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DIPLOMA THESIS

Diversity and distribution study of viruses in the entomopathogenic fungus *Beauveria bassiana* in the Czech Republic

Studium diversity a rozšíření virů entomopatogenní houby *Beauveria bassiana* v České republice

Thesis Supervisor: Noemí Herrero Asensio, Ph.D.
Institute of Entomology, Biology Centre of the CAS

Co-Supervisor: prof. Ing. Vladislav Čurn, Ph.D.
Faculty of Agriculture, University of South Bohemia

Author: Bc. Petr Vaněček

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Zadávající katedra:	**Katedra speciální produkce rostlinné**

Zásady pro vypracování:

Cílem této práce je studium prevalence, variability a distribuce jednotlivých vzorů virových dsRNA fragmentů ve sbírce izolátů *B. bassiana* získané na různých lokalitách a přírodních stanovištích v České republice.
Stručný úvod do problematiky a nástin významu tématu.
Literární přehled: charakteristika entomopatogenních hub a jejich využití v biologické ochraně; charakteristika a systematika mykovirů; principy použitých metod.
Materiál a metody: popis a charakteristika izolátů *B. bassiana*; metody kultivace, izolace a analýzy dsRNA.
Výsledky:Detekce dsRNA molekul ve velikosti 1 až 16 kbp, rozšíření, distribuce a vzory dsRNA fragmentů.
Diskuze: porovnání vlastních výsledků s literárními údaji, posouzení možností praktického uplatnění dosažených výsledků a poznatků.
Závěr: přehledné shrnutí nejdůležitějších poznatků a závěrů vyplývající z řešené problematiky.

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Charnley AK, Collins SA (2007) Entomopathogenic fungi and their role in pest control. Enviromental and Microbial Relationships, pp 159-187. In Esser K (ed) The Mycota Vol 4. Springer.
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Konzultant diplomové práce:	**prof. Ing. Vladislav Čurn, Ph.D.**
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JIHOČESKÁ UNIVERZITA
V ČESKÝCH BUDĚJOVICÍCH
ZEMĚDĚLSKÁ FAKULTA
studijní oddělení
Studentská 13
370 05 České Budějovice

L.S.

prof. Ing. Miloslav Šoch, CSc., dr. h. c.
děkan

prof. Ing. Vladislav Čurn, Ph.D.
vedoucí katedry

V Českých Budějovicích dne 2. února 2015

Declaration of the author's diploma thesis

I hereby declare that this diploma thesis has been carried out in the Institute of Entomology, Biology Centre of the Czech Academy of Sciences in České Budějovice, Czech Republic under the guidance of Noemí Herrero Asensio, Ph.D., and in Faculty of Agriculture, University of South Bohemia, České Budějovice, Czech Republic under the guidance of Professor Vladislav Čurn. The work is original and has not been submitted in part or full by me for any degree or bachelor thesis at any other University. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

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Abstract

Mycoviruses are viruses that infect and replicate in fungal cells, but unlike most known viruses of plants and animals, they exceptionally produce deleterious effects on their host. Nonetheless, the last discoveries showed that some mycoviruses can decrease the virulence of their phytopathogenic fungal hosts, making them very attractive for their possible use as biological control agents. Most mycoviruses have dsRNA genomes and are widespread in all major taxa of fungi. *Beauveria bassiana* is one of the most studied species of entomopathogenic fungi; it has a cosmopolitan distribution and is used as biocontroller against invertebrates in agriculture.

In the present work, a collection of 137 isolates of *B. bassiana* obtained at different locations and from different habitats in the Czech Republic was analysed. These isolates were analysed for the presence of dsRNA elements indicative of viral infections. The results revealed a high prevalence of viral infections in Czech *B. bassiana* isolates, with 22.6% of the isolates containing dsRNA elements with viral characteristics. Obtained dsRNA electropherotypes showed that virus diversity in infected isolates was high and that mixed virus infections occurred among them. Based on the characteristics of the electrophoretic band patterns, it could be hypothesized that *B. bassiana* isolates collected in the Czech Republic could harbour members of the viral families *Totiviridae*, *Partitiviridae*, *Chrysoviridae* and *Hypoviridae*.

Keywords: *Beauveria bassiana*, entomopathogenic fungi, mycoviruses, dsRNA

Abstrakt

Mykoviry jsou viry, které infikují houbové buňky, kde se i replikují, ale na rozdíl od většiny známých virů rostlin a živočichů mají jen výjimečně škodlivý vliv na jejich hostitele. Nicméně, poslední objevy ukazují, že některé mykoviry mohou snížit virulenci jejich fytopatogenních hostitelů, což dělá mykoviry velmi atraktivními z důvodu jejich možného využití v biologické ochraně. Většina mykovirů má dsRNA genomy a jsou rozšířené ve všech hlavních taxonech hub. *Beauveria bassiana* je jednou z nejvíce studovaných entomopatogenních hub. Má kosmopolitní rozšíření a v zemědělství je využívána v rámci biologické ochrany rostlin proti bezobratlým škůdcům.

V této práci byla analyzována sbírka 137 vzorků *B. bassiana* získaných z odlišných lokalit a přírodních stanovišť v České republice. Tyto izoláty byly testovány na přítomnost dsRNA fragmentů indikujících virovou infekci. Výsledky odhalily vysoký výskyt virové infekce mezi českými izoláty *B. bassiana*, bylo detekováno 22,6% vzorků obsahujících dsRNA fragmenty s virovými charakteristikami. Získané elektroforeotypy dsRNA ukazují, že virová rozmanitost u infikovaných izolátů byla vysoká, a že se mezi izoláty objevily smíšené virové infekce. Na základě charakteristik elektroforetických profilů může být předpokládáno, že izoláty *B. bassiana* nasbírané v České republice mohly být infikovány zástupci virových čeledí *Totiviridae, Partitiviridae, Chrysoviridae* a *Hypoviridae.*

Klíčová slova: *Beauveria bassiana*, entomopatogenní houby, mykoviry, dsRNA

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1. Introduction

Social development is imminently connected with changes in nature which are perceived at global scale. The world is modernized and changed in many ways every day. These changes sometimes lead to side effects that are materialized in the increase of environmental pollution. Therefore, nowadays more and more efforts are spent in keeping pollution at acceptable levels. These efforts are extended to many different sectors, including agriculture. According to this, there is a tendency in modern agriculture to limit the usage of chemical pesticides replacing them by integrate pest management practices, which include the usage of biological control agents. Actually, entomopathogenic fungi are becoming in the recent years a very attractive method as an alternative to the usage of chemical pesticides, since they do not have hazard effects on human health and environment, meeting challenges of globalisation, climate change and plant protection policies. They cause lethal infections and regulate insect and mite populations in nature by causing epizootics. *Beauveria bassiana* is one of the most studied entomopathogenic fungal species and is commercialized under many different formulations as a biological control agent against different invertebrate pest species.

Mycoviruses, are viruses that infect and replicate in fungal cells. They have been detected in many fungal species, covering all four phyla of true fungi: *Zygomycota, Chytridiomycota, Ascomycota* and *Basidiomycota*. Mycovirology is a quite young field within general virology, being only active since 1962 when Hollings described the first virus particles infecting mushrooms. Hence, only few studies exist until the date reporting viruses infecting entomopathogenic fungi. Mycoviruses does not produce obvious symptoms on their hosts, but some of them can decrease the virulence of their phytopathogenic fungal hosts, or produce malformations in mushrooms. Nonetheless, the cases in which mycoviruses are detrimental to their hosts could be the exception to the rule. Indeed, the high prevalence and persistence of mycoviruses among the major groups of fungi could indicate that their presence could be beneficial to their fungal hosts, as is known to occur with a number of non-fungal viruses that are able to establish mutualistic or neutral relationships with their hosts.

In this thesis a collection of 137 isolates of the entomopathogenic fungus *B. bassiana* will be analysed for the presence of dsRNA molecules indicative of mycovirus infections. New advances in the study of the entomopathogenic fungus

B. bassiana and their associated mycoviruses will help to better comprehend the biology and ecology of this attractive entomopathogenic fungal species, which is crucial for understanding its role in managed and natural ecosystems, and hence, for their successful development as a biological control agent.

2. REVIEW OF LITERATURE

2.1 Entomopathogenic fungi

It is known that fungi and insects can establish relationships that can go from mutualistic or commensal to obligate pathogenic. Fungi which cause lethal infections and regulate insect and mite populations in nature by causing epizootics are called entomopathogenic fungi (CARRUTHERS & SOPER, 1987; MCCOY *et al.*, 1988, CHARNLEY & COLLINS, 2007).

The earliest studies in entomopathogenic fungi were carried out in the 1800s, and they were focused on the development of mechanisms to avoid the pest that was devastating the silkworm industry of that time (STEINHAUS, 1975).

Entomopathogenic fungi group includes about 700 species belonging to approximately 100 orders, nevertheless, only a few of these species have been subjected to deep study. Most of these entomopathogenic species belong to orders *Hypocreales* (phylum *Ascomycota*) and *Entomophthorales* (phylum *Entomophthoromycota*) (HIBBETT *et al.*, 2007; SUNG *et al.*, 2007, HUMBER, 2012).

The occurrence of entomopathogenic fungi is quite wide and they can be found almost all over the world (e. g., tropical rainforest, Antarctica or Arctic), so they are a worldwide spread group of fungi (BRIDGE *et al.*, 2005; EILENBERG *et al.*, 2007; AUNG *et al.*, 2008; AUGUSTYNIUK-KRAM & KRAM, 2012).

The species diversity of entomopathogenic fungi in nature is influenced by several factors, like the altitude, the latitude, the ecosystem type or the temperature. Sun and Liu (2008) found that entomopathogenic fungi inhabit wide range of altitudes (even more 5200m) and latitudes. It is important to mention that not all entomopathogenic fungi inhabit the same range of altitudes and latitudes, or at least differences arise at the level of frequency of occurrence of individual species. For example, *Metarhizium anisopliae* occurs more frequently in northern latitudes than *Beauveria bassiana* (QUESADA-MORAGA *et al.*, 2007). In the same way, ecosystem types significantly affects species diversity of entomopathogenic fungi, for example, in temperate forests it is found higher diversity than in agriculture lands, but lower than in the tropical forests. As it was mentioned earlier, temperature is another factor which affects the occurrence of entomopathogenic fungi, because

species have different optimum growth temperatures. For example, the optimum growth temperature of *Hyphomycetes* is generally between 20-30°C while for *Entomophthorales*, it is considered as 15-25°C. Additionally, it was found that entomopathogenic fungi occurring in forests are more tolerant to lower temperatures (about 8°C), and those growing in agriculture lands are more tolerant to higher temperatures (about 37°C). (BIDOCHKA *et al.*, 2002; SOSNOWSKA *et al.*, 2004; AUGUSTYNIUK-KRAM & KRAM, 2012; GUL *et al.*, 2014).

An important part of the entomopathogenic capability of these fungi lies in their ability for the production of different enzymes and secondary metabolites which help with the insect infection process and also play an important insecticidal role. As insect cuticle comprises up to 70% of protein, the penetration process is facilitated by extracellular fungal proteases like subtilisins, chymotrypsins, trypsins and metalloproteases, usually with multiple isoforms of each. Toxic metabolites with insecticidal effect include substances as destruxins (produced by *Metarhizium* species), beauvericins, isarolides, bassianolides, beauverolides, oosporein (produced by *Beauveria* species or *Isaria fumosorosea*), efrapeptins (produced by *Tolypocladium* species) and hirsutellin (from *Hirsutella thompsonii*) (MAZET & VEY, 1995; WEISER & MATHA, 1988; CHARNLEY, 2003; GUL *et al.*, 2014). These substances enable to kill many different species of insects and arthropods thanks to their high variability, their ability to facilitate penetration of insect cuticle and because they are able to overcome the insect immune system. For example, destruxins mostly cause an initial tetanic paralysis leading to insect's death. The tetanic paralysis is attributed to muscle depolarization by direct opening of the Ca^{2+} channels in membrane (SAMUELS *et al.*, 1988; GABARTY, 2014).

The production of the secondary metabolites is not only important for infecting and killing insects, this substances are also crucial for the competition for the niche of entomopathogenic fungi with other fungi. This strategy is known as antibiosis. During this fight for the space, fungi actively compete with each other for carbon, nitrogen, and various microelements. The site of competition is often the rhizosphere, phyllosphere, or the intercellular space of a plant. Successful competition is often a matter of timing, as resources are likely to go to the initial colonizer (FRAVEL, 1988). Nonetheless, there are other known mechanisms that entomopathogenic fungi use in their competition for the space. Mycoparasitism is one of these mechanisms and consists in the direct parasitism of one fungus by another. This strategy normally

starts with the production of extracellular exochitinases, which diffuse and catalyse the release of cell-wall oligomers from the target host fungus (HARMON *et al.*, 2004).

