

Review

Analysis of *Trichoderma* as an effective biological control agent against the honey fungus (*Armillaria* spp.)



Jorge Poveda^{a,b,*}, Morgan R. Millen^b, Andy M. Bailey^{b,*}

^a Recognised Research Group AGROBIOTECH, UIC-370 (JCyL), Department of Plant Production and Forest Resources, Higher Technical School of Agricultural Engineering of Palencia, University Institute for Research in Sustainable Forest Management (iuFOR), University of Valladolid, Avda. Madrid 57, 34004 Palencia, Spain

^b School of Biological Sciences, University of Bristol, Life Sciences Building, 28 Tyndall Ave, Bristol BS8 1TQ, UK

HIGHLIGHTS

- *Trichoderma* spp. is a BCA widely studied and used in agriculture.
- *Armillaria* spp. is a globally important pathogen of agricultural/forestry species.
- So far, there are 30 studies on the effective control of ARR by *Trichoderma*.
- *Trichoderma* effectively control *Armillaria* in different experimental conditions.
- So far, there are four mechanisms of action identified in this biological control.
- More research is needed on the use of *Trichoderma* against *Armillaria*.

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ABSTRACT

Trichoderma is a genus of filamentous fungi with several species widely studied and used as biological control agents (BCAs) in agriculture. With respect to fungal disease control, *Trichoderma* spp. can use different mechanisms of action, including mycoparasitism, antibiosis, competition and/or activation of plant defences. *Armillaria* spp. are the causal pathogens of Armillaria root rot (ARR) disease, which is widely distributed and of great economic and environmental importance in agriculture and forestry. ARR can be devastating to plant health, through colonisation and rotting of the root system and collar of the infected plant/tree, which can cause a reduction in yield and eventually death. *Armillaria* can live as a saprophyte that survives on dead plant material, making eradication of ARR extremely difficult. In an exhaustive analysis of work published to date on the control of *Armillaria* spp. by *Trichoderma* spp., using the WoS and Scopus databases, we identified 31 papers. We discuss these reports in light of their key findings, including number of publications per year, the countries where they were published, the journals and the citations obtained so far. Although the vast majority do not identify the mechanism of action involved in control, some describe mycoparasitism, antibiosis, competition or modification of the rhizospheric microbiota as being responsible for effective biological control of the pathogen. Further research into these mechanisms and additional in-depth analysis of less studied or currently unidentified mechanisms, such as the activation of plant defences, would be highly beneficial to our understanding of *Trichoderma* as a BCA of *Armillaria*.

* Corresponding authors at: Recognised Research Group AGROBIOTECH, UIC-370 (JCyL), Department of Plant Production and Forest Resources, Higher Technical School of Agricultural Engineering of Palencia, University Institute for Research in Sustainable Forest Management (iuFOR), University of Valladolid, Avda. Madrid 57, 34004 Palencia, Spain (J. Poveda).

E-mail addresses: jorge.poveda@uva.es (J. Poveda), andy.bailey@bristol.ac.uk (A.M. Bailey).

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1. Introduction

1.1. *Trichoderma*: A multifunctional biological control agent

Trichoderma is a genus of filamentous fungi included in the family Hypocreaceae, order Hypocreales, class Sordariomycetes, and division Ascomycota (Adnan et al., 2019). Morphologically, *Trichoderma* is easily distinguishable from many other fungi, due to the bright green colour of its conidia, its rapid growth and the repeated branching of its hyphae (Zin & Badaluddin, 2020). Molecular analyses in 2021 proposed some 300 different species within the genus *Trichoderma* (Del Carmen et al., 2021). Whilst this highlights the incredible diversity within the genus, many of these species names were only recently ascribed, and some older literature may be misleading regarding specific species involved in each study.

Trichoderma species are found in many different ecosystems. Their capacity to grow on different substrates and tolerance to pollutants and pesticides allows for survival in forests and agricultural soils (Zin & Badaluddin, 2020; Poveda, 2022). They are commonly found free-living as saprophytes, typically within the rhizosphere, but can also be found living within plants as endophytes, or even as pathogens of cultivated mushrooms and other fungi and possibly as a pathogen of nematodes (Druzhinina & Kubicek, 2013). In this sense, *Trichoderma* displays a range of different lifestyles and very varied metabolic and enzymatic machinery, which makes the genus interesting for industrial production of secondary metabolites and hydrolytic enzymes (Sharma et al., 2019) or for soil and water mycoremediation (Poveda, 2022).

In agriculture, *Trichoderma* species have been widely studied and have shown considerable opportunity as biological control agents (BCAs), abiotic stress relievers, and plant growth promoting fungi (PGPF) (Sood et al., 2020; Abdullah et al., 2021). A number of products commercialised based on various *Trichoderma* species for crop protection. Under abiotic stresses such as drought, salinity or cold, some *Trichoderma* are able to increase plant tolerance. This can be from a range of methods including by direct production of phytohormones, osmoprotectants and antioxidants or by inducing plant responses against stress, as has been seen in crops such as wheat, tomato, rapeseed, maize, rice or cucumber (Abdullah et al., 2021). Some *Trichoderma* are PGPF that also improve the nutraceutical characteristics of the crops they colonise (Velasco et al., 2021). This can be due to the production of organic acids that solubilise nutrients such as P and K, the production of siderophores that chelate Fe, Cu and Zn, the production of plant growth-promoting phytohormones and/or the induction of systemic changes in the plant (Sood et al., 2020). Moreover, *Trichoderma* species are widely used as BCAs against many phytopathogens and pests, such as insect pests (Poveda, 2021), nematodes (Poveda et al., 2020a), fungi (Ali et al., 2021), oomycetes (Liu et al., 2022), bacteria (Khan et al., 2021) and viruses (Rochal et al., 2021). The method of control by *Trichoderma* varies by plant host as well as by pest/pathogen (Ferreira & Musumeci, 2021).

Trichoderma are not universally beneficial to plants. While many species are studied for their positive effect on plant health, *Trichoderma* can be plant pathogens (Poveda et al., 2020b). In 2014, Alonso-Ramírez et al. described how in the absence of salicylic acid (SA) the fungus reaches the vascular bundles and behaves as a systemic pathogen (Alonso-Ramírez et al., 2014). Subsequently, it has been possible to determine how evolutionarily, *Trichoderma* was likely a phytopathogen of the first plants that colonised the earth's surface (bryophytes), and how the development of a SA-mediated plant defence response allowed the mutually beneficial *Trichoderma*-plant symbiosis (Poveda et al., 2023).

