Ph.D. THESIS

Viry obilnin přenosné Polymyxa graminis
Cereal viruses transmitted by Polymyxa graminis

DISSERTATION
Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Plant Protection Sciences,
Czech University of Life Sciences, Prague

By
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2011
"The great aim of education is not knowledge but action"

Herbert Spencer
Declaration

I declare that I have elaborated my dissertation work aimed at (Cereal viruses transmitted by Polymyxia graminis). I have used only the sources quoted in References.

This PhD thesis is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Protection Sciences, Czech University of Life Sciences, Prague, 2011.

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Prague, 2011

In Prague, date 13. 12. 2011, ..............................................
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I am grateful to my parents and my wife for their help and supporting me during all stages of the research work.

I thank also all the colleagues and technicians of the Department of Plant Protection for their assistance and friendship, that made me feel home.
ABSTRACT

Barley mild mosaic virus (BaMMV) and Barley yellow mosaic virus (BaYMV) that belong to Bymovirus genus, family Potyviridae, are causal agents of soil-borne mosaic disease in winter barley in the UK, China, Japan and several European countries, but they are not yet reported in the Czech Republic. This disease is economically important and in years particularly favourable for disease development is known to cause crop yield losses of up to 50-70%. Mechanical transmission of BaMMV into barley seedlings is generally inefficient and is the major constraint for testing cultivar resistance to the virus. Both viruses are transmitted to plants exclusively by a vector, Polymyxa graminis, which is a relatively poor characterised eukaryotic fungal-like micro-organism. It survives in the soil as resting spores (cystosori) containing the viruses within their protective ‘shells’. When the host plants are present and the environmental conditions are favourable, these spores release swimming zoospores which invade and develop in the roots of barley and some other cereal crops. During root invasion, the viruses are transmitted to healthy root cells from where they move upwards systemically into the shoots and leaves where they cause mosaic symptoms, general stunting of the crop and poor seed set.

There are no known chemical control agents against P. graminis, and control of BaYMV and BaMMV is only possible by deploying virus resistant cultivars. Thus, the cultivation of resistant varieties is the only way to prevent the considerable yield losses caused by the pathogen. Czech isolates of P. graminis may be able to transmit BaYMV and BaMMV.

To explore mechanical transmission, BaMMV-infected barley plants were grown at glasshouse conditions and used as inoculum sources to inoculate seedlings of susceptible winter barley cv. Florian. Extracts were prepared from BaMMV-infected Florian plant leaves at 30 and 60 days after symptom appearance (DASA) and grown at 12 to 14 °C. High infection rates were attained in addition of some compounds in inoculation buffer (potassium phosphate buffer 0.04 M). Especially addition of sodium diethyldithiocarbamate (Na-DIECA) was found to increase infectivity in mechanical inoculation of BaMMV as polyphenoloxidase inhibitor and chelator of copper. Inoculated plants showed visible symptoms after four weeks from inoculation, symptomatic plants were tested by ELISA (DAS) to confirm the results.

From the results of first experiment we observed, that the chemical additives Na-DIECA 0.01 M and Na-DIECA 0.001 M were more effective during the mechanical
inoculation than other chemical additives. For the previous reason we designed the second experiment of mechanical inoculation with potassium phosphate buffers 0.04 M and potassium phosphate buffers 0.04 M with additives (Na-DIECA 0.001 M and Na-DIECA 0.01 M) in different pH values (6.5, 7, 7.5 and 8). The obtained results confirmed, that the chemical additive Na-DIECA 0.001 M with pH value 8 was the most effective chemical substance for mechanical inoculation of BaMMV to susceptible barley plants cv. Florian.

For the monitoring of BaMMV and BaYMV in some localities in the Czech Republic, seventeen soil samples, which collected separately from different localities of cereal production (barley) during April 2008 using a digging fork were placed separately and planted to barley in pots in a greenhouse at 12-14 °C and fifty-six tested barley plant samples, planted into soil samples collected from different localities of sugar beet production during 1989 and 1999 were infected neither by BaMMV nor BaYMV.

From the obtained results, the preservation method with CaCl₂ was the best way for BaMMV-infected tissues preservation for a long-term (13 and 14 months).

Homogenization method of plant materials by the machine (RETsch Mixer Mills MM 400) was much easier and quicker than other used methods of homogenization for the reason of BaMMV detection by ELISA. Detection sensitivity using the classical ELISA method reported that BaMMV was detected using ELISA in the three greatest concentrations. A sample was considered positive for BaMMV when absorbance at 405 nm was more than three times the mean of the negative control.

Although the using of mortar and pestles with liquid nitrogen for homogenization of plant materials for RNA extraction is a labor intensive and it takes much time, it was the best method for homogenization of plant material. All tested RNA extraction methods, the isolation of RNA using phenol-chloroform isolation, isolation with GeneJET RNA kit and magnetic isolation were used for the detection of BaMMV in the infected leaves of barley, were on the bases of RT-PCR analysis to be reliable. The presence of BaMMV was confirmed in all samples analyzed.

Purity of RNA expressed as an A₂₆₀/A₂₈₀ coefficient ranged between 2.02 - 2.06 with the isolation method GeneJET RNA kit and isolation by magnetic method, while the method of phenol-chloroform isolation values ranged between 1.59 – 1.65. In terms of the time consumption and total steps during RNA isolation was evaluated as the optimal method for the isolation of total RNA using the magnetic method. The separated fragments of cDNA on the gel were so sharp and clear with the extraction method by magnetic isolation in comparison with other methods of extraction.
For the monitoring of *P. graminis* in some localities in the Czech Republic, seventeen soil samples from different localities of cereal fields (barley) with prediction of *P. graminis* occurrence were collected during April 2008 and fifty-six soil samples collected from different localities of sugar beet production during 1989 and 1999. All collected soil samples were planted to barley. The protist was identified as *P. graminis* on the basis of morphology of resting spores (cystosori) and sporangia and the size of individual cystosori (4 to 5 μm in diameter). Cystosori of *P. graminis* were observed in the roots of barley plants grown in 2 of the 17 soil samples and 20 of the 56 soil samples especially the samples from České Meziříčí. The presence of *P. graminis* in the roots of plants grown in the soil samples and the positive control sample versus the absence of the vector in roots of plants in the negative control soil was verified by PCR assay with DNA extracts. The method of extraction and homogenization of barley roots material by using RETSCH Mixer Mills MM 400 was more suitable for obtaining the DNA of *P. graminis* cystosori and the time consumption was shorter in comparison with the first method of extraction and homogenization.

To characterize the *P. graminis* isolates, the amplified PCR product (a DNA fragment of 472 bp) was sequenced and blasted for each of the samples that tested positive. These sequences were aligned with a known sequence (GenBank Accession No. AM259276) for *P. graminis*. The sequences from *P. graminis* on barley were 100 % homologous to the published sequence of *P. graminis* f. sp. temperata. To our knowledge, this is the first report of *P. graminis* f. sp. temperata in the Czech Republic.

The obtained results from the experiments of the sand culture indicated that all tested barley cv. Florian roots were parasitized by *P. graminis*, but were not infected by BaMMV after one and half month of growing. The chosen plants were tested by ELISA and RT-PCR. Later after more than three and half months we obtained clear symptoms of BaMMV with the seedlings of barley cv. Florian. The results confirmed that the Czech isolates of *P. graminis* are able to transmit the BaMMV.
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<th>Definition</th>
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<tr>
<td>A$_{260}$</td>
<td>UV in wave length 260 nm (Maximum absorption of nucleic acid)</td>
</tr>
<tr>
<td>AMV-RT</td>
<td>AMV Reverse Transcriptase</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Chl:Iaa</td>
<td>Chloroform-isoamylalcohol</td>
</tr>
<tr>
<td>CP</td>
<td>Capsid (Coat Protein)</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>cv.</td>
<td>Cultivar</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Dinucleotid tri phosphate</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double strand DNA</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double strand RNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>g</td>
<td>Times gravity</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton (molecular weight of proteins)</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>ME</td>
<td>Mercaptoethanol (2- Mercaptoethanol)</td>
</tr>
<tr>
<td>min.</td>
<td>Minute</td>
</tr>
<tr>
<td>MP</td>
<td>Movement protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
</tr>
<tr>
<td>Ribolock</td>
<td>Ribonuclease inhibitor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sec.</td>
<td>Second</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single strand DNA</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single strand RNA</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>Tab.</td>
<td>Table</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate-EDTA</td>
</tr>
<tr>
<td>TGB</td>
<td>Triple gene block</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-hydroxymethyl-aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Sustainable production of crop plants is essential for human development. The major challenges to fulfil the current and future needs of feeding the world population are the:

- Limited availability of arable land and pastures,
- Further increase in world population from the current 6.6 billion to as estimated 9.1 billion in 2050,
- Increasing consumption of meat in emerging nations with high populations like China, India and Brazil,
- Rapidly increasing demand for crop plants as sources of energy and industrial raw materials.

To meet this challenge agriculture in general and plant production in particular must be developed into sustainable systems, which achieve further increases in crop yield and improved product quality. Despite the achievements made over the last 50 years in protecting crop plants by improved chemical control, the development and cultivation of resistant cultivars and introduction of new production technologies, plant diseases still remain a major factor causing severe reductions in yield and quality. This can be attributed mainly to the general genetic flexibility of pathogens and their ongoing dissemination into new geographic regions by global trade and travel and also as a consequence of climatic changes. Small grain cereals, especially wheat and barley are of outstanding importance for global crop production. Like all cultivated plants they are affected by many diseases and are attacked by numerous pests.

*Barley mild mosaic virus* is one of two agents (the other is *Barley yellow mosaic virus*) responsible for yellow mosaic disease of barley. Both viruses are transmitted in soil by the plasmodiophorid protist *Polymyxa graminis*. The viruses occur, either separately or together, in autumn-sown barley and cause similar symptoms. Affected fields show yellow patches or appear entirely yellow, depending on the distribution of infested soil. The initial symptoms are irregularly distributed chlorotic streaks of varying size on the youngest emerging leaves, often associated with upward rolling of leaf margins. The streaks are most distinct on the youngest leaves, and develop into a mosaic pattern. The mosaic symptoms are occasionally associated with necrotic patches, yellow discoloration, and/or rapid death of the older leaves. At temperatures above 20 °C, new leaves are free from symptoms. The growth of infected plants is greatly diminished by temperatures in the range 12-14 °C, especially when this
happens under high soil moisture conditions. The virus has a narrow host range, limited to the Gramineae. The only known natural host is *Hordeum vulgare* (barley). The virus is transmissible by mechanical inoculation to *Hordeum* spp., *Aegilops* spp., *Eremopyrum hirsutum*, *Lagurus ovatus*, *Triticosecale* (triticale), *Secale cereale* (rye) and *Triticum durum*, but not to *Triticum aestivum* (wheat) or *Avena sativa* (oat). Vector transmission of the virus has been possible only to barley. The only practical way to control the disease is to grow resistant (immune) barley cultivars.

The information about the ability of Czech isolates of *P. graminis* to transmit BaMMV and/or BaYMV is essential for the growers of winter barley as in this case both viruses could easily spread in the country. More detailed knowledge of the biology and molecular interaction between soil-borne viruses, *P. graminis* and cereal crops is urgently required to help develop novel options for control or even to prevent these diseases before they become widespread. Cystosori of *P. graminis* in infested soils can persist for 20 years. *P. graminis* cannot be controlled once plants are infected, so methods to prevent transmission and limit spread are required. Cleaning cultivation equipment between fields will reduce the risk of the virus spreading by this vector.
2. REVIEW OF LITERATURE

Soil-borne viruses have been known for a long time and some for more than 60 years. This review will briefly summarize the results of research on some soil-borne cereal viruses, mechanical transmission, Polymyxa graminis as a vector and detection methods of P. graminis and Barley mild mosaic virus/Barley yellow mosaic virus. Based on current knowledge of the various pathosystems and considering the progress made in resistance breeding the question is discussed as to whether soil-borne viruses are a serious threat for cereal production now and in future.

Soil-borne viruses represent a group of particularly important pathogens (Table 1), which cause severe yield losses in cereal crops (Kühne, 2009). Under natural conditions all the viruses listed are transmitted to their host plants by the root-inhabiting plasmodiophorid P. graminis (Adams et al., 1988; Canova, 1966; Rao and Brakke, 1969; Slykhuis and Barr, 1978).

Table 1. Important soil-borne viruses of cereals (Kühne, 2009).

<table>
<thead>
<tr>
<th>Virus species</th>
<th>Acronym</th>
<th>Genus</th>
<th>W</th>
<th>B</th>
<th>T</th>
<th>R</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley yellow mosaic virus</td>
<td>BaYMV</td>
<td>Bymovirus</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley mild mosaic virus</td>
<td>BaMMV</td>
<td>Bymovirus</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil-borne wheat mosaic virus</td>
<td>SBWMV</td>
<td>Furovirus</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil-borne cereal mosaic virus</td>
<td>SBCMV</td>
<td>Furovirus</td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese wheat mosaic virus</td>
<td>CWMV</td>
<td>Furovirus</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Wheat spindle streak mosaic virus</td>
<td>WSSMV</td>
<td>Bymovirus</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat yellow mosaic virus</td>
<td>WYMV</td>
<td>Bymovirus</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Oat golden stripe virus</td>
<td>OGSV</td>
<td>Bymovirus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Oat mosaic virus</td>
<td>OMV</td>
<td>Furovirus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

W= wheat, B = barley, T = triticale, R = rye, O= oats.

