National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Exploring the necessity of additional data requirements under the pesticide regulation to take into account endophytes

RIVM letter report 2021-0056
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Colophon

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Synopsis

Exploring the necessity of additional data requirements under the pesticide regulation to take into account endophytes

Fungal and bacterial-based plant protection products are used in agricultural crops, such as maize and wheat. The main criterion looked at when risk assessments are carried out on these ‘microbial plant protection products’ is whether the micro-organisms concerned can grow on the outside of plants.

It has, however, recently become apparent that some of these micro-organisms can also grow inside plants. When bacteria and fungi grow, potentially harmful substances (metabolites) may be released. It has been suggested that when micro-organisms grow in plants people who eat the plants could then be exposed to these metabolites.

RIVM has investigated whether micro-organisms that are introduced into plants via plant protection products and grow in them produce different or more substances than we are aware of. This research has shown that various of these micro-organisms can grow in plants but only in small quantities and that these quantities subsequently decrease rapidly. No indications have been found that the quantities of metabolites they produce are harmful for people. The risks microbiological plant protection products entail can therefore be determined using existing risk assessment methods. No different or additional information is needed to this end.

RIVM has arrived at this conclusion after an exploratory literature review carried out at the request of the Ministry of Agriculture, Nature & Food Quality (LNV).

Keywords: Endophytic growth, microbiological plant protection products, endophytes, metabolites, risk assessment

Publiekssamenvatting

Onderzoek naar de noodzaak voor extra datavereisten voor endofyten in de gewasbeschermingsmiddelenverordening

Gewasbeschermingsmiddelen op basis van schimmels en bacteriën worden gebruikt om insecten, bacteriën en schimmels te bestrijden bij de teelt van gewassen als mais en tarwe. Bij de risicobeoordeling van deze 'microbiële gewasbeschermingsmiddelen' wordt vooral gekeken of ze aan de buitenkant van een plant kunnen groeien.

Sinds kort is bekend dat sommige micro-organismen ook in planten kunnen groeien. In het algemeen is het zo dat bacteriën en schimmels schadelijke stoffen kunnen maken als ze groeien (metabolieten). Wanneer micro-organismen in planten groeien, zouden deze schadelijke stoffen in de plant kunnen ontstaan. In dat geval zouden mensen die deze planten eten blootgesteld kunnen worden aan deze metabolieten.

Het RIVM heeft verkend of micro-organismen die via gewasbeschermingsmiddelen in planten groeien, andere of meer stoffen produceren dan bekend is. Voor zover bekend kunnen verschillende micro-organismen die als gewasbeschermingsmiddel worden gebruikt, in planten groeien. Maar dat gebeurt in kleine hoeveelheden die na toepassing snel afnemen. Er zijn geen aanwijzingen gevonden dat de hoeveelheid metabolieten die ze produceren schadelijk is voor de mens. De risico's van microbiologische gewasbeschermingsmiddelen kunnen daarom met de bestaande risicobeoordeling worden bepaald. Daarvoor is geen andere of extra informatie nodig.

Het RIVM concludeert dit na een verkennend literatuuronderzoek. Dat is in opdracht van het ministerie van Landbouw, Natuur en Voedselkwaliteit (LNV) uitgevoerd.

Kernwoorden: Endofytische groei, microbiologische gewasbeschermingsmiddelen, endofyten, metabolieten, risicobeoordeling,

Contents

Summary

The current risk assessment of microbial control agents (MCAs) is based on their epiphytical growth. This may be inadequate if MCAs are also able to grow endophytically and produce a higher amount of metabolites inside the plant, which could result in negative effects on human health. Using literature research, this report investigated whether MCAs can grow endophytically, if that leads to human health risks and if adaptations to current data requirements of MCAs are necessary.

Several examples of fungal and bacterial MCAs with endophytic lifestyles were found in literature and presented in this report. Endophytic growth during the lifespan of the MCAs in or on crops cannot be excluded, regardless of changing the method of MCA application on crops. The available literature indicates that MCAs are only transiently present in plants as endophytes and that they are present in low concentrations. There is also no indication that endophytically growing MCAs produce metabolites or concentrations of metabolites that are harmful to human health.

Based on an exploratory literature research, the conclusion of this report is that MCAs are able to live endophytically, without indicating harmful health effects in humans. Therefore there is no need for adjustment of the current data requirements as the current framework covers potential risks of MCAs that grow endophytically.

1 Framing of the question

1.1 Question of LNV

The ministry of Ministry of Agriculture, Nature and Food Safety (further referred to as LNV) poses the question whether the current risk assessment of microbial control agents (MCAs) is adequate in case an MCA, in addition to growing epiphytically, is also able to grow endophytically (inside the plant). Endophytically growing MCAs might produce a higher amount of metabolites than epiphytically growing MCAs. To determine if the current risk assessment is still adequate, the possibility of MCAs to grow endophytically needs to be investigated. In case MCAs can grow endophytically, it needs to be considered whether the production of secondary metabolites results in significant additional risks for human health/food quality. If so, this should be taken into consideration in the data requirements. As the question is complex and not easily answered, an exploratory literature search is needed.

1.2 Background

The data requirements for MCAs were developed by the end of the 1990’s and published in Commission Directive 2001/36/EC (EC 2001a). At the time of its inception, regulatory experts did not have much experience with MCAs, presumably because the biological control industry was still in its infancy. In the nineties of the previous century, limited knowledge was available on endophytes in general (Figure 1) and probably none at all on endophytic growth of MCAs in particular. As many of the current data requirements do not suit the risk assessment of MCAs, updated and simplified data requirements are necessary. This is considered feasible as knowledge on MCAs has considerably increased in the last 20 years.

The data requirements are currently under review by the EC workgroup on biopesticides (chaired by EU commission – DG Sante). During this process several questions have been posed by National Authorities. One of the questions was whether endophytically growing MCAs pose a risk for human health. The main worry of risk assessors is that endophytically growing MCAs, despite poor growing conditions inside the plant, may have many nutrients at their disposal and can therefore produce high levels of toxic metabolites in plant tissues.
This question was also discussed in the Dutch working group on microbial agents in which members of WUR, NIOO, Utrecht University, Ctgb, RIVM take place. This group was established on demand of LNV in 2019.

The current data requirements and the Uniform Principles assess the risks of secondary metabolites, irrespective of the exact location of production. In principle, metabolites produced by endophytically growing MCAs are included. However, endophytic growth of MCAs was not anticipated at the time of conception of the data requirements. During the revision of the data requirements the question now arises if additional questions should be posed for MCAs that are able to grow endophytically.

A first scan of the literature in Scopus demonstrates that there is much interest in bacterial and fungal endophytes. Numbers of publications on fungal endophytes reach 1250 a year in 2020 (Figure 1) and almost 900 for bacterial endophytes (Figure 3 in Appendix 1).