As a BCA, *Trichoderma* excels in its use against fungi and oomycetes, significantly reducing the impact of crop diseases, which untreated, can account for 10–35 % losses in the four major human feeding crops (rice, wheat, maize and soybean) (Godfray et al., 2016). The known range of mechanisms utilized by *Trichoderma* against plant pathogenic fungi are:

mycoparasitism, antibiosis, competition and induction of plant defences (Asad, 2022; Tyśkiewicz et al., 2022). It is unclear whether all species can use all of these methods, and thus whether use of a particular mechanism is specific to its interaction with certain plant hosts or phytopathogens. Mycoparasitism is the mechanism by which *Trichoderma* directly attacks plant pathogenic fungi and obtains nutrients from their hyphae until they are killed. Here, *Trichoderma* first recognises its prey and grows by chemotropism towards it. Depending on the interacting fungi, it may coil around the pathogen's hyphae, form appressoria and/or releases cell wall degrading enzymes (CWDEs) such as chitinases, glucanases and proteases to attack the target fungus mycelium. Several species of *Trichoderma* now have genome sequences available and these all show abundant potential for CWDE production, although these gene repertoires do not correlate with biocontrol ability. Once "inside" the pathogen, *Trichoderma* feeds on its internal contents, killing the host (Asad, 2022; Tyśkiewicz et al., 2022). The interaction with *Armillaria* has recently been investigated *in vitro* by RNAseq and has highlighted expression of such fungal cell wall degrading enzymes amongst numerous genes differentially regulated during infection (Chen et al., 2023). Perhaps given their wealth of CWDEs it is no surprise that *Trichoderma* species are widely studied for industrial purposes, notably as a valuable source of enzymes for generating fungal protoplasts (Lalithakumari, 2019). Antibiosis is based on the inhibition of growth/development or death of a pathogen through the release of secondary metabolites (volatile and non-volatile) by *Trichoderma*. In this regard, the ability of the genus *Trichoderma* to produce about 400 secondary metabolites with antagonistic properties has been described, among which peptaibols, polyketides, terpenoids, pyrones and anthraquinones stand out for their fungicidal activity (Asad, 2022; Tyśkiewicz et al., 2022). The non-volatile metabolites reported to be produced by *Trichoderma* have been extensively reviewed by Li et al., (2019), and volatile compounds by Gualtieri et al., (2022) and Lee et al., (2016). Whilst these lists are extensive, few if any of these molecules have proven roles in biocontrol ability and many of the studies involved a limited set of *Trichoderma* species, and limited growth conditions so whether these compounds are relevant in a natural biocontrol context is far from clear.

Trichoderma can grow very quickly on various substrates due to its high adaptive capacity, chemical tolerance and ability to mobilise nutrients. This growth rate is often superior to most other soil microorganisms, allowing *Trichoderma* to compete effectively for space and resources in the rhizosphere. This has an impact on the ability of phytopathogens to establish in the rhizosphere (Asad, 2022; Tyśkiewicz et al., 2022). Furthermore, *Trichoderma* is often able to induce defences (local and systemic) in its associated plants, through processes such as the recognition of microbe-associated molecular patterns (MAMPs), or via use of volatile metabolites (Lee et al., 2016). This molecular dialogue leads to hormonal and metabolic changes that reduce the development of the phytopathogenic fungus in plant tissues and the damage it causes (Asad, 2022; Tyśkiewicz et al., 2022). Through both competition and induction of plant defences, *Trichoderma* can indirectly antagonize phytopathogens. Fig. 1 is an infographic that summarises the various mechanisms of action available to *Trichoderma* when acting as BCA against plant pathogenic fungi. It should be remembered that whilst these gene pathways are available to *Trichoderma*, the gene repertoire varies between *Trichoderma* species, and evidence of their expression in biocontrol situations is very limited, probably the best of these to date is the RNAseq analysis of *Trichoderma virens* against *Rhizoctonia solani* by Halifu et al., (2020).

For practical use of *Trichoderma* as a BCA in the field, mass production of conidia and their formulation into bio-inoculants is necessary. For the industrial production of *Trichoderma* conidia, solid state fermentation in sterilised grains, such as rice, maize, sorghum, rye or millet, is mainly used (Poveda & Eugui, 2022). Subsequently, these conidia are used in the formulation of bio-inoculants, mainly using talc, vermiculite or wheat bran as carriers. These are typically able to maintain the viability of the fungus for up to 18 months (Poveda &

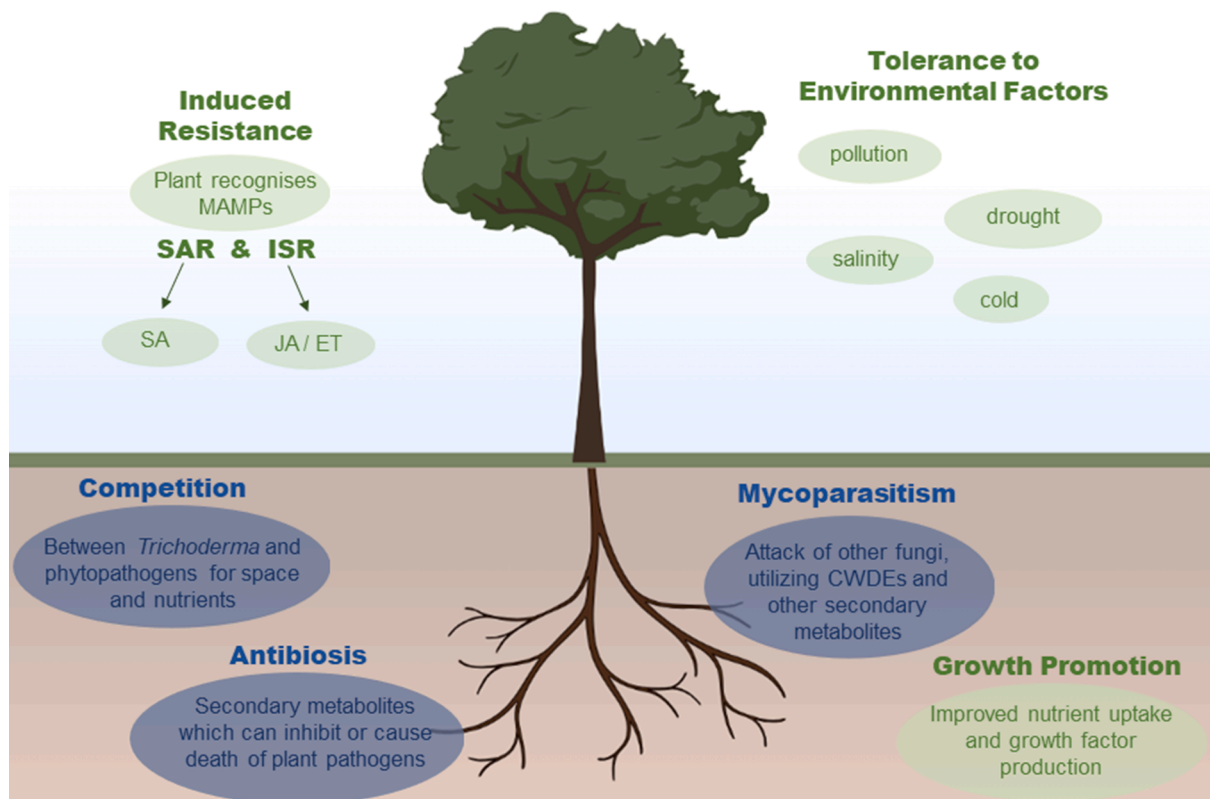


Fig. 1. Infographic summarising the mechanisms of action of *Trichoderma* as a BCA against plant pathogenic fungi. MAMPs: microbe-associated molecular patterns; SAR: systemic acquired resistance; ISR: induced systemic resistance; SA: salicylic acid; JA: jasmonic acid; ET: ethylene; CWDEs: cell wall-degrading enzymes.