*Rice necrosis mosaic virus* and *Sorghum necrotic spot virus* as soil-borne pathogens of the small grain cereal species rice and sorghum, respectively.

According to published literature.
2.1. Family Potyviridae

The Potyviridae is the largest of the plant virus families currently recognized. It contains almost 200 definite and possible members (about 20% of all classified plant viruses), many of which cause significant losses in agricultural, pasture, horticultural and ornamental crops. The family consists of 6 genera based on their transmission by protists (Bymovirus), whiteflies (Ipomovirus), aphids (Macluravirus, Potyvirus) or mites (Rymovirus, Tritimovirus) (Shukla et al., 1998). All members have filamentous particles 650–900 nm or 500–600 and 200–300 nm in length and 11–13 nm in width, made up of about 2000 units of a single structural coat protein surrounding a linear, single-stranded positive sense monopartite or bipartite RNA genome of 8,500–12,000 nucleotides with a poly (A) tail at the 3′-terminus and probably a genome-linked protein (VPg) at its 5′-terminus. The genome or genome segments are translated into polyproteins which are subsequently processed by virus encoded proteases into functional proteins. The viruses induce characteristic pinwheel or scroll-shaped inclusion bodies in the cytoplasm of the infected cells (Edwardson and Christie, 1996). These cylindrical inclusion (CI) bodies are formed by a virus encoded protein and can be considered as the unique phenotypic criterion for assigning viruses to the family (Ward and Shukla, 1991).

2.2. Bymoviruses

Bymoviruses have particles and cytopathologies similar to those of Potyviruses. However, they have their own different vectors. Bymoviruses, named after BaYMV, also affect cultivated grain crops and grasses. This genus includes e.g., Oat mosaic virus (OMV), Rice necrosis mosaic virus (RNMV), Wheat spindle streak mosaic virus (WSSMV), Wheat yellow mosaic virus (WYMV), Barley mild mosaic virus (BaMMV) and Barley yellow mosaic virus (BaYMV). They are soil-borne, transmitted by P. graminis. Each bymovirus consists of two different particles, one about 500 to 600 by 12 nanometres and the other 275 to 300 by 12 nanometers (Agrios, 2005).

They are mostly serologically related. Because WYMV and WSSMV show very similar features they may be considered as strains of the same virus (Usugi and Saito, 1976; Usugi et al., 1989), while BaMMV and BaYMV are considered as distinct viruses (Huth and Adams, 1990). The host range of bymoviruses is quite limited, although BaMMV has been mechanically transmitted to triticale (Kegler et al., 1985), rye (Ordon et al., 1992) and Triticum turgidum (Proeseler, 1993). Despite of the limited host range, these viruses are infectious to crops of major agronomic importance, i.e., wheat, barley, oats and rice.
Depending on the climatic conditions they cause high grain yield losses in susceptible crops, e.g., OMV in oats 66-78 % (Catherall and Boulton, 1969) and up to 100 % (Coffmann et al., 1962), WSSMV in wheat 3-87 % (Miller et al., 1992; Cunfer et al., 1988) or BaYMV/BaMMV in barley 50-100 % (Huth et al., 1984; Friedt et al., 1990).

Due to the transmission by the soil-borne organism P. graminis chemical measures against the diseases are neither efficient nor acceptable for economical and ecological reasons. Therefore, breeding for resistance is the only way to prevent yield reductions in infested growing areas. Since RNMV is limited to Japan and India (Gosh, 1981), the following considerations on resistance breeding will focus on the viruses present in Europe with special emphasis on the type member of bymoviruses, Barley yellow mosaic virus.

2.3. Distribution of soil-borne barley viruses

Barley yellow mosaic (BaYMV) and Barley mild mosaic (BaMMV) bymoviruses are vectored by the soil-borne protist P. graminis Led. (Toyama and Kusaba 1970) and have the potential to cause important damage to winter barley crops. BaYMV and BaMMV have been reported in Europe Germany, (Huth and Lesemann, 1978, Proeseler et al., 1984, Katis et al., 1997); United Kingdom (Hill and Evans, 1980), France (Lapierre, 1980), Belgium (Maroquin et al., 1982), The Netherlands (Langenberg and van der Wal, 1986), Hungary (Szunics et al., 1989), Italy (Rubies-Autonell et al., 1995), Ukraine (Fantakhun et al., 1987), Greece (Katis et al., 1997), Spain (Achon et al., 2003, 2005) and Bulgaria (Bakardijeva et al., 2004) and recently also Poland (Jeżewska et al., 2009). Huth et al., (1984) first described the occurrence of a mixture of two strains of BaYMV in field infected barley in the Federal Republic of Germany. The two strains were reported to differ in serology, buoyant density and mechanical transmissibility but not particle morphology or symptomatology. Strain BaYMV-M was mechanically transmissible to barley, the other, BaYMV-NM was not. These strains were also reported in the UK (Adams et al., 1987), East Asia (Ikata and Kawai, 1940), Japan (Kashiwazaki et al., 1990), South Korea (Kashiwazaki et al., 1989, Lee et al., 1996, 2006; Park et al., 2005a) and China (Chen et al., 1992), but these strains are now recognised as different viruses (Huth and Adams, 1990).

The results of a systematic survey of the occurrence of the yellow mosaic viruses of barley in Germany are presented schematically in Fig. 1.

Barley yellow mosaic virus and Barley mild mosaic virus in the Czech Republic have not yet been recorded.
2.4. Biological and serological properties of BaMMV and BaYMV

The first report of a yellow mosaic disease of barley was from Japan (Ikata and Kawai, 1940) and the causal agent was designated BaYMV. The biological (Miyamoto, 1958a, b) and, after successful virus purification, physical and serological properties have since been determined (Usugi and Saito, 1976). Analyses of 23 isolates of BaYMV collected from various sites in Japan distinguished six strains on the basis of pathogenicity to a number of barley cultivars. Despite the different biological behaviour all isolates were serologically indistinguishable (Kashiwazaki et al., 1989). In China the first recognized outbreak of BaYMV was in the 1950s and it caused serious losses in the mid-1970s. As in Japan indications of strain differences were obtained in screening and breeding programmes (Chen et al., 1992, 1996).

Whilst investigating the biological properties of the causal agent it became obvious that two viruses or virus strains are associated with the yellow mosaic disease and they clearly differ in their transmission rate after mechanical inoculation of barley plants (Friedt, 1983). Only one of the viral pathogens reacted with an antiserum against a Japanese isolate of BaYMV (Huth et al., 1984). Similar observations were made in the UK (Adams et al., 1987).

In Japan Kashiwazaki et al. (1989) concluded that the readily transmissible European isolate differs from the local isolates and therefore should be excluded from the taxon BaYMV. Additional results of Northern blot experiments (Batista et al., 1989) led to the suggestion that in Europe, in addition to BaYMV, a second virus, named BaMMV can induce
the yellow mosaic in winter barley (Huth and Adams, 1990). Soon after this first description the virus was also detected in Japan (Kashiwazaki et al., 1990), where two pathotypes could be discriminated. Pathotype Ka1 was serologically indistinguishable from a German isolate of BaMMV, while the second (Na1) was clearly different (Kashiwazaki et al., 1993).

The biological and serological properties of two Japanese Barley mild mosaic virus (BaMMV) strains (BaMMV-Kal and BaMMV-Nal) and a German BaMMV strain (BaMMV-M) were compared. Mechanical inoculation experiments showed that these three strains differed from one another in their ability to infect specific barley cultivars. BaMMV-Kal and BaMMV-M caused similar symptoms, but BaMMV-Nal clearly differed from them in its symptoms on some barley cultivars. The three BaMMV strains efficiently infected barley plants at 15 °C, whereas at 20 °C BaMMV-Kal and BaMMV-M also infected many plants but BaMMV-Nal infected only a few. BaMMV-Kal and BaMMV-M were indistinguishable by ELISA, while BaMMV-Nal was distinguished from both (Nomura et al., 1996).

Barley mild mosaic virus has a narrow host range, limited to the Gramineae. The only known natural host is Hordeum vulgare (barley). The virus is transmissible by mechanical inoculation to Hordeum spp., Aegilops spp., Eremopyrum hirsutum, Lagurus ovatus (Proeseler, 1988), Triticosecale (triticale) (Kegler et al., 1985), Secale cereale (rye) (Ordon et al., 1992) and Triticum durum (Proeseler, 1993), but not to Triticum aestivum (wheat) or Avena sativum (oat) (Ordon et al., 1992). Vector transmission of the virus has been possible only to barley. The virus is transmitted by the plasmodiophorid protist P. graminis. Zoospores released either from resting spores (cystosori) or from zoosporangia in barley roots, transmit the virus. The virus was detected by ELISA in extracts of dried roots heavily infected with cystosori and in zoospores liberated from the cystosori (Adams et al., 1988). Immunogold-labelled bundles of virus-like particles were observed in the cytoplasm of about 1% of zoospores liberated from plant roots and in zoospores inside zoosporangia (Chen et al., 1991). Resting spores remain viruliferous in dried roots and dried soil for several years (Huth, 1991; Chen et al., 1991). Transmission through seed has not been reported yet. BaMMV is moderately immunogenic. A rabbit polyclonal antiserum with a titre of 1/1280 in ring precipitin tests has been obtained. In double diffusion tests in 1% agar containing 0.5% lithium 3, 5-diiodosalicylate, the virus particles form a single precipitin band (Kashiwazaki et al., 1989). Immunosorbsent electron microscopy (ISEM), ELISA and tissue print immunoassay are useful for virus detection (Huth et al., 1984; Adams et al., 1987; Laing & Coutts, 1988; Huth, 1997).
The only host known for BaYMV is *Hordeum vulgare* (barley). Transmitted through infested soil, and (with difficulty) by inoculation of sap. The species of plants tested but not infected include *Triticum aestivum* (wheat), *Avena sativa* (oat), *Oryza sativa* (rice), *Chenopodium amaranticolor* and *Nicotiana tabacum* (tobacco). *Hordeum spontaneum* and *H. agriocrithon* were susceptible when planted in infested soil (Miyamoto, 1958b). Reports of successful mechanical inoculation of the virus to other species of *Hordeum* and to a few other species of the Poaceae (Proeseler, 1988) refer to what was then described as a strain of *Barley yellow mosaic virus* but which is now recognised as *Barley mild mosaic virus*. Serological properties are almost the same as mentioned above with the BaMMV. ISEM and ELISA are useful for routine diagnosis (Adams, 1991). Monoclonal antibodies have been prepared (Hariri et al., 1996a; 1996b).

### 2.5. Controlling of soil-borne barley viruses

The only effective means of controlling these viruses is through the use of resistant cultivars. Thus, the cultivation of resistant varieties is the only way to prevent the considerable yield losses caused by the pathogens (Ordon et al., 1996).

In Europe, resistant cultivars grown since the disease first appeared in the late 1970s have all possessed the same resistance gene, *ym4*, located on chromosome 3 (Graner and Bauer, 1993). Since 1988, a resistance-breaking strain, usually called BaYMV-2, has been reported from several European countries (Huth, 1989; Hariri et al., 1990; Adams, 1991).

### 2.6. Genome and coat protein of BaYMV

BaYMV has a single-stranded, positive-sense RNA genome with two components of approximately 7.6 and 3.5 kb. The nucleotide sequences of isolates from Japan (strain II-1, designated BaYMV-J) and Germany (BaYMV-G) have been determined (Kashiwazaki et al., 1990, 1991; Davidson et al., 1991; Peerenboom et al., 1992) and were found to have homologies of 93.6 % (RNA 1) and 90.4 % (RNA 2). Each RNA encodes a single polypeptide which is then processed into at least 5 (RNA 1) and 2 (RNA 2) products. The capsid protein is at the C-terminus of the RNA 1 polypeptide, while the RNA 2 products are a 28 - kDa protein with homologies to the helper component proteinase of *potyviruses* and a 70 - kDa protein of unknown function (Fig. 2).

Subsequently a German isolate of BaYMV-2 (designated BaYMV-G2) was cloned and compared by restriction mapping with the previously published data (Bendiek et al., 1993). There was a high level of restriction site conservation for RNA 1 and for the majority of RNA
2 but a region with several differences was detected at the N-terminus of the 70-kDa protein on RNA 2. The sequence of 621 nucleotides in this region was determined and, while most of the nucleotide differences were silent, a difference of 3 amino acids between BaYMV-G2 and BaYMV-G was predicted. The coat protein coding region on RNA 1 was also sequenced and two amino acid differences between BaYMV-G2 and BaYMV-G were predicted by Nong-nong Shi et al., (1995).