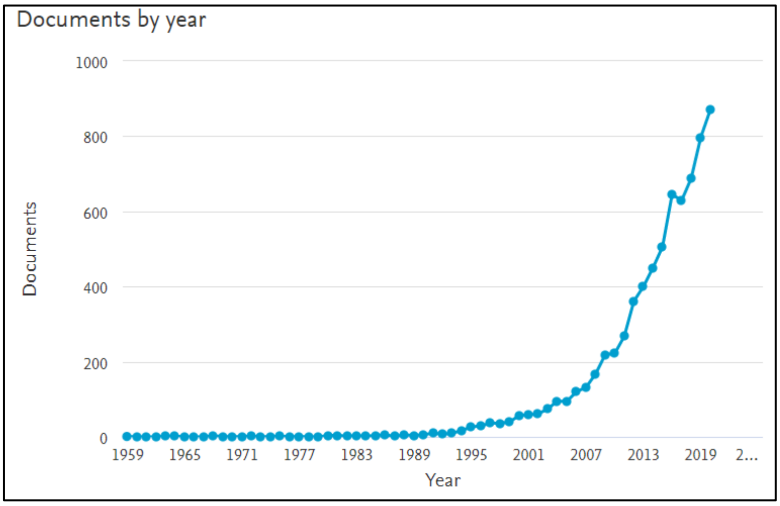

Figure 1 Scopus search string ((fungus OR fungal OR fungi) AND endophyt).*

These publications include several areas of research interests. Endophyte biology is even mentioned to be an emerging field (Strobel 2018). In particular, there is much interest in the metabolites produced by endophytes for pharmaceutical purposes (Smith et al. 2008). Secondary metabolites derived from endophytes comprise classes of compounds such as steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides, and cytochalasins, which are identified to possess biological activities with antibiotic, antiviral, volatile antibiotic, anticancer, antioxidant, insecticidal, antidiabetic, and immunosuppressive properties (Deshmukh et al. 2018, Zhang et al. 2012).
Considering the large number off hits not all studies could be evaluated in details and therefore this study focussed mainly on studies relating to micro-organisms used in plant protection (MCAs).
Concerning agricultural purposes, much research is performed on the beneficial effects of endophytes in crops (see paragraph 8.2). There is a strong indication that some of the previously mentioned secondary metabolites have antibiotic and insecticidal properties that benefit the crops. In contrast, it seems that no literature is available on the subject of risks for human health caused by endophytic MCAs and their metabolites as a search with Scopus did not yield any publications.

1.3 Research questions

To determine if additional questions should be posed for MCAs that are able to grow endophytically, several questions need to be answered first using literature research. The necessity of further data requirements will depend on the available information in literature.

In this report the following nine questions are answered:
- What is the definition of an endophyte? *(Chapter 2)*

- Which regulations may be relevant for endophytes? *(Chapter 3)*
- What are methods to determine or exclude that a microorganism can also live as an endophyte? *(Chapter 4)*
- Which bacterial and fungal MCAs are known to have an endophytic lifestyle? *(Chapter 5)*
- What is the role of the application method? *(Chapter 6)*
- Which harmful metabolites are produced by MCAs growing endophytically? Is a Maximum Limit (ML) available? *(Chapter 7)*
- Can metabolites have beneficial effects? (*Chapter 8*)
- Based on the current information, is it necessary to develop data requirements? *(Chapter 9)*
- Which recommendations can be given for an adequate human risk assessment? *(Chapter 10)*

2 Definition of endophyte

In the literature definitions of endophytic fungi are linked to non-pathogenic properties. In these definitions, different forms of fungal relationships with plants are:

a) phytopathogenic (obligate biotrophic, necrotrophic, hemibiotrophic),
b) saprophytic, and
c) endophytic (Pusztahelyi et al. 2016).

These three forms are described below.

a) *Phytopathogens* constitute one of the primary infectious agents in plants, causing alterations during developmental stages including the postharvest stage, gaining nutrients from the plants they invade, and resulting in crop losses.
b) Saprophytes
   Saprotrophs derive energy from non-living, organic material. They do not induce symptoms in living organisms.
c) Endophytes
   Endophytes occupy hosts without causing adverse symptoms in the host.

In the literature many other definitions of an endophyte can be found. The definitions are highly variable concerning the life history strategies of the symbiosis. This may range from facultatively saprobic to parasitic to exploitive to mutualistic. Distinctions between life history strategies are however not always clear-cut and may even vary within the life span of the microorganism.

The concept of Hardoim et al. (2015) opposes this function based concept. They suggest that endophytes should be defined by their colonization niche e.g. the inside of plants, as determined after surface sterilization of plants. In this view endophytes can be pathogenic or non-pathogenic. This concept was also embraced by Brader et al. (2017).

The word ‘endophyte’ comes from the Greek endon (within) and phyton (plant). An endophyte is a microorganism, usually a bacterium or a fungus, that lives within a plant. Following this semantic interpretation of the word endophyte, an endophyte also includes pathogenic microorganisms, even necrotrophs that produce mycotoxins.

Arguments to support the Hardoim concept is that

- a particular endophyte may cause pathogenic effects in a certain plant host while it does not show any effects in other plant species, so there is no strict relation between endophytic lifestyle and lack of phytopathogenicity,
- a microorganism can have several life styles within its life span. Some species such as *Alternaria* range from saprophytes to endophytes and pathogens. The position of the endophyte in the continuum is not necessarily strict. The current assumption is that most microorganisms do not have an obligate endophytic lifestyle.

Although this definition is considered to be the best, it is impractical as the literature already adopted the definition that endophytes occupy hosts without causing symptoms in the host.

Conclusion:
The current definition of the endophyte is that it occupies the host plant without causing symptoms. Mycotoxin producing necrotrophs are excluded from this definition. Whatever definition of the endophyte is adopted, it should be realized that endophytes have a broad range of lifestyles.

3 Regulations that may be relevant for endophytes

There are several regulations that deal with microorganisms, irrespective of their lifestyle. In this exploration the most likely regulations were investigated for mentioning endophytes.

- Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market (EC 2009);
  - Commission Regulation (EU) No 283/2013 of 1 March 2013 (EC 2013) setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009;
- Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products (EC 2012) (Biocidal Product Regulation);
- Directive 2001/18/EC on the deliberate release of GMOs into the environment (EC 2001b);
- Regulation (EC) 1829/2003 on genetically modified food and feed
- Council Regulation (EEC) No 315/93 of 8 February 1993 laying down Community procedures for contaminants in food (EC 1993);
- Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (EC 2006).

Part B, Chapter 2 of Commission Regulation 283/2013 (EC 2013) asks information on the place in the ecosystem, including the plant:

> *“The geographical region and the place in the ecosystem (e.g. host plant, host animal, or soil from which the micro-organism was isolated) must be stated. The method of isolation of the micro-organism shall be reported.*
> *The natural occurrence of the micro-organism in the relevant environment shall be given if possible at strain level”*

The Biocidal Product Regulation (BPR) asks under Chapter 8.1.7:

> *“If the active substance is to be used in products for action against plants including algae then tests shall be required to assess toxic effects of metabolites from treated plants, if any, where different from those identified in animals”.*

The BPR asks under Chapter 5.3:

> *“Analytical methods for monitoring purposes including recovery rates and the limit of quantification and detection for the active substance, and for residues thereof, in/on food of plant and animal origin or feeding stuffs and other products where relevant”*

This data requirement probably concerns products based on chemical substances and their metabolites that may come in contact with feed and food as in product type 4 (food and feed). For this product type products based on MCA do not exist.