Eugui, 2022). Commercial BCA based on *Trichoderma* are available, either as single strains or indeed as mixtures.

2. *Armillaria*: The honey fungus causing Armillaria root rot

Armillaria species are infamous for causing disease in numerous trees and shrubby plants, both in agriculture and in horticulture. The genus *Armillaria*, as well as its sister genus *Desarmillaria*, are included in the so-called armillarioid genera, belonging to the family Physalacriaceae, order Agaricales and class Basidiomycetes (Coetzee et al., 2018; Kedves et al., 2021). The difference between the two genera is the absence of an annulus, or ring, on the stipe (stem) of the basidiocarp (in the case of *Desarmillaria*) or the presence of the annulus (*Armillaria*), the latter include 50–60 different species worldwide (Kim et al., 2022). The armillarioid fungi include ecologically and economically important pathogens distributed worldwide, causing Armillaria root rot (ARR) disease in more than 500 different plant species, mainly woody species (conifers, fruit trees, grapevine, etc.), but also affecting herbaceous species (Ford et al., 2017; Devkota & Hammerschmidt, 2020; Kim et al., 2022). Indeed, *Armillaria* typically top the ranked lists of enquiries to the Royal Horticultural Society (RHS) for fungal diseases of concern to gardeners in the UK (RHS, 2023).

Armillaria live as facultative necrotrophs and saprophytes. Initially, they need to colonise living roots and kill tissues in order to feed on their cells. Subsequently, they survive in wood-decaying mode as white-rot saprophytes within the decaying root system (Devkota & Hammerschmidt, 2020). The presence of *Armillaria* in an ecosystem is not always a threat to plant health, like many fungi they may aid in the breakdown and utilization of dead material. However, ARR disease can be severe and devastating to agriculture. In the northern hemisphere two species stand out for their virulence and importance: *A. mellea* and *A. ostoyae* (syn. *A. solidipes*). *A. mellea* widely attacks forest trees, urban trees, nut crops and grapevine, with 10–40 % annual yield loss; while *A. ostoyae*

mainly attacks conifers and fruit trees (Devkota & Hammerschmidt, 2020).

Other than the honey-coloured basidiocarps (Fig. 2), the most characteristic structure of *Armillaria* spp. is the rhizomorph, an agglomeration and intertwining of hyphae, up to 5 mm in diameter (Devkota & Hammerschmidt, 2020). These structures can grow through the soil in search of new hosts, often several metres from the initial infection, spreading disease to nearby plants. The life cycle of *Armillaria* is divided into 5 distinct phases (Fig. 3). Stage 1 encompasses the contact of basidiospores with a healthy plant, where they germinate and originate a mycelium that can be haploid (n) in heterothallic species, or diploid (2n) in homothallic species (Kim et al., 2022). In stage 2, the mycelium colonises plant/tree tissues, being always diploid mycelium, as in heterothallic species the previous fusion of two compatible haploid hyphae has occurred. Stage 3 involves transmission of the pathogen to other neighbouring plants/trees via root-root contact or by the growth of rhizomorphs in the soil. In stage 4, the pathogen invades the entire root system and stem base of its host, killing and rotting its cambium and pith. Finally, in stage 5, fruiting bodies develop on the dead tissues/wood, which release basidiospores (Heinzelmann et al., 2019). The mechanisms by which various *Armillaria* cause disease is starting to be elucidated, but the pathogen is slow, and the hosts are typically long-lived making these studies time-consuming. Genomic data for *Armillaria* are becoming available and these show complex genome structures, likely due to substantial gene acquisition by horizontal gene transfer, but highlight a considerable repertoire of CWDE enzymes (Sahu et al., 2023).

The symptoms of ARR disease are dependent on both the host and environmental conditions. Symptoms of root infection can be confused with the effect of abiotic stress such as drought, especially in the early stages of infection. As disease progresses, there is typically a reduction in plant growth, chlorotic and slow loss of foliage, distress cone production on conifers, slow crown decline, abundant basal resin flow on conifers,



Fig. 2. *Armillaria* basidiocarps showing typical honey-coloured fruiting bodies of *A. mellea*.

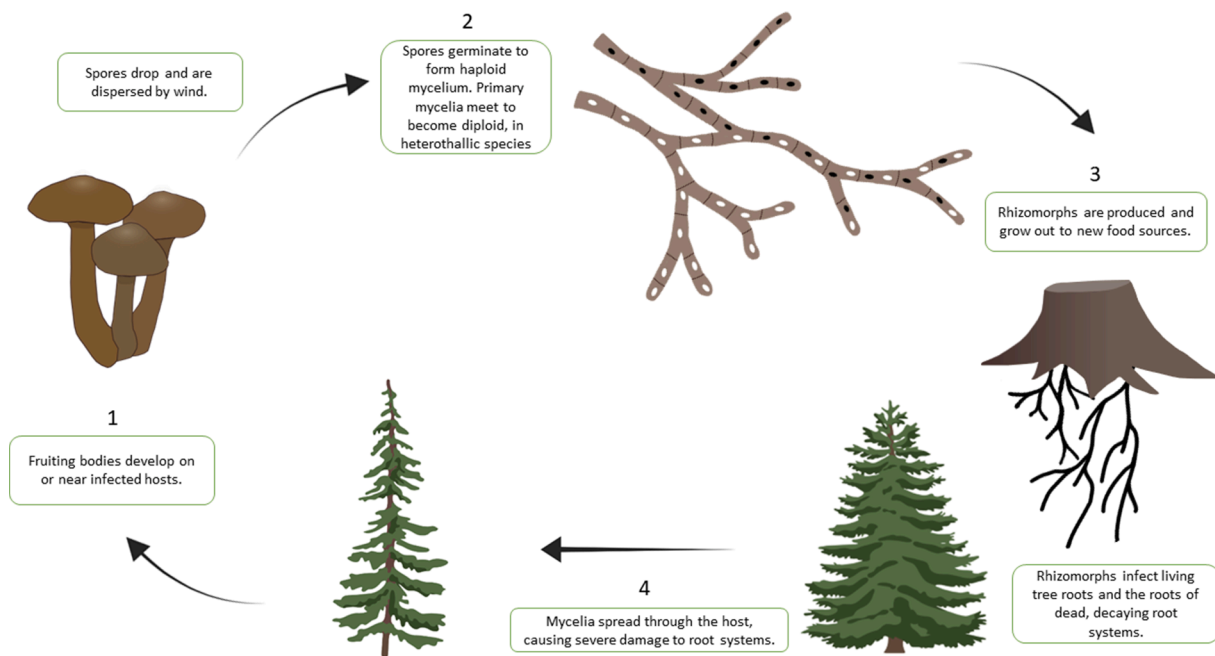


Fig. 3. *Armillaria* spp. life cycle.

gummosis on hardwood trees, and sudden or slow death of tree can be observed. In addition, the presence of the ARR disease can be diagnosed by visual signs such as the presence of mycelial fans under the bark, root-like rhizomorphs on the root surface or soil, yellow wet/stringy wood decay with zone lines, and seasonally produced honey-coloured basidiocarps at the tree base (Kim et al., 2022). Some evidence suggests that ARR infection is more likely on trees that are already stressed, so issues such as soil compaction, recurring drought or frequent pruning may predispose plants to infection.