Fig. 2. Map of BaYMV genome and putative protein products showing the genome fragments (A-G) amplified and/or sequenced. CP coat protein. Other proteins with functions tentatively assigned from homologies with potyviruses are: CI, cytoplasmic inclusion; NIa, nuclear inclusion (proteinase); NIb, nuclear inclusion (polymerase); HCPro, helper component proteinase.

The bipartite genomes of both viruses consist of single-stranded, polyadenylated RNA. Each RNA encodes a polyprotein which is subsequently processed into several putative smaller proteins. The full-length sequences of both RNAs of BaYMV isolates from Japan and Germany have been determined (Kashiwazaki et al., 1990, 1991; Davidson et al., 1991; Peerenboom et al., 1992). For BaMMV RNA-1, only partial sequences of the 3'-terminal half have been published (Kashiwazaki et al., 1992; Foulds et al., 1993; Schlichter et al., 1993), while for RNA-2, the sequence of a German field isolate has been reported (Timpe and Kühne, 1994). Later, Peerenboom et al. (1996) reported the sequence of a UK isolate (UK-
M), which has been mechanically passaged for several years and which can no longer be transmitted by the its vector (Jacobi et al., 1995). Compared with the German field isolate, this lacked 1092 nucleotides in the 3'-terminal part of the genome. An RNA-2 from a mechanically passaged isolate with an apparently similar deletion has also been reported from France (Dessens et al., 1995). Peerenboom et al., (1996) presented the complete nucleotide sequence of RNA-2 of the protist-transmitted UK isolate of BaMMV from which the mechanically-transmitted isolate was derived and report the results of an intensive search for similarities between the encoded proteins of BaMMV, BaYMV and the furoviruses, which also have plasmodiophorid vectors.

2.7. Genome organization and coat protein of BaMMV

The schematic genome organization of bymoviruses, exemplified for BaMMV, is presented in Fig. 3. An isolate of BaMMV, which was derived from a wild-type isolate by repeated mechanical transmission over a period of more than 5 years, was found to have lost the ability to be transmitted by the fungal vector (Adams et al., 1988). Sequence analysis of the genome revealed that RNA2 lacked c. 1000 nt of its C-terminal protein (P2) gene. In contrast, RNA1 of the isolate showed a sequence identity of 99.3 % to RNA1 of a transmissible isolate, and the few resulting amino acid differences were thought unlikely to affect vector transmission (Peerenboom et al., 1997).

There are more reports on spontaneous deletions of different size in the P2 coding region as a result of the repeated mechanical passage of BaMMV (Dessens et al., 1995; Dessens and Meyer, 1995; Timpe and Kühne, 1994). Moreover, an RNA2 of reduced length was also detected in a field-grown plant (Cartwright et al., 1996). In parallel, maintenance of BaYMV on barley plants in the absence of P. graminis also led to isolates with deleted P2 coding regions (Kühne et al., 2003). These observations strongly suggested that the P2 protein is involved in fungus transmission of bymoviruses. However, only a few experimental data that demonstrate the significance of its integrity for the interaction with the vector have been published so far. The spontaneously deleted parts of RNA2 can differ in size and ranged from c. 50 to 1000 nt in different isolates. In all cases only the 3_ half of the P2 coding region was affected. So far no data exist on a possible correlation between the size of deletion and the efficiency of virus transmission by P. graminis. The reason for this is the extremely low protist transmission rate even of wild-type virus isolates to susceptible barley plants under controlled conditions.
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![Genome organization of BaYMV and BaMMV](image)

Fig. 3. Genome organization of *Barley mild mosaic virus* (BaMMV). P1, P2, P3, 7K1, 7K2 = viral proteins, VPg = genome-linked protein, CI = cytoplasmatic inclusion protein, NIa = protease, NIb-RNA polymerase, CP = coat protein.

BaYMV and BaMMV are identical in morphology. They contain two species of ssRNA (Huth et al., 1984). Determination of the complete nucleotide sequences indicated that RNA 1 and RNA2 of BaMMV (Dessens et al., 1995; Kashiwazaki, 1996; Meyer and Dessens, 1996; Subr et al., 2000; Timpe and Kühne, 1994) have similar genetic organization, but only a low level of sequence identity to the RNAs of BaYMV (Davidson et al., 1991; Kashiwazaki et al., 1990, 1991; Peerenboom et al., 1992). Thus, the two viruses are related but distinct members of the family Potyviridae.

2.8. Phylogenetic analysis

Phylogenetic analysis of nucleotide sequences demonstrated a clear separation between Asian and European isolates of BaYMV (Fig. 4). The corresponding analysis of the predicted peptide sequence of the P2 region also demonstrated a similar separation. There were some significant subgroupings revealed by the boot strap analysis. In particular, the isolates from Yangzhou, Rudong and Shanghai formed a distinct cluster in the P2 fragment analysis. However, in data from field experiments using a large number of barley cultivars, these three sites did not group together on the basis of cultivar response (Chen et al., 1996). It has already been shown that the variation in the P2 region of European BaYMV isolates is not linked to virulence on European cultivars (Shi et al., 1995).

Molecular differences between isolates from ten sites widely distributed in Eastern China were studied by sequencing RNA regions coding for the CP (RNA1) and the N-terminus of the P2 protein (RNA2). The P2 fragment was more variable than the CP, and phylogenetic analysis of both regions showed that Asian and European isolates formed distinct clusters (Chen et al., 1999).
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Fig. 4. Phylogenetic trees of the coat protein (top) and the P2 fragment (bottom) coding regions of BaYMV isolates obtained using NEIGHBOR analysis and displayed as an unrooted Phylogram from VIEWTREE. The values at the forks indicate the number of times out of 100 trees that this grouping occurred after bootstrapping the data. Bars show distances as substitutions per base according to Chen et al., (1998).

2.9. Mechanical inoculation and tests for resistance

The transmissibility of these viruses by mechanical transmission through finger rubbing or air spray is not always reliable and is cumbersome. Production of large quantities of inoculum with high virus infectivity to provide a continuous supply of inoculum of the same source is still a major problem. Previous studies showed that virus infectivity in infected
leaves was reduced by 20% after freezing (Adams et al., 1986b; Friedt, 1983). For these reasons, screening for resistance is commonly done in the field.

Field resistance tests however had some drawbacks. **First,** infection and symptom appearance is delayed in the field. The incubation period of virus when transmitted by *P. graminis* requires approximately 5-6 months before resistance evaluation can be assessed (Kashiwazaki et al., 1989). **Secondly,** field testing allows only one cycle of resistance test per growing season and that only winter season (Huth et al., 1984). **Third,** field resistance evaluation may vary from year to year due to changing environmental condition (Friedt, 1983). **Finally,** which is the most important of all is the problem of mixed infection (Kanyuka et al., 2003). Considering that BaMMV is transmitted by *P. graminis*, the resistance might not only be due to resistance to the virus but be due to resistance to the fungal vector, or both. Comparison of cultivar reactions by mechanical and natural transmissions would answer this question. At present, the only practical control measure is to grow resistant barley cultivars.

Groups of plants are inoculated with a series of dilutions of the inocula to be compared. The dilutions must span the range for which the test plants are neither all infected nor all healthy. Presence or absence of systemic symptoms is subsequently recorded. This procedure is known as a quantal assay, distinct from a quantitative assay based on local lesions. Various means have been used to improve the precision of quantal assays (Matthews, 1991). When plants are inoculated with small amounts of virus, systemic symptom development takes longer time than with heavy inocula. A record of the time taken for systemic symptoms to appear, combined by some arithmetic manipulation with the proportion of plant infected, may give increased precision to a quantal assay (Diener and Hadidi, 1977).

BaMMV is readily transmissible, while BaYMV infection rate is often very low (Adams, 2000), discouraging in adapting mechanical inoculation in testing for cultivar resistance. The reason of its difficulty in transmission by mechanical means is not well understood. Virus infection by mechanical means is affected with virus concentration, host susceptibility, stability of the virus, and environmental conditions. Several studies have been done on BaYMV focusing on these factors. Infection rates so far obtained for mechanical transmission of BaYMV was relatively low (below 50%) (Kashiwazaki, et al., 1989; So et al., 1997) however, if two times of inoculation were applied at 7 days interval, transmission rate was improved (Friedt, 1983).

In the case of WSSMV another member of Bymovirus genus showed that age of virus source and temperature are essential for virus transmission (Slykhuis, 1974). Jonson et al., (2007) reported that extracts from tillering stage of plants (approximately 78 days old) when
used as inoculum showed significantly higher transmission than extracts from elongated stem and heading stages. Also, growing the source plants at temperature above 15 °C decreased transmission efficiency. Field tests in South Korea showed that the barley cultivars Baegdong and Jinyangbori were susceptible (Park et al., 2005b) and Hopumbori was resistant (Hyun et al., 2006) to BaYMV. Seedlings were grown at 20 – 25 °C in a greenhouse and used for inoculation at the 3 - 4 leaf stage (approximately 3 weeks from sowing). The virus sources were collected at Haenam, South Korea and tested in ELISA for the presence of BaYMV, BaMMV and soil-borne wheat mosaic virus (SBWMV). Plants infected with BaYMV alone were selected and used as the inoculum source for mechanical inoculation of Baegdong seedlings. Inoculated seedlings were grown in a growth chamber controlled the temperature at 10 and 12 °C (10/12 °C) for 12 hours each under 2000 and 500 lux, respectively. Inoculum preparation for BaMMV (Jonson et al., 2006) was also applied for BaYMV.

2.9.1. Modified inoculation technique

The extracts were thoroughly shaken to achieve even distribution of carborundum and inoculated to test seedlings using stick with gauze (SWG) method. SWG method is a modified inoculation technique making used of gauze rolled in a wooden chopstick and was applied by leaf rubbing (Jonson et al., 2006). Inoculated seedlings were maintained in the growth chambers. In each test, 20 seedlings per cultivar were inoculated and experiment was repeated twice. At 45 days after treatments the inoculated seedlings showing typical BaYMV symptoms on the leaves were further tested by RT-PCR using primer pair S13 and S14 designed by Lee et al. (1998).

2.9.2. Reducing agents and substances protecting against phenolic compounds

Reducing agents such as sodium sulphite, sodium thioglycollate, 2-mercaptoethanol, or cysteine hydrochloride are frequently added to extraction media. These materials assist in preservation of viruses that readily lose infectivity through oxidation. They also may reduce adsorption of host constituents to the virus. Phenolic materials may cause serious difficulties in the isolation and preservation of viruses. Several methods have been used more or less successfully to minimize the effects of phenols on plant viruses during isolation. Cysteine or sodium sulphite added to the extraction medium both probably act by inhibiting the phenol oxidase and by combining with the quinine (Pierpoint, 1966).

Polyphenoloxidase is a copper-containing enzyme. Two chelating agents with specificity for copper, diethyldithiocarbamate and potassium ethyl xanthate have been used to
obtain infectious preparations of several viruses (e.g., *Prunus necrotic ring spot Ilavirus*; Barnett and Fulton, 1971). Materials that compete with the virus for phenols have sometimes been used. For example, Brunt and Kenten (1963) used various soluble proteins and hide powder to obtain infective preparations of swollen shoot virus from cocoa leaves. Synthetic polymers containing the amide link required for complex formation with tannins have been used effectively to bind these materials. The most important of these is polyvinyl pyrrolidone (PVP). On the other hand, Takanashi *et al.*, (1967) indicated that low infectivity of BaYMV and *Wheat yellow mosaic virus* (WYMV) by mechanical inoculation was due to the action of polyphenol oxidase. High infection rates were attained in addition of potassium cyanide (KCN) in extraction buffer. While in another study, addition of phenylmethylsulfonyl fluoride (PMSF) was found to increase infectivity in mechanical inoculation of BaYMV-2 (Kuntze *et al.*, 2000). Consequently, in an effort to increase BaYMV transmission by mechanical means, Jonson *et al.*, (2007) are reporting effects of age of source plants grown at varying temperatures. Also, effects of enzyme inhibitors such as KCN and PMSF added in the inoculum were further confirmed.

In brief, BaYMV infected leaves showing symptoms such as elongated chlorotic streaks were harvested, cut into small pieces, and then ground vigorously using mortar and pestles with 4 times in weight of 0.04 M phosphate buffer (pH 7.0) containing 0.001 M KCN. Extracts were filtered through double layered gauzes, transferred to falcon tubes, and mixed with carborundum (600 mesh) at a rate of 1 mg/ml.

### 2.10. Long-term preservation of virus-infected tissues

The use of cooling to prolong the survival of isolated tissues depends on the effect that a reduction in temperature has an effect on chemical reaction rates and hence on metabolism, thereby reducing the demand for oxygen and substrates. Unfortunately, however, cooling to temperatures above 0 °C does not provide adequate storage periods for many practical purposes, and there are no common cells for which long-term preservation can be obtained in this way. There are many reasons why this is so: metabolism does not cease at 0 °C, nor are all reactions slowed to the same degree; consequently, inter-related metabolic pathways may be 'dislocated' by cooling.