2.1.1 Life cycle

Life cycles of entomopathogenic fungi are synchronized with insect host stages and environmental conditions (SHAHID *et al.*, 2012). This way, the infection process of fungal entomopathogens to their hosts could be summarized in six main steps: adhesion, germination, differentiation, penetration, development, colonization, sporulation and dispersion.

The degree of pathogenicity of an entomopathogenic fungus depends on several factors which influence each of the cited steps. During the adhesion, the spore has to adhere or attach to the insect cuticle, which is facilitated by a layer of mucus composed of proteins and glucans that cover the whole spore. After the adhesion, the spore begins to germinate and then it occurs the differentiation. At this moment, the germinating spore produces specialized structures called appressoria, which fix the spore to the epicuticular surface. Next step of the infection is penetration of the insect cuticle or entrance through natural openings of the insect host such mouth or anus. The effects of mechanical pressure and enzymatic activity of the germ tube help to the penetration. A crucial point in this step is the secretion of several proteases, lipases and chitinases. As soon as entomopathogenic fungi penetrate the insect body, they grow and spread through the hemocoel as yeast-like propagules (blastospores), hyphal bodies or protoplast lacking a cell wall (Fig. 1) (ROBERTS, 1981; HAJEK & ST. LEGER, 1994; BOUCIAS *et al.*, 1998; MACIÁ-VICENTE *et al.*, 2011).

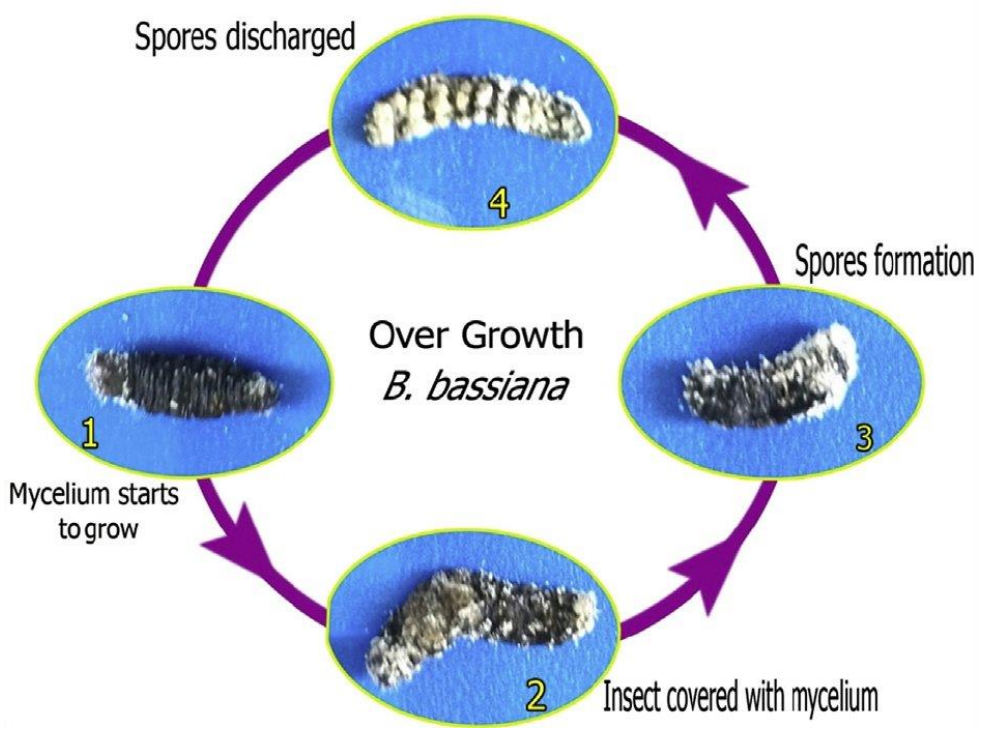


Fig. 1. The growth of *B. bassiana* on larvae of *Agrotis ipsilon* (GABARTY *et al.,* 2014)

Entomopathogenic fungi kill the insect by mechanical damage when mycelia grow inside the insect or by the production of toxins. (ROBERTS, 1981; HAJEK & ST. LEGER, 1994; BOUCIAS *et al.,* 1998).

After host death fungi colonize the cadaver, and during 2-3 days they form aerial hyphae and then sporulate (AUGUSTYNIUK-KRAM & KRAM, 2012). The sporulation takes place through a massive production of conidia that will be dispersed in different ways. Species like *Metarhizium* and *Beauveria* spp. produce hydrophobic conidia, which are passively spread, while fungi of the order *Entomophthorales* produce conidia actively discharged under hydrostatic pressure that are carried by the wind or by co-occurring insects (HEMMATI *et al.*, 2001; ROY *et al.,* 2001).

If the primary conidia of entomophthoralean species are not on suitable host substrates, where they can germinate, then secondary conidia can be formed and be actively discharged (e.g., *Pandora* ssp.), or passively held on long stalks (e.g., *Zoophthora* ssp.) (SHAHID *et al.,* 2012).

2.1.2 Lifestyles of entomopathogenic fungi

Entomopathogenic fungi are known as saprotrophic soil-borne fungi that can infect insects and arthropods, but it is important to remark that they are not obligate saprotrophs. There are many ways in which entomopathogenic fungi can receive nutrients from their host. These ways include biotrophy (nutrition derived only from living cells, which ceases once the cell has died), necrotrophy (killing and utilization of dead tissues), and hemibiotrophy (initially biotrophic and then becoming necrotrophic) (VEGA *et al.,* 2009).

Recently, it has been discovered that many entomopathogenic fungi play additional roles in nature besides infecting arthropods or being saprotrophs. Actually, they can colonize plants as endophytes, act as plant pathogen antagonists, as rhizosphere colonizers or even as plant growth promoters (VEGA *et al.,* 2009).

Fungal endophytes are fungi which live within a plant for at least part of their life cycle without causing apparent disease to the plant host. Endophytes can infect either above or below ground internal plant tissues. Genera as *Beauveria*, *Isaria* and several others, traditionally known as insect pathogens, have been naturally isolated as endophytes (VEGA, 2008; VEGA *et al.,* 2008).

Fungal endophytes can protect host plants against pathogens and herbivores (ARNOLD *et al.,* 2003; SCHULZ & BOYLE, 2005; ARNOLD & LEWIS, 2005; RUDGERS *et al.,* 2007; VEGA *et al.,* 2009). They use different strategies for this purpose, as production of metabolites, space competition, parasitism, hypovirulence, induced systemic resistance or the increase of plant growth response (OWNLEY & WINDHAM, 2007).

2.1.3 The entomopathogenic fungi as biocontrol agents in agriculture

Entomopathogenic fungi effectively infect a wide range of insect and arthropod species, which make them very interesting for their usage as biological control agents (AUGUSTYNIUK-KRAM & KRAM, 2012).

Eilenberg *et al.* (2001) defined biological control as the use of living organisms to suppress the population density or the impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be. The most important goal is to reduce the population of the pest below the economic threshold of harmfulness.

The use of entomopathogenic fungi as biological control agents constitute an important component of what is known as integrated pest management (IPM). These fungi have great potential in the management of insect pests infecting different crop systems, since they have a big impact on the population dynamics of many economically important species of insect, arthropods, spiders and mites. Although entomopathogenic fungi cannot be massively produced as easily and efficiently as chemical insecticides, and it is usually hard to meet optimal environmental and host conditions for their application in the field, investing more in entomopathogenic fungi research will place them in a better position at the IPM than they are now. This way, new paradigms in the development, utilization and regulation of these insecticides will be established (LIU, 2012).

Biological control is more and more necessary mainly because of the protection of the environment. Chemical pesticides unlike biopesticides cannot be perceived only as pest control agents, it is necessary to realize that they are also contaminants of the environment. Although chemical pesticides are used mostly in a direct control of pests on above-ground plant parts, a large amount of these pesticides can reach the soil, groundwater or the atmosphere. They can also cause long-term negative effects and move over long distances (ocean fog, arctic snow) (ELMOHOLT *et al.*, 1991; VAN DER WERF, 1996).

Another reason why chemical pesticides need to be replaced by biological pesticides, is the increasing amount of resistances that insects are developing to various chemical substances contained in plant protection products (MOTTA-SANCHEZ *et al.,* 2002). Additionally, since many invasive species have reached new countries or continents where they do not have natural pathogens or predators, the use of biological control agents could help to solve this problem (AUGUSTYNIUK-KRAM & KRAM, 2012).

2.2 *Beauveria bassiana*

The fungi subject of this study *Beauveria bassiana* received this name in honour of the Italian scientist Agostino Bassi who in 1835 formulated the germ theory of disease after using the white muscardine fungus (*B. bassiana*) to treat diseased silkworms (AUGUSTYNIUK-KRAM & KRAM, 2012). Bassi found that the infectious agent of this worms was related to a white structures developed on dead silkworms. He proved that the transference of a small amount of this “white

material" caused the death of healthy silkworms, after sprouting, growing and mummifying them (PORTER, 1973). This discovery constituted the first step on the utilization of fungi as biological control agents against insect pests (GILBERT & GILL, 2010).

The genus *Beauveria* belongs to phylum *Ascomycota*, more precisely to the order *Hypocreales* and family *Cordycipitaceae*. *Beauveria* is a cosmopolitan anamorphic genus of soil-borne entomopathogenic fungi, which is facultative necrotroph (ROBERTS & HAJEK, 1992; GOETTEL *et al.*, 2005), but that can also occur as saprotroph or as a plant endophyte (VEGA *et al.*, 2008).

This genus is characterized morphologically by globose to flask-shaped conidiogenous cells (Fig. 2a and 2b) from which one-celled terminal holoblastic conidia are produced in sympodial succession on an indeterminate denticulate rachis. *Beauveria* species are distinguished principally by the morphology of their conidia, which are typically smooth-walled, hyaline, 1.5–5.5 μm and globose to cylindrical or vermiform (REHNER *et al.*, 2011).

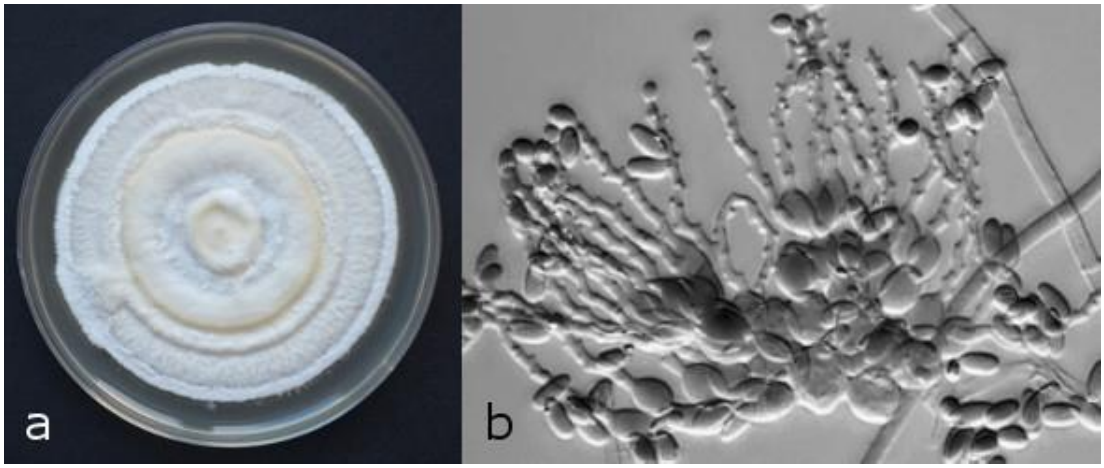


Fig. 2. a) The growth of *B. bassiana* on potato dextrose agar (Source: author). b) Conidiogenous cells and conidia of *Beauveria* sp. (REHNER *et al.*, 2011).

B. bassiana can be reproduced sexually or asexually. The major reproductive form is the asexual since the sexual stage of *B. bassiana* is rarely observed. The sexual stage (the telemorph) of *B. bassiana* has been identified as *Cordyceps bassiana* (LI *et al.*, 2001).

B. bassiana is one of the most widespread entomopathogenic fungi. It is a cosmopolitan fungi but it is important to mention that *B. bassiana* seems to be very sensitive to the disturbance effects of cultivation (GABARTY *et al.*, 2014). It was

found that *B. bassiana* inhabits also the Arctic Circle in Norway and Finland (VÄNNINEN, 1995; KLINGEN *et al.,* 2002).

Fungi of the genus *Beauveria* are also well-known for their production of biologically active secondary metabolites as polyketides (e.g., oosporein, bassianin and tennelin), non-ribosomally synthesized peptide antibiotics (e.g., beauvericin, bassianolides and beauverilides), non-peptide pigments and other metabolites (e.g., oxalic acid). These secondary metabolites are mainly implicated in insect pathogenesis and virulence and can be applied in agriculture. Actually, *Beauveria bassiana* is commercialized under many different formulations as a biological control agent against different invertebrate pest species. Nevertheless, some of the metabolites produced by *B. bassiana* can have also medical applications and they are exploited by chemical and pharmaceutical industries (GRIFFITH *et al.,* 1993; VEY *et al.,* 2001; XU *et al.,* 2009).