The possibility of endophytic growth of MCAs in plants is not explicitly mentioned in Commission Regulation 283/2013. The possibility that MCAs applied for epiphytic growth and control can also live endophytically was probably not anticipated at the time the data requirements were drafted.
In the BPR endophytic survival or growth is also not envisaged as biocides based on micro-organisms are only used in the control of non-agricultural insects and will not be used in close contact with feed and food. Therefore potential risks for human consumption do not need to be considered.
The GMO regulations do not mention endophytic growth of the genetically modified microorganisms.
Regulation No 315/93 concerns microorganisms in food and feed but does not mention endophytes.
Interestingly, regulation No 1881/2006 mentions levels of toxins in food. These are typically produced by pathogenic fungi that live endophytically. These toxins will be discussed in Chapter 7.

Conclusion:
No regulation specifically addresses potential risks of endophytes.

4 Methods to determine or exclude that a microorganism with an epiphytic lifestyle can also live as an endophyte

Endophytes can be differentiated into obligate, facultative or passenger endophytes (Hardoim et al. 2008).

Obligate endophytes are those that are not culturable, or require more specific conditions for their growth. Few obligate endophytes have been developed into products. Some *Epichloë* species have been successfully marketed for decades in New Zealand and Australia and the Americas (particularly the USA, Argentina, Brazil, Chile and Uruguay) for their insect deterrent properties via production of alkaloids. Most notable commercially developed species are *E. festucae* var. *lolii* that associates with perennial ryegrass and *E. coenophiala* that associates with tall fescue (Card et al. 2016). Products with arbuscular mycorrhiza are marketed as biofertilizer and are not further included in this report (see also paragraph 5.1).

Facultative endophytes are those that are able to survive in soil, artificial nutrient medium, plants surface and inside the plants. The advantage of facultative endophytes is that their potential for the development of commercial products can be exploited, as they can be isolated easily compared to obligate endophytes (Tidke et al. 2017). Most MCAs are considered to be facultative or passenger endophytes. For example, entomopathogenic fungi do not thrive in plants – their presence in plant tissue appears to be ephemeral (transitory, short-lived) and at extremely low quantities (Cai et al. 2019).

Clearly, a risk assessment of metabolites produced by endophytes can be omitted if it can be demonstrated that the MCA will not be present in the interior parts of the plant at any time after application of the product. This will require not only a reliable method to detect endophytes inside tissues, but also that those measurements have to be performed during the complete lifespan of the crop that is treated with the MCA. There are methods to determine the presence of endophytes in plants (see Appendix 2). However, due to the erratic occurrence of endophytes, in particular the facultative and passenger endophytes, it cannot be excluded that an alleged epiphytic microorganism can also live endophytically in the plant at some time after application. Demonstration of their complete absence in the plant tissue during the lifetime of the crop will therefore be very difficult, if not impossible.

Conclusion:
There are methods to determine the presence of endophytes in plants. However, as the presence of MCA endophytes is erratic and they are present in low levels, their presence as endophytes cannot be fully excluded as they might have been missed in case their numbers were too low at the time of determination.

5 Bacterial and fungal MCAs known to have an endophytic lifestyle

Already in 1991 it was estimated that there might be as many as one million different bacterial and fungal endophyte species. However, only very few of them were described by that time (Petrini 1991). This chapter focuses on bacterial and fungal MCAs that can live endophytically as described in peer-reviewed journals.

Firstly, the frequency of phyla in which bacteria and fungi endophytes occur is given in Figure 2. Then, lists of classes in which endophytes are presented are given in Table 1 for fungi and in Table 3 for bacteria. Lastly, MCAs that are known to grow endophytically are listed in Table 2 for fungi and Table 4 for bacteria. These lists give examples and are certainly not exhaustive.

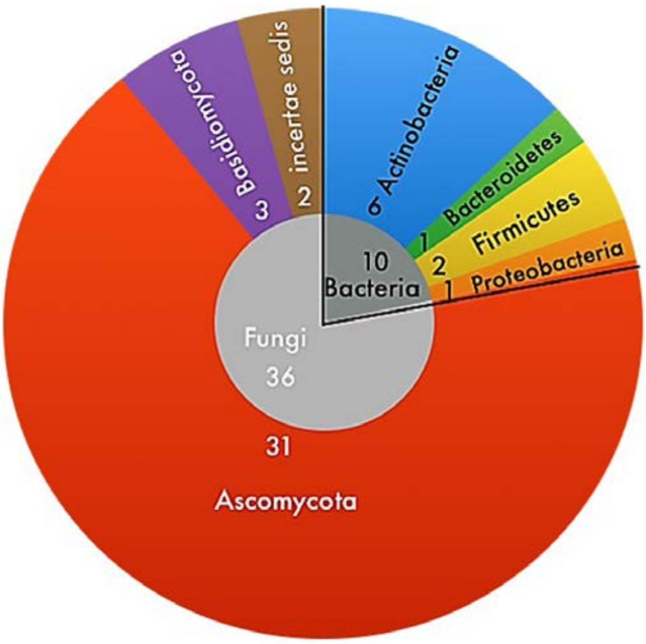

Figure 1 Frequency of phyla of endophytes. Outer circle represents the frequency of each phylum of the endophytes. Inner circle indicates whether the phyla belong to super kingdom Bacteria or to kingdom Fungi. Numbers indicate the number of genera listed within each phylum (copied from Martinez-Klimova (2017)). Note that the Glomeromycota holding arbuscular mycorrhizal fungi are not mentioned in this figure but are included in Table 1 as being endophytic.

5.1 Fungal endophytes

Fungal endophytes are a diverse, polyphyletic group of microorganisms. Hardoim et al. (2016) presented an overview of endophytes by building a data set of eukaryotic endophytic full-length internal transcribed spacer (ITS) regions. Table 1 shows that fungal endophytes mainly belong to the Glomeromycota (40%), Ascomycota (31%), Basidiomycota (20%), unidentified phyla (8%), and, to a lesser extent, Zygomycota (0.1%).

Table 1 Summary of the endophytic data set on fungi from all peer-reviewed eukaryotic full-length ITS sequences (as of 1 August 2014).a

Taxonomic assignment	No. of sequences	% of sequences
Total	**8439**	
Ascomycota	2610	30.92
Archaeorhizomycetes	2	0.02
Dothideomycetes	1272	15.07
Eurotiomycetes	54	0.64
Incertae sedis	2	0.02
Lecanoromycetes	5	0.06
Leotiomycetes	171	2.03
Orbiliomycetes	0	0
Pezizomycetes	112	1.33
Saccharomycetes	11	0.13
Sordariomycetes	785	9.3
Unidentified	196	2.32
Basidiomycota	1712	20.3
Agaricomycetes	1560	18.49
Atractiellomycetes	26	0.31
Cystobasidiomycetes	3	0.04
Exobasidiomycetes	0	0
Microbotryomycetes	23	0.27
Pucciniomycetes	1	0.01
Tremellomycetes	30	0.36
Ustilaginomycetes	0	0
Unidentified	69	0.82
Glomeromycota	3390	40.17
Glomeromycetes	3294	39.03
Unidentified	96	1.14
Zygomycota		
Incertae sedis	5	0.06
Unidentified	722	8.56

a: Copied from Hardoim et al. (2015).
b: Fungal endophytes mentioned in this table have proven biocontrol capacities (see product names) and are also mentioned to be an endophyte. Endophytic growth was indicated in the references.