Storage of virus isolates: Short term storage (minutes or hours) during experiments in the laboratory or glass house is normal at or close to 0 °C. This is usually accomplished by plugging the tube containing the virus in an ice-bucket. If the virus isolates are continually
sub-cultured in the laboratory/green house host plants, problems may occur. For long term it is bedevilled with several problems:

(1) The virus may become contaminated by another virus this can occur in the glasshouse by insect transmission or even by plants touching or rubbing together.

(2) Secondly, mutation or attenuation of the isolate may occur, with the progressive selection of a typical strain during sub-culture.

(3) Thirdly, the isolate may be lost through death of the host plant.

(4) Finally, the propagation host may occupy valuable glasshouse space over long periods of time (NOUN)

Methods of long-term storage of virus isolates: To maintain isolates in their original uncontaminated condition, the following methods are adoptable

(1) Dry the virus-infected leaves rapidly over calcium chloride (CaCl$_2$). The dried materials may then be ground to a powder and stored (McKinney and Silber, 1968; Bos, 1969)

(2) A more efficient method that is quite effective for some, but not all viruses is to freeze-dry (Lyophilisation) infected leaves in the presence of glucose and peptone. A suitable method is to add 0.7 % (w/v) of D-glucose and peptone to filtered sap in a glass ampoule (Hollings and Stone, 1970). After lyophilisation the ampoule is covered and stored at a room temperature.

(3) Lyophilised small samples of infected leaves are stored in an ampoule or a plastic bag without grinding or the addition of other chemicals (Walkey, 1991).

(4) Preservation in glycerol at 4 to 5 °C.

2.11. Plasmodiophorid taxonomy

After more than a century of research on plasmodiophorids, their systematic affinities are still a matter for debate. Traditionally considered as fungi, analysis of their small subunit rDNA sequences has shown that they are a monophyletic group that is not closely related to the true Fungi or to other zoosporic plant parasites (the straminipiles, which include the oomycetes and the chytridiomycetes). Their most appropriate classification appears to be within the protists, a highly diverse group of eukaryotic organisms. They seem to be most closely related to the Phagomyxida (Phagomyxa sp.) and Maulinia ectocarpii, parasites of diatoms and filamentous algae (Bulman et al., 2001; Maier et al., 2000).

There are more distant relationships to the chlorarachneans and sarcomonads, e.g. Chlorarachnion sp., Cercomonas sp., Heteromita globosa, Paulinella chromatophora and
Euglypha rotunda (Bulman et al., 2001; Cavalier-Smith, 2000; van de Peer et al., 2000). Plasmodiophorid taxonomy and morphology have been most recently reviewed by Braselton (1995) and Dick (2001). They have been classified in the order Plasmodiophorales (Plasmodiophorida), and family Plasmodiophoraceae (Plasmodiophoridae) and a total of 10 genera are recognized: Polymyxa, Spongospora, Plasmodiophora, Ligniera, Membranosorus, Octomyxa, Sorodiscus, Sorosphaera, Tetramyxa and Woronina. P. graminis and several other species of the first three genera are of significant agronomic importance. For example Plasmodiophora brassicae causes the important club-root disease of brassicas, whilst Spongospora subterranea is the agent of powdery scab of potato and is also a virus vector.

Within the genus Polymyxa, two species have been recognized largely on the basis of host range. P. graminis primarily multiplies in grass and cereal species while P. betae is a parasite of species in the family Chenopodiaceae and some related plants, and is also a virus vector, e.g. Beet necrotic yellow vein virus causing rhizomania disease in sugar beet.

2.12. Plasmodiophorid distinctive features

All plasmodiophorids share the following distinctive features: (i) an unusual ‘cruciform’ type of nuclear division whereby the nucleolus is elongated perpendicularly to the plane of the metaphase chromatin (Braselton, 1995); (ii) zoospores with two anterior whiplash flagella of unequal length; (iii) multinucleated plasmodia (protoplasts); (iv) obligate, intracellular parasitism; and (v) resting spores ‘cysts’ (Fig. 5) that can survive for many years in various environments (Braselton, 1995).

2.13. Polymyxa graminis a vector of soil-borne barley viruses

P. graminis is worldwide in distribution and primarily multiplies in the roots of grass and cereal species. It is an obligate, intercellular parasite with thick-walled resting spores and motile primary and secondary zoospores. The organism provides protection for the viruses against unfavourable environmental conditions and during long rotations of non-host crops, and allows the viruses to persist almost indefinitely once a field has become infested (Adams et al., 1993; Braselton, 1995; Kanyuka et al., 2003; Linford and McKinney, 1954). In the presence of a susceptible host and near saturated soil moisture conditions, resting spores each release one primary zoospore, which moves actively toward the host and enters root hair cells. If the plant is infected, P. graminis acquires the viruses into these cells (Campbell, 1996). The virus particles are thought not to be attached to the surface of the spores but are incorporated in resting spores and zoospores (Rao and Brakke, 1969). The question of whether spores contain the viruses as intact virions or in intermediate form (e.g., infectious nucleoprotein) has
not yet been answered conclusively. Particles of the bymovirus BaMMV have been detected by electron microscopy inside zoospores and zoosporangial plasmodia, but this could not be demonstrated for resting spores (Chen et al., 1991). Driskel et al., (2004) recently observed accumulation of the RNAs of the furovirus *Soil-borne wheat mosaic virus* (SBWMV) in resting spores from virus-infected plants. The researchers were also able to detect the viral movement protein (MP), but not the coat protein (CP). Based on these data they conclude that *P. graminis* might not transmit SBWMV virion particles to host cells, but a ribonucleoprotein complex consisting of SBWMV MP and RNA. Because in roots infected with the bymovirus *Wheat spindle streak mosaic virus* (WSSMV) the viral CP was detected in resting spores. The mechanism for transmission of WSSMV and bymoviruses generally may be unrelated to that of SBWMV and other furoviruses. *P. graminis* is non-pathogenic, but has the ability to acquire and transmit a range of plant viruses which cause serious diseases in cereal crop species and result in significant yield reductions. The viruses are protected from the environment within *P. graminis* resting spores (‗cysts‘) that may remain dormant but viable for decades (probably until a suitable host plant is encountered). The persistent, soil-borne nature of these diseases makes the use of virus-resistant crop varieties currently the only practical and environmentally friendly means of control.

A serious ‘mosaic-like leaf mottling’ or ‘rosette disease’ of winter wheat was first reported from the USA in the early 1920s (McKinney, 1923a). It was noticed that the causal agent of this disease appeared to be carried over from year to year in the soil, and that soil treatments with formaldehyde or steam prevented infection (McKinney, 1923b). In 1925, McKinney demonstrated that this disease could also be transferred from diseased to healthy wheat in infectious sap by needle-pricking inoculation, proving that it is caused by a virus, now named *Soil-borne wheat mosaic virus* (SBWMV). It was not clear, however, how this virus could survive in soil for many years and more importantly how it gained an entrance to the root cells. Various soil organisms and microorganisms such as nematodes, fungi and bacteria were considered as possible carriers of SBWMV. It took more than 40 years from the first report of disease to establish a correlation with the presence of *P. graminis* (Canova, 1966; Estes and Brakke, 1966; Rao, 1968). It was only in 1969a that Rao and Brakke demonstrated in controlled laboratory conditions that *P. graminis* grown in roots of plants mechanically inoculated with SBWMV could acquire the virus and transmit it to healthy plants. At present SBWMV is considered as one of the most important diseases in winter wheat in central and Eastern USA, because it can practically destroy an entire crop of a susceptible variety.
2. REVIEW OF LITERATURE

*P. graminis* is now recognized as a vector for many other plant viruses (Table 2) that belong to at least three separate genera, and are serious pathogens of several cereal crop species (Kanyuka et al., 2003). For example, the winter barley disease caused by *Barley yellow mosaic virus* (BaYMV) and/or *Barley mild mosaic virus* (BaMMV) is widespread in Europe, Japan and China where it is of great concern to farmers and the agricultural industry. Yield losses of > 50% may occur when susceptible barley varieties are grown on severely infested soils (Plumb et al., 1986). Chemical control of these soil-borne virus diseases is neither efficient nor acceptable for economic and ecological reasons. Therefore, considerable scientific efforts have been directed in recent years mainly towards breeding for disease resistant varieties of cereal crops. However novel pathotypes of the viruses that can overcome resistance genes available in breeding programmes are continually emerging (Adams, 1991; Hariri et al., 1990, 2003; Huth, 1989; Steyer et al., 1995). First report of *P. graminis* in the Czech Republic was reporteded by Rýšánek et al., (2009). In addition, *P. graminis* f. sp. *temperata* was firstly reported in the Czech Republic by Ketta et al., (2011a). Therefore, more detailed knowledge of the biology and molecular interaction between soil-borne viruses, *P. graminis* and cereal crops is urgently required to help develop novel options for control or even to prevent these diseases before they become widespread.

Table 2. Cereal viruses transmitted by *Polymyxa graminis*, designed by Kanyuka et al., (2003).

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Acronym</th>
<th>Genus</th>
<th>Natural hosts</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice stripe necrosis virus</td>
<td>RSNV</td>
<td>Benyviridae</td>
<td>Rice</td>
<td>West Africa, South and Central America</td>
</tr>
<tr>
<td>Barley mild mosaic virus</td>
<td>BaMMV</td>
<td>Bymovirus</td>
<td>Barley</td>
<td>Europe, Japan, China, Korea</td>
</tr>
<tr>
<td>Wheat yellow mosaic virus</td>
<td>WYMV</td>
<td>Bymovirus</td>
<td>Wheat, rye, triticale</td>
<td>North America, Europe</td>
</tr>
<tr>
<td>Chinese wheat mosaic virus</td>
<td>CWMV</td>
<td>Furovirus</td>
<td>Wheat</td>
<td>China</td>
</tr>
<tr>
<td>Oat golden stripe virus</td>
<td>OGV</td>
<td>Furovirus</td>
<td>Oats</td>
<td>Europe, USA</td>
</tr>
<tr>
<td>Soil-borne cereal mosaic virus</td>
<td>SBCMV</td>
<td>Furovirus</td>
<td>Wheat, rye, triticale</td>
<td>Europe</td>
</tr>
<tr>
<td>Sorghum chlorotic spot virus</td>
<td>SrCSV</td>
<td>Furovirus</td>
<td>Sorghum</td>
<td>USA</td>
</tr>
<tr>
<td>Peanut clump virus</td>
<td>PCV</td>
<td>Pecluvirus</td>
<td>Peanut, sorghum</td>
<td>India, West Africa</td>
</tr>
<tr>
<td>Aulian wheat mosaic virus</td>
<td>AWMV</td>
<td>?</td>
<td>Wheat</td>
<td>France, UK</td>
</tr>
</tbody>
</table>

*Formally accepted virus species appear in italics, and tentative species are in the regular font. Also includes isolates previously named European wheat mosaic virus or Soil-borne rye mosaic virus.

2.14. Life cycle of *Polymyxa graminis*

*P. graminis* has a life cycle characteristic for most of the plasmodiophorids which consists of two phases (Fig. 5): (i) sporangial (primary), resulting in the production of zoospores, and (ii) sporogenic (secondary), resulting in the production of resting spores. Each
phase is initiated by the attachment of zoospores and penetration of epidermal or root hair cells (Fig. 5). This latter process is unique for plasmodiophorids and involves: (i) encystment of the zoospore at the surface of the host cell wall; (ii) development in the encysted zoospore of a tubular structure (Rohr) that contains a dense dagger-like body (Stachel); (iii) production of an adhesive outgrowth (adhesorium/appresorium) from the encysted zoospore; (iv) almost instantaneous injection of the Stachel and zoospore contents through the adhesorium, host cell wall and plasma membrane into the cytoplasm of plant root cell (Aist and Williams, 1971; Keskin and Fuchs, 1969).

The zoospore contents enlarge within the host cell, undergo several cycles of synchronous mitotic ‘cruciform’ nuclear divisions and reduction in nuclear size (Braselton, 1995), and eventually develop into a multinucleate sporangial plasmodium separated from the host cytoplasm by a distinct cell wall. Several septa are formed within the zoosporangium, dividing it into lobes that expand in volume followed by several cycles of ‘non-cruciform’ mitotic nuclear divisions, and many form exit tubes extending to the host cell wall. Then the secondary zoospores are cleaved apart, become rounded when they mature, and the septa between zoosporangial segments disintegrate. Mature secondary zoospores are released either outside of the root, or into the adjacent root cells via specialized exit tubes that dissolve an opening in the host cell wall (Littlefield et al., 1998). Secondary zoospores either initiate a new sporangial phase resulting in the production of a new generation of secondary zoospores, or develop into sporogenic plasmodia and resting spores. Factors that determine the sporangial vs. sporogenic development phase for P. graminis are unknown, and there is an apparent overlap of the presence of the two phases. During the first 3–5 weeks after primary infection, both types of plasmodia can often be seen within the same host root in adjacent cells.