2.3 Mycoviruses

Mycoviruses are viruses that infect and replicate in fungal cells, but unlike viruses of plants and animals, most known mycoviruses lack an extracellular phase in their replication cycle (NUSS, 2011). This viruses have been detected in many fungal species, covering all four phyla of true fungi: *Zygomycota, Chytridiomycota, Ascomycota* and *Basidiomycota*. Typical representatives of fungal viruses are isometric particles of 25±50 nm in diameter and with dsRNA genomes (GHABRIAL, 1998; PEARSON *et al.,* 2009). Until the date more than 250 mycovirus species have been sequenced and registered in the NCBI database.

The first presence of mycovirus was found in 1962 by Hollings in diseased mushroom *Agaricus bisporus,* and later they were detected in the fungus *Penicillium* spp., which it is well known by its capacity of stimulating the production of interferon in mammals (BOZARTH, 1972). In 1978, Sanderlin and Ghabrial discovered mycoviruses in plant filamentous fungal pathogens, more precisely, they were found in the cereal fungal pathogen *Helminthosporium victoriae*. These discoveries constituted the establishment of a new branch of research within general virology: mycovirology.

2.3.1 Taxonomy and genomic organization

Although most mycoviruses have double stranded RNA (dsRNA) or single stranded RNA (ssRNA) genomes, a DNA virus was recently described infecting the fungal plant pathogen *Sclerotinia sclerotiorum*, it means that fungi may harbour both, RNA and DNA viruses (XIE & JIANG, 2014). Most mycoviruses have quite simple genomes and they can range from 2 to 14 kb in size. These genomes usually encode for two genes, one encodes the capsid protein and the other a RNA-dependent RNA polymerase (RdRp), but some mycoviruses encode only for a single RdRp gene. Regarding to genome organization, mycoviruses can have non-segmented or segmented genomes. According to this, members of *Reoviridae* family can have genomes made up of 10 to 12 segments, while *Totiviridae* members have non-segmented genomes. Another important feature of mycoviruses is that not all of them have their genomes encapsidated within a protein capsid (Fig. 3). For example, members of the *Hypoviridae* family present their genomes within unencapsidated membrane vesicles, and *Narnaviridae* family is characterized because its members have naked (+) ssRNA genomes (GHABRIAL *et al.*, 2015). This absence of capsids, which protect viral genetic information, is possible because most mycoviruses lack an extracellular phase on their replication cycle (NUSS, 2011).

Even if the genomes of these viruses seem very unique, Liu *et al.* (2010) found that the capsid protein and RdRp genes from totiviruses and partitiviruses have widespread homologs in the nuclear genomes of eukaryotes like plants, arthropods, fungi, nematodes, and protozoa. These transfers could have been possibly mediated by retrotransposons. Horizontally transfer of the viral genes of fungal partitiviruses between viruses and plants was also found by Chiba *et al.* (2011). Liu *et al.* (2010) also discovered that the amino acid sequence of the coat protein of *Sclerotinia sclerotiorum* partitivirus-S (SsPV-S) has the highest amino acid sequence similarity to the IAA-leucine-resistant protein 2 (ILR2) of *Arabidopsis thaliana*.

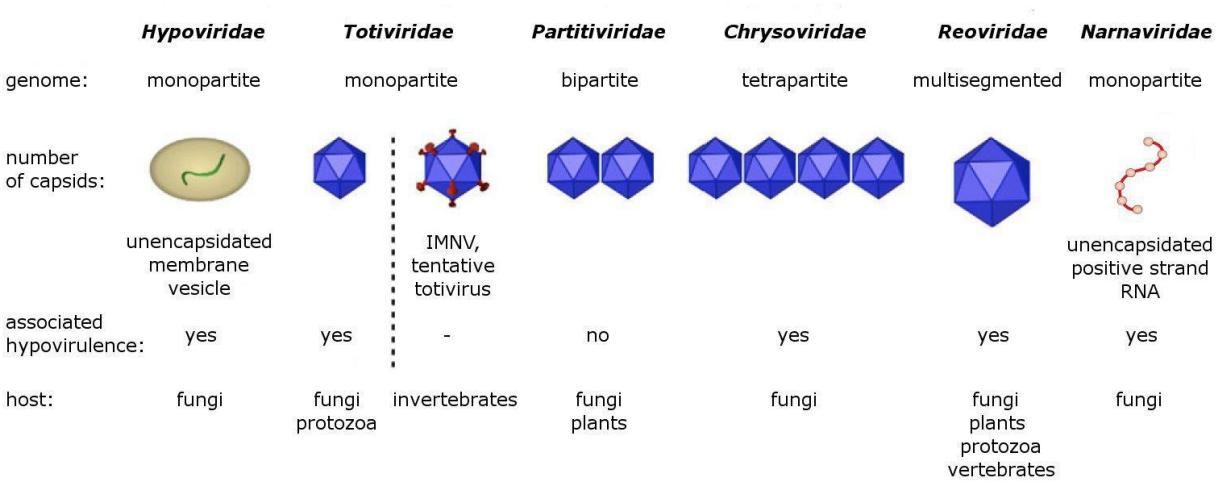


Fig. 3. Characteristics of the most studied taxonomic families of mycoviruses (NUSS, 2005; VAN DE SANDE *et al.*, 2010)

According to all commented features regarding to genome organizations of mycoviruses, they are classified within the following 13 families, (GHABRIAL *et al.*, 2015) dsRNA: *Totiviridae*, *Partitiviridae*, *Megabirnaviridae*, *Chrysoviridae*, *Quadriviridae*, *Reoviridae*, *Endornaviridae*; (+)ssRNA: *Alphaflexiviridae*, *Barnaviridae*, *Gammaflexiviridae*, *Hypoviridae*, *Narnaviridae;* (-)ssRNA: propose family *Mycomononegaviridae*; circular ssDNA (unclassified). Nonetheless, these mycovirus families are not all exclusively from fungi. Some virus from these families can be harboured by other organisms (XIE & JIANG, 2014).

The most studied families of mycoviruses are *Totiviridae*, *Partitiviridae* *Chrysoviridae*, *Reoviridae*, *Narnaviridae* and *Hypoviridae* (HULL, 2014; XIE, JIANG, 2014), so I will describe below the characteristics of these families in detail (GHABRIAL *et al.* 2015).

Family: ***Totiviridae***

Members of this family have monosegmented (i.e., nonsegmented) bicistronic genomes, 4.6–7.0 kbp in length and usually encompassing two large, partially overlapping open reading frames (ORFs) on one strand. The 5´-proximal ORF encodes the capsid/coat protein (CP) and the 3´-proximal ORF encodes the RdRp (GHABRIAL, 2008; WICKNER *et al.*, 2011).

Members that infect fungi are currently grouped in two genera: *Totivirus* and *Victorivirus*. Viruses in genus *Totivirus* have been found to infect, for example, the yeast *Saccharomyces cerevisiae*, fungi *Ustilago maydis* and *Tuber aestivum*

representing the first evidence for mycoviruses in ectomycorrhizal fungi (ectophytes of plant roots). Mycoviruses belonging to genus *Victorivirus*, in contrast, have been found to infect only filamentous fungi (GHABRIAL & NIBERT, 2009; STIELOW & MENZEL, 2010; GHABRIAL *et al.*, 2015).

Family: ***Partitiviridae***

Members of this family have bisegmented genomes, 1.4–2.4 kbp in length and encompassing one large ORF per segment. Generally the smaller segment (dsRNA2) encodes the CP and the larger segment (dsRNA1) encodes the RdRp. These two genome segments are packaged into separate virus particles. Following are centre organization of this family to reflect phylogenetic relationships, members that infect fungi are now grouped in three genera: *Alphapartitivirus*, *Betapartitivirus* and *Gammapartitivirus* (NIBERT *et al.*, 2014). Alphapartitiviruses and betapartitiviruses infect not only filamentous fungi but also plants, whereas gammapartitiviruses infect only filamentous fungi. In general, partitivirus infections are largely symptomless (GHABRIAL *et al.*, 2015).

Family: ***Chrysoviridae***

Penicillium chrysogenum virus (PcV) is the prototype of genus *Chrysovirus*, the only current genus in this family (GHABRIAL & CASTÓN, 2011). It has four monocistronic genome segments, 2.4–3.6 kbp in length and separately encapsidated in virus particles. dsRNA1 encodes the RdRp, and dsRNA2 encodes the major CP (GHABRIAL *et al.*, 2015).

Family: ***Reoviridae***

Genus *Mycoreovirus* was created to accommodate three species, Mycoreovirus1 (MyRV1) to Mycoreovirus3 (MyRV3). MyRV1 and MyRV2 were isolated from Cryphonectria parasitica, and MyRV3 from *Rosellinia necatrix* (HILLMAN & SUZUKI, 2004; WEI *et al.*, 2004).

All mycoreoviruses confer hypovirulence to their respective natural hosts. Mycoreovirus genome segments are monocistronic with 5′ caps on their positive strands. MyRV1 and MyRV2 have 11 genome segments (S1–S11) whereas MyRV3 has 12 segments, 0.7–4.1 kbp in length for each virus. (KANEMATSU *et al.*, 2004; GHABRIAL *et al.*, 2015).

Family: ***Narnaviridae***

Members of this family contain the simplest genomes of any autonomous RNA virus, each a single linear molecule of (+)ssRNA, 2.3–3.6 kbp in length and encompassing a single ORF that encodes the RdRp (HILLMAN & CAI, 2013; WICKNER *et al.,* 2013).

The family comprises two genera based on subcellular location. Members of genus *Narnavirus* have been found in the yeast *S. cerevisiae* as well as in the protistan water mold *Phytophthora infestans*, and are confined to the cytosol. Members of genus *Mitovirus*, in contrast, have been reported only in filamentous fungi to date and are localized to the mitochondria. Lacking a CP, their genomes are confined within intracellular lipid vesicles, as in the case of several other RNA viruses of "lower" eukaryotes including hypoviruses (GHABRIAL *et al.,* 2015).

Family: ***Hypoviridae***

Cryphonectria hypoviruses1 to 4 (CHV1 to CHV4) are currently grouped in sole genus *Hypovirus* within this family (NUSS & HILLMAN, 2011). They infect the chestnut blight fungus *C. parasitica*, where they cause hypovirulence. Infection of fungal mycelium is known to occur only through hyphal contact. Transmission rate through conidiospores varies greatly but could be as high as 100% in some cases, whereas transmission through ascospores is not known to occur. No true virions areas sociated with members of this family. Instead, pleomorphic vesicles containing viral RNAs and replication-associated proteins can be isolated from infected mycelia (GHABRIAL *et al.,* 2015).

The hypovirus genomes were originally thought to be dsRNA but are now considered to be (+)ssRNA, ~ 9–13 kbp in length excluding the 3′ poly(A) tail and encompassing two minimally overlapping ORFs in CHV1 and CHV2 but only one ORF in CHV3 and CHV4. CHV3 and CHV4 are also distinct in encoding a putative glycosyltransferase domain. Based on these differences as well as phylogenetic analyses, it seems proper to divide the Cryphonectria hypoviruses into two new genera: *Alphahypovirus* containing CHV1 and CHV2, and *Betahypovirus* containing CHV3 and CHV4 (GHABRIAL *et al.,* 2015).

2.3.2 Transmission of mycoviruses

Mycoviruses can be vertically transmitted via host spores. This transmission is mainly through asexual spores, but they can be also transmitted by sexual spores (ascospores and basidiospores). However, the sexual reproduction of some species (specially, of the phylum *Ascomycota*) constitute a barrier for the mycoviral transmission (ROMO *et al.,* 2007). Actually, some studies have shown that mycoviruses are more frequently transmitted through basidiospores than ascospores. (MCFADDEN *et al.,* 1983; CHUN & LEE, 1997; PEARSON *et al.,* 2009; TUOMIVIRTA *et al.,* 2009). Additionally, mycoviruses can be transmitted horizontally via hyphal anastomosis (Fig. 4). When a virus-infected strain contacts a virus-free strain, either hyphal anastomosis or an incompatibility response occurs (Fig. 3), and the incompatibility response often leads to programmed cell death (PCD), which limits the transmission of mycoviruses (CHOI *et al.,* 2012).

Although it was believed that mycoviruses are limited to individuals with the same or closely related vegetative compatibility groups, it was found that a vegetative-incompatibility between two fungal strains, a virus-infected strain and a virus-free strain, is not likely to be insurmountable barrier for mycovirus transmission in nature. Therefore, they were described few cases in which the host vegetative-incompatibility is supressed, but this case of mycovirus transmission is less frequent than transmission between vegetative-compatible individuals (GHABRIAL, 1998; XIE & JIANG, 2014). The degree of suppression depends on individual mycoviruses and their host. Most likely because of the attenuation of the vegetative-incompatibility reaction of host fungi, the co-infection of mycoviruses is a common phenomenon (XIE & JIANG, 2014).