Although the phylum of the Glomeromycota holds by approximation 40% of all endophytes, these endophytes are not dealt with in this report as they are arbuscular mycorrhizal fungi (AMF). Products based on AMF are not marketed as MCAs but as biofertilizers improving plant health (Berruti et al. 2016). Although AMF do have biocontrol capacities (Baum et al. 2015) the producers probably refrain from a biocontrol claim to avoid the stringent pesticide regulation.

Within the Ascomycota the endophytes are mostly present in the classes of the Dothideomycetes and the Sordariomycetes, Leotiomycetes and

Pezizomycetes. In Table 2 an overview is given of MCAs that are able to live endophytically. For this purpose the Annex to the Working Document on the Risk Assessment of Secondary Metabolites of Microbial Biocontrol Agents (OECD 2018) was used, supplemented with extra literature searches to confirm the endophytic lifestyle of the MCA.

Biocontrol agents with herbicidal action were not included as their targets are weeds which are not used for human consumption.

Table 2 MCAs with endophytic potential in the division of the Ascomycota.

Species	**Order / family**	**Example[a] disease / insect**	**Example[a] host plant**	**Reference[a]**
Dothideomycetes				
Aureobasidium	Dothideales/ Dothioraceae	pathogenic fungi	Apple, grape, tomato	(Schena et al. 1999)
Sordariomycetes				
Chaetomium globosum	Hypocreales/ Chaetomiaceae	Nematodes, aphids, beet army worm	Facultative in cotton	(Zhou et al. 2016)
Isaria fumosorosea	Hypocreales/ Clavicipitaceae	locust *Locusta migratoria*	Leaves of *Ricinus communis*	(Laib et al. 2020)
Metarhizium anisopliae	Hypocreales/ Clavicipitaceae	*Plutella xylostella* larvae	*Brassica napus*	(Batta 2013)
Beauveria bassiana	Hypocreales/ Cordycipitaceae[c]	Soil borne diseases and insects	Wide array of plant species, monocots and dicots	(Castillo Lopez et al. 2014); (Ownley et al. 2010); (Card et al. 2016)
Beauveria brongniartii	Hypocreales/ Cordycipitaceae[c]	The scarab *Melolontha melolontha*	Broad bean *Vicia faba*	(Jaber and Enkerli 2017)
Lecanicillium muscarium	Hypocreales/ Cordycipitaceae[c]	Aphids and scale insects	Cucumber	(Ownley et al. 2010)
Lecanicillium lecanii	Hypocreales/ Cordycipitaceae[c]	Aphids and nematodes	Cotton, cucumber	(Card et al. 2016)
Trichoderma hamatum[b]	Hypocreales/ Hypocreaceae	Fungal diseases	Cacao	(Bailey et al. 2008); (Card et al. 2016)
Fusarium oxysporum	Hypocreales/ Nectriaceae	Root pathogens	e.g. Watermelon, asparagus, tomato	(de Lamo and Takken 2020)
Paecilomyces licacinus (Purpureocillium lilacinum)	Hypocreales/ Ophiocordycipitaceae	Aphids	Facultative in cotton	(Castillo Lopez et al. 2014)
Acremonium alternatum	Hypocreales/ Hypocreaceae	Moth *Plutella xylostella*		(De Silva et al. 2019)
Acremonium alternatum	Hypocreales/ Hypocreaceae	Clubroot control	Oil seed rape	(Auer and Ludwig-Müller 2014)

a: Examples derive from one reference and demonstrate that the MCA is able to live endophytically in this MCA/plant combination. There are other references in which MCA/plant combination are studied. Examples are therefore not exhaustive.

b: The majority of *Trichoderma*–plant interactions currently described in the literature are considered to be successful BCAs that associate closely with plants and are not considered to have not endophytic associations (Card et al. 2016).
c: Formerly within the Clavicipitaceae.

The class of the Sordariomycetes holds many of the most well-known biocontrol agents such as the entomopathogenic fungi *Isaria fumosorosea*, *Paecilomyces* spec., *Metarhizium anisopliae* and *Beauveria* spec. These fungi are characterized by showing high diversity, having a broad host range, colonizing foliar tissues and having limited growth within the plant (Gange et al. 2019). The majority of the biocontrol products are based on these entomopathogenic fungi (Maina et al. 2018). They are also found to be able to exist as endophytes in a wide variety of crops (Bamisile et al. 2018).

Within the Basidiomycetes the endophytes are mainly present in the class of the Agaricomycetes. A search in Scopus did however not yield species with clear biocontrol capacities.

5.2 Bacterial endophytes

According to Hardoim et al. (2015) most bacterial endophytes belong to mainly four phyla, but they encompass many genera and species. Prokaryotic 16S rRNA gene sequences showed that Proteobacteria, Actinobacteria, Firmicutes and Bacterioides harbor most endophytes with 54%, 20%, 15% and 6% respectively (Table 3).

Table 3 Summary of the endophytic data set on bacteria from all peer-reviewed publications with prokaryotic 16S rRNA gene sequences[a].

Phylogenetic affiliation	No. of sequences	% of sequences
Bacteria	**7319**	
Acidobacteria	53	0.72
Actinobacteria	1461	19.88
Armatimonadetes	6	0.08
Bacteriodetes	465	6.29
GOUTA4[c]	1	0.01
OD[c]	6	0.08
TM7[c]	2	0.03
Chlamydiae	8	0.11
Chlorobi	5	0.07
Chloroflexi	3	0.04
Cyanobacteriaa	102	1.39
Deinococcus-Thermus	7	0.1
Elusimicrobia	1	0.01
Firmicutes		
Bacilli	1132	15.41
Clostridia	68	0.93
Fusobacteria	3	0.04
Nitrospirae	3	0.04
Planctomycetes	5	0.07

Phylogenetic affiliation	No. of sequences	% of sequences
Proteobacteria		
Alpha	1337	18.2
Beta	736	10.02
Delta	26	0.35
Epsilon	3	0.04
Gamma	1878	25.56
Spirochaetae	3	0.04
Tenericutes	2	0.03
Verrucomicrobia	6	0.08
Archaea	**29**	
Euryarchaeota	23	0.31
Thaumaarcheota	6	0.08
Total	**7348**	

a: Copied from Hardoim et al. (2015).
b: Bacterial endophytes mentioned in this table have proven biocontrol capacities (see product names) and are also mentioned to be an endophyte. Endophytic growth was indicated in the references.

Functions of the genera within the Proteobacteria, Actinobacteria, Firmicutes and Bacterioides cannot be assigned clearly to taxonomy and seem to depend on the host and environmental parameters. For instance Gammaproteobacteria also comprise a large number of genera such as the *Enterobacter* and species which are known as phytopathogens (Bull et al. 2010, Bull et al. 2012).
Some well-known endophytic bacterial MCAs are mentioned in Table 4. This list is not meant to be exhaustive.

Table 4 Bacterial MCAs with an endophytic lifestyle[a] and product names.

