorhagia* spp.). But if the predation of these organisms is indisputable, their effects on nematode populations are unknown.

MAIN AGENCIES FOR THE HOMOLOGATION OF BCA'S, AND THEIR MARKET

Biocontrol agents and biopesticides must undergo an approval process before being placed on the market. This is a long and costly process but makes sure that the product brought to the field is both safe for humans and the environment. There are government organizations (agencies) responsible for defining the rules for using these products that come into contact with the food chain.

THE ENVIRONMENTAL PROTECTION AGENCY (EPA)

This U.S. government agency (United States 2014) is responsible for assessing the risks of the products used in the crops for food. Its mission is to protect both human health and the environment. When the Congress passes an environmental law, the EPA writes the regulations that strengthen it. This is the organization that certifies "biopesticide" products in the U.S., a leader in the field of biopesticides. In 2008, 279 biopesticides were registered in the U.S. compared to 77 in Europe, due to a procedure which is more complex and time consuming for the latter. However, the EPA does not require testing efficiency in its reviewing process. One can thus register a biopesticide on the sole basis of its safety for human health and the environment without demonstrating its effectiveness under different conditions for which the product may have to be used (Caron et al. 2006).

The Pest Management Regulatory Agency (ARLA)

The ARLA (Santé - Canada 2014) of Canada is committed to promoting, verifying and ensuring the compliance with the Act on the label. This is equivalent to the U.S. EPA. Unlike the EPA, the ARLA requires that the pesticide potential of a product be demonstrated, and that, over several seasons and over all particular geographical areas of Canada. This requirement leads to huge additional costs for the companies, which often cause the product not to meet the market's expectation. However, authorization by ARLA can be granted on the sole basis of EPA's registration of the same product (Caron et al. 2006).

The French Ministry of Agriculture and Fishing

It is responsible for assigning the AMM (Marketing Authorization) after assessing the risks of the product under investigation. For this, it relies on the Office of Pesticides and Fertilizers. Risk assessment is carried out by a committee of experts. The work of the experts and of the Commission for the Study of Toxicity is coordinated by the SSM (Joint Scientific Structure). The Accreditation Committee then makes a proposal, based on risk and efficiency assessments. The AMM, preceded by a provisional marketing authorization, is issued by the State and is valid throughout the European territory (France 2014).

The Organic Materials Review Institute (OMRI)

Founded in 1997, the Organic Materials Review Institute (OMRI) is a nonprofit organization (Omri 2014), which provides organic certifiers, growers, manufacturers, and suppliers an independent review of products intended for use in certified organic production, handling, and processing. When companies apply, OMRI reviews their products against the National Organic Standards. Acceptable products are OMRI-listed® and appear on the *OMRI Products List*. OMRI also provides subscribers and certifiers guidance on the acceptability of various material inputs, in general under the National Organic Program.

The market for Biological Control Agents

The global pesticide market is constantly changing and was estimated at USD 43 billion in 2009, with predictions for an annual increase of 5 to 8% for each subsequent year. Two competitors, chemical pesticides and biopesticides, share this market. With recent developments in global market requirements for pesticide residues, and the desire for products from organic agriculture, biopesticides have gained much attention. Exponential growth is predicted in this sector, which currently accounts for USD 1.6 billion. The United States are a leader in this area but the most important market growth for biopesticides is in Europe. The market in Europe was USD 270 million in 2010 and a 15% increase was expected in this sector. France ranked third on this market, behind Spain and Italy (Source: Europe: Pesticides Biomarket, CPL Consultants 2010). In Europe, several companies compete in this market: Novartis (18.4%), Koppert (18.2%), Abbot Laboratories (12.5%), AgrEvo Intrachem, BASF, Bayer cropscience, etc.

MAJOR FUNGAL PREPARATIONS ACTIVE ON MELOIDOGYNE AVAILABLE ON THE MARKET

In the U.S., more than 279 biopesticides (all inclusive) were approved in 2009 against only 77 in France. This was due to a more restrictive policy on the part of the European Union. Fourteen bacterial and twelve fungi have been registered with the EPA for the control of plant diseases (Fravel 2005). Among the products available on the market, only a few of the best-known biological nematicides are given below.