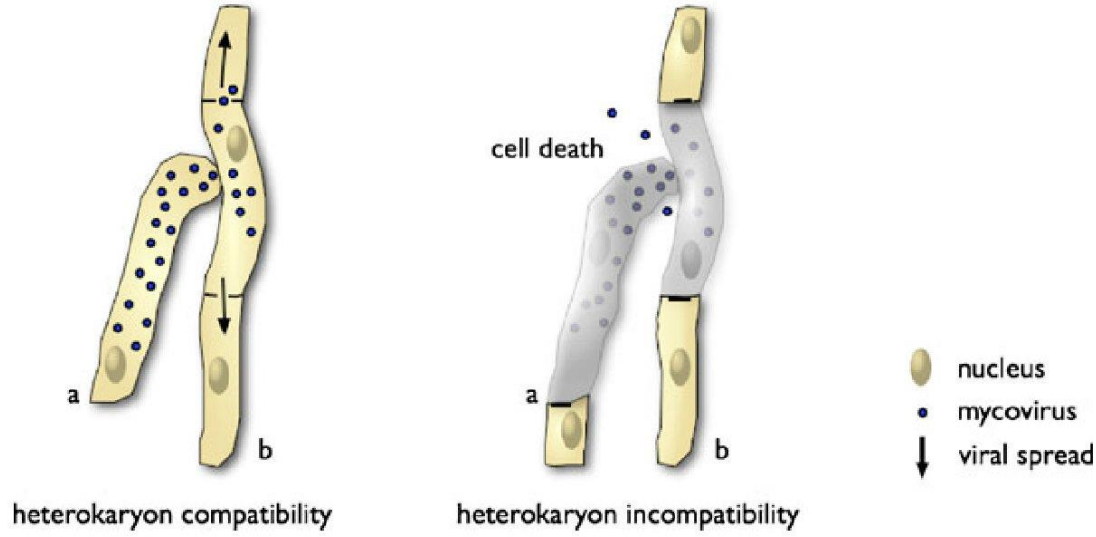


Fig. 4. Horizontal transmission of mycoviruses (VAN DE SANDE *et al.*, 2010)

The vegetative incompatibility could be considered as an antiviral defence mechanisms in fungi, but not only at the individual level, a defence mechanism can also occur at the population level, as well as at the cellular level (RNA silencing) (NUSS, 2011).RNA silencing probably arose as an ancestral surveillance system to protect against invading nucleic acids, including viruses of plants, insects, mammals and fungi. It is important to mention that some fungi, for example *Ustilago maydis*, seem to have completely lost the genes for the RNA silencing machinery, but these fungi do not seem to suffer unusually high incidence of virus-induced symptoms (NAKAYASHIKI & NGUYEN, 2008; NUSS, 2011).

2.3.3 Viral effect on the fungal host

Mycoviruses can change phenotypes of their hosts providing some advantages or deleterious effects to them, but normally mycovirus does not produce obvious symptoms on their hosts. Because of the lack of an infectivity assay and the frequent occurrence of mixed infections, there is a problem with assigning any fungal phenotypic change to a particular mycovirus (ROMAINE & SCHLAGNHAUFFER, 1995; MCCABE *et al.*, 1999; HOWITT *et al.*, 2006).

Symptomless or cryptic infections

Many investigators reject the possibility of the effect on fungal biology caused by mycoviruses, because they observe only symptomless infections, but it can happen that the virus might induce symptoms under some unexplored environmental

conditions. These symptoms that appear after altering some growth conditions of the fungi, for example the abundance of available nutrients, are termed cryptic. It is reasonable to assume that many mycovirus infections will have some slightly effect on growth, although many mycoviruses produce no obvious phenotypic changes on their hosts (BUCK, 1998; VAN DIEPENINGEN *et al.*, 2006; PEARSON *et al.*, 2009).

Detrimental infections

Some mycoviruses are able to negatively affect their hosts, this is the case of the La France disease of the commercial mushroom *Agaricus bisporus* (HOLLINGS, 1962; RO *et al.*, 2006) caused by La France isometric virus (LIV) (ROMAINE & SCHLAGNHAUFFER, 1995) which affects to the basidiocarp formation. Another example are oyster´s diseases (with similar symptoms the La France disease) caused by the Oyster mushroom spherical virus (OMSV) (YU *et al.*, 2003) and Oyster mushroom isometric virus (RO *et al.*, 2006). These diseases constitute one of the most economically important diseases caused by mycoviruses.

Hypovirulence

It was found that some mycoviruses reduce the ability of their fungal phytopathogenic hosts to cause disease in plants. This ability is referred as hypovirulence. This phenomenon is very attractive due to the importance of fungal diseases in agriculture and the limited strategies that are available to control them. This property is very original, since one "pathogen" can be used to control another one (NUSS, 2005). Hypovirulence of the chestnut blight fungus *Cryphonectria parasitica* caused by hypoviruses is the best known case of hypovirulence used in biological control (ANAGNOSTAKIS, 1982; NUSS, 2011).

Beneficial infections

Some mycoviruses have probably coevolved in parallel with their hosts to limited detrimental effects or to mutual benefit. According to this, some mycoviruses are linked with killer phenotypes in yeast and smuts that confer a strong advantage to their hosts in interference competition by encoding toxins to which they are immune, but which are lethal to sensitive cells. This killer phenomenon in the yeast (*Saccharomyces cerevisiae*) and in the smut fungus (*Ustilago maydis*) was discovered in 1960´s. Genetic and biochemical studies in yeast have conclusively shown that toxin

production and immunity are cytoplasmically inherited, and that dsRNAs of viral origin comprise the cytoplasmic determinants (GHABRIAL, 1998). And finally, one of the most remarkable and fascinating case within this category of viral effects is the case of the virus infecting the root endophytic fungus *Curvularia protuberata*, which confer heat tolerance to the panic grass *Dichanthelium lanuginosum* that hosts the fungal endophyte (MARQUÉZ *et al.*, 2007).

2.3.4 Origins of mycoviruses

Two main hypotheses exist which can explain origins and evolutionary history of mycoviruses: the ancient coevolution hypothesis and the plant virus hypothesis.

The ancient coevolution hypothesis proposes that these infections are ancient, coming from an unknown source, and have coevolved along with their hosts. It means that mycoviruses infected ancestors of theirs present fungal hosts and have evolved with them to give rise to present-day diversity (VOTH *et al.*, 2006). This long time of co-evolution could also explain the existence of asymptomatic phenotypes in many of the infections caused by mycoviruses. The case of symptomatic infections found in the phytopathogenic fungus *Cryphonectria parasitica* are hard to explain by this hypothesis, in fact, from this example arises another hypothesis for the explanation of mycovirus origin, the plant virus hypothesis.

The plant virus hypothesis proposes that the viruses have moved relatively recently from the host plant to the fungus, what means that evolution of mycoviruses and their fungal hosts is not probably congruent.

This theory is supported by a study of sequence comparisons between mycoviruses and plant viruses which revealed the relatedness of hypoviruses to several species of the ssRNA plant virus genus *Potyvirus* (FAUGUET *et al.*, 2005; LINDER-BASSO *et al.*, 2005). Actually, these studies show that a higher similarity exist between hypoviruses and plant viruses, than between hypoviruses and other asymptomatic fungal viruses. According to this, the hypothesis proposes that a common ancestral between hypoviruses and plant potyviruses existed, in the way that saprophytic or pathogenic fungi acquired ssRNA viruses from plants, and after they lost their protein capsid and evolved to dsRNA forms due to different evolution pressures (PEARSON *et al.*, 2009).

Both theories ('the ancient coevolution' and 'the plant virus' hypotheses) are needed to explain the full range of mycovirus diversity that is being revealed. It is also important to realize that not only these theories have to be true, but it is possible that mycoviruses have moved from their fungal host into the plant host acting like vectors (PEARSON *et al.,* 2009).

2.3.5 Mycoviruses infecting entomopathogenic fungi

In the last 20 years, it is gradually increasing the interest in the detection of mycoviruses in entomopathogenic fungi, due to the use of entomopathogenic fungi as part of the IPM, as mentioned earlier. The more information we will have about entomopathogenic fungi, the more effectively we will be able to use them. Till the present time, only few studies have been developed in mycovirus of entomopathogenic fungi research. *Beauveria bassiana* and *Metarhizium anisopliae* are among the most tested entomopathogenic fungi for the presence of virus-like dsRNA molecules (LEAL *et al.,* 1994; BOGO *et al.,* 1996; MELZER & BIDOCHKA, 1998; CASTRILLO *et al.,* 2004; DALZOTO *et al.,* 2006; HERRERO *et al.,* 2009; HERRERO *et al.,* 2012; YIE *et al.,* 2014).

The first research on the presence of dsRNA in *B. bassiana* was reported by Melzer and Bidochka in 1998. They found dsRNA elements in 2 out of 12 Canadian soil isolates. The presence of viral dsRNAs in *B. bassiana* were also detected in 7 out of 34 North American insect isolates (CASTRILLO *et al.,* 2004), and Dalzoto and collaborators detected dsRNA elements in 2 out of 13 Brazilian insect isolates (DALZOTO *et al.*, 2006). The most extensive research on the presence of dsRNA virus-like molecules in *B. bassiana* was made in Spain and Portugal were 40 out of 73 soil and endophytic isolates resulted positive for the presence of viral dsRNA (HERRERO *et al.*, 2012). The latest research was conducted by Yie and collaborators in 2014, in this study 8 out of 10 isolates from New Zealand were found harbouring dsRNA elements. Till the date, only three mycoviruses have been completely characterized infecting *B. bassiana*, two victorivirus and an unclassified virus related to *Partitiviridae* family (HERRERO *et al.*, 2012, YIE *et al.*, 2014 and KOTTA-LOIZOU *et al.*, 2015)

The presence of mycovirus and dsRNA virus-like molecules was also found in other species of entomopathogenic fungi as *Tolypocladium cylindrosporus* (HERRERO & ZABALGOGEAZCOA, 2011).

2.3.6 Applications and possible usages of mycoviruses

It is important to notice that mycovirology is a relatively new scientific field. That is the reason why only a small percentage of the possibilities of mycoviruses are known. Moreover, most of mycovirus research is focused in those who infect economically important fungi, such as yeasts, cultivated mushrooms and pathogens of plants and animals (PEARSON *et al.*, 2009).

Using mycoviruses to control crop disease

The usage of mycoviruses to control crop diseases started more than 50 years ago, when mycoviruses were first employed to control chestnut blight disease in Europe. Nevertheless, the use of mycoviruses against phytopathogenic fungi depends a lot on the crop or disease to be treated. In fact, the pathosystem that can be found among forests, orchards or agricultural differs a lot. In agricultural lands for example, the high crop density, low species diversity and unique environmental conditions, gives to the pathogen excellent conditions for its development, but at the same time, these conditions can also help mycoviruses to establish prevalence in the host populations in fields (XIE & JIANG, 2014).

Controlling crop fungal diseases with mycoviruses has also some disadvantages compared to the use of chemical insecticides. Whereas insecticides act relatively fast, mycoviruses need sufficient time for their establishment among the fungal population in the field, which is vital for a successful control of the fungal pest. Viral transmission between vegetative-incompatible individuals would make easier this establishment of mycovirus among the fungal population, but this needs more time and the efficiency of the proccess is very low. It should be pointed that the transmission between vegetative-incompatible individuals is very often impossible and it leads to PCD. (XIE & JIANG, 2014). However, Ikeda *et al.* (2013) recently found that it may be situations, where vegetative-incompatibility reaction is attenuated by amending chemical compounds. He found that zinc compounds could attenuate the heterogenic incompatibility of *Rosellinia necatrix.* Other ways to overcome fungal vegetative incompatibility could be to find mycoviruses with strong infectivity abilities that could be used as a universal donor, creating a kind of “vector” for mycoviruses (XIE & JIANG, 2014).

Future usage of mycoviruses as therapeutic agents in medicine

A thought to use viruses in medicine as biological control agents of human diseases is not so new, actually, since the early 20th century, it has been known that some viruses (bacteriophages) can specifically and uniquely search and destroy bacteria (SULAKVELIDZE *et al.*, 2001). Until the date, they have been conducted several studies on viruses for their development as therapeutic agents in medicine, e.g., a bacteriophage of vancomycin resistant *Enterococcus faecium* (BISWAS *et al.*, 2002).

Because of ongoing research based on the study of bacteriophages as therapeutic agents for bacterial infections in humans, it has been derived the idea that even mycoviruses would be able to suppress some medically important fungi (VAN DE SANDE *et al.*, 2010).

Invasive fungal infections are relatively common opportunistic infections in immunocompromised patients and are still associated with a high mortality rates. These infections are often accompanied by complications like resistance or refractoriness to current antimicrobial agents, so it is very important to find new therapeutic strategies based on the identification of new microbial targets and novel antimicrobial agents (VAN DE SANDE *et al.*, 2010).

One of these hypothetical therapeutic strategies may involve the use of mycoviruses that are able to selectively infect fungi, but such mycoviruses are not still identified (VAN DE SANDE *et al.*, 2010).