In addition to including important pathogenic fungi, the genus *Armillaria* also has plant-symbiotic organisms. The first description of *Armillaria* as a plant symbiont (published in English) was made in 1996, reporting that *Armillaria jezoensis* is able to endophytically colonize the roots of the orchid *Galeola septentrionalis* (Cha & Igarashi, 1996). However, the most studied symbiotic interaction so far has been with *A. gallica* and the orchid *Gastrodia elata*. In this regard, it is known that *A. gallica* is able to increase the growth of its host plant (Wang et al., 2023), probably due to an increase in C, Na, Mg, Ca, Cr, Cu, Zn and Rb available to the plant (Yu et al., 2022) through intracellular digestion of fungal hyphae by plant cells (Zhan et al., 2020).

Armillaria has been described as an important symbiont of the fungus *Polyporus umbellatus*, whose sclerotia are widely used in traditional medicine for their diuretic, antitumor and renal protection activity (Liu et al., 2015; Xing et al., 2022). In medicine, different molecules and compounds isolated from the mycelium and/or fruiting bodies of *Armillaria* are used, such as polysaccharides to reduce the risk of cardiovascular disease (Chen et al., 2015; Sośnicka et al., 2018; Ren et al., 2023), sphingolipids with antihepatotoxic, antitumor and immunostimulatory activities (Sośnicka et al., 2018; Ren et al., 2023), sesquiterpenoids as potent antimicrobials (Sośnicka et al., 2018; Ren et al., 2023), or serotonin (Sośnicka et al., 2018; Ren et al., 2023). Other uses of *Armillaria* are as a source of lignin- and cellulose-degrading enzymes (Sośnicka et al., 2018) and as a mycoremediator of pollutants, such as polycyclic aromatic hydrocarbons (benzoates) (Sośnicka et al., 2018; Champramary et al., 2023).

3. Control of *Armillaria* root rot

The control of *Armillaria* spp. is complicated, due to its saprophytic life form and its long viability in the soil (up to decades) (Heinzelmann

et al., 2019). The most commonly used strategy is based on reducing the *Armillaria*-inoculum present in the soil, by removing infected plants and roots, and/or mass application of chemical fungicides. Soil fumigants were used historically, but now have largely been prohibited due to environmental pollution and impacts on soil health. Other strategies aim to reduce the damage of the disease on the plant/tree by improving soil structure, by planting more resistant plant species (Heinzelmann et al., 2019; Kim et al., 2022). Due to the environmental and health problems resulting from the use of chemical fungicides and the difficulty of using these on tree root systems, there is a need for safer alternatives that can effectively control ARR in the agricultural and forestry system and biological control is one such approach (Kim et al., 2022). So far, BCAs effective in the control of *Armillaria* spp. have been described in different taxonomic groups, such as bacteria (*Pseudomonas fluorescens*, *Bacillus simplex*, *Erwinia billingiae*, *Streptomyces aurantiacogriseus*, etc.), cyanobacteria (*Nostoc* sp.), fungi (*Trichoderma* sp., *Ganoderma lucidum*, *Hypholoma fasciculare*, *Xylaria hypoxylon*, etc.), nematodes (*Ditylenchus* sp., *Neotylenchus* sp., *Aphelenchus* sp., etc.) and plants (biofumigation with *Brassica* crops tissues) (Kedves et al., 2021). Whilst there are convincing reports of success in a lab situation or in short-duration outdoor experiments, their durability in the field is far from clear.

4. Analysis conducted

A literature review was performed together with a quantitative analysis of publications according to year, journal and countries. The compilation of all publications was done with the keywords “*Trichoderma* AND *Armillaria*”. The bibliographic database Web of Science™ (Web of Science Core Collection - WoS) (<https://www.webofscience.com>) and the Elsevier® Scopus library services metabase (<https://www.scopus.com>) were used, due to the advantages of scientific rigor compared to other free and more open databases, such as Google Scholar (Martín-Martín et al., 2021).

In WoS, after searching for keywords in “All Fields”, without time restriction, 74 results were retrieved (search performed on October 7,

2023). Of these 74 papers, 9 are conference papers and 36 are not related to the subject, therefore, 29 papers were included in the review. On the other hand, after searching for keywords in “Title, Abstract and Keywords”, without time restriction, 64 results were retrieved in Scopus (search performed on October 7, 2023), of which 4 are conference papers and 32 are not related to the subject of this work, therefore, 27 papers were included in the review. It is important to note the overlapping results between the two databases. Of the 29 papers used from WoS and the 27 from Scopus, 22 coincide, contributing 7 and 3 unique papers, respectively. Therefore, the total number of final papers of the review on the use of *Trichoderma* spp. as BCAs against *Armillaria* spp. was 34 papers. However, after analysing these 34 studies, 3 of them showed no control of the pathogen by the antagonistic fungus. Of course, these papers need to be discussed (which will be done at the end of this section), but in the following data analysis only the 31 papers where *Trichoderma* spp. effectively control *Armillaria* spp. are considered.