At early developmental stages, sporangial and sporogenic plasmodia are anatomically very similar, but at later stages sporogenic plasmodia can be distinguished as they frequently fill the whole cross-section of host cells. Moreover, sporogenic plasmodia are separated from the host cell cytoplasm by only a thin membrane layer (Littlefield et al., 1998). In addition, the ‘noncruciform’ meiotic nuclear divisions characterized by the formation of synaptonemal complexes can only be observed in mature sporogenic plasmodia immediately prior to or during cleavage into immature resting spores (Braselton, 1995). Immature resting spores are tightly packed and angular shaped, but they become more rounded with a multilayered cell wall when they mature. Mature resting spores are usually grouped in clusters (cystosori/sporosori) with characteristic morphology (Fig. 5). The resting spores can survive in soil for
several decades, and in suitable environmental conditions they each release one primary zoospore upon germination.

![Diagram of Polymyxa graminis life cycle](image)

2.15. Virus acquisition by *Polymyxa graminis*

How and when *P. graminis* acquires viruses, and how viruses enter the host plant cell cytoplasm is unknown. However, it is likely that these processes are taking place either when zoospores penetrate the host cells and transfer their contents into the host cell cytoplasm or at the sporogenic plasmodia stage of *P. graminis* development when there is only a thin membrane boundary separating the plasmodiophorid from the host cell cytoplasm. Acquired viruses are thought to be carried inside the *P. graminis* resting spores and zoospores. Viruses cannot be removed from zoospores by washing or inactivated by application of antiserum. Moreover, resting spores remain viruliferous, even after treatments with diluted NaOH and HCl (Rao and Brakke, 1969). BaMMV particles have been observed inside zoospores and zoosporangial plasmodia (Chen *et al.*, 1991), but this has not been demonstrated for resting spores mainly because of the impermeability of their multilayered wall that renders their ultrastructure difficult to study.
It is not known whether the viruses are able to multiply inside *P. graminis*, but there is indirect evidence that suggests that they do not, at least for BaMMV. When viruliferous isolates of *P. graminis* have been grown in the roots of a virus-resistant host, the released zoospores no longer contain or transmit virus (Adams et al., 1987; McGrann and Adams, 2003). Ketta et al., (2011b) reported that Czech isolates of *P. graminis* were able to transmit the BaMMV.

### 2.16. Risk factors

- Previous infection that can persist for 25 years.
- Soil movement, especially during cultivations spreading within and between fields.

Disease cannot be controlled once plants are infected, so methods to prevent transmission and limit spread are required. Cleaning cultivation equipment between fields will reduce the risk of the virus spreading.

### 2.17. Molecular diversity and phylogenetic relationships of Plasmodiophoromycetes

*Polymyxa* sp. is a genus of obligate biotrophs and can only be maintained in the roots of host plants. Therefore it is generally difficult to obtain good quality DNA for molecular studies that is free from the contaminating DNA of the host plant or other root parasites, especially when samples are collected in the field. Moreover, even in experimental glasshouse conditions, *P. graminis* multiplication is slow, taking 3–4 weeks to produce zoospores and approximately 2–3 months to produce resting spores. *Polymyxa* isolates for molecular studies preferably need to be initiated from a single cystosorus, and propagated on host plant roots in semi-sterile sand cultures (Adams et al., 1986). Several reliable protocols have been developed for the preparation of DNA from zoospores and resting spores of *Polymyxa* sp. (Mutasa et al., 1993; Ward et al., 1994). A subtractive hybridization approach has also been used in an attempt to obtain *P. graminis*-specific DNA from plant roots (Subr et al., 2002). Molecular diversity and phylogeny of isolates of *Polymyxa* species have been studied using RFLPs and sequencing of transcribed regions of ribosomal RNA genes (nuclear ribosomal DNA, rDNA). Fragments of nuclear 18S, 5.8S and internal transcribed spacers (ITS1 and ITS2) rDNA regions have been analysed (Legrèве et al., 2002; Morales et al., 1999; Ward and Adams, 1998; Ward et al., 1994). In these studies, the two *Polymyxa* species can be clearly distinguished, and there are several subgroups of *P. graminis*. 
These distinct rDNA sequence types appear (to some extent at least) to be related to the host range, temperature requirements and geographical origin, and this has prompted Legrève et al. (2002) to propose classifying them as different formae speciales (Table 3). The variation observed is far greater than would generally be expected between isolates of the same species; some differences of more than 20% across the two ITS regions can be seen between some isolates. Ribosomal DNA sequences have also been used to develop Polymyxa- and *P. graminis*-specific PCR tests that allow *P. graminis* subgroup determination by amplicon size or RFLP analysis (Morales et al., 1999; Ward and Adams, 1998; Ward et al., 1994). *Polymyxa*-specific antibodies have also been developed (Delfosse et al., 2000; Mutasa-Gottgens et al., 2000). These antibodies showed little cross-reactivity to other root parasites and may be useful in the quantification of *Polymyxa* in plants and soil, and in aiding localization of *Polymyxa* sp. in their hosts by microscopy.

Table 3. Subgroups of *Polymyxa graminis* (after Legrève et al., 2002)

<table>
<thead>
<tr>
<th>Subgroup of Polymyxa graminis</th>
<th>rDNA subgroup*†</th>
<th>EMBL accession no.</th>
<th>Natural host</th>
<th>Optimum temperature</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>temperata I</td>
<td>Y12824, AJ311572-4</td>
<td>15–20 °C</td>
<td>Belgium, Canada, China, France, Germany, UK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tepida II</td>
<td>Y12826</td>
<td>15–20 °C</td>
<td>Canada, UK, France, Germany†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tropicalis III</td>
<td>Y12825, AJ311575-6, AJ31580</td>
<td>&gt; 23 °C</td>
<td>Tropical regions—India, Senegal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>subtropicalis IV</td>
<td>AJ311577-9</td>
<td>&gt; 23 °C</td>
<td>Subtropical regions—India, Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>colombiana V</td>
<td>AJ010424</td>
<td>&gt; 23 °C</td>
<td>Colombia†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Legrève et al. (2002).*
§Subgroup inferred by RFLP analysis only, not from sequencing (Ward et al., 1994).
¶Subgroup inferred by Pgfwd2/Pxex7 PCR product size only (Suber et al., 2002).
¶Morales et al. (1999).

For the phylogenetic analysis, *Polymyxa* sequences were first aligned with homologous sequences from three other plasmodiophorids. In the tree generated by the neighbour-joining method (Fig. 6), the eight sequences from *Polymyxa* grouped together. They were clearly separated from the three other plasmodiophorids and showed a closer association with *Ligniera* than with the two other species. Within *Polymyxa*, *P. betae* was separated from *P. graminis*. Within the cluster formed by the *P. graminis* sequences, the isolates from northern India (type Pg-IVa, isolates I9 and I10) were obviously associated with the isolate from Pakistan (type Pg-IVb, isolate P1) in cluster A, seeing the minor differences between their respective sequences. Similarly, the isolates originating from southern India (type Pg-IIIa,
isolates I\textsubscript{1:1}, I\textsubscript{1:20} and I\textsubscript{1:229}) grouped with the isolate from Senegal (type Pg-III\textsubscript{b}, isolate S\textsubscript{6}) in cluster B.

These two clusters were not associated, although they both included isolates originating from warm areas and growing well on common host species such as sorghum. In the phylogenetic tree, cluster A was associated with \textit{P. graminis} type Pg-II (isolate F\textsubscript{51}; Ward and Adams 1998) in cluster C, which in turn was associated with type Pg-V (Colombian \textit{Polymyxa}; Morales \textit{et al}. 1999) in cluster E. The isolates B\textsubscript{1}, C\textsubscript{1} and F\textsubscript{11} from type Pg-I, originating from temperate areas, grouped with cluster B (type Pg-III\textsubscript{a} and Pg-III\textsubscript{b}) to form cluster D. Clusters D and E grouped together and included all \textit{P. graminis} isolates. Phylogenetic analysis of rDNA suggests that \textit{Ligniera} and \textit{Sorosphaera} are very closely related to \textit{Polymyxa}, while \textit{Spongospora} and \textit{Plasmodiophora} are more distantly related (Bulman \textit{et al}., 2001; Ward and Adams, 1998). Several branches of this tree are supported by high bootstrap values (Fig. 6). This was the case for the association between Pg-III\textsubscript{a} and Pg-III\textsubscript{b}, between Pg-IV\textsubscript{a} and Pg-IV\textsubscript{b}, and between Pg-IV and Pg-II, as well as for the branch joining \textit{P. betae} to \textit{P. graminis}. In contrast, other branches within \textit{P. graminis} were not well supported after bootstrapping. Two other tree-building methods were therefore tested. The tree generated according to the maximum-likelihood approach had a topology similar to that obtained using the neighbour-joining method. However, the tree produced by the parsimony method was slightly different and the relative position of some \textit{P. graminis} types differed from those illustrated in Fig. 6 (Legrève \textit{et al}., 2002).
Fig. 6. Phylogenetic analysis of Plasmodiophoromycetes on the basis of the rDNA ITS1-5.8S gene-ITS2 sequences. For *P. graminis*, the sequence types (see text); the origin of associated isolates and the proposed new special forms are given. Tree built by NEIGHBOR analysis using *S. subterranea* as outgroup. The numbers above and below the internodes indicate bootstrap frequencies obtained using the neighbour-joining method and the parsimony method, respectively. In the absence of a number below the internodes, the bootstrap tree was distinct from the one illustrated (see the text). The numbers in italic indicate the bootstrap frequencies when *Polymyxa*-sequences were rooted only with *Ligniera* spp., (Legrève, *et al.*, 2002).

In the parsimony tree, *P. graminis* cluster E was associated with cluster B, and afterwards with *P. graminis* type I. The bootstrap values for these branches were 35.5 % and 40.8 %, respectively. All the other branches were similar to those generated using the two other methods but the low bootstrap values produced by the neighbour-joining method within the *Polymyxa* clustering were also low when using parsimony.

To better determine the inter-relationship between isolates within *Polymyxa* and to reduce the sequences alignment problems generated when different sequence types are
superimposed, the phylogenetic analysis was conducted again with the eight sequence types from *Polymyxa* spp. and with the sequence from *Ligniera* spp. used as the outgroup since the analyses with *S. subterranea* and *P. brassicae* suggest that it is the sister group of *Polymyxa*. In this case, the topologies of the trees built using parsimony, maximum likelihood and neighbour-joining were identical and were similar to those generated by the neighbour-joining and maximum likelihood methods when including *S. subterranea* and *P. brassicae* (Fig. 6). However, even when the tree is rooted only with *Ligniera* spp. some bootstrap values (in italic in Fig. 6) within the *Polymyxa* clustering remained low for branches that also appeared uncertain in the tree where *Polymyxa* is compared with the 3 other plasmodiophorids.

### 2.18. Enzyme-linked immunosorbent assay (ELISA) procedures

Clark and Adams (1977) showed that the microplate method of ELISA could be very effectively applied to the detection and assay of plant viruses. Since that time the method has come to be more and more widely used. Many variations of the basic procedure have been described, with the objective of optimizing the tests for particular purposes. The method is very economical in the use of reactants and readily adapted to quantitative measurement. It can be applied to viruses of various morphological types in both purified preparations and crude extracts. It is particularly convenient when large numbers of tests are needed. It is very sensitive, detecting concentrations as low as 1-10 ng/ml. Detailed protocols are given by Van Regenmortel (1982), Koenig and Paul (1982), Hill (1984), and Clark and Bar Joseph (1984). Two general procedures have been developed.

#### 2.18.1. Direct Double Antibody Sandwich Method

The principle of the direct double antibody sandwich procedure is described by Clark and Adams (1977). It has been widely used, but suffers two limitations: (i) It may be very strain specific. For discrimination between virus strains this can be a useful feature; however for routine diagnostic tests it means that different viral serotypes may escape detection. This high specificity is almost certainly due to the fact that the coupling of the enzyme to the antibody interferes with weaker combining reactions with strains that are not closely related. (ii) It requires a different antivirus enzyme-antibody complex to be prepared for each virus to be tested.

#### 2.18.2. Indirect Double Antibody Sandwich Methods

In the indirect procedure, the enzyme used in the final detection and assay step is conjugated to an antiglobulin antibody. For example, if the virus antibodies were raised in a rabbit, a chicken antirabbit globulin might be used. Thus one conjugated globulin preparation
can be used to assay bound rabbit antibody for a range of viruses. Furthermore, indirect methods detect a broader range of related viruses with a single antiserum (Koenig, 1981). Many variations of these procedures are possible. Koenig and Paul (1982) studied nine of them with the aim of optimizing the tests for different objectives. Their results emphasized the versatility of the assay for different applications. They concluded that the direct double antibody sandwich method is the most convenient for the routine detection of plant viruses in situations where strain specificity and very low virus concentrations cause no problems. The broadest range of serologically related viruses is detected by indirect ELISA using unprecoated plates, but this procedure is open to interference by crude plant sap. Precoating of plates with antibodies or their F(\text{ab}')_2 fragments (Barbara and Clark, 1982) eliminated the interference problem but narrowed the specificity. Other forms of interference may occur. For example, roots of herbaceous plants contain a factor that makes the use of protein A-horseradish peroxidase unsatisfactory as an enzyme conjugate (Jones and Mitchell, 1987). Other viral-coated proteins such as the cylindrical inclusion body protein produced by potyviruses can be used for diagnosis with ELISA methods (Yeh and Gonsalves, 1984). Newer ELISA-like techniques utilize fluorogenic, electrochemiluminescent, and real-time PCR reporters to create quantifiable signals. These new reporters can have various advantages including higher sensitivities and multiplexing (Leng, et al., 2008 and Alder, et al., 2009). Technically, newer assays of ELISAs are not "enzyme-linked" but are instead linked to some non-enzymatic reporter. However, given that the general principles in these assays are largely similar, they are often grouped in the same category as ELISAs.