3. Objectives and aims

As it was showed in the introduction, viruses have been discovered in numerous fungal species, but unlike most known animal or plant viruses, they are rarely associated with deleterious effects on their hosts. *Beauveria bassiana* is one of the most studied species of entomopathogenic fungi; it has a cosmopolitan distribution and is used as a biological control agent against invertebrates in agriculture.

According to this, the main objectives of this work are to study the prevalence, variability, and patterns of distribution of virus like-dsRNA elements in a collection of soil isolates of the entomopathogenic fungus *B. bassiana* obtained at different locations and habitats in the Czech Republic.

4. Materials and methods

4.1 Fungal isolates

A total of one hundred thirty-seven isolates of the entomopathogenic fungus *B. bassiana* were analysed for the presence of dsRNA-virus like molecules. All analysed strains were isolated from different types of soils collected from cultivated and non-cultivated habitats in the Czech Republic (Fig. 5; Tab. 1). All the isolates analysed in this study belong to the collection of entomopathogenic fungi from the Faculty of Agriculture of the University of South Bohemia and from the Institute of Entomology of the Biology Centre of the Czech Academy of Sciences.

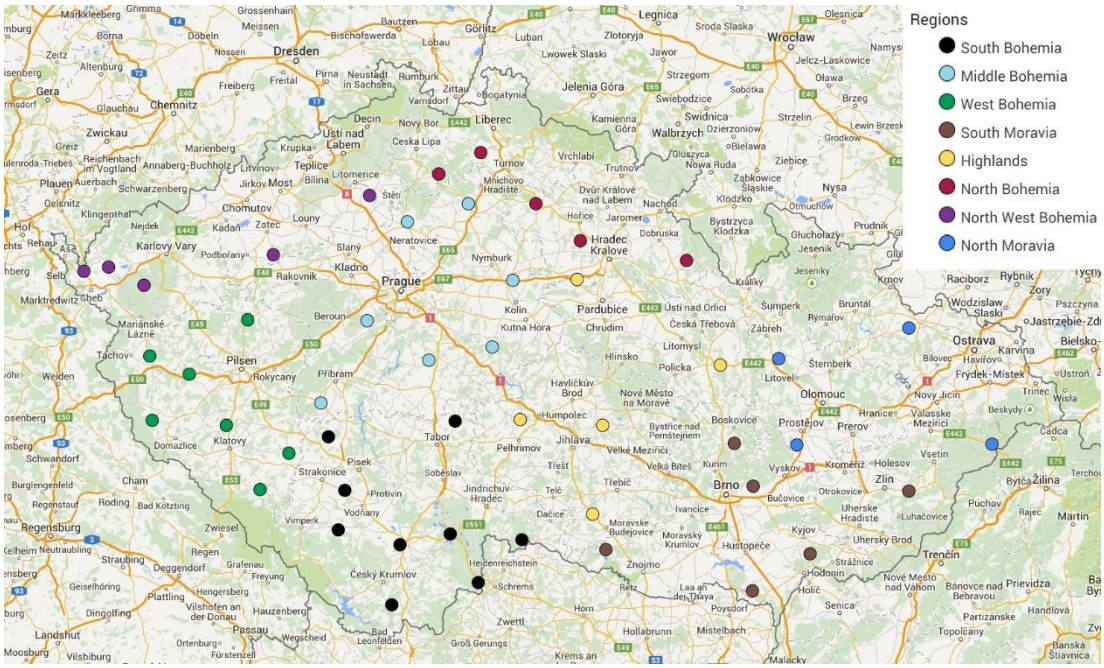


Fig. 5. Isolation points of *B. bassiana* strains analysed in the study.

Samples of *B. bassiana* were isolated from soil following 2 different methods:

Selective medium isolation method, this method is based on the ability of microorganisms to grow on specific artificial media whose composition have been developed in order to select certain groups of microorganisms. When using this method it is important to inhibit bacterial growth, mostly by the application of broad-spectrum antibiotic such as chloramphenicol, tetracycline or streptomycin (GOETTEL & INGLIS, 1997). In the present work, *B. bassiana* strains were isolated in selective medium containing dodine (SYLLIT 65 WP), and antibiotics (cyclohexamide and chloramphenicol).

Tab. 1. Soil isolates of *B. bassiana* collected in the Czech Republic and analysed for the presence of dsRNA virus-like molecules

Sample name	IMFS	Region	Locality	Habitat	Subhabitat	Sample name	IMFS	Region	Locality	Habitat	Subhabitat
NBBBA-1	SM	North Bohemia	Doksy	N	Mixed forest	SMBBA-15	TBM 15	South Moravia	Vranovská Ves	N	Conifers forest
NBBBA-2	SM	North Bohemia	Jičín	C	Meadow	SMBBA-16	TBM 15	South Moravia	Hlohovec	C	Plum crop
NBBBA-3	SM	North Bohemia	Jičín	N	Fallow land	SMBBA-17	TBM 15	South Moravia	Vacenovice	N	Hedgerow
NBBBA-4	SM	North Bohemia	Jičín	N	Hedgerow	SMBBA-19	GBM 15	South Moravia	Hostěnice	N	Mixed forest
NBBBA-5	SM	North Bohemia	Knežničky	N	Mixed forest	HBBA-1	SM	Highlands	Kasalice	C	Meadow
NBBBA-6	SM	North Bohemia	Nechanice	N	Mixed forest	HBBA-2	SM	Highlands	Kasalice	C	Cereal field
NBBBA-7	SM	North Bohemia	Jaroslav	N	Hedgerow	HBBA-3	SM	Highlands	Rohovládova Bělá	N	Mixed forest
NBBBA-8	TBM 15	North Bohemia	Doksy	N	Mixed forest	HBBA-4	SM	Highlands	Kasalice	N	Hedgerow
NBBBA-9	TBM 15	North Bohemia	Doksy	N	Mixed forest	HBBA-5	SM	Highlands	Koclířov	C	Cereal field
NBBBA-10	TBM 15	North Bohemia	Doksy	N	Mixed forest	HBBA-6	SM	Highlands	Koclířov	N	Hedgerow
SMBBA-1	SM	South Moravia	Vranovská Ves	N	Conifers forest	HBBA-7	SM	Highlands	Poděšín	N	Mixed forest
SMBBA-2	SM	South Moravia	Vacenovice	N	Hedgerow	HBBA-8	SM	Highlands	Poděšín	N	Hedgerow
SMBBA-3	SM	South Moravia	Hostěnice	N	Mixed forest	HBBA-9	SM	Highlands	Želetava	C	Cereal field
SMBBA-4	SM	South Moravia	Hostěnice	N	Mixed forest	HBBA-11	SM	Highlands	Želiv	N	Hedgerow
SMBBA-5	SM	South Moravia	Hostěnice	N	Mixed forest	HBBA-12	TBM 15	Highlands	Želiv	N	Hedgerow
SMBBA-7	SM	South Moravia	Hostěnice	N	Hedgerow	MBBBA-1	SM	Middle Bohemia	Skruhrov	N	Riverbank
SMBBA-8	SM	South Moravia	Hlohovec	C	Plum crop	MBBBA-2	SM	Middle Bohemia	Skruhrov	N	Riverbank
SMBBA-9	SM	South Moravia	Vizovice	N	Hedgerow	MBBBA-3	SM	Middle Bohemia	Čistá	C	Cereal field
SMBBA-10	SM	South Moravia	Vizovice	N	Meadow	MBBBA-4	SM	Middle Bohemia	Čistá	N	Hedgerow
SMBBA-11	SM	South Moravia	Vizovice	N	Hedgerow	MBBBA-5	SM	Middle Bohemia	Mělník	N	Mixed forest
SMBBA-12	SM	South Moravia	Rajec Jestřebí	C	Cereal field	MBBBA-7	SM	Middle Bohemia	Vlková	N	Mixed forest
SMBBA-13	TBM 15	South Moravia	Vranovská Ves	N	Conifers forest	MBBBA-8	TBM 25	Middle Bohemia	Mořina	C	Corn field
SMBBA-14	TBM 15	South Moravia	Vranovská Ves	N	Conifers forest	MBBBA-9	GBM 15	Middle Bohemia	Čistá	C	Cereal field

Sample name	IMFS	Region	Locality	Habitat	Subhabitat	Sample name	IMFS	Region	Locality	Habitat	Subhabitat
MBBBA-10	GBM 25	Middle Bohemia	Vatěkov	C	Meadow	NWBBBA-7	SM	North West Bohemia	Sádek	N	Mixed forest
MBBBA-11	SM	Middle Bohemia	Velký Osek	N	Meadow	NWBBBA-8	TBM 15	North West Bohemia	Krásno	N	Fallow land
MBBBA-12	SM	Middle Bohemia	Velký Osek	N	Mixed forest	NWBBBA-9	TBM 15	North West Bohemia	Libotenice	C	Hop field
MBBBA-13	SM	Middle Bohemia	Velký Osek	N	Mixed forest	NWBBBA-10	TBM 25	North West Bohemia	Horka	C	Rapeseed field
MBBBA-14	GBM 25	Middle Bohemia	Velký Osek	N	Mixed forest	WBBBA-1	SM	West Bohemia	Janov	C	Meadow
NMBBA-1	SM	North Moravia	Bílá	N	Conifers forest	WBBBA-2	SM	West Bohemia	Janov	C	Cereal field
NMBBA-2	SM	North Moravia	Staré Hamry	N	Conifers forest	WBBBA-3	SM	West Bohemia	Janov	N	Fallow land
NMBBA-3	SM	North Moravia	Slavkov	C	Cereal field	WBBBA-4	SM	West Bohemia	Vřeskovice	N	Mixed forest
NMBBA-4	SM	North Moravia	Mohelnice	C	Corn field	WBBBA-5	SM	West Bohemia	Drahotín	N	Mixed forest
NMBBA-5	SM	North Moravia	Stavenice	N	River edge	WBBBA-6	SM	West Bohemia	Bezděkov	N	Mixed forest
NMBBA-6	SM	North Moravia	Mohelnice	N	Hedgerow	WBBBA-7	SM	West Bohemia	Janov	N	Hedgerow
NMBBA-8	SM	North Moravia	Dětkovice	N	Hedgerow	WBBBA-8	SM	West Bohemia	Plasy	N	Mixed forest
NMBBA-9	TBM 15	North Moravia	Stavenice	N	Hedgerow	WBBBA-9	SM	West Bohemia	Plasy	N	Hedgerow
NMBBA-10	GBM 15	North Moravia	Stavenice	N	River edge	WBBBA-10	SM	West Bohemia	Sušice	N	Mixed forest
NMBBA-11	GBM 15	North Moravia	Mohelnice	N	Hedgerow	WBBBA-11	SM	West Bohemia	Velký Bor	N	Hedgerow
NMBBA-12	GBM 25	North Moravia	Stavenice	N	River edge	WBBBA-12	SM	West Bohemia	Drahotín	N	Hedgerow
NMBBA-13	GBM 25	North Moravia	Mohelnice	N	Hedgerow	WBBBA-13	SM	West Bohemia	Sušice	N	River side
NMBBA-14	GBM 15	North Moravia	Stavenice	N	Hedgerow	WBBBA-14	SM	West Bohemia	Sušice	N	Hedgerow
NWBBBA-1	SM	North West Bohemia	Františkovy Lázně	C	Meadow	WBBBA-15	SM	West Bohemia	Velký Bor	N	Conifer forest
NWBBBA-2	SM	North West Bohemia	Krásno	N	Fallow land	WBBBA-16	GBM 25	West Bohemia	Vřeskovice	N	Mixed forest
NWBBBA-3	SM	North West Bohemia	Krásno	N	Fallow land	WBBBA-17	GBM 25	West Bohemia	Drahotín	N	Hedgerow
NWBBBA-4	SM	North West Bohemia	Krásno	N	Hedgerow	WBBBA-18	GBM 25	West Bohemia	Lhota u Stříbra	N	Conifer forest
NWBBBA-5	SM	North West Bohemia	Želkovice	C	Apple tree field	WBBBA-19	GBM 15	West Bohemia	Drahotín	N	Hedgerow
NWBBBA-6	SM	North West Bohemia	Horka	N	Hedgerow	WBBBA-20	TBM 15	West Bohemia	Drahotín	N	Hedgerow