BIO-ACT®/ MELOCON WG (Prophyta 2014)

Marketed by Prophyta (acquired in 2013 by Bayer CropSscience Biologics GmbH), BioAct® is a biological nematicide used for biocontrol of the root-knot nematodes (Brand et al. 2009). It contains 10^{10} spores per dry weight gram of the ovicidal fungus, *Paecilomyces lilacinus* strain 251, which is a highly effective parasite of all the stages of development of common plant-infecting nematodes, especially the eggs and infectious juveniles. Formulated as a water-dispersible granule, BioAct®/MeloCon can be applied through conventional methods, using the irrigation system. *Paecilomyces lilacinus* is filamentous Ascomycete fungus that controls phytoparasitic nematodes such as *Meloidogyne* spp. (Kiewnick and Sikora 2004; Kiewnick and Sikora 2006; Khan et al. 2006; Mukhtar et al. 2013), *Radopholus similis* (Mendoza et al. 2007), *Heterodera* spp., *Globodera* spp. and *Pratylenchus* spp. Direct interactions between *P. lilacinus* strain 251 and eggs but also females of *M. javanica* were demonstrated *in vitro* by Holland et al. (1999).

BOTANIGARD®; MYCOTROL®

In 2009, the ARLA, under the authority of the Pest Control Products Act and Regulations, made a full registration of the Technical *Beauveria bassiana*, named Botanigard® ES or Botanigard®22WP (Laverlam 2014) and Mycotrol O® or Mycotrol ES® (Laverlam 2014), containing the *B. bassiana* strain GHA, a technical-grade active ingredient for sale and use in against whiteflies, aphids and thrips in vegetable and ornamental greenhouses. These products are also registered by the EPA. The *B. bassiana* GHA is found naturally in the soils throughout the world and parasitizes various insect species. It is a "generalist entomopathogenic fungus" - a fungus that causes disease in many types of insects. As the insects that live in the ground or near the ground have evolved natural defenses against *B. bassiana* because it is common in the wild, this fungus can be used as a biological insecticide against most other insects. Although its approval does not stipulate it, *B. bassiana* has a repressive action on nematodes of the genus *Meloidogyne* spp (Bradley et al. 1992; Bekanayake and Jayasundar 1994; Liu et al. 2008).

BIOSTAT®

BIOSTAT® (EPA approved) contains a biotype of *Purpureocillium lilacinum*, selected for its capacity to control plant pathogenic nematodes, such as: *Meloidogyne* sp., *Radopholus* sp., *Pratylenchus* sp., *Tylenchus* sp., *Ditylenchus* sp., *Helicotylenchus* sp. and *Rotylenchulus* sp. It parasitizes all reproductive stages of plant pathogenic nematodes, especially eggs and females, causing deformations, destruction of ovaries and reducing egg fertility. Also, under acid pH conditions, it produces toxins that affect the nematode's nervous system (Laverlam 2014).

MYCOTAL® (Koppert 2014)
Developed by KOPPERT, Mycotal® contains an entomopathogenic fungus, *Verticillium lecanii*. It is formulated as a wettable powder. It also acts against the larval stages of whiteflies, with a strong secondary action on adult thrips and red spiders. It has an action on the suppression on *M. incognita* on tomatoes, as reported by Meyer (1999).

TRIANUM® (koppert 2014)
The use of *Trichoderma* spp. as biological control agents has been investigated for over 70 years but it is only relatively recently that strains have become commercially available. Many *Trichoderma* strains, mainly *T. harzianum*, *T. viride* (Masadeh et al. 2004) and *T. virens* (formerly *Gliocladium virens*) have been identified as having potential applications in biological control (Lumsden et al. 1993; Monte and Llobell 2001). Trianum® is a product marketed by KOPPERT based on *T. harzianum* T-22. This filamentous fungus has various advantages for the protection of plants and the fight against nematodes (Widden and Abitbol 1980; González et al. 2012): it produces molecules such as 6-pentyl α-pyrone, VOCs and enzymes (Samson et al. 1996) that can attack the cuticle of nematodes. Also, its hyphae form a physical barrier, which is a difficult step for nematodes, since the fungus grows along with the plant roots. *T. harzianum* has a beneficial effect on the stimulation of the defense system of the plants (Samuels 1996; Harman 2000).

MET52® (Novozymes 2014)
Metarhizium anisopliae is an entomopathogenic fungus used against thrips, termites and mosquitoes. Studies have shown its nematicide potential as well (Mahmoud 2009). The PMRA-ARLA granted conditional approval in 2010 for the sale and use of *M. anisopliae* strain F52 and the associated end-bio-Met52 Granular Insecticide. Its active ingredient is the F52 strain of *M. anisopliae* in order to remove the root weevils, particularly black vine weevils and strawberry root weevils, which infest ornamental plants grown in pots.

NEMAXXION BIOL® (Greencorp 2014)
Nemaxxion Biol® has been commercialized by GreenCorp (Mexico). It is a liquid-formulated large-spectra product that is active against nematodes, composed of a consortium of microorganisms (*Bacillus subtilis*, *Trichoderma* spp, *Paecilomyces* spp.) and extracts (tagetes) that are active against RKN.