The indirect F(\text{ab}')_2 fragments of immunoglobulin method of Barbara and Clark (1982) but with a protein A alcaline-phosphatase assay was used as described by Adams et al. (1987). However, although this gave good results with BaMMV, BaYMV antiserum that was effective in Immunospecific electron microscopy (ISEM) showed strong non-specific reactions that hindered the assay. Experiments were therefore made using modified buffers to extract the leaf sap and for dilution of the second antiserum and the protein A conjugate. The usual buffer (0.02 M phosphate-buffered saline containing 0.5 ml/1 Tween 20, 20 g/l polyvinylpyrrolidone and 2 g/l ovalbumin) was amended to give 10 g/l ovalbumin or the ovalbumin was replaced by skimmed milk powder (Five Pints, St Ivel, Swindon) or full cream milk powder (Nido, Nestle, Croydon) at either 2 or 10 g/l. Plates were precoated with F(\text{ab}')_2 fragments at 1/5000 and samples of healthy leaves or those with BaMMV or BaYMV were prepared at dilutions of 1/10, 1/100 and 1/1000 and applied to three replicate wells per treatment. Second antisera (partially purified IgG) were applied at 1/1000 (Adams, 1991).
2.19. Molecular methods

2.19.1. RNA extraction methods for viral detection

Several cetyltrimethylammonium bromide (CTAB)-based methods have been developed for RNA extraction from tissues containing high levels of polysaccharides and phenols. Moreover, two extraction reagents, TRIzol (Sigma) and Concert Plant RNA reagent (Invitrogen) are commercially available and can be used for extracting total RNA from plant tissues. RNA extraction has some methods as:

- Phenol-chloroform RNA extraction;
- Extraction of total RNA by kit;
- Extraction by silica (Rott and Jelkmann, 2001).

2.19.2. Concentration of total RNA

The concentration of an RNA or DNA sample can be checked by the use of UV spectrophotometry. Both RNA and DNA absorb UV light very efficiently making it possible to detect and quantify either at concentrations as low as 2.5 ng/µl. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. Diluting samples for measuring were in rate 1/24 (1 part of total RNA and 24 part of water treated by DEPC). Using a 1cm light path, the extinction coefficient for nucleotides at this wavelength is 20. Based on this extinction coefficient, the absorbance at 260 nm in a 1cm quartz cuvette of a 50 µg/ml solution of double stranded DNA or a 40 µg/ml solution of single stranded RNA is equal to 1.

Concentration of the DNA or RNA in samples measured as follows:

DNA concentration (µg/ml) =

\[(A_{260}) \times \text{(dilution factor)} \times (50 \, \mu\text{g DNA/ml}) / (1 \, A_{260} \, \text{unit}) \]

RNA concentration (µg/ml) =

\[(A_{260}) \times \text{(dilution factor)} \times (40 \, \mu\text{g RNA/ml}) / (1 \, A_{260} \, \text{unit}) \] (Hofstra, 1996).

2.19.3. Reverse transcription (RT)

**RT-PCR** (Reverse Transcription-Polymerase Chain Reaction):

Reverse Transcription (RT reaction) is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA or poly (A) RNA, a reverse transcriptase enzyme, a primer, dNTPs and an RNase inhibitor. The resulting
cDNA can be used in RT-PCR reaction. RT reaction is also called first strand cDNA synthesis. RT-PCR can also be carried out as one-step RT-PCR in which all reaction components are mixed in one tube prior to starting the reactions. Although one-step RT-PCR offers simplicity and convenience and minimizes the possibility for contamination, the resulting cDNA cannot be repeated used as in two step RT-PCR. The possibility that genetic information could be passed on in this manner was finally accepted, when the scientists (Temin et al., 1970) both independently discovered the enzyme responsible for reverse transcription, named reverse transcriptase.

2.19.4. Polymerase Chain Reaction (PCR)

The advent of Polymerase Chain Reaction (PCR) by Kary B. Mullis in the mid-1980s revolutionized molecular biology as we know it. Since its introduction, the polymerase chain reaction has found a solid niche in broad areas of agriculture, industrial, forensic and medical communities where people routinely handle nucleic acids. Applications in these areas have developed mainly as a result of PCR’s high sensitivity (Lee et al., 2003). A critical step in developing a new PCR assay is to optimize the reaction conditions to obtain maximum specificity and sensitivity. The primary reason for these optimization steps is determine what deviations from the standard reaction conditions are necessary to promote functional primer annealing and extension (Miesfeld, 1999). Amplification efficiency is influenced by a number of factors including target length and sequence, primer sequence, buffer conditions, sample impurities, cycling conditions and PCR enzyme. PCR consists of many amplification steps, adjusting reaction conditions to achieve even slight improvements in amplification efficiency can lead to dramatic increase in PCR product yield (Arezi et al., 2003).

Although PCR is now a routine technique in many laboratories, there are still a considerable number of problems in getting good, reproducible amplifications (Seal and Cotes, 1998). Unquestionably, no single protocol will be appropriate to all situations (Innis and Gelfand, 1998). In diagnostic laboratories the use of PCR is limited by cost and sometimes the availability of adequate test sample volume (Usta et al., 2005). Recently, increasing interest and efforts from a variety of approaches have been directed to the quality assurance and standardization of the PCR technique (Yang et al., 2004). PCR is a technique for genetic screening, detection studies and microsatellite applications where it is necessary to amplify specific products in a single or double reaction. The technique often requires extensive optimization because of non-specific products, which may interfere with the amplification process. Although the mis-annealing temperature can usually be avoided by using gradient functioned PCR cyclers, amplification specificity is also influenced by other
factors such as primer, enzyme, dNTP and Mg\(^{++}\) ion concentration. The availability of a wealth of genomic sequence information has led to the generally straightforward development of sequence-targeted detection assays based on the polymerase chain reaction (PCR) for many viruses. An alternative but somewhat less developed strategy is the development of PCR-based assays using primers targeting conserved regions of viral genomes, thus allowing the simultaneous detection of a number of different viruses in a single PCR assay.

Following the initial demonstration that degenerate primers can amplify a range of potyviruses (Langeveld et al., 1991), extensive efforts in a number of laboratories have led to the development of numerous broad-spectrum PCR assays allowing the amplification of a few viruses or of even most or all members of a genus, including, for example, closteroviruses (Karasev et al., 1994), tymoviruses and marafiviruses (Sabanadzovic et al., 2000), mastreviruses (Willment et al., 2001), begomoviruses (Brown, 2000; Rojas et al., 1993), cucumoviruses (Choi et al., 1999), carmoviruses (Morozov et al., 1995), badnaviruses (Thompson et al., 1997), tobamoviruses (Dovas et al., 2004), or potyviruses (Pappu et al., 1993). Despite their potential usefulness, there are, however, few examples of primer pairs or PCR-based amplification assays with a specificity extending above the genus level, a notable exception being the development of assays to amplify all members of the family Potyviridae, including members of the genera potyvirus, macluravirus, ipomovirus, rymovirus, tritimovirus, and bymovirus (Gibbs and Mackenzie, 1997). An assay allowing the amplification of members of the family Comoviridae has also been reported (Maliogka et al., 2004). A number of PCR-based DNA markers have been developed in recent years to evaluate genetic variation at the intraspecific and interspecific levels (Wolfe and Liston 1998). Molecular markers allow the selection of desired traits based on genotype and can therefore complete and accelerate plant breeding programs. They can be also used for the early selection of traits, which are not expressed during the juvenile phase such as persistence, competitive ability and seed yield (Kölliker et al., 2001).

2.19.4.1. PCR Modifications

- **RAPD**: (random amplified polymorphic DNA) technology is a reliable method for characterizing variation within and among species and populations (Gustine and Huff 1999).

- **PCR-RFLP**: (PCR/restriction fragment length polymorphism). PCR-RFLP is suitable for rapid identification of origin and specific parentage of hybrids or allopolyploid species (Chen and Sun, 1998).
• **PCR-SPLAT**: (PCR specific polymorphic locus amplification test), (D’Ovidio, Anderson, 1994).

• **PCR-In situ**.

• **PCR-Real Time**: Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection (Higuchi, 1992; Higuchi, 1993).

### 2.19.5. Separation of nucleic acids by electrophoresis

Electrophoretic mobility is now usually determined by migration in agarose or polyacrylamide gels. Thus this property will depend both on the size of the particle and on the net charge at its surface. While strains of the same virus will usually have very similar mobilities, different viruses within a group may be distinguished as was shown for the *cucumovirus* group by Hanada (1984). A book on electrophoresis by Bier, (1959) cites references from the 1800s. Introduction of acrylamide gels (Raymond and Weintraub, 1959); accurate control of parameters such as pore size and stability. Disc gel electrophoresis (Ornstein and Davis, 1964). Introduction of denaturing agents especially SDS separation of protein subunit (Weber and Osborn, 1969). Gel electrophoresis is a technique used for the separation of deoxyribonucleic acid, ribonucleic acid, or protein molecules using an electric current applied to a gel matrix (Berg *et al*., 2002). In the case of nucleic acids, the direction of migration, from negative to positive electrodes, is due to the naturally-occurring negative charge carried by their sugar-phosphate backbone (Lodish *et al*., 2004).

#### 2.19.5.1. Agarose gel electrophoresis

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. The DNA is visualized in the gel by addition of ethidium bromide. Agarose gel is useful to check whether the DNA is degraded or not (Semagn *et al*., 2006). The most common stain is ethidium bromide, which intercalates into the double-stranded DNA (and some RNA) strands. Supply power of electrophoresis is usually between 2 to 5 V/cm of the gel between cathode and anode (Vejl, 1997).

#### 2.19.5.2. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis has been used to separate a wide variety of macromolecules such as proteins, RNA, DNA, and cellular components such as ribosomes (Talens *et al*., 1973). It has also been used to separate and measure the isoelectric points of
some small viruses (Beaudoin et al., 1974; Hutchison et al., 1967). The gel can be run native (TBE) or denaturing (TBE + 8.3 M urea), (Maniatis, et al., 1975). Polyacrylamide gels with pores of a much more controlled and uniform size than doe’s agarose. Acrylamide is particularly superior when very small pore sizes are needed. It can be used to effectively separate small molecules (i.e., DNA oligonucleotides of 100 bases or less) that tend to run right through agarose gels. Another advantage of acrylamide as a gel matrix is that it is much stronger than agarose, producing gels that do not tear as nearly as easily. It is also possible to load larger quantities of material onto acrylamide gels and (because of the relative purity of the gel ingredients).
3. THESIS OBJECTIVES

Two related viruses, *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV), are causal agents of soil-borne mosaic disease in winter barley in several European countries. The hypotheses are:

- *P. graminis* is present in Czech Republic
- Czech isolates of *P. graminis* are able to transmit *Barley mild mosaic virus* and *Barley yellow mosaic virus*.

The experimental and scientific works have been devoted to achieve the following aims:

1) Using improved techniques of mechanical inoculation method like SWG and comparison it with Finger rubbing for inoculation of BaMMV.

2) Adding some chemical substances such as phenylmethylsulfonyl fluoride (PMSF), sodium diethyldithiocarbamate (Na-DIECA) and other reducing agents of polyphenoloxidase activity to grinding buffer to protect the particles of BaMMV.

3) Preservation of BaMMV infected barley plant tissues for a long term with high virus infectivity to provide a continuous supply of inoculums of the same source.

4) Monitoring of BaMMV and BaYMV in barley in the Czech Republic.

5) Monitoring of *P. graminis* in the arable land of cereals in the Czech Republic.

6) Developing some reliable protocols for the preparation of DNA from zoospores and resting spores of *P. graminis* to obtain *P. graminis*-specific DNA from plant roots.

7) Testing the ability of Czech *P. graminis* isolates to transmit BaMMV.
4. MATERIALS AND METHODS

As experimental material plants of the highly BaMMV-susceptible winter barley cv. Florian were used.

4.1. Inoculum source

For growing the virus, winter barley Maris Otter was cultivated during 2008 in soil infested by viruliferous *P. graminis* with BaMMV, obtained from Dr Habekuss (JKI Quedlinburg, Germany) at glasshouse condition 12-14 °C. The two or three youngest leaves of BaMMV-infected Maris Otter plants (donor), which had visual symptoms, were cut into approximately 5 mm pieces and homogenized into potassium phosphate buffer 0.04 M, pH 7 for the reason of mechanical inoculation to the winter barley cv. Florian.