Sample name	IMFS	Region	Locality	Habitat	Subhabitat	Sample name	IMFS	Region	Locality	Habitat	Subhabitat
WBBBA-21	TBM 25	West Bohemia	Vřeskovice	N	Mixed forest	SBBBA-11	GBM 15	South Bohemia	Ostrov	C	Meadow
WBBBA-22	TBM 25	West Bohemia	Plasy	N	Hedgerow	SBBBA-12	GBM 15	South Bohemia	Ostrov	C	Corn field
WBBBA-23	GBM 25	West Bohemia	Švihov	N	Hedgerow	SBBBA-13	SM	South Bohemia	Prachatice	N	Conifer forest
WBBBA-24	TBM 15	West Bohemia	Vřeskovice	N	Mixed forest	SBBBA-14	SM	South Bohemia	Prachatice	N	Hedgerow
WBBBA-25	TBM 15	West Bohemia	Janov	N	Hedgerow	SBBBA-15	SM	South Bohemia	Mladá Vožice	C	Cereal field
WBBBA-26	TBM 15	West Bohemia	Sušice	N	Hedgerow	SBBBA-16	SM	South Bohemia	Šindlovy Dvory	N	Fallow land
WBBBA-27	TBM 15	West Bohemia	Velký Bor	N	Fallow land	SBBBA-17	GBM 15	South Bohemia	Sedliště	N	Hedgerow
WBBBA-28	TBM 25	West Bohemia	Janov	N	Hedgerow	SBBBA-18	GBM 25	South Bohemia	Ostrov	N	Mixed forest
WBBBA-29	TBM 15	West Bohemia	Velký Bor	N	Conifer forest	SBBBA-19	TBM 25	South Bohemia	Staré město pod L.	N	Hedgerow
WBBBA-30	TBM 25	West Bohemia	Velký Bor	N	Hedgerow	SBBBA-20	TBM 15	South Bohemia	Vahlovice	N	Hedgerow
WBBBA-31	GBM 25	West Bohemia	Janov	C	Cereal field	SBBBA-21	TBM 15	South Bohemia	Halámky	N	Mixed forest
WBBBA-32	GBM 25	West Bohemia	Plasy	N	Mixed forest	SBBBA-22	GBM 15	South Bohemia	Prachatice	N	Conifer forest
SBBBA-1	SM	South Bohemia	Sedliště	N	Hedgerow	SBBBA-23	TBM 15	South Bohemia	Ostrov	N	Mixed forest
SBBBA-2	SM	South Bohemia	Staré město pod L.	N	Conifer forest	SBBBA-24	TBM 15	South Bohemia	Prachatice	N	Conifer forest
SBBBA-3	SM	South Bohemia	Ostrov	N	Mixed forest	SBBBA-25	TBM 15	South Bohemia	Čeřín	N	Conifer forest
SBBBA-4	SM	South Bohemia	Sedliště	N	Mixed forest	SBBBA-26	TBM 25	South Bohemia	Ostrov	N	Mixed forest
SBBBA-5	SM	South Bohemia	Vahlovice	N	Hedgerow	SBBBA-27	TBM 25	South Bohemia	Čeřín	N	Conifer forest
SBBBA-6	SM	South Bohemia	Mladá Vožice	N	Hedgerow	SBBBA-28	GBM 15	South Bohemia	Halámky	N	Mixed forest
SBBBA-7	GBM 15	South Bohemia	Třeboň	C	Meadow	SBBBA-29	TBM 15	South Bohemia	Halámky	N	Mixed forest
SBBBA-8	GBM 25	South Bohemia	Sedliště	N	Hedgerow	SBBBA-30	GBM 15	South Bohemia	Dobrotín	N	Edge of river
SBBBA-9	SM	South Bohemia	Vahlovice	N	Mixed forest	SBBBA-31	GBM 15	South Bohemia	Halámky	N	Hedgerow
SBBBA-10	SM	South Bohemia	Halámky	N	Hedgerow						

NOTES: IMFS – isolation method from soil; SM – selective medium; TBM 15 and TBM 25 – *Tenebrio* bait method (isolation performed at 15 or 25°C), GBM 15 and GBM 25 – *Galleria* bait method (isolation performed at 15 or 25°C); N – non-cultivated; C – cultivated

Insect bait method, it is based on the ability of entomopathogenic fungi to infect an insect host. *Galeria melonella* and *Tenebrio molitor* are the most commonly used bait species (ZIMMERMANN, 1986; KLINGEN *et al.*, 2002). Both insect species were used for the isolation of *B. bassiana* in this work.

Obtained soil isolates of *B. bassiana* were cultured for 3 weeks over cellophane disks layered on top of potato dextrose agar (Sigma Life Science) Petri dishes (6 Petri dishes per isolate). Cultures were grown in incubators at 23°C for 3 weeks. After that, isolates were harvested and stored at -20°C.

4.2 Analyses of the presence of dsRNA

The presence of dsRNA molecules of sizes ranging from 1 to 14 kbp in fungal isolates was used as an indicator of virus infection. This type of nucleic acid can represent the genomes of dsRNA mycoviruses, as well as replicative forms of viruses with ssRNA genomes (MORRIS & DODDS, 1979). However, DNA viruses recently discovered in fungi, will not be detected by this technique (YU *et al.*, 2010).

This viral dsRNA purification procedure relies on the property of dsRNA to bind to cellulose (cellulose chromatography) when the concentration of ethanol in the buffer solution is 15%. DNA and ssRNA have lower affinity for cellulose at this ethanol concentration, allowing the selective purification of dsRNA. dsRNA is eluted from the cellulose with ethanol free STE 1X buffer. Isolated dsRNAs were subjected to agarose gel electrophoresis and visualized after staining with ethidium bromide. Electrophoresis is based on the different movement of charged molecules in an electric field. This way, negatively charged molecules, as DNA, RNA or proteins, migrate through the agarose matrix towards positively charged pole (anode) (YILMAZ *et al.*, 2012).

All positive dsRNA extractions were independently repeated twice.

Steps of the procedure

About 1 g of each analysed fungal isolate was pulverized with liquid nitrogen using mortar and pestle. After that, 4 ml of extraction buffer (compositions of all solutions can be find at Supplementary materials section), 0.1 ml of 60 mg/ml bentonite aqueous suspension, 2 ml of phenol (phenol/TRIS saturated solution for molecular biology, Acros Organics) and 2 ml of chloroform (Fisher Scientific) were added to the pulverized mycelium. The mix was homogenized with pestle,

transferred to a 12 ml plastic tube and centrifuged at 8000 rpm (Hettich Universal 320R) for 20 minutes at 4°C (all centrifugations were made at 4°C).

The upper aqueous phase was recovered in a new 12 ml tube, 1.5 ml of absolute ethanol were added and the volume was adjusted to 10 ml using STE 1X buffer. It means that the final ethanol concentration of the solution was 15%. After this, 0.2 g of cellulose (fibres (medium), Sigma Life Science) were added and the suspension was shaken for 5 minutes in a rotator (Multi Bio RS-24; BioSan).

After shaking, the suspension was centrifuged at 4000 rpm for 5 minutes. Then the supernatant was discarded and the cellulose was resuspended in 10 ml of STE 15 buffer, shook for 5 minutes, and centrifuged as before. After that, the supernatant was discarded again. This washing process with STE 15 buffer was repeated three times.

To elute the dsRNA from the cellulose, all remaining STE 15 buffer was removed with a pipette and the cellulose was resuspended in 2 ml of STE 1X (without ethanol). It was shaken for 5 minutes and centrifuged at 5000 rpm during 8 minutes. Then the supernatant containing dsRNA was transferred to a new 12 ml tube. The extracted dsRNA was precipitated with 2.5 volumes of cold ethanol at -20°C for at least 30 minutes.

To pellet the dsRNA, the tube was centrifuged at 8000 rpm for 20 minutes. The supernatant was discarded and the tube was dried. Then the pellet was resuspended in 20 µl of distilled and sterile water.

Finally, the extracted dsRNA was loaded and ran in a 1% agarose (Sigma Life Science) gel prepared with TAE buffer and stained with ethidium bromide to visualize the dsRNA. The agarose gel electrophoresis was carried out at a voltage of 200 V, 600 mA and 300 W (MS 500V Power Supply; Major Science). Most mycoviruses have genomes smaller than 10 kbp, so it was used a molecular weight ladder appropriate for this range (e.g. 1-10 kbp DNA ladder; GeneRuler^{TM} 1 kb DNA Ladder). dsRNA elements in gel were visualized in UV light transilluminator Slite 200 SW (Life Science Avegene).

4.3 Enzymatic treatment with DNase I and S1 nuclease

In order to prove the double stranded nature of the extracted RNA, and to eliminate possible traces of ssRNA or DNA that could have co-purified with the dsRNA, enzymatic treatments with Dnase I and S1 nuclease were performed.

DNase I degrades DNA and S1 nuclease degrades all single-stranded nucleic acids (DNA, RNA) (YU & MANLEY, 1986; KISHI *et al.*, 2001).

Steps of the procedure of the DNase I treatment of dsRNA

DNase I treatment of dsRNA was performed in 20 µl of reaction mixture. 5 µl of dsRNA, 2 µl of DNase I Buffer 10X, 5 µl of DNase (1u/ µl) and 8 µl sterile and distilled water were mixed in a 1.5 ml tube. The reaction was incubated at 37°C for 30 minutes. After that, a phenol-chloroform extraction was performed in order to remove enzyme rests. 180 µl of sterile and distilled water were added to the reaction and the extraction was carried out by adding 200 µl of phenol, gentle vortexing and centrifugation at maximum speed (MiniSpin®, Eppendorf) for 15 minutes. After that, the supernatant was removed and transferred into a new 1.5 ml tube. 200 µl of chloroform were added to the obtained supernatant from the previous step, vortexed gently and centrifuged at maximum speed for 5 minutes. The supernatant was carefully removed and transferred into a new 1.5 ml tube. The sample was precipitated with 2.5 volumes of absolute ethanol at -20°C overnight.

Steps of the procedure of the S1 nuclease treatment of dsRNA

Precipitated dsRNA from DNase I treatment was pelleted at maximum speed (Hettich Universal 320R) for 25 minutes and then the supernatant was discarded. The tube was dried and the pellet was resuspended in 10 µl of sterile and distilled water. Then the reaction mixture was prepared in a total volume of 15 µl containing 10 µl of dsRNA, 1.5 µl of S1 N Buffer 10X, 1 µl of S1 N (1u/ µl) and 2.5 µl of sterile and distilled water. The reaction was incubated at 37°C for 15 minutes. After that, a phenol-chloroform extraction followed by ethanol precipitation was performed as explained before. Precipitated dsRNA was centrifuged at maximum speed for 25 minutes, pellet was dried and resuspended in 10 µl of sterile and distilled water. Enzymatically treated dsRNA was loaded and ran in a 1% agarose gel prepared with TAE buffer (as it was explained before).

5. Results

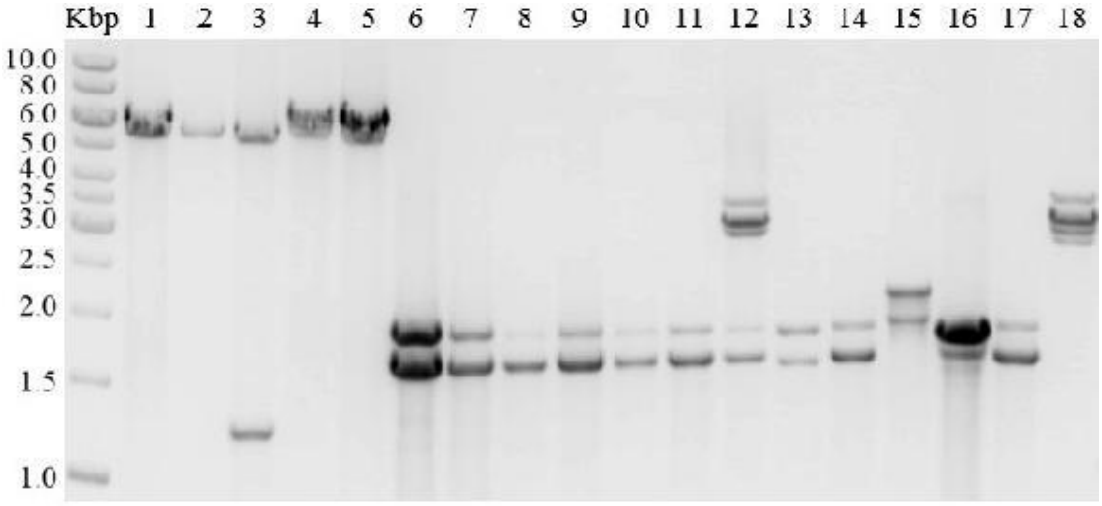
A total of 137 *B. bassiana* isolates collected from soils of the Czech Republic were analysed for the presence of dsRNA virus-like molecules, 31 isolates harboured dsRNA elements (22.6%). It was proved after the enzymatic treatments that all the nucleic acids observed in the gels were indeed dsRNA (Fig. 6a and 6b) (Electrophoretic patterns obtained before enzymatic treatments were placed in Supplementary Material section as Fig. 9a and 9b).

Regarding to the habitat where the fungal cultures were obtained, 5 (20%) of the 25 isolates from cultivated soils and 26 (23.2%) from the 112 isolates collected in non-cultivated soils contained dsRNA elements. Almost 55% (17 out of 31) of the isolates harbouring dsRNA elements were isolated from forests. It should be noticed that none dsRNA elements were detected in fungal strains isolated from fallow lands or river edges.