The first publication on the effective control of *Trichoderma* against *Armillaria* dated to 1973 (Ohr et al., 1973), but no further publications were found until 1990 (Reaves et al., 1990). Between 1990 and 1997 there was a period of slow but continuous publications on this subject (7 in total), followed by a period of absence between 1998 and 2002. From 2003 to the present, there was a steady flow of publications, with 22 papers out of the 30 analysed. Furthermore, it is important to note that in 2003 and 2004 we found a period of great scientific interest in the subject, with 4 publications each year (Fig. 4a). With respect to the country of papers publication (where the research group was based, being able to be more than one if there are authors from several countries in the same paper), the UK stands out as the country publishing the most research (12 papers). Next in importance are Pakistan and Italy, with 7 and 5 papers, respectively. Tied in fourth and fifth place are Kenya and the USA (with 4 papers), and Hungary, Switzerland and the Netherlands (with 2 papers). With one publication with authors from this position we found a total of 12 countries from Oceania (Australia and New Zealand), Europe (Belgium, Czech Republic, France, Germany, Poland and Turkey), America (Canada), Africa (South Africa) and Asia

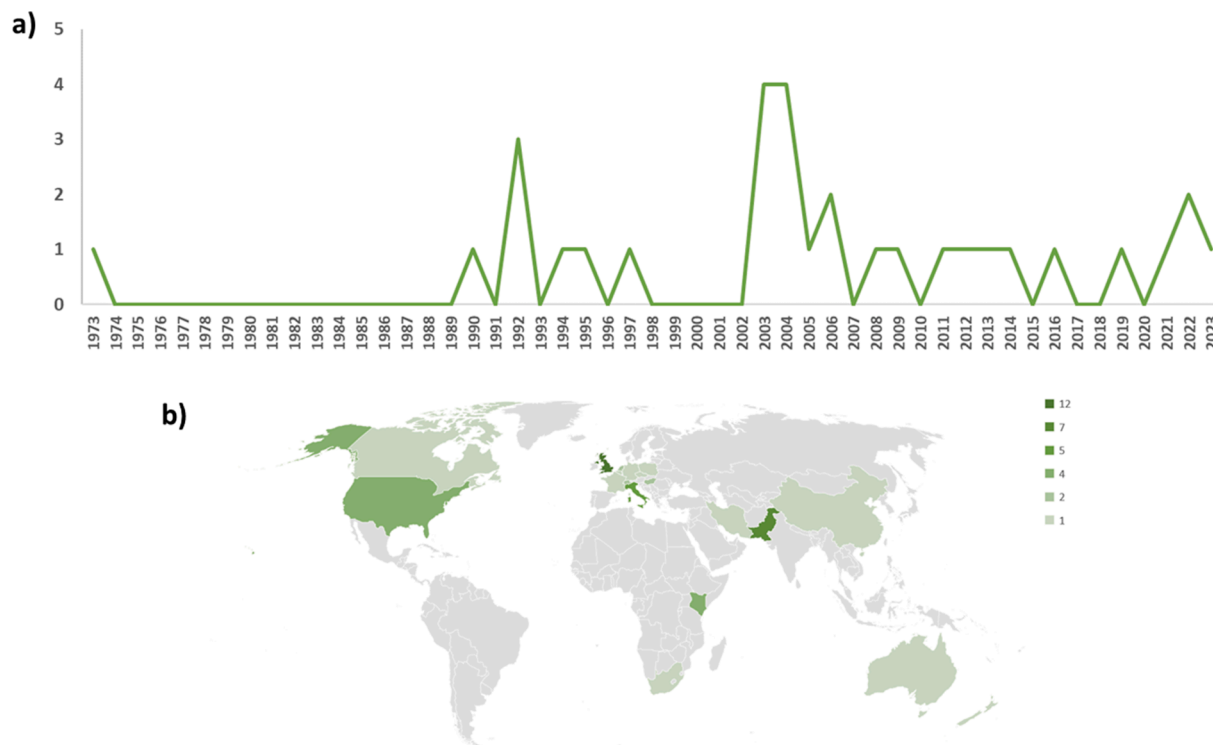


Fig. 4. Graphical representation of the data obtained in the bibliographic search. Number of papers per year (a) and countries of authors (b), the legend represents the number of papers per country.

(China, Iran and Turkey) (Fig. 4b).

These authors published their works in 16 different journals and in one book (as a chapter). Of all the journals, *Biological Agriculture & Horticulture* (Taylor & Francis) with 7 papers, *Crop Protection* (Elsevier) with 2 papers and *Northwest Science* (BioOne) with 2 papers stood out. The remaining journals only published one paper (Table 1). Regarding the number of citations per paper, the most cited paper is the one by Savazzini et al. (2009), with 43 citations in WoS and 48 in Scopus. However, these papers do not have a high number of citations compared to other papers related to biocontrol. For example, the third most cited paper is more than 30 years old and only accumulates 20 citations in WoS and 22 in Scopus. Among the top 10 most cited papers is one less than 5 years old, published in the journal *Forests* by Chen et al. in 2019, which accumulates 14 citations in WoS and 19 in Scopus (Table 2). In this sense, given how slow *Armillaria* grows, and the challenges in performing meaningful bioassays where symptoms can take months to years to develop, before any control effect can be assessed, perhaps the slow impact of these papers is not surprising.

As indicated in the classification of results obtained in the databases, several studies have shown that *Trichoderma* spp. had no effect against *Armillaria* spp. as BCA. Some species even caused an increase in ARR disease. Interestingly, *Trichoderma* has been described as a promoter of *Armillaria* development. Specifically, the application *in vitro* of non-volatile metabolites from *T. harzianum* significantly increased the number and length of rhizomorphs formed by *Armillaria borealis*, *A. gallica* and *A. ostoyae* but the impacts of this on virulence are unclear (Przybyl & Manka, 2004). In contrast, soil application of non-volatile metabolites of *T. viride* did not modify the development of *A. ostoyae* rhizomorphs and mycelium (Henryk-Szewczyk et al., 2013). In field conditions, it has been reported that the combined application of *T. harzianum* and *T. viride* did not improve the survival of nectarine trees affected by *A. mellea*; the disease could not be controlled by the use of

Table 1
Journals where the reviewed papers were published.

Journal	Number of papers	Papers references
<i>Biological Agriculture & Horticulture</i>	7	Raziq & Fox, 2003, 2004a, 2004b, 2004c, 2005, 2006a, 2006b
<i>Crop Protection</i>	2	Otieno et al., 2003a; Pellegrini et al., 2014
<i>Northwest Science</i>	2	Reaves et al., 1990; Filip & Yang-Erve, 1997
<i>Applied Soil Ecology</i>	1	Pellegrini et al., 2013
<i>Arboricultural Journal</i>	1	Percival et al., 2011
<i>Australasian Plant Pathology</i>	1	Li & Hood, 1992
<i>Biological Control</i>	1	Otieno et al., 2003b
<i>Biological Control of Plant Diseases</i> (Book)	1	Davet et al., 1992
<i>BMC Genomics</i>	1	Perazzolli et al., 2016
<i>Bulletin of the Chemical Society of Ethiopia</i>	1	Tarus et al., 2003
<i>European Journal of Forest Pathology</i>	1	Dumas & Boyonoski, 1992
<i>Forests</i>	1	Chen et al., 2019
<i>Forest Pathology</i>	1	Kwaśna et al., 2004
<i>International Journal of Pest Management</i>	1	Onsando et al., 1994
<i>Journal of Applied Microbiology</i>	1	Rees et al., 2021
<i>Journal of Plant Protection Research</i>	1	Asef et al., 2008
<i>Kastamonu University Journal of Forestry Faculty</i>	1	Aslan et al., 2022
<i>Microbiology Spectrum</i>	1	Chen et al., 2023
<i>Mycological Research</i>	1	Nelson et al., 1995
<i>Phytopathology</i>	1	Ohr et al., 1973
<i>PLoS One</i>	1	Rees et al., 2022
<i>Rapid Communications in Mass Spectrometry</i>	1	Pellegrini et al., 2012
<i>Soil Biology & Biochemistry</i>	1	Savazzini et al., 2009

Table 2

Number of citations of the 10 most cited articles.