*Barley yellow mosaic virus* inocula were obtained from DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German collection of microorganisms and cell cultures) in the form of young infected barley plants.

4.2. Mechanical inoculation

Depending on the experiment 1 g of plant material and 5 ml of inoculation buffer were used for subsequent inoculation of one treatment of susceptible winter barley cv. Florian (15 – 17 plants). Seeds of winter barley cv. Florian were sown into plastic pots 10 cm diameter filled with commercial peat substrate Stender, 15 to 19 seeds per pot at glasshouse condition 12-14 °C and 12 hour day length (natural light supplement). BaMMV-infected leaves of barley Maris Otter were homogenized by hand in sterilized mortars and pestle. Mortars and pestle were chilled before using in freeze box for 30 min. For inoculation, tested plants in the 3-4 leaves stage (2 – 3 weeks old) of each seedling were held flat on the plastic glove-covered palm and dusted with the inoculum contains carborundum abrasive (600 mesh) in a rubbing motion starting at the base to the leaf tips (Fig. 8), afterwards the experiment was divided into two groups and two inoculation techniques (stick with gauze (SWG) and finger rubbing (FR)) were used. The SWG is an improvised inoculator made of ordinary wooden chopsticks with gauze folded 3 times (4 cm wide and 15 cm long). Gauze was rolled at the end of the stick then secured with a string (Fig. 8). Group (A) thoroughly rubbed off with SWG soaked in the respective inoculum fluid starting at the base to the leaf tips and group (B) thoroughly rubbed off with FR. For healthy controls, grinding buffer without infected plant tissue was inoculated similarly. Inoculated plants were placed in the shade overnight. The inoculums were allowed
to dry on the treated leaves without water post-washing. Plants were grown in the growth chamber at 12-14 °C. Watering was done once per week. SWG was compared with finger rubbing for their efficiency, handling and amount of inoculum required per inoculation. One month after inoculation, plants were visually scored for infection and individual leaf per plant were sampled and tested by RT-PCR. All experiments were repeated once. In some experiments, the inoculation procedure was repeated seven days intervals from the first treatment.

All experiments were designed as Randomized Complete Block Design (RCBD) and repeated once. Data from all experiments were analyzed by statistical program (SAS).

4.3. Means of effective mechanical transmission of BaMMV

Potassium phosphate buffers 0.04 M with and without chemical additives were applied for mechanical inoculation of BaMMV (Table 4). Potassium phosphate buffer 0.04 M was applied for mechanical inoculation without additives at pH 7. Na-DIECA and PMSF were added to the potassium phosphate buffer 0.04 M in different concentrations 0.01 M and 0.001 M. There were five treatments with two replications per treatment, and the experiment was repeated once.

Table 4. Chemical additives to the inoculation buffer

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>pH values</th>
<th>Effective as</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄ + KH₂PO₄</td>
<td>0.04 M</td>
<td>7.0</td>
<td>Buffer</td>
</tr>
<tr>
<td>Additive Na-DIECA*</td>
<td>0.01 M</td>
<td>8.1</td>
<td>Chelating agent</td>
</tr>
<tr>
<td>Additive Na-DIECA*</td>
<td>0.001 M</td>
<td>7.4</td>
<td>Chelating agent</td>
</tr>
<tr>
<td>Additive PMSF**</td>
<td>0.01 M</td>
<td>8.3</td>
<td>Inhibitor of polyphenoloxidase</td>
</tr>
<tr>
<td>Additive PMSF**</td>
<td>0.001 M</td>
<td>7.6</td>
<td>Inhibitor of polyphenoloxidase</td>
</tr>
</tbody>
</table>

* Sodium diethyldithiocarbamate: chelates copper and inhibit polyphenoloxidase.

** Phenylmethylsulfonyl fluoride.
Potassium phosphate buffers 0.04 M and potassium phosphate buffers 0.04 M with additives (Na-DIECA 0.001 M and Na-DIECA 0.01 M) were applied for mechanical inoculation in different pH values (Table 5).

Table 5. Chemical additives to the inoculation buffer in different pH values

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>pH values</th>
<th>Effective as</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$ + KH$_2$PO$_4$</td>
<td>0.04 M</td>
<td>6.5 7 7.5 8</td>
<td>Buffer</td>
</tr>
<tr>
<td>Additive Na-DIECA</td>
<td>0.01 M</td>
<td>6.5 7 7.5 8</td>
<td>Chelating agent</td>
</tr>
<tr>
<td>Additive Na-DIECA</td>
<td>0.001 M</td>
<td>6.5 7 7.5 8</td>
<td>Chelating agent</td>
</tr>
</tbody>
</table>

4.4. Monitoring of BaMMV and BaYMV in barley in the Czech Republic

For the monitoring of BaMMV and BaYMV in some localities in the Czech Republic, seventeen soil samples, which collected separately from different localities of cereal production (barley) during April 2008 using a digging fork were placed separately and planted to barley in pots (12 x 12 cm) in a greenhouse at 12-14 °C and fifty-six tested barley plant samples, planted into soil samples collected from different localities of sugar beet production during 1989 and 1999 when Polymyxa betae was monitored. All plant samples were tested by DAS-ELISA using positive and negative control (ADGEN Phytodiagnostics - Protocol 1) and by RT-PCR assay.

4.5. Long-term preservation of BaMMV-infected tissues

The following methods were used for long-term storage of BaMMV-infected tissues:

Calcium chloride (CaCl$_2$):

The most widely used storage method is to keep the virus in suitable, actively growing storage hosts. Leaf material can also be rapidly dried and stored over calcium chloride at 0 - 4 °C.

Materials needed:

- plastic jar or Petri dish
4. MATERIALS AND METHODS

- granular anhydrous calcium chloride (5-15 mm)
- cotton, tissue paper, or gauze
- tape or parafilm

Method: A few granules of anhydrous calcium chloride were placed in the bottom of a plastic jar or Petri dish. Then the plastic jars were covered with a thin layer of gauze. On top of this layer were placed 2 to 4 g sample of a virus-infected barley cv. Florian leaf which had been finely chopped with a clean razor blade. The plastic jars were covered and sealed with tape or parafilm and the samples were put in refrigerator. In order to dry the leaf sample completely, it may be necessary to open the container several times and replace the calcium chloride crystals (McKinney and Silber, 1968).

Lyophilisation (freeze-drying):

Lyophilisation remains the virus-infected plant tissues stable and is easier to store at room temperature (surrounding air temperature). The process consists of three separate, unique, and interdependent processes; freezing, primary drying (sublimation), and secondary drying (desorption).

To remove water from virus-infected plant tissues, the process of lyophilisation consisted of:

1. Freezing the virus-infected plant tissues so that the water in it become ice;
2. Under a vacuum, sublimating the ice directly into water vapour;
3. Drawing off the water vapour;
4. Once the ice is sublimated, the virus-infected plant tissues were freeze-dried and stored at room temperature.

Freezing:

BaMMV-infected plant materials were wrapped in plastic bags and preserved in a freezer at -20 °C.

Glycerol (or glycerine):

BaMMV-infected plant materials were put in plastic jars filled with glycerol 100% and preserved in a refrigerator at 4 °C. Glycerol were removed from the plastic jars and filled again by fresh glycerol three times at the first three weeks of preservation.

For testing of all previous methods of preservation, BaMMV-infected barley cv. Florian tissues were preserved for 13 months. Barley seedlings (3-4 leaves stage) of cv. Florian were used for mechanical inoculation in six replicate pots (5 seedlings per pot) for each method of preservation. Positive control (fresh BaMMV-infected leaves) and negative control
(inoculation by potassium phosphate buffer only) were used. The experiment was repeated again after one month with BaMMV-infected barley cv. Florian tissues preserved for 14 months.

4.6. DAS-ELISA protocol (PAb)

For homogenization the barley plant materials were used three methods: homogenization by using mortar and pestles, homogenization by using extraction bags 120 x 140 mm, plastic intermediate layer with hand homogenizer (Bioreba) see appendix (Fig. 1) and the third method was by the machine (RETSCH Mixer Mills MM 400) see appendix (Fig. 2). As experimental material plants of the highly BaMMV-infected winter barley cv. Florian were used. ELISA tests for BaMMV detection were performed using ADGEN (Neogen Europe Ltd.) kits, following the manufacturer’s instructions. The positive control provided in the ADGEN ELISA. The optical density (OD) was measured at 405 nm using the TECAN MAGELLAN ELISA-reader, after 120 min from the addition of the p-nitrophenyl phosphate (pNPP). Two repetitions were performed on the tested samples.

1. Coating antibody was diluted in coating buffer (see buffers list below) as recommended on the bottle label and added 100 µl to the required numbers of wells for the test.
2. The plate was tightly wrapped in plastic box with some damp paper towels and closed. The plate was incubated at 37 °C for 4 hours.
3. The plate was washed three times with phosphate buffered saline + Tween 20 (0.05 %)-PBST (see buffers list below). The wells of the plate was filled with PBST and inverted to remove the buffer. This step was repeated five times; the plate was dried on paper towels.
4. Leaves of infected plant samples were extracted by grinding 1 g of tissue with 10 ml of general extraction buffer (see buffers list below) in a mortar and pestle (or an alternative method of grinding). Then the samples were filtered through a layer of muslin (or similar fine cotton gauze).
5. 100µl of each plant sample, positive and negative control were added to the coated wells. All samples and controls were added in duplicate for testing.
6. The plate was wrapped as described in (step 2) above and incubated at 4 °C overnight (at least 16 hours).
7. The plate was washed as described in (step 3) above.
8. The antibody-enzyme conjugate was diluted as recommended on the bottle label in conjugate buffer (see buffers list below) and 100 µl of it was added to each test well.

9. The plate was wrapped as described in (step 2) above and incubated at 37 °C for 1 hour.

10. The plate was washed five times as described in (step 3) above. An extra wash is included at this stage to ensure that all unbound antibody-enzyme conjugate is removed from the wells.

11. The substrate buffer (see buffers list below) was prepared just before using. The pNPP was added at 1mg/ml to substrate buffer.

12. 100µl of prepared substrate buffer were added to each test well.

13. The plate was wrapped as described in (step 2) above and incubated in the dark at room temperature for at least 1 hour.

The absorbance was measured by using a spectrophotometer at 405 nm or 595-650 nm.

List of used buffers

**Coating Buffer (Carbonate Buffer)**

Sodium carbonate 1.59 g  
Sodium hydrogen carbonates 2.93 g

Make up to 1 litre with dH2O. The pH of this buffer is 9.6 and does not require to be adjusted.

**Phosphate buffered saline (PBS) x 10**

Sodium chloride 80 g  
Potassium diHydrogen orthophosphate 2 g

DiSodium Hydrogen orthophosphate 11.5 g  
Potassium chloride 2 g

Make up to 1 litre with dH2O. The pH of this solution when diluted to 1xs is 7.2

**Wash buffer (PBS + Tween 20)**

Phosphate buffered saline 1 litre  
Tween 20 0.5 ml

**General Extraction Buffer**

Polyvinylpyrrolidone (PVP) 20 g  
Ovalbumin 2 g

Sodium sulphite (anhdyrous) 1.3 g  
Sodium azide 0.2 g

Tween 20 0.5 ml  
Sodium chloride 8 g

Potassium diHydrogen orthophosphate 0.2 g  
Potassium chloride 0.2 g

DiSodium Hydrogen orthophosphate 1.15 g
Make up to 1 litre with distilled/deionised water. This buffer can be difficult to get into solution and it is easier if the PVP is mixed into a "paste" with a small volume of water before adding the other components and the remainder of the water.

**Conjugate buffer**

Bovine serum albumin 0.2 g  PBST 100 ml

**Substrate buffer (Diethanolamine buffer 1M)**

Diethanolamine 90.39 g  Diethanolamine-HCl 19.82 g

Magnesium chloride 0.1 g

Make up to 1 litre with ddH₂O. The pH of this buffer is 9.8 and it does not require to be adjusted. (The diethanolamine and diethanolamine-HCl are liquids however; it is easier to weigh them out than to measure their volumes as they are extremely viscous.) pNPP is added to the above buffer at 1mg/ml to make up the substrate for alkaline phosphatase.

**Sensitivity of ELISA**

The sensitivity of ELISA was evaluated using the German isolate of BaMMV (DSMZ) as the reference sample. ELISA extracts from fresh-infected leaves of this isolate were diluted 8 times (vol/vol), and these dilutions were tested simultaneously using the ELISA method. Two repetitions were performed on the sample and its dilutions for all the tests conducted. For ELISA, 50 mg of barley cv. Florian fresh leaves infected by BaMMV were crushed in a 100 µl extraction buffer. From this extract, 8 serial dilutions were carried out in the extraction buffer and 100 µl of each dilution and concentrated extract were added per well to the coated plates.

### 4.7. RNA extraction of BaMMV

For homogenization the barley plant materials were used three methods: homogenization by using mortar and pestles with liquid nitrogen, homogenization by using extraction bags 120 x 140 mm, plastic intermediate layer with hand homogenizer (Bioreba) and the third method was by the machine (RETSCH Mixer Mills MM 400).