The majority of fungal isolates harbouring dsRNA elements came from the region of South Bohemia, 10 out of 31 (32.3%). Nonetheless, the region with the highest percentage of occurrence of isolates harbouring dsRNA elements was North Bohemia, since 50% (5 out of 10) of the isolates collected in this region were infected by dsRNA elements. On the other hand, only 1 of the 32 analysed fungal isolates from West Bohemia region harboured dsRNA molecules. None dsRNAs were found in any of the 10 isolates from North West Bohemia region (Fig.7).

Regarding to the method used for the isolation of the *B. bassiana* cultures tested in this study, it was found that 9 (15.3%) of the 59 fungal strains isolated by bait methods harboured dsRNA molecules; and 22 (28.2%) from the 78 strains isolated by selective media method contained dsRNA elements.

a)



b)

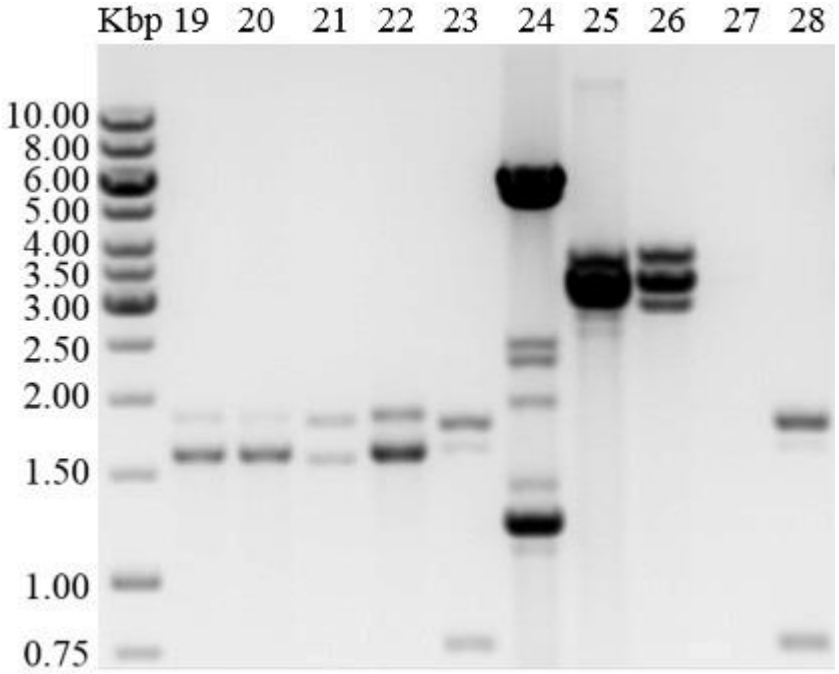


Fig. 6. a) Electrophoretic profiles of dsRNA elements present in several isolates of *B. bassiana* after enzymatic treatments. Lanes: 1, NBBBA-4; 2, HBBA-5; 3, HBBA-9; 4, MBBBA-11; 5, SBBBA-7; 6, NBBBA-5; 7, NBBBA-6; 8, NBBBA-8; 9, NMBBA-1; 10, MBBBA-4; 11, MBBBA-5; 12, SBBBA-1; 13, SBBBA-4; 14, SBBBA-5; 15, SBBBA-6; 16, SMBBA-3; 17, WBBBA-5; 18, SBBBA-2. b) Electrophoretic profiles of dsRNA elements present in several isolates of *B. bassiana* after enzymatic treatments. Lanes: 19, isolate NBBBA-1; 20, NBBBA-9; 21, MBBBA-13; 22, SMBBA-2; 23, SBBBA-25; 24, SBBBA-10; 25, NMBBA-4; 26, SMBBA-19; 27, NMBBA-9; 28, SBBBA-27. Lane Kbp contains molecular size markers, and the values on the left are sizes in kilobase pairs.

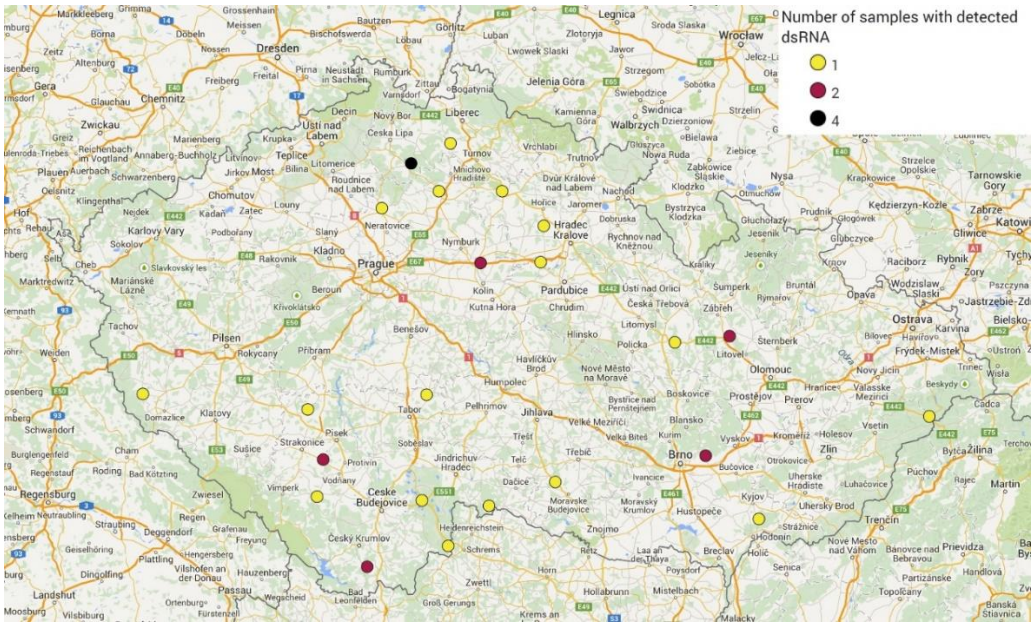


Fig. 7. Isolation points of *B. bassiana* cultures harbouring dsRNA virus-like molecules.

The diversity of dsRNA elements observed was high and 15 different dsRNA profiles were found among the infected isolates. Twenty six different dsRNA elements with estimated sizes ranging from 0.8 to 12 kbp were detected. The number of elements harboured by each fungal isolate varied from 1 to 7 (Fig. 6a and 6b; Fig. 8). It was found that some dsRNA elements were always or often together in different isolates: sets of three dsRNA molecules of 3.15, 3.35 and 3.75 kbp were detected in 4 isolates; and sets of two dsRNA molecules of 1.50 and 1.75 kbp occurred in 4 isolates; 1.55 and 1.80 kbp sets were detected in 9 isolates; 1.60 and 1.65 kbp occurred in 3 isolates; and 1.70 and 1.95 kbp sets were found in 3 isolates (Fig. 8).

dsRNA sets made up of two molecules, were detected in isolates coming from different parts of the Czech Republic but that were collected only from hedgerows and forests (this was the only concordance found between the type of habitat where the fungal host was isolated and the dsRNA patterns discovered).

On the other hand, the 5.8 kbp dsRNA element was found in isolates collected from diverse habitats, as hedgerow, forest, field or meadow all around the Czech Republic (Fig. 8, Tab. 1).

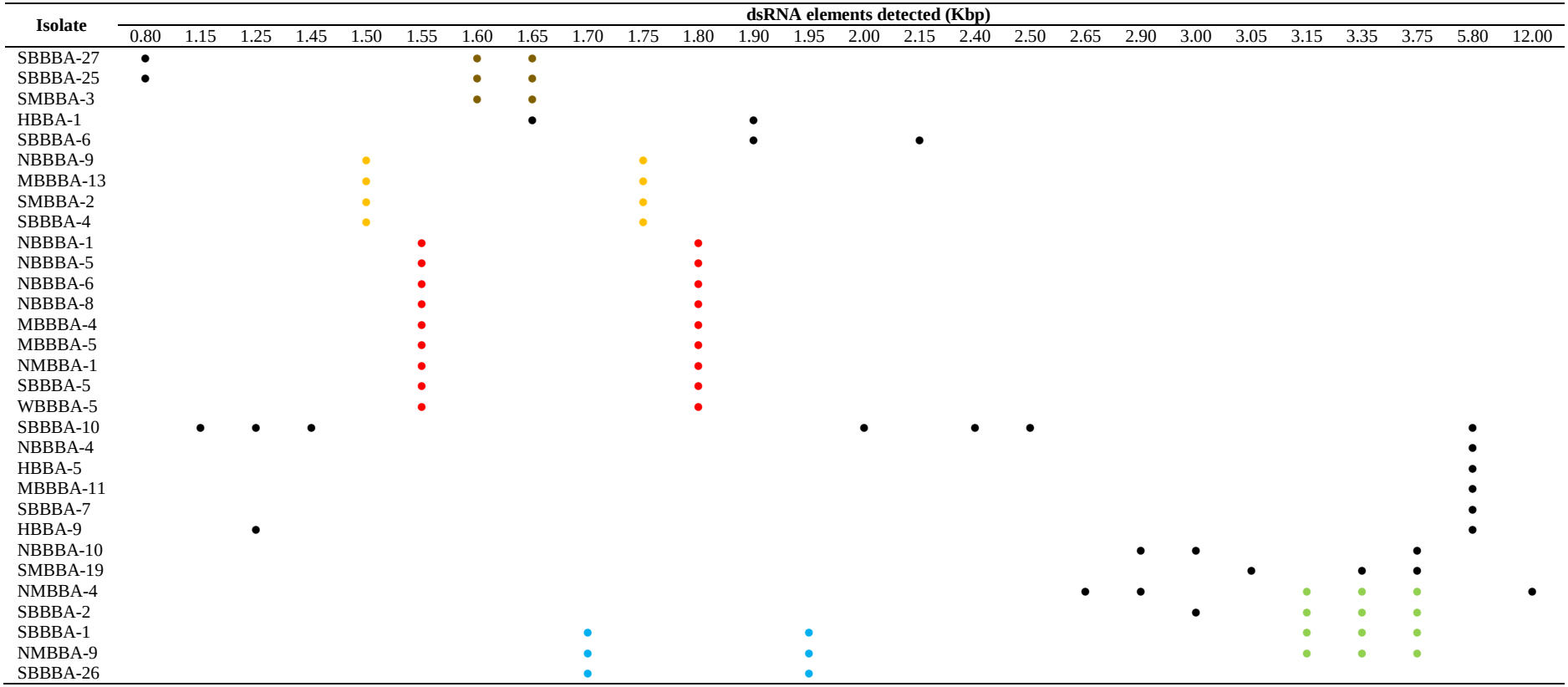


Fig. 8. dsRNA electropherotypes observed in soil isolates of *B. bassiana*. The circles indicate the presence in an isolate of a dsRNA molecule of the size shown at the top of each column. Similar sets of two or three dsRNA elements observed in different isolates are indicated by identical colours.

6. Discussion

The incidence of dsRNA virus-like molecules found in *B. bassiana* isolates collected in different parts of the Czech Republic indicates that mycovirus infections are common among *B. bassiana* soil isolates from this country. dsRNA elements were detected in 22.6% of the 137 analyzed isolates. Higher incidences were found in previous works, for example, in *B. bassiana* isolates from Spain and Portugal incidences of 54.8% were found (40 out of 73 isolates) (HERRERO *et al.,* 2012) and even higher in isolates from New Zealand 77.8% (7 out of 9 isolates) (YIE *et al.,* 2014). Nevertheless, other studies achieved lower incidences of dsRNA elements among *B. bassiana* isolates, Melzer and Bidochka (1998) found dsRNA presence only in 2 isolates of the 12 analysed in Canada (16.7 %); similar results were found in Brazil with 2 out of 13 isolates infected (DALZOTO *et al.,* 2006) and a 20.6% incidence was found by Castrillo *et al.* (2004) in isolates of *B. bassiana* from North America. Differences between the percentages of infected isolates could be explained by differences in the number of tested isolates, the habitat, or natural conditions of countries and regions from which isolates were collected.

Infected isolates were found in *B.bassiana* isolates coming from all around the Czech Republic, except from the North West Bohemia region where not infected isolates were found, or West Bohemia region where only one isolate harbouring dsRNA was detected. This finding could indicate the direction of propagation of the virus-like dsRNA molecules in the Czech Republic could have been from east to the west.

The prevalence of virus-like dsRNA molecules in *B. bassiana* isolates from cultivated soils was found slightly lower (20%) than in those from non-cultivated ones (23.2%), which shows that this feature does not seem to be related with the incidence of dsRNA elements in *B. bassiana.* Nevertheless, the majority (54.8%) of infected isolates coming from the non-cultivated areas were isolated from forests, which could be explained by the larger number of isolates analysed from this type of habitat. Herrero *et al.* (2012) evaluated the prevalence of dsRNA in *B. bassiana* strains isolated from soil or plants as endophytes and it was shown that the prevalence was lower (51.7%) in soil isolates than in the endophytic ones (66.7%). According to this, the niche from which the fungal host was isolated (soil, plant, insect…) could influence the incidence of dsRNA elements. Additionally, it was also

found a difference in the occurrence of dsRNA elements between *B. bassiana* strains isolated with bait methods (15.3%) and strains isolated with selective media (28.2%). It could indicate that some mycoviruses adversely affect the ability of entomopathogenic fungi to infect insects.