Ranking	Paper reference	Journal	WoS citations	Scopus citations
1	Savazzini et al., 2009	<i>Soil Biology & Biochemistry</i>	43	48
2	Ohr et al., 1973	<i>Phytopathology</i>	36	Not indexed
3	Dumas & Boyonoski, 1992	<i>European Journal of Forest Pathology</i>	20	22
4	Otieno et al., 2003a	<i>Crop Protection</i>	17	20
5	Reaves et al., 1990	<i>Northwest Science</i>	16	20
6	Chen et al., 2019	<i>Forests</i>	14	19
7	Pellegrini et al., 2014	<i>Crop Protection</i>	14	15
8	Onsando et al., 1994	<i>International Journal of Pest Management</i>	13	19
9	Raziq & Fox, 2005	<i>Biological Agriculture & Horticulture</i>	11	11
10	Otieno et al., 2003b	<i>Biological Control</i>	11	12

mycorrhizal fungi either (Baldi et al., 2016).

These studies often use different strains or species of *Trichoderma*, different species of *Armillaria*, and different plant hosts, so perhaps it is not surprising that there is no consistent pattern of control. A successful biocontrol strategy appears specific to each fungal and plant combination. This highlights the importance of further testing, to understand the interactions between organisms involved in the strategy.

5. *Trichoderma* as BCA against *Armillaria*

In the studies analysed we found results of *Armillaria* spp. control by *Trichoderma* spp. under very different experimental conditions (*in vitro*, in laboratory, *in planta*, in field) and with different plant species and negative effects on the pathogen (Table 3). The vast majority of these studies (20 out of 30) describe the biological control of the pathogen by *Trichoderma* but do not identify the mechanism of action responsible. *In vitro*, the ability of different *Trichoderma* species (*T. hamatum*, *T. harzianum*, *T. viride* or *T. atroviride*) to inhibit the growth of *A. mellea* and *A. gallica*, and the viability of their inoculum has been described (Raziq & Fox, 2003, 2004a; Pellegrini et al., 2014). Similarly, it has been reported that *Trichoderma* spp. can reduce *Armillaria* spp. inoculum present on different plant materials, such as oak wood discs, pine twig segments, citrus roots or hazelnut pieces in the laboratory (Ohr et al., 1973; Li & Hood, 1992; Kwaśna et al., 2004), even in chips or sections of woody tea stems, karri stumps or red alder stem segments in the field (Filip & Yang-Erve, 1997; Nelson et al., 1995; Otieno et al., 2003a, 2003b). Indeed, *in vitro* studies have reported killing of the pathogen, both in Petri dishes and on hazel disks (Rees et al., 2021).

Trichoderma spp. has also been described as an effective *in planta* BCA against *Armillaria* spp. In this regard, increased survival of different plant species subjected to ARR after application of *Trichoderma* spp. has been reported, such as maritime pine against *A. ostoyae* (Davet et al., 1992), privet against *A. mellea* (Rees et al., 2022), but especially in strawberry. Various *in planta* studies were carried out in greenhouses with strawberry as a host, showing a reduction in disease incidence and an increase in plant survival when attacked by *A. gallica* or *A. mellea*, due to the action of different *Trichoderma* species (*T. atroviride*, *T. hamatum*, *T. harzianum*, *T. viride* or *T. atroviride*) (Raziq & Fox, 2003, 2004b, 2004c, 2005; Pellegrini et al., 2014; Rees et al., 2022). Given how few host plant species have been assessed in detail, it remains unclear whether a particular strain of *Trichoderma* will be successful on all hosts, and against all species of *Armillaria*, or whether there will be specificity between host plant and strain of *Trichoderma*.

Table 3
Biocontrol of *Armillaria* spp. by *Trichoderma* spp., indicating the effect obtained and the mechanisms of action involved.

<i>Armillaria</i> species	<i>Trichoderma</i> species	In combination with	Plant and experiment	Effect	Mechanism of action	Reference
<i>Armillaria</i> sp.	<i>T. harzianum</i>	Solarization	Tea woody chips – In field	Reduction of inoculum viability	Not indicated	Otieno et al., 2003a
	<i>T. harzianum</i>	–	Tea stem sections – In field	Reduction of inoculum viability	Not indicated	Otieno et al., 2003b
<i>A. gallica</i>	<i>T. polysporum</i> <i>T. harzianum</i> <i>T. viride</i> <i>T. atroviride</i>	–	<i>In vitro</i>	Pathogen death	Mycoparasitism	Dumas & Boyonoski, 1992
	<i>T. virens</i> <i>T. atrobrunneum</i>	–	<i>In vitro</i> Strawberry – In greenhouse Oak – In field	Reduction of inoculum viability (<i>in vitro</i>) Reduction of disease incidence (<i>in planta</i>) Inhibited pathogen growth (<i>in vitro</i>) Increased plant survival (<i>in field</i>)	Not indicated Siderophores production	Pellegrini et al., 2014 Chen et al., 2019
<i>A. heimii</i>	<i>T. koningii</i> <i>T. longibrachiatum</i> <i>T. harzianum</i>	–	<i>In vitro</i>	Inhibited pathogen growth	Antibiosis by non-volatile compounds	Onsando et al., 1994
<i>A. lilionea</i>	<i>Trichoderma</i> sp.	–	Pine branch segment – In laboratory	Reduction of inoculum viability	Not indicated	Li & Hood, 1992
<i>A. luteobubalina</i>	<i>Trichoderma</i> spp.	–	Stumps of karri – In field	Reduction of inoculum viability	Not indicated	Nelson et al., 1995
<i>A. mellea</i>	<i>Trichoderma</i> sp.	–	Citrus roots – In laboratory	Reduction of inoculum viability	Not indicated	Ohr et al., 1973
	<i>T. koningii</i> <i>T. longibrachiatum</i> <i>T. harzianum</i> <i>T. harzianum</i> <i>T. longibrachiatum</i> <i>T. harzianum</i>	–	<i>In vitro</i>	Inhibited pathogen growth	Antibiosis by non-volatile compounds	Onsando et al., 1994
	<i>T. hamatum</i> <i>T. harzianum</i> <i>T. viride</i>	–	<i>In vitro</i> Strawberry – In greenhouse In hazel billets – In laboratory	Inhibited pathogen growth (<i>in vitro</i>) Increased plant survival (<i>in planta</i>) Inhibited pathogen growth (<i>in vitro</i>) Reduction of inoculum viability (<i>in vivo</i>)	Not indicated	Tarus et al., 2003 Raziq & Fox, 2003 Raziq & Fox, 2004a
	<i>T. harzianum</i>	–	Strawberry – In greenhouse	Increased plant survival	Not indicated	Raziq & Fox, 2004b
	<i>T. hamatum</i> <i>T. harzianum</i> <i>T. viride</i> <i>T. harzianum</i>	–	Strawberry – In greenhouse	Increased plant survival	Not indicated	Raziq & Fox, 2004c
	<i>T. harzianum</i>	Fenpropidin	Strawberry – In greenhouse	Increased plant survival	Not indicated	Raziq & Fox, 2005
	<i>T. harzianum</i> <i>T. hamatum</i> <i>T. virens</i> <i>T. harzianum</i>	Fenpropidin	Strawberry – In greenhouse Apple trees – In field	Increased plant survival	Not indicated	Raziq & Fox, 2006a Raziq & Fox, 2006b Asef et al., 2008
	<i>T. atroviride</i>	–	<i>In vitro</i>	Pathogen death	Mycoparasitism Antibiosis by volatile compounds	
	<i>T. atroviride</i>	–	Vineyard – In field	Not indicated	Increased soil fungi and bacteria diversity	Savazzini et al., 2009
	<i>T. harzianum</i>	–	Strawberry – In field	Reduced disease severity	Not indicated	Percival et al., 2011
	<i>T. atroviride</i>	–	<i>In vitro</i>	Inhibited pathogen growth	Mycoparasitism	Pellegrini et al., 2012
	<i>T. harzianum</i>	–	<i>In vitro</i>	Inhibited pathogen development	Mycoparasitism Chitinase activity	Pellegrini et al., 2013
	<i>T. atroviride</i>	–	In soil	Inhibited pathogen activity	Modified soil microbial activity Lytic enzymes production Antibiosis by non-volatile compounds Siderophores production	Perazzolli et al., 2016 Chen et al., 2019
	<i>T. virens</i> <i>T. atrobrunneum</i>	–	<i>In vitro</i> Oak – In field	Inhibited pathogen growth (<i>in vitro</i>) Increased plant survival (<i>in field</i>)	Not indicated	Rees et al., 2021
	<i>T. virens</i> <i>T. hamatum</i> <i>T. harzianum</i> <i>T. viride</i>	–	<i>In vitro</i> Hazel disks – In laboratory <i>In vitro</i>	Pathogen death Inhibited pathogen growth	Mycoparasitism	Aslan et al., 2022