Species	Order / family	Disease / insect	Crop	Reference
Gammaproteobacteria				
Pseudomonas fluorescens PICF7	Pseudomonadales/ Pseudomonoadaceae	*Verticillium dahliae*	olive	(Gómez-Lama Cabanás et al. 2014)
Pseudomonas spp.: AtEze, Bio-save, BlightBan, Frostban, Spot-Less	Pseudomonadales/ Pseudomonoadaceae			(Berg and Hallmann 2006)
Alphaproteobacteria				
Rhizobium radiobacter (was *Agrobacterium radiobacter* strain 84) Galltrol, Nogall	Rhizobiales/ Rhizobiaceae	Crown gall disease	fruit, nut, and ornamental nursery stock	(Kerr 1980)
Betaproteobacteria				
Burkholderia cepia Deny, Intercept	Burkholderiales/ Burkholderiaceae	Phytopathogenic fungi		(Parke and Gurian-Sherman 2001, OECD 2018)

Species	Order / family	Disease / insect	Crop	Reference
Actinobacteria				
Streptomyces spp. Actinovate, Mycostop	Actionomycetales/ Streptomycetaceae	Powdery and Downy Mildew, Botrytis spp., Alternaria spp.		(Parke and Gurian-Sherman 2001)
Bacilli				
Bacillus thuringiensis	Bacillales/Bacillaceae	Insects and nematodes		(Tao et al. 2014)
Bacillus spp. BioYield, Companion, EcoGuard, HiStick N/T, Kodiak, Mepplus, Serenade, Sonata, Subtilex, YieldShield	Bacillales/Bacillaceae	Phytopathogenic fungi		(Card et al. 2016)
Bacillus pumilus QST2808 Ballad	Bacillales/Bacillaceae	Phytopathogenic fungi		(Yi et al. 2013)

a: Bacterial endophytes mentioned in this table have proven biocontrol capacities (see product names) and are also mentioned to be an endophyte. Endophytic growth was indicated in the references.

In particular, *Burkholderia* strains have the potential to colonize a wide range of hosts and environments, suggesting a great metabolic and physiological adaptability of endophytes belonging to this genus (Parke and Gurian-Sherman 2001). Members of the genus *Streptomyces* are well known for their capacity to synthesize antibiotic compounds (Watve et al. 2001). Within the genus *Bacillus*, the species *Bacillus thuringiensis* is well known for its production of parasporal crystal proteins with insecticidal properties (Tao et al. 2014).

6 Role of the application method

MCAs can be applied to crops using different methods such as sprays or seed dipping (see 6.1). The method of application may determine whether an MCA can colonize the plants as an endophyte or not.

Points of entrance have been shown in detail for plant growth promoting rhizobacteria (PGPR). They may enter the plant through tissue wounds, stomata, lenticels, root cracks, root hair cells and germinating radicles (see references in Ali, Duan et al. (2014)).
Points of entrance are probably the same for other types of MCAs. A study with the entomopathogen *B. bassiana* showed that hyphae grew randomly across the surfaces of corn leaves (Wagner and Lewis 2000). Upon encountering a natural opening (e.g. stomata) *B. bassiana* was shown to enter and invade the plant. Mechanical force or enzymatic activity are suggested to play a role (Wagner and Lewis 2000).

6.1 Different types of application methods

Several inoculation routes for bacterial and fungal endophytes have been described by Rao et al. (2020) such as foliar and stem inoculation, seed dipping and soil spray. Besides the route of inoculation there are also other factors that influence the success of the inoculation such as biotic and abiotic factors, growth media and the density of inoculum (Ownley et al. 2008). The plant itself is an important determinant of successful colonization (Germida et al. 1998). Plants that have an evolutionary relationship with endophytes seem to facilitate the growth of endophytes while they seem to have defense mechanisms against unknown endophytes.

A literature search performed by Bamisile et al. (2018) on endophytic entomopathogens yielded 88 publications with among them seed treatments, soil drenching, foliar spraying, solid substrate method, stem injection, seed coatings, radicle dressings, root and rhizome immersions and flower sprays. These authors concluded that in general, root and soil inoculation methods show less endophytic growth than foliar and stem injection methods. Presumably, in the first methods other fungi and bacteria already present in the soil prior to inoculum application will inhibit fungal entrance into the plant roots.

Some examples of entomopathogens are given below for each application method.

Seed dipping
Seed dipping with *Beauveria bassiana* strains (GHA, PTG4, and PTG6 results in different colonization rates of root, stems and leaves of *Z. mays*.
Results showed 100% endophytic root colonization, regardless of adherent type or strain tested. Colonization was variable in shoots (63-100%) and leaves (25-75%) and also depended on the adherent. Also the type of adherent (methyl cellulose or cornstarch) played a role (Kuzhuppillymyal-Prabhakarankutty et al. 2020).

Foliar inoculation
Foliar inoculation of sorghum seedlings with conidia of several strains of *Beauveria bassiana*, *Isara fumosorosea* and *Metarhizium anisopliae* showed that *B. bassiana* and *Isaria* strains were detected in the roots, stems and leaves while *M. anisopliae* was confined to the root (Borisade 2016). Confinement of *M. anisopliae* to roots was confirmed by Behie (Behie et al. 2015) in haricot beans. Also variability in the frequency of detection in different parts of the plant was strain dependent.
A foliar application with *B. bassiana* for inoculation of the common bean resulted in approximately 30% of the leaf samples to be colonized after 7 days whereas no colonization was found in roots samples.
Foliar application with *B. bassiana* strain LPSC 1067 of tobacco, corn, wheat and soybean seedlings, by means of leaf spray, resulted in higher colonization rates of the leaves than seed inoculation and root immersion (Russo et al. 2015). There were large differences within the four plant species. Foliar applications resulted after 7 days in 100% of the tobacco leaf samples to be colonized by *B. bassiana* whereas colonization in corn leaf samples was less than 10%.

Soil spray/drench
A soil drench with *B. bassiana* for inoculation of the common bean resulted in approximately 25% colonization of the roots whereas the colonization of the leaves was less than 5% (Parsa et al. 2013).

Conclusion:
Based on above examples it is not possible to determine whether there is a particular application method that favours endophytic growth of MCAs. Endophytic colonization largely depends on the combination of factors that determine whether a bacterium or fungus gains access to the plant and whether it is able to propagate endophytically. Endophytic colonization by *B. bassiana*, for instance, depends on the inoculation method, fungal isolate and plant species.

6.2 Colonisation rate and duration of entomopathogens

Russo et al. (2015) showed that, independent of the application method and initial endophytic colonization rate, colonization rate of *Beauveria* decreases in time. Colonization rates in tobacco and wheat declined sharply from 100 and 40% at day 7 and the fungus could no longer be detected at day 28 after application. For another entomogenous fungus, *Metarhizium* spec., Cai et al. (2019) concluded that no reliable evidence of colonization of this species within plant cells has been documented and colonization might be restricted to the intercellular space of plant cells. This characteristic of the slow proliferation of hyphae may be caused by the plant host regulating the growth of the fungus (Saikkonen et al. 2004), or multitrophic interactions with other microorganisms present in the plant (van Overbeek and Saikkonen 2016). Further, the type and success of the relationship between both plant and fungus is strongly influenced by environmental variables, and plant physiological and genetic traits (references in Cai et al. 2019).

Conclusion:
There is not much information on the rate in which plants are colonized by entomopathogenic fungi (as demonstrated by the percentage of tissue samples in which the fungus is detected) and the duration of their presence in plants. Variation may exist between strains of the fungus and plant species.