In this work, 15 different dsRNA profiles were observed among *B. bassiana* isolates, slightly less than in similar studies carried out in Spain and Portugal (19 different profiles observed), but significantly more than in studies by Melzer and Bidochka (1998), Dalzoto *et al.* (2006) and Yie *et al.* (2014), nonetheless, in these studies lower number of isolates were analysed. There were also detected twenty six different dsRNA elements in Czech *B. bassiana* isolates, this is the same number of different dsRNA elements detected in isolates from Spain and Portugal (HERRERO *et al.*, 2012). However, they detected more infected isolates, which could explain the greater variability of dsRNA elements obtained in that study. All the variability of dsRNA profiles obtained in the present work as well as in similar previous studies, could be probably influenced by the virus way of transmission among different isolates from different or the same compatibility groups. Additionally, by hyphal anastomoses, different rates of transmission of the different viruses could occur.

The estimated sizes of dsRNA elements detected in this work range from 0.8 to 12 kbp. This is the largest range detected among similar published studies in *B. bassiana*. Nonetheless, this difference comes mainly from the detected 12 kbp dsRNA element. This could correspond to the replicative form of a (+) ssRNA member of family *Hypoviridae* (monopartite genomes of 9-13 kbp) (ZHANG & NUSS, 2008). The most common dsRNA profile detected in *Beauveria* isolates from the Czech Republic is a set of two dsRNA molecules of 1.55 and 1.80 kbp detected in 9 of the 137 analysed isolates, which roughly corresponds the sizes of 2 dsRNA elements (1.6 and 1.80 kbp respectively) detected also in 9 *B. bassiana* isolates from Spain by Herrero *et al.*, (2012). Similar profiles to this one were found infecting other 8 isolates but with slightly different sizes of the dsRNA elements making up the sets: 1.60 and 1.65 kbp, 1.65 and 1.90 kbp, 1.70 and 1.90; 1.90 and 2.15 kbp. These profiles could correspond to members of the family *Partitiviridae* (bipartite genomes of 1.4-2.4 kbp) (NIBERT *et al.*, 2014). Another dsRNA element of approximately 5.8 kbp was detected in 6 of the analysed isolates, it could correspond to a member of the *Totiviridae* family (monopartite genomes 4.6-7 kbp) (GHABRIAL *et al.*, 2015). This 5.8 dsRNA element could be similar to any of the victorivirus infecting

B. bassiana that were recently sequenced and characterized (HERRERO *et al.*, 2012; YIE *et al.*, 2014). Four additional infected isolates showed a profile of a set of three dsRNA elements of 3.15, 3.35 and 3.75 kbp respectively. They could correspond to members of the *Chrysoviridae* family (genomes composed of 4 segments of 2.4 to 3.6 kbp) (GHABRIAL *et al.*, 2015). It could happen that the fourth segment was not able to be distinguished in the electrophoresis gel, since the sizes of the four segments of chrysovirus genomes are very close. According to this, thanks to the characteristics of the electrophoretic band patterns, like estimated size and number of the dsRNA elements, it can be hypothesized that *B. bassiana* isolates collected in the Czech Republic could harbor members of the viral families *Totiviridae*, *Partitiviridae*, *Chrysoviridae* and *Hypoviridae.* Nevertheless, complete or partial sequences of all the dsRNAs detected in this study would be necessary for their correct classification. Additionally, it is important to mention that dsRNA elements similar in size do not always have the same nucleotide sequence. It means that virus species diversity among Czech *B. bassiana* isolates could be higher than that estimated by dsRNA electropherotypes in this work.

The presence of mixed infections could be also inferred from the dsRNA patterns observed in this study; in some isolates, one or several dsRNA elements similar in size occurred, but in other isolates, those elements were accompanied by different dsRNAs. For example, the dsRNA element of 5.8 kbp which is alone in 4 of the infected isolates, it is found also accompanied by other 6 different dsRNA elements in SBBBA-10 isolate or by one different element in HBBA-9 isolate (Fig. 8). Similar mixed virus infections were also detected in *B. bassiana* isolates from Spain and New Zealand (HERRERO *et al.* 2012; YIE *et al.*, 2014) as well as in some isolates of entomopathogenic species *Tolypocladium cylindrosporum* and in other fungal species (HERRERO & ZABALGOGEAZCOA, 2011; GHABRIAL & SUZUKI, 2010).

A clear geographical or habitat distribution of isolates harbouring similar viral infections was not found, except for some sets of two dsRNA molecules that were hypothesised as possible members of family *Partitiviridae* (1.50 and 1.75 kbp, 1.55 and 1.80 kbp, 1.70 and 1.95 kbp) which were detected in isolates coming only from hedgerows and forests.

In the conditions that all *B. bassiana* isolates were cultured in the laboratory, not obvious phenotype associated with virus-infected isolates was observed, but as was

commented in the introduction, lack of obvious symptoms is common among virus-infected fungi. Nonetheless, it can happen that these viruses might induce symptoms under some unexplored environmental conditions. These effects that dsRNA virus-like molecules could produce in their *Beauveria* host were not tested in this study, but it could be really interesting for the future to assess how these dsRNAs affect *B. bassiana* biology in order to improve its use as biological control agent.

7. Conclusions

Based on the results of the analysis of the presence of virus like-dsRNA molecules in a collection of soil isolates of *B. bassiana* isolated from different locations and habitats of the Czech Republic, it can be concluded:

1. The incidence of dsRNA virus-like molecules found in the 137 *B. bassiana* isolates analysed in this study (22.6%) indicate that mycovirus infections are common among *B. bassiana* isolates from all around the Czech Republic.
2. dsRNA electrophoretic profiles of infected isolates indicated high virus diversity in Czech *B. bassiana* isolates, nonetheless, this diversity could be higher, since similar band patterns could correspond to different nucleotide sequences.
3. A clear geographical or habitat distribution of isolates harbouring similar viral infections was not found, except for some sets of two dsRNA molecules, which were hypothesised as possible members of family *Partitiviridae*, and occurred only in *B. bassiana* cultures isolated from forest of hedgerows.
4. Mixed viral infections occurred in several *B. bassiana* isolates.
5. Under the conditions of cultivation in the laboratory, *B. bassiana* isolates harbouring dsRNA virus-like molecules did not show any obvious phenotype due to this infection.

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9. Supplementary materials

Solutions

STE 10X (400 ml):

40 ml Tris-HCl 1M (tris base for molecular biology, Fisher Science),

80 ml NaCl 5M (sodium chloride, Fisher Science),

4 ml EDTA (edetic acid G. R., Lach-Ner),

276 ml sterile water

STE 1X: from STE 10X

BENTONITE (60 mg/ml) (80 ml):

4.8 g bentonite (bentonite, Acros Organics),

80 ml sterile water

SDS 10 % (80 ml):

8g SDS (sodium dodecyl sulphate, Fisher Science),

80 ml sterile water

Extraction buffer (50 ml):

10 ml STE 10X,

5 ml SDS 10%,

50 µl 2-mercaptoethanol (for electrophoresis, Fisher Science),

Sterile water to a final volume of 50 ml

STE 15 (200 ml):

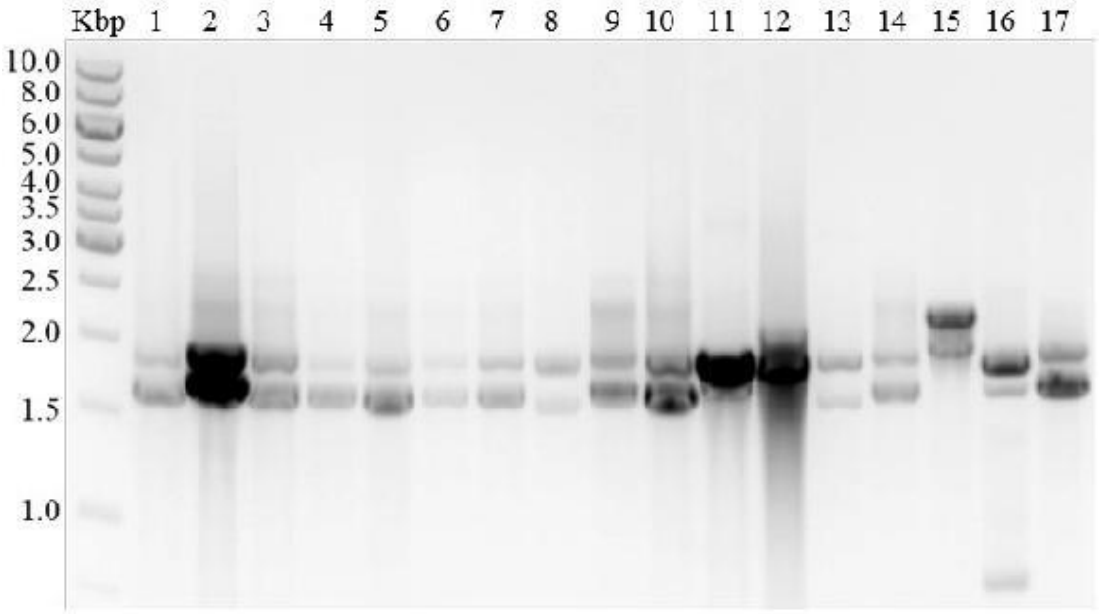
30 ml absolute ethanol (HPLC grade absolute ethanol, Fisher Science),

20 ml STE 10X,

Sterile water to a final volume of 200 ml

Pictures

a)



b)

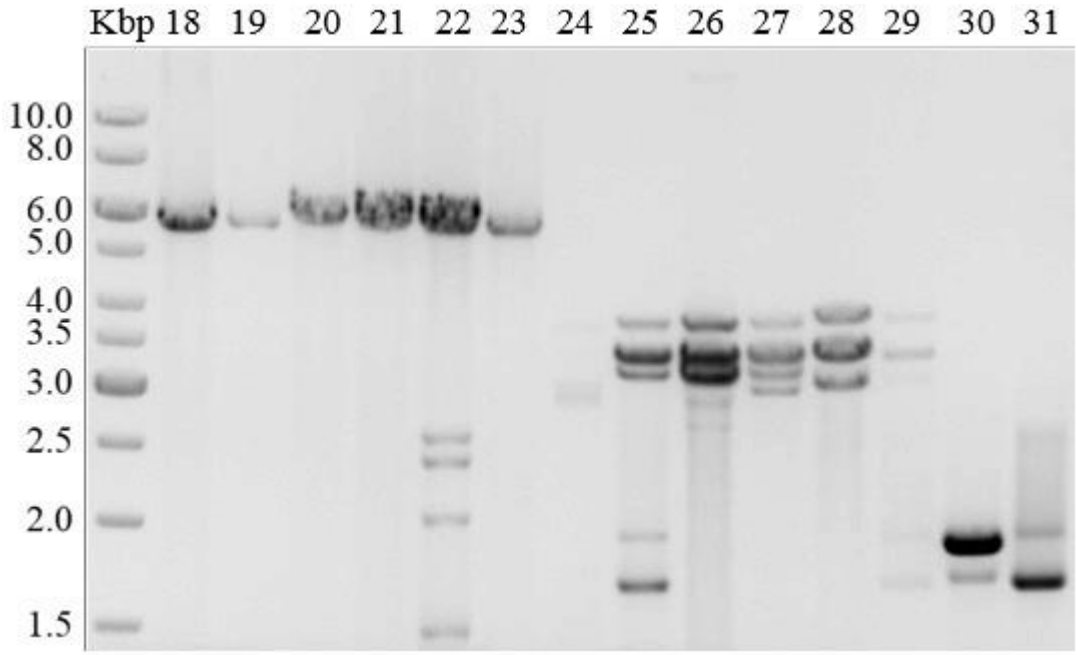


Fig. 9. a) Electrophoretic profiles of dsRNA elements present in isolates of *B. bassiana* before enzymatic treatment. Lanes: 1, isolate NBBBA-1; 2, NBBBA-5; 3, NBBBA-6; 4, NBBBA-8; 5, NBBBA-9; 6, MBBBA-4; 7, MBBBA-5; 8, MBBBA-13; 9, NMBBA-1; 10, SMBBA-2; 11, SMBBA-3; 12, HBBA-1; 13, SBBBA-4; 14, SBBBA-5; 15, SBBBA-6; 16, SBBBA-25; 17, WBBBA-5. b) Electrophoretic profiles of dsRNA elements present in isolates of *B. bassiana* before enzymatic treatment. Lanes: 18, isolate NBBBA-4; 19, HBBA-5; 20, MBBBA-11; 21, SBBBA-7; 22, SBBBA-10; 23, HBBA-9; 24, NBBBA-10; 25, SBBBA-1; 26, NMBBA-4; 27, SBBBA-2; 28, SMBBA-19; 29, NMBBA-9; 30, SBBBA-27; 31, SBBBA-26. Lane Kbp contains molecular size markers, and the values on the left are sizes in kilobase pairs.