(continued on next page)

Table 3 (continued)

Armillaria species	Trichoderma species	In combination with	Plant and experiment	Effect	Mechanism of action	Reference
	<i>T. atrobrunneum</i>	–	Strawberry – In greenhouse	Reduced disease severity	Not indicated	Rees et al., 2022
	<i>T. hamatum</i>		Privet – In greenhouse			
	<i>T. harzianum</i>		Pine branch segment – In laboratory			
<i>A. novae-zeelandiae</i>	<i>Trichoderma</i> sp.	–	Pine branch segment – In laboratory	Reduction of inoculum viability	Not indicated	Li & Hood, 1992
<i>A. ostoyae</i>	<i>T. citrinoviride</i>	–	<i>In vitro</i>	Inhibited pathogen growth	Antibiosis by non-volatile compounds	Reaves et al., 1990
	<i>T. hamatum</i>	–	Maritime pine – In greenhouse	Increased plant survival	Not indicated	Davet et al., 1992
	<i>T. harzianum</i>					
	<i>T. viride</i>	Burning	Red alder stem segments – In field	Reduction of inoculum viability	Not indicated	Filip & Yang-Erve, 1997
	<i>T. harzianum</i>	–	Oak-wood discs – In laboratory	Reduction of inoculum viability	Not indicated	Kwaśna et al., 2004
<i>A. tabescens</i> (syn. <i>Desarmillaria tabescens</i>)	<i>T. atroviride</i>	–	<i>In vitro</i>	Inhibited pathogen growth	Mycoparasitism	Chen et al., 2023
	<i>T. harzianum</i>	–	<i>In vitro</i>	Inhibited pathogen growth	Mycoparasitism	Aslan et al., 2022
	<i>T. viride</i>					

An important aspect to highlight about the use of *Trichoderma* as a BCA is its great capacity to tolerate different fungicides, which allows its application in an integrated manner (Poveda, 2022). In this regard, it has been described how the application of *T. hamatum* and *T. harzianum* on strawberry plants and apple trees, together with the systemic fungicide fenpropidin, synergistically reduces ARR mortality caused by *A. mellea* (Raziq & Fox, 2006a, 2006b), but such fungicide application may not be realistic in forestry situations.

Although there are many studies that fail to describe the mechanism of action involved in the effective control of *Armillaria* spp. by *Trichoderma* spp., we found several studies that suggested mycoparasitism, antibiosis, competition and modification of the rhizospheric microbiota as the mechanisms of action involved. With regard to mycoparasitism, we found 7 papers describing the ability of *Trichoderma* spp. to control *Armillaria* spp. by this mechanism of action. Mycoparasitism of *Trichoderma* spp. on *Armillaria* spp. was first confirmed in 1992, when Dumas and Boyonoski tested *T. polysporum*, *T. harzianum* and *T. viride* against *A. gallica* *in vitro* and analysed their interaction by scanning electron microscopy. This technique confirmed hyphae of these three *Trichoderma* species coiling around the rhizomorphs and showed penetration of the melanized outer tissue, consuming the pathogen completely within a week (Dumas & Boyonoski, 1992). Subsequently, using the same technique, Asef et al. (2008) described how *T. virens* and *T. harzianum* mycoparasitize *A. mellea* by penetrating through the meristematic centre of the apex and the apical buds of the rhizomorphs, colonizing the entire interior of the rhizomorphs and lysing all their tissue, to sporulate on their surface after 5–7 days (Asef et al., 2008). Scanning electron microscopy also identified that *Trichoderma* spp. grow by chemotropism towards *Armillaria* spp. through recognition of metabolites released by the pathogen, presumably volatiles (Aslan et al., 2022).

For mycoparasitism to occur, the production and release of CWDEs by *Trichoderma* upon contact with the pathogen is necessary. In this regard, it has been determined by RNAseq how the interaction between *T. atroviride* and *A. mellea* induces in the BCA the expression of genes related to the synthesis of lytic enzymes (Perazzolli et al., 2016). Also, by RNAseq, studying the mycoparasitism of *T. atroviride* on *A. ostoyae*, it has been identified how *Armillaria* attempts to defend itself from its antagonist (increased expression of genes related to oxidative stress relief, biosynthesis of antibiotics and neutralization of toxic compounds) and how *Trichoderma* mycoparasitises its prey (increased expression of genes related to CWDEs and toxic secondary metabolites) (Chen et al., 2023). In the case of *T. harzianum*, chitinases have been described as the main enzymes involved in *A. mellea* mycoparasitism (Pellegriani et al., 2013). In this and a previous work by Pellegriani et al., (2012), mycoparasitism

was analysed by isotope ratio mass spectrometry (IRMS), labelling *A. mellea* with ^{13}C . This technique confirmed that *Trichoderma* spp. is able to metabolise and assimilate molecules extracted from pathogen hyphae into its own hyphae (Pellegriani et al., 2012, 2013).