7 Production of harmful metabolites by MCAs growing endophytically

This chapter investigates if harmful metabolites can be produced by microorganisms that have biocontrol potential. This question is of particular interest when assessing the risk of MCAs that still need authorization.

MCAs produce a plethora of secondary metabolites. The EU guidance on the risk assessment of metabolites (EC 2020) indicates that it is not straightforward whether a metabolite can be classified as toxic or not. This depends on produced quantities and the way of exposure. In the EU guidance it is first determined whether there is an indication that a metabolite is toxic (hazard). In the second and more difficult step it is determined whether it is produced in quantities that cause effects (depending on the way of exposure).

The OECD background document on secondary metabolites (OECD 2018) lists MCAs and their known metabolites. According to the Norine database (Flissi et al. 2019) some of these metabolites are considered toxins based on the data added to the database. These metabolites and their MCA producers are listed in Table 5. Information on endophytic growth of these MCAs in one crop was added to this table proving that the MCA is able to grow in at least one crop.

Table 5 Endophytic fungal MCAs and concentrations of toxic metabolites.

Species	**Toxic secondary metabolite**[a]	**Quantity (µg / kg plant tissue)**[e]	**Reference**[b]
Beauveria bassiana	beauvericin bassianin	n.a[f]	(Rao et al. 2020); (Parsa et al. 2013); (Vega et al. 2008)
Beauveria brongniartii	beauvericin	n.a	(Petkova et al. 2020)
Isaria tenuipes	beauvericin	n.a	(Zhang et al. 2019)
Isaria fumosorosea[c]	beauvericin	n.a	(Mantzoukas et al. 2015)
Lecanicillium longisporum	destruxin B	n.a	(Ownley et al. 2010)
Metarhizium anisopliae *M. flavoviride*	destruxin B	n.a	(Rao et al. 2020); (Vega et al. 2008)
Trichoderma gamsii[d] *T. brevicompactum*	trichodermin	n.a	(De Silva et al. 2019)
Trichoderma virens	gliotoxin	n.a	(Schweiger et al. 2020)

a: Toxic metabolites based on (OECD 2018)
b: Reference proofing endophytic growth of the MCA
c: Formerly *Paecilomyces fumosoroseus*
d: Formerly *T. viride*
e: Data on endophytic production of metabolites were searched for in Scopus: ((beauvericin OR bassianinn OR trichodermin OR gliotoxin) AND (isaria OR lecanicillium OR metarhizium OR beauveria OR trichoderma) AND endophyt*))
f: n.a means 'not available'

Toxins produced by MCAs in Table 5 do not appear in the list of mycotoxins produced by the WHO given in Table 6. All mycotoxins in Table 6 are produced by notorious phytopathogens such as *Aspergillus* and *Fusarium*. Maximum Limits (MLs) have been determined for these mycotoxins.

Table 6 Mycotoxins produced by pathogenic endophytes and their ML (WHO 2019).

Mycotoxins	**Produced by**	**Class, order, family**	**ML[a] (µg/kg)**
Aflatoxin (B1)	*Aspergillus* spec.	Eurotiomycetes, Eurotiales, Trichocomaceae	2–12
Citrinin	*Penicillium* spec.	Eurotiomycetes, Eurotiales, Trichocomaceae	2000
Ochratoxin A	*Aspergillus* spec. *Penicillium* spec.	Eurotiomycetes, Eurotiales, Trichocomaceae	2–80
Patulin	*Aspergillus* spec. *Penicillium* spec. *Byssochlamys nivea*	Eurotiomycetes, Eurotiales, Trichocomaceae	10-50
Deoxynivalenol	*Fusarium* spec.	Sordariomycetes, Hypocreales, Nectriaceae	500–1750
Fumonisins	*Fusarium* spec.	Sordariomycetes, Hypocreales, Nectriaceae	800–4000
Zearalenone	*Fusarium* spec.	Sordariomycetes, Hypocreales, Nectriaceae	50–400

a: ML is determined for a specific crop. The range of MLs indicates that MLs are determined for several crops. Crops are not specified in this table.

Other toxins such as apicidin, aurofusarin, fusaproliferin, beauvericin, butanolide, culmorin, enniatins, fusaric acid and moniliformin have been indicated as emerging mycotoxins (Afzal et al. 2014, Jestoi 2008, Khoshal et al. 2019). These metabolites are produced by one of the most common grain-contaminating genus of fungi, *Fusarium* spp.

Table 5 shows that beauvericin is the only emerging toxin that is not only produced by *Fusarium* but also by the MCAs *Beauveria* spec. and *Isaria* spec., both belonging to the Hypocreales although separated in their lineage. Luangsa-ard et al. (2009) assume that beauvericin production within the family of *Beauveria* has either arisen independently or arose in an ancestor (possibly deeper within the Hypocreales) and has been subsequently lost from many genera/taxa. The production of beauvericin has been measured in some insects (OECD 2018). Literature searches on quantities of beauvericin in plants (produced by endophytically growing *Beauveria* or *Isaria)* did not yield any results. In Table 5 the column on measured quantities of metabolites produced by endophytic MCAs in plants therefore remains empty.

Entomopathogenic fungi only occur in plants at extremely low quantities (Cai et al. 2019). For this reason and the fact that concentrations are not measured up till now, the production of beauvericin by *Beauveria* is not thought to significantly contribute to quantities of beauvercin produced by *Fusarium* in food. Therefore extra data requirements are not considered necessary. This conclusion is substantiated by the fact that the EFSA panel on contaminants in the Food Chain concluded that there is no risk due to acute exposure to *Fusarium* produced beauvericin for human health. This was based on the evaluation of a total of 12685 analytical results for beauvericin in food, feed and unprocessed grains (EFSA 2014). Follow-up in vivo studies, however, seem to indicate low genotoxic potential of beauvericin after chronic exposure (EFSA, 2018).

Lastly, there are some examples of *Fusarium* and *Aspergillus* genotypes that do not produce mycotoxins. The biocontrol species *Aspergillus flavus* AF36 is registered with USEPA for prevention of contamination of food with aflatoxin (Ortega-Beltran et al. 2016). It is an atoxigenic genotype.
Some non-pathogenic strains of *Fusarium oxysporum* such as strain Fo47 can control *Fusarium* diseases responsible for severe damages in many crops (Edel-Hermann et al. 2009).

Conclusion:
Literature research performed in this chapter shows that some MCAs can produce toxins. Although none of them are considered to be a mycotoxin, attention was payed to beauvericin as this toxin is assigned as an emerging mycotoxin. References to quantities of beauvericin produced by MCAs in plants were however not found in the literature. The EFSA panel on contaminants in the Food Chain concluded that there is no risk for human health due to acute exposure to beauvericin produced by *Fusarium*, but there may be low risk after chronic exposure. It can be assumed that quantities of beauvericin in plant tissue, if produced by MCAs, will not be proportionate to the quantities produced by pathogenic *Fusarium* species. These low quantities may therefore not pose a risk to human health, even not after chronic exposure.
The production of beauvericin by both *Fusarium* as *Beauveria* raises the question whether other MCAs could be distantly related to mycotoxin producing necrotrophic fungi. It is advised to pay attention to these distant relationships in the risk assessment.