REM G®
REM G® is a new product commercialized by Green Solutions in Italy. Formulated as a consortium of nematophagous fungi such as *Arthrobotrys* spp, *Dactyllela* spp., *Paecilomyces* spp., Mycorrhiza (*Glomus* spp.), and bacteria (*B.* spp. and *Pseudomonas* spp.), it is supplemented with chitinolytic, proteolytic and lipolytic enzymes to specifically target the nematodes' walls. It is currently showing good results in tomato crops in the Souss-Massa Drâa aera in Morocco (personal information).

PRODUCTION OF BCAs

There are two ways of producing filamentous fungi (or bacteria, such as *Pasteuria penetrans*), by Liquid-State Fermentation (LSF) or Solid-State Fermentation (SSF). The choice of the method depends on the desired (liquid or wettable powder) final formulation. It also depends on the strain and on production costs.

PRODUCTION BY LIQUID-STATE FERMENTATION

Fermentation in a liquid medium is conducted in large, agitated, temperature-controlled and aerated tanks, and is perfectly suited to the production of single-celled organisms such as bacteria or yeasts. Some fungi can be produced by LSF such as *Fusarium venenatum*, used as Qorn®, for human food. Examples of microorganisms produced by LSF include *P. penetrans*, *B. thuringiensis*, etc.

PRODUCTION BY SOLID-STATE FERMENTATION

SSF is generally known as a culture of microorganisms (preferably filamentous fungi) on a solid substrate without water flow (Hesseltine 1987; Roussos et al. 1993; Mitchell 2002; Barrios-González 2012). It exploits the metabolism and growth mechanism of these microorganisms to degrade the solid substrates to produce biopesticides (biomass and secondary metabolites). Microbial growth occurs on the surface and within the solid matrix in the absence of any liquid flow. The porous matrix may be formed from a natural substrate or from an inert support capable of absorbing the nutrients found in dissolved state in a solution. For their mass multiplication, Selvakumar and Srivastava (2000) and Zaidi and Singh (2004), multiplied *T. harzianum* on pre-soaked and autoclaved Jhangora seeds for 12 days at 28ºC, then air-dried, ground and passed them through 50 and 80 mesh sieves simultaneously to obtain spore powder. The commercial formulation was prepared by diluting this powder with talcum powder containing 1% carboxymethyl-cellulose to get the desirable concentration of biocontrol agent.

FORMULATION OF BCAs

Formulation is the blending of active ingredients, such as fungal spores, with an inert material, such as diluents or surfactants, in order to preserve the viability and virulence of the strain used. Commercial products also have to present an appropriate structure (powder or liquid) for their application in the field. Formulation must ensure the viability of the strain, preserving its germinating power; it must help the strain keep its virulence against the pest involved. The material can be mineral such as talc (Chaube et al. 2003) or zeolites (Küçük and Kivanç 2005). It is also essential to protect the BCA from the UV rays of the sun and to stabilize the product for both storage and utilization conditions. A granular starch matrix encapsulating *B. thuringiensis* was patented in 1997 (Quimby et al. 1996). One of the technologies for the formulation of biocontrol organisms is the immobilization of wet or dry biomass within cross-linked polymers such as alginate and carrageen (Cho and Lee 1999). Incorporating fungal mycelia in alginate pellets has been found to be successful for the delivery of biocontrol fungi (Papavizas et al. 1987; Küçük and Kivanç 2005). The biocontrol microorganisms were immobilized, wet or dry, as formulated pellets (Walker and Connick 1983). Alginate-type pellets were used in the formulations of chemical and microbial herbicides (Walker and Connick 1983). In the biotechnology industry, cell entrapment is often used to enhance the production rates of bioproducts, to reduce the mortality of cells, and to facilitate their recovery. Alginate pellets containing the spores of various biocontrol fungi (Lewis and Papavizas 1983; 1985) and of yeast cells have been formulated (Serp et al. 2000). Such preparations offer many advantages compared with the conidial suspensions, e.g., pellets can be stored dry.

CONCLUSION

Biological control is a sustainable alternative to chemical control. The use of fungi to control the pests like root-knot nematodes may be developed to protect the environment from the pesticides. Unless they are more difficult to produce, store and/or use, they can be a suitable solution to protect the crops. Currently, some products are available on the market and more are being developed. Isolating indigenous strains is the best way to ensure the success of the use of these biological control agents, because they are then adapted to the pest they have to control and to the environment they will be used in. Formulation of these strains should be adapted to ensure both good conservation of the microorganisms and high virulence against the pests.

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