As discussed above, the *Trichoderma* genus includes fungal species with a large repertoire of metabolic machinery, allowing synthesis and excretion of a large diversity of secondary metabolites with biological activity, e.g. antifungals (Rashad & Abdel-Azeem, 2020). In this regard, so far we have found 5 studies describing antibiosis using volatile and non-volatile metabolites as a mechanism involved in the control of *Armillaria* spp. by *Trichoderma* spp. In 1994, Onsando et al. tested *T. koningii*, *T. longibrachiatum* and *T. harzianum* *in vitro* against *A. mellea* and *A. heimii*, reporting a high growth inhibitory capacity of the pathogen and the production of a pigment released into the culture medium by the BCA. They cultured the BCAs in liquid medium and filtered them, obtaining cell-free filtrates rich in metabolites. After the application of these filtrates on the pathogens, the production of non-volatile metabolites was confirmed as one of the mechanisms involved in the antagonistic capacity of the BCAs used (Onsando et al., 1994). Similarly, Reaves et al. (1990) confirmed that cell-free filtrates obtained from *T. citrinoviride* were able to inhibit rhizomorph formation and hyphal growth of *A. ostoyae* (Reaves et al., 1990). In particular, Tarus et al. (2003) identified a metabolite involved in the antagonistic ability of *T. harzianum* and *T. longibrachiatum* against *A. mellea* to be 6-pentyl-2-pyrone. Furthermore, RNAseq analysis performed on the *T. atroviride*-*A. mellea* interaction confirmed that pathogen recognition by BCA resulted in an increase in the expression of genes related to the synthesis of antifungal non-volatile compounds (Perazzolli et al., 2016). With regard to the production of volatile metabolites, only one study has been carried out on the control of *Armillaria* spp. by *Trichoderma* spp., in the control of *A. mellea* by *T. virens* and *T. harzianum* (Asef et al., 2008). In the area of fungal secondary metabolites it is well established that the biosynthetic machinery is often tightly regulated and only active under very specific conditions, so things observed in a lab may not be relevant to the field and vice versa.

Other mechanisms of action identified for the control of *Armillaria* spp. by *Trichoderma* spp. are competition and effect of the BCA on the rhizospheric microbiota, with one and two papers, respectively. Specifically, the competition for nutrients of *T. virens* and *T. atrobrunneum* over *A. gallica* and *A. mellea* has been identified. The BCAs produce siderophores that “trap” the metals present in the substrate (mainly Fe) preventing their use by the pathogen, which then hinders the establishment and growth of *Armillaria* spp. This mechanism has been related to a reduction in the growth of the pathogen *in vitro* and to an increase in the survival of oak trees in the field (Chen et al., 2019).

Regarding the action of *Trichoderma* spp. on the rhizospheric microbiota, it has been described how *T. atroviride* is capable of increasing the diversity of bacteria and fungi in soils where *A. mellea* is also present, although the exact mechanisms involved are not known (Savazzini et al., 2009). In addition, through RNAseq, it has been determined that *T. atroviride* also promotes the defensive activity of these microorganisms, through the synthesis of antifungal compounds that inhibit the development of *A. mellea* in these soils (Perazzolli et al., 2016).

With so many species of *Trichoderma* being reported to have activity against *Armillaria*, there are likely to be a number of different successful strategies, and each strain or species of *Trichoderma* is likely to be able to deploy more than one approach to combat *Armillaria*. The majority of these research papers focus on just one aspect for the interaction, and thus may be overlooking other approaches being used in parallel by the BCA. It is likely that each *Trichoderma* can use several complementary methods to attack *Armillaria*, but these may vary between environments, growth stages, etc. This is further complicated by whether the *Trichoderma* is free-living or root-endophytic, or whether the control required is preventative rather than curative.

6. Conclusions and future perspectives

The present work represents a comprehensive analysis of all existing work on the control of ARR disease by *Trichoderma* spp. The genus *Trichoderma* includes numerous species of effective BCAs. However, despite the great economic and environmental importance of *Armillaria* spp., only 31 studies have been carried out so far on the effective control of the pathogen with this BCA.

Historically, one of the main strategies used in the effective control of *Armillaria* spp. Has been the use of chemical fungicides, however, the widespread use of these compounds has potential to cause serious environmental and health problems. Therefore, the search for new effective control strategies using BCAs such as *Trichoderma* spp. is absolutely necessary.

The bibliographic analysis carried out has obtained 31 published works on the effective control of *Armillaria* spp. by *Trichoderma* spp. Although the publication of works has been constant over the last two decades, the years 2003–2004 were the most productive in this area. These papers have been published mostly by UK authors and in the journal *Biological Agriculture & Horticulture* (Taylor & Francis). However, despite the great importance of the problem worldwide, these papers have not yet been widely cited, which suggests that a strong interest in the use of this BCA against *Armillaria* spp. has not yet developed.

It is interesting to note that *Trichoderma* spp. is not always an effective BCA against *Armillaria* spp. As discussed here, there are several cases where non-volatile metabolites of *Trichoderma* favour the growth and development of the pathogen or, at least, have no detrimental effect against it. Similarly, the direct application of the BCA does not always benefit plant health. Furthermore, given the interest in using *Trichoderma* for control of other infections, particularly foliar infections, there is a risk that a BCA that provides protection against one pathogen might inadvertently make the host more susceptible to another and this requires investigation.

Although there are several works on the effective control of *Armillaria* spp. with *Trichoderma* spp., only in a few of them were the mechanisms of action involved identified. Therefore, there is a wide field yet to be explored, especially in the mechanism of antibiosis, where only one non-volatile metabolite with antifungal capacity against *Armillaria* spp. has been identified and where the production of volatile metabolites is yet to be intensively studied. Similarly, other mechanisms of biocontrol which are not well understood include competition and the modification of the rhizospheric microbiota. Even more remarkable is the absence of studies carried out on the activation of plant defenses by *Trichoderma* spp. against *Armillaria* spp. a widely known mechanism of action in this BCA against a wide variety of fungal plant diseases. The in

depth study of all these mechanisms of action is likely to be needed if we are to understand how *Trichoderma* works and how it can be applied effectively against the pathogen.

Authorship contribution statement

JP wrote the first version of the manuscript and performed all analyses. MRM made the infographics. MRM and AMB contributed to the manuscript correction and critical reading. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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JP wrote the first version of the manuscript and performed all analyses. MRM made the infographics. MRM and AMB contributed to the manuscript correction and critical reading. All authors have read and agreed to the published version of the manuscript.

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