8 Beneficial effects of metabolites of endophytes

Endophytes can be diversified into three different groups. Some endophytes have no apparent effects on plant performance but live on the metabolites produced by the host. These are termed commensal endophytes, whereas other endophytes confer beneficial effects to the plant, such as protection against invading pathogens and (arthropod) herbivores, either via antibiosis or via induced resistance, and plant growth promotion. A third group includes latent pathogens.

Many publications on endophytic relationships focus on the beneficial effects of endophytes. Some examples are given below.

8.1 Bacterial endophytes

Many bacterial endophytes are also known as PGPR. They are free living, soil-born bacteria, which enhance the growth of the plant either directly or indirectly. The direct mechanisms involve ammonia production, nitrogen fixation, phosphorus solubilization, siderophore production and the production of plant hormones triggering the plant's immune system (see references in Ali, Duan et al. (2014)). Some endophytic bacteria degrade pollution in partnership with plants (Afzal et al. 2014). PGPR may also have biocontrol capacities which are exerted through antagonism against phytopathogens. Antimicrobial metabolites are assumed to be one of the possible factors.

Fluorescent *Pseudomonas* are considered to be the most promising group of PGPR involved in the biocontrol of plant diseases. Significant control by PGPR in laboratory and greenhouse studies have been demonstrated. Results in the field are however inconsistent.

8.2 Fungal endophytes

There is ample literature proving beneficial effects of endophytic MCAs. Some examples are given below.

Beneficial effects on plant growth, fitness, and productivity
The endophytic fungus *Clonostachys rosea*, a hyperparasite, has beneficial effects on plant growth, fitness, and productivity of cucumber, even in absence or near-absence of pathogens (Sutton et al. 2008).

Defense against insects
Endophytic entomopathogenic fungi defend plants against insects (Gange et al. 2019) and pathogens (Ownley et al. 2010). Some endophytic entomopathogenic fungi have been reported to produce metabolites that can reduce insect infestations on their host plants (Jaber and Ownley 2018). It is believed that an increase in quantity and diversity of secondary metabolites in endophyte-containing plants is the cause of the reduction of insect herbivory on plants (Hartley and Gange 2009) and references in (Vega et al. 2008).

Antibiotic properties against fungi
Endophytic entomopathogenic fungi have antibiotic properties against pathogenic fungi (Martinez-Klimova 2017).

Tolerance against drought
Endophytic entomopathogenic fungi also give tolerance against drought (Kuzhuppillymyal-Prabhakarankutty et al. 2020).

Conclusion:
Publications investigating potential adverse effects of endophytically growing MCAs to consumers seem to be absent in the literature. This may be explained by the fact that there is not much information on endophytic growth of MCAs in the first place. Moreover, animal tests are not suitable as these only detect effects of highly toxic metabolites. It is very unlikely that these tests would be used as they are too expensive and the chance that they would detect an effect is almost absent. Instead the literature focuses on beneficial effects of endophytically growing bacteria and fungi. This indicates that metabolites of MCAs in general are not toxic in the concentrations produced in/on crops.

9 Are adaptations to current data requirements of MCAs necessary based on current information?

Adaptation to the current data requirements would only be necessary when there are indications that endophytically growing MCAs produce higher concentrations of harmful metabolites than during their anticipated epiphytic growth.
Based on our exploratory survey we conclude that there are no reasons to adjust the current data requirements based on the endophytic potential of an MCA. First of all, the available literature indicates that MCAs are only transiently present in plants as endophytes and are only detected in low concentrations. Secondly, there are no indications that MCAs produce metabolites, or concentrations of metabolites that are harmful for human health when they grow endophytically. Thus, it is concluded that endophytic growth of MCAs do not pose an extra risk to human health/food quality.

10 Conclusions

There are questions posed by National Authorities if current data requirements may need to be adapted for MCAs that can live endophytically in plants. These questions are based on the concern that MCAs may produce higher concentrations of metabolites if they are able to grow endophytically, which could result in negative effects on human health.
This report investigated whether MCAs are able to grow endophytically, as they were initially thought to only grow epiphytically, and if endophytic growth poses an extra risk to human health/food quality that needs to be taken into consideration.

Our report indicates that MCAs are able to live both epiphytically as well as endophytically and that it is not possible to exclude endophytic growth during the lifespan of the MCA in/on the crop, for example by changing the method of MCA application on crops.
However, based on our limited survey, we conclude that there are no reasons to adjust the current data requirements based on the potential of an MCA to be able to live endophytically. First of all, the available literature indicates that MCAs are only transiently present in plants as endophytes and are detected at low concentrations. Secondly, there are no indications that MCAs produce metabolites, or concentrations of metabolites that are harmful for human health in case they grow endophytically.

The provisional conclusion is that, based on this exploratory literature search, the current framework is also adequate to assess potential risks of MCAs that may grow endophytically in addition to the expected epiphytic growth.

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12 Abbreviations

Ctgb	College voor de toelating van gewasbeschermingsmiddelen en biociden - Dutch Board for the Authorisation of Plant Protection Products and Biocides
LNV	Ministerie van Landbouw, Natuur en Voedselkwaliteit, Ministry of Agriculture, Nature and Food Safety
MCA	Microbial control agent
ML	Maximum limit
WUR	Wageningen University & Researh
NIOO	Nederlands Instituut voor Ecologie – Netherlands Institute of Ecology

13 Appendix

13.1 Appendix 1

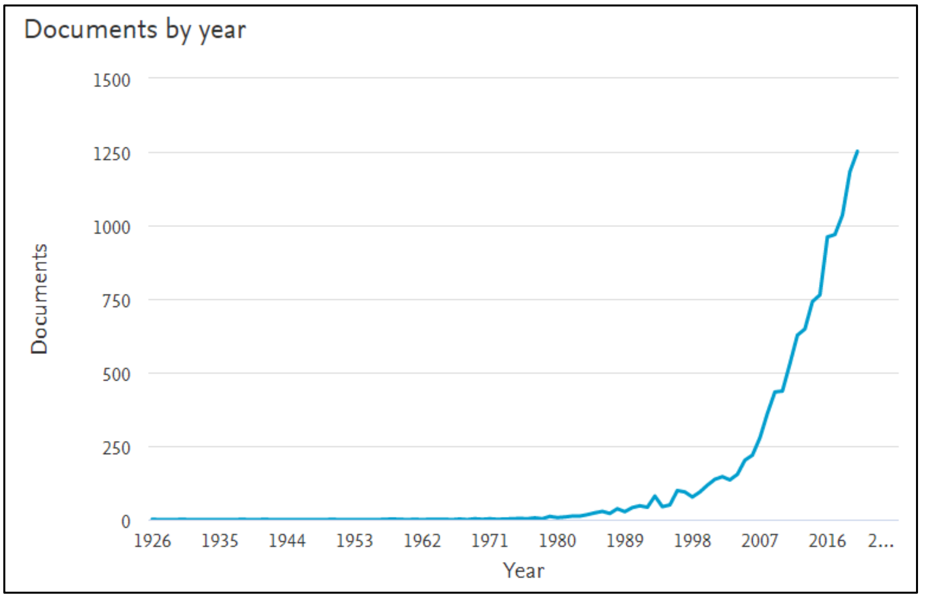

Figure 2 Scopus search string (Bacteri AND endophyt*).*

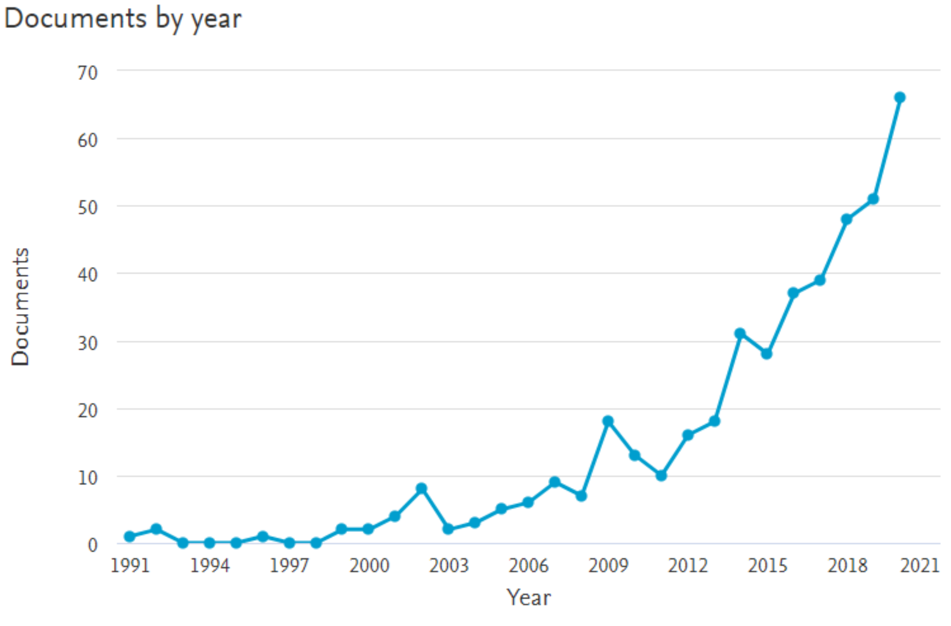

Figure 3 Scopus search with string: (entomopathogen OR Beauveria OR Metarhizium OR Isaria OR Acrimonium OR Paecilomyces OR Lecanicillium) AND endophyt).*

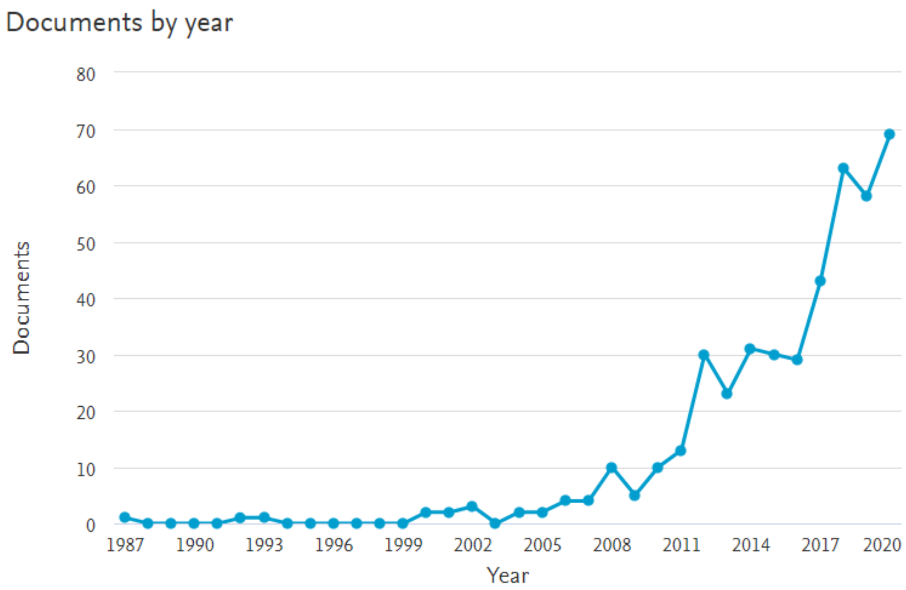

Figure 4. Scopus search with string: (endophyte AND (bioactive* OR metabolite*) AND (pharmaceut* OR medicine OR therap*)).*

13.2 Appendix 2

Isolation and detection techniques for bacteria and fungi

Techniques for bacteria

The following steps have been described by Hallman et al. (2006). These methods were valid for the last three decades. Each one has advantages and disadvantages and the best technique should be selected to meet the research objectives.

Surface disinfection and trituration

Surface disinfection is the first step in this procedure. Various disinfectants such as sodium hypochlorite, ethanol or hydrogen peroxide can be used. In the second step the plant material is washed repeatedly in sterile water or buffer solutions. In the third step the plant tissue is triturated with a mortar by any suitable device in sterile water, buffer solution or liquid media. In the last step the triturate is processed for bacterial enumeration. The researcher needs to balance the risk of penetration of the disinfectant into the plant tissue, hence killing endophytes, against incomplete sterility of the plant tissue.

Vacuum and pressure extraction

The vacuum extraction and pressure bomb both collect root sap from plants. This plant sap mainly consists of fluid from the conducting elements and adjacent intercellular spaces, representing two physical niches often considered favorable for systemic colonizing bacteria. These methods also have disadvantages. The pressure bomb technique predominantly recovers *Pseudomonas* spp., a subset of the endophytes. These techniques give lower number of endophytic bacteria compared to the trituration technique. Moreover, the pressure and vacuum techniques cannot be applied to soft plant tissues as they do not withstand such a harsh treatment. This narrows down the plant species that can be studies.

Centrifugation
The centrifugation technique is of interest as, opposed to the vacuum and pressure extraction, it is suitable for soft plant tissue. The method however requires surface disinfection.

Recognition, localization and enumeration of endophytic bacteria
Several methods are described by Hallman et al. (1997) to recognize, localize and count endophytic bacteria, such as the use of artificial media, viable staining procedures in combination with light microscopy, electron microscopy, immunological staining and quantification by ELISA, nucleic acid hybridization and autoradiography.

Other techniques such as SCAR (sequence characterized amplified region) markers and qPCR protocols were developed for the detection and quantifications of the presence of Streptomyces strains in plant material (González-García et al. 2019).

Techniques used for fungi
Techniques for specific fungal groups have been reviewed extensively. The abundance, diversity and species composition of endophyte assemblages and infection frequencies vary according to host species, issue type and tissue age, site characteristics, local microclimate conditions, anthropogenic factors. For the specific MCAs of this report, the search can be focused at cultivable species mentioned in Table 2. These are predominantly entomopathogenic species. Table 7 gives some examples.

Table 7 Techniques to determine presence of fungal endophytes.

Species	**Plant**	**Technique**	**Reference**
Metarhizium robertsii and *Beauveria bassiana*	haricot bean	PDA medium and GFP[a] labelling, followed by fluorescent microscopic analysis	(Behie et al. 2015)
Metarizhium anisopliae	maize	Egfp-labelling[b] followed by confocal microscopy[c]	(Cai et al. 2019)
Metarizhium anisopliae; *Beauveria bassiana*; *Isaria fumosoroseus*	sorghum	Plating stems on Sabouraud Dextrose Agar (SDA) medium modified with chloramphenicol followed by assessment of occurrence of mycelial growth	(Borisade 2016)

a: Green fluorescent protein labelling. The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range.
b: Egfp-labelling = enhanced green fluorescent protein-labelling.
c: Most frequently confocal laser scanning microscopy (CLSM).

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