For RNA extraction of 50 mg BaMMV-infected tissues from 7 samples were used three methods of extraction: extraction by phenol chloroform, GeneJET RNA purification kit and magnetic isolation of RNA.

The first method of RNA extraction: phenol chloroform

Phenol for RNA extraction (pH 4.3)
4. MATERIALS AND METHODS

Chloroform

Chloroform-isoamylalcohol (Chl: Iaa) (24: 1 [v/v]);

- 3 M sodium acetate (pH 5.5);
- DEPC-treated water;
- Absolute and 70 % (v/v) ethyl alcohol;

1. Plant tissues approximately 0.050 g were homogenized as rapidly in sterilized mortars and pestle with adding of liquid nitrogen to each sample;
2. RNA extraction buffer was added to each sample;
3. Each sample sap (known amount) was transferred from the mortar to 2 ml eppendorf tube;
4. The same amount of phenol (pH 4.3) and chloroform-isoamyl alcohol (24:1) was added in the rate of 1:1;
5. All samples were shaken on vortex machine for 10 min., and then centrifuged (Jouan MR 23 I) for 10 min. 7000 g, at 4 °C. After centrifugation the supernatant of samples were taken to new eppendorfs and the pellets were removed;
6. Steps number 4 and 5 were repeated twice;
7. To the supernatant of each sample was added the same amount of chloroform-isoamyl alcohol (24:1), and samples were shaken on vortex machine for 10 min., and then centrifuged for 10 min. 7000 g, at 4 °C;
8. To the supernatant (known amount) was added double amount of icy absolute ethanol and 1/10 of sodium acetate (3M, pH 6);
9. Mild shuffle by hand was done to all samples and stored over night in freeze box (-28 °C);
10. Samples were centrifuged after one night in freeze box on 10000 g, for 10 min. and then the supernatants were removed;
11. To pellets of samples in each eppendorf were added 100 - 200 µl 70 % ethanol (70 % ethanol + 30 % water treated with DEPC –diethylpyrocarbonate) with gently mix;
12. Centrifugation for 10 min. on 10000 g/min.;
13. Supernatants were removed and the eppendorfes left to dry at laboratory temperature on paper tissue for 15 - 20 min.;
14. To each eppendorf of samples were added 200 µl of water treated with DEPC and mixed by hand (gently mix) for dissolving the pellets.
All times of centrifugation should be at 4 °C. Prepared samples could be immediately used for reverse transcription or stored in freeze box (-28 °C) until using again. In this method of extraction RNA by phenol-chloroform isolation was used this buffer:

**Buffer for isolation of RNA (pH 7) according to Robinson, 1992:**

- 50 mM Tris
- 100 mM NaCl
- 20 mM EDTA
- 1 % SDS

All of them dissolved in dd (double distilled) H₂O and then sterilized by autoclave at 120 °C for 30 min.

The second method of RNA extraction: GeneJET RNA purification kit, Mammalian tissue and insect total RNA

1. Fresh plant infected sample tissues with BaMMV, approximately 50 mg were homogenized as rapidly in sterilized mortars and pestle with adding of liquid nitrogen to each sample;
2. The powder of each crushed sample was transferred to 1.5 ml eppendorf tube with 300 µl of lysis buffer with β-mercaptoethanol;
3. All samples were shaken on vortex machine for 10 sec.;
4. 600 µl of diluted proteinase-K were added to each sample;
5. All samples were shaken on vortex machine for 10 min., and then centrifuged (Jouan MR 23 I) for 10 min. 12000 g, at 4 °C. After centrifugation the supernatant of samples were taken to new eppendorfs and the pellets were removed;
6. To the supernatant of each sample was added 450 µl ethanol 96 % and mixed thoroughly by pipette;
7. Up to 700 µl of each sample were transferred to the GeneJET purification column;
8. Samples were centrifuged for 1 min. 12000 g, at 4 °C;
9. The flow-throughs were discarded and the centrifugation was repeated once more again;
10. The columns of GeneJET purification tube were replaced into 2 ml collection tubes;
11. 700 µl of wash buffer 1 were added to each sample, then centrifuged for 1 min. 12000 g, at 4 °C;
12. The flow-through were discard and 600 µl of wash buffer 2 were added to each sample, then centrifuged for 2 min. 12000 g, at 4 °C;
13. Samples were centrifuged again for 1 min. 14000 g, at 4 °C;
14. The columns of GeneJET purification tube were replaced into 1.5 ml collection tubes;
15. 100 µl of water nuclease free were added to each sample and left 10 min on the table.

The third method of RNA extraction: magnetic isolation of RNA

1. Fresh plant infected sample tissues with BaMMV, approximately 50 mg were homogenized as rapidly in sterilized mortars and pestle with adding of liquid nitrogen to each sample;
2. The powder of each crushed sample was transferred to 2 ml eppendorf tube with 450 µl of lysis solution RL;
3. All samples were shaken on vortex machine for 10 min., and then centrifuged (Jouan MR 23 I) for 1 min. 15000 g, at 4 °C. After centrifugation the supernatant of samples were taken to new eppendorfs (spin filter D) and the pellets were removed;
4. Samples were centrifuged for 2 min. 12000 g, at 4 °C;
5. The spin filters D were discarded;
6. The 50 µl of MAG suspension was added to each hole (Pack A reagent cavity);
7. The filtrated solutions of each sample were transferred into the cavity of reagent Pack A;
8. Reagent Pack B were put into machine (InnuPure® C12), see Fig. 7;
9. MFR tips were put into machine (InnuPure® C12) see appendix (Fig. 5);
10. Elution tubes were put into machine (InnuPure® C12);
11. The rank was put into machine (InnuPure® C12) and the programme started.
4. MATERIALS AND METHODS

11 Preliminary steps of the InnuPure® C12

11.1 Consumables
During the first sample load the following consumables to the InnuPure® C12 sample tray, as shown below as an example of 3 preparations in parallel.

44 Fig. 7. Preliminary steps of the InnuPure® C12

Total RNA purity:

Total RNA purity and concentration was measured by the NanoDrop2000. NanoDrop2000 (see appendix Fig. 4) is a full-spectrum spectrophotometer that measures 1 µl samples with high accuracy and reproducibility. It utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place.

- The NanoDrop 2000 software was started,
- The instrument using a lightly moistened kimwipe was cleaned,
- Blank the instrument was done by using 2 µL of solution. For better accuracy blank was used the solution (ex. Water, TE, etc) that samples were suspended with.
- The instrument using a dry kimwipe was cleaned,
- 1 µl of sample was read,
- The instrument using a dry kimwipe was cleaned between measurements.

260/280: ratio of sample absorbance at 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the
ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

**260/230:** ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

### 4.8. Reverse transcription (RT)

Transcription of tRNA to cDNA method was modified by Department of Plant Protection in Prague, Faculty of Agrobiology, Food and Natural Resources (FAPPZ, ČZU). Reverse primer was designed according to (Hariri et al., 2000).

For one reaction was used eppendorf tubes 0.5 ml with total volume of reaction mixture 25 µl:

- 15.35 µl dd water;
- 5 µl AMV-RT buffer (Promega) 5x;
- 0.25 µl dNTP (0.4 mM);
- 0.4 µl Reverse primer (25 pmol) (5′ GCGTCCGTTGCAA CTGA 3′);
- 0.5 µl AMV Reverse transcriptase (5 U) (Promega);
- 0.5 µl Ribolock (Ribonuclease Inhibitor 20 u/µl);
- 3 µl tRNA from the testing sample.

Firstly, amounts of all components excepting tRNA were multiplied (depending, on the number of samples), then put in one eppendorf 1.5 ml (Premix). This mixture was closely vortexed and separated to eppendorf tubes 0.5 ml, for each one is 22 µl, then added to each eppendorf 3 µl of tRNA (samples).

Whole process of preparation was done on ice. Samples were transferred immediately into thermo block machine for incubation at temperature 42 °C for 60 min., then for 5 min. at 100 °C.

### 4.9. Polymerase Chain Reaction (PCR)

PCR method was used for DNA amplification with programme for BaMMV using the primers P1 and P2 (according to Hariri et al., 2000). Steps were optimized by Department of Plant Protection in Prague, Faculty of Agrobiology, Food and Natural Resources (FAPPZ, ČZU).
4. MATERIALS AND METHODS

For one reaction was used eppendorf tubes 0.5 ml with total volume of reaction mixture 25 µl:

- 16.35 µl dd water;
- 3 µl MgCl₂ 25mM (Promega);
- 2.5 µl 10x Taq buffer + (NH₄)₂SO₄ without MgCl₂ (Promega);
- 0.25 µl dNTP (10 mM);
- 0.4 µl Reverse primer (25 pmol) (5' GCGTCCGTTGCAACTGA 3') + Forward primer (25 pmol) (5' GAATTGCTTGTGCAACA 3');
- 0.5 µl Taq DNA polymerase (Promega 5u/ µl);
- 2 µl cDNA from samples;

All amounts of previous components excepting cDNA were multiplied (according to the number of samples), then put in one eppendorf 1.5 ml (Premix). This mixture was closely vortex and separated to eppendorf tubes 0.5 ml, for each one is 23 µl, then added to each eppendorf 2 µl of cDNA (samples).

The PCR was realized using the Thermocycler MJ research PTC-200 (Peltier Thermal Cycler) as follows: Step by step of PCR program BaMMV:

1. 95 °C for 5 min.
2. 94 °C for 1 min.
3. 52 °C for 30 sec.
4. 72 °C for 1 min.
5. Repeating previous steps No. 2, 3 and 4, usually 35 times.
6. 72 °C for 4 min.
7. 4 °C forever.

The annealing temperature was optimized by testing a gradient of temperatures on a set of cDNA samples in a Thermocycler MJ research PTC-200 (Peltier Thermal Cycler). After ending the thermocycler program, eppendorfe tubes were convoluted by mild centrifugation, and then the PCR products were immediately separated by gel electrophoresis.

4.10. Agarose gel electrophoresis

The amplified samples were loaded on 1 % agarose gel in Tris–borate–EDTA buffer and electrophoresis was performed in Agarose Gel Electrophoresis Systems.

For preparation of agarose gel was used 10x TBE buffer (Tris-Borate-EDTA):

- 108 g Tris base
4. MATERIALS AND METHODS

- 55 g Boric acid
- 9.3 g Na_{4}EDTA

Previous amounts were added to 1 liter dd H_{2}O, adjusted to pH 8.3, and then sterilized by autoclave at 120 °C for 30 min.

Agarose gel (100 ml) was prepared as:

- 1 g Agarose;
- 10 ml TBE buffer 10x + 90 ml dd H_{2}O (for prepare TBE buffer 1x);
- This solution transferred to Erlenmeyer flask, and then agarose was melt in a microwave oven. After cooling pure molten agarose to 60 °C was added 50 µl of ethidium bromide, and then put onto gel plate to a depth of 4 - 8 mm.;
- 10 µl (of PCR product) + 2 µl loading dye (6x loading dye solution, Fermentas), were mixed well and put in the microfuge desk;
- Samples were added to wells of gel;
- Marker (MassRuler™ DNA Ladder, Low Range, ready-to-use (#SM0383) (Fermentas) see appendix (Fig. 3), was added for bands comparison in the first and last well of gel;
- Electrophoresis machine with tension (electric volt) 5 V/cm^{2} (62 V) for one hour was turned on.

The gel was transferred to desk of Transiluminator for visualizing bands of DNA under UV light (280 nm).

4.11. Monitoring of *P. graminis*

Field observations

Following the submission of poorly growing barley (*Hordeum vulgare*) from a cereal fields, the field sites were then visited and seventeen soil samples were collected on April 2008.

Soil collection

For the monitoring of *P. graminis* in some localities in the Czech Republic, seventeen soil samples from different localities of cereal fields (barley) with prediction of *P. graminis* occurrence were collected during April 2008 and fifty-six soil samples collected from different localities of sugar beet production during 1989 and 1999 when *P. betae* was monitored. No weeds were present among the soils in the area sampled. Soil from each sample was placed in five replicate pots (12 × 12 cm) in a greenhouse at 22 to 25 °C. Seeds of
barley cv. Florian were sown into the soil (10 seeds per pot). Negative control soil (non-infested soils from the Czech Republic) and positive control soil (known *P. graminis*-infested soil from Germany) were also used in five replicate pots. After 90 days, plants were collected; the roots were washed thoroughly in sterilized water and examined with a light microscope without staining.

For testing the surviving of *P. graminis* cystosori in soils for several years, 46 and 10 soil samples were collected from different localities of cereals in the Czech Republic in 1999 and in 1988-1989 respectively.

Root microscopy

The soil with plant roots were soaked in water for one hour then washed repeatedly on a 425 μm sieve to collect clean roots. The roots were placed in 500 ml plastic dishes for additional washing. The roots were cleared for 30 min in diluted household bleach (sodium hypochlorite 5 % and sodium hydroxide 1 %) 1:9. Roots without staining were checked under an inverted microscope Nikon at magnifications up to x20. Photomicrographs were taken with Olympus BX51 microscope-camera system at magnifications up to x100.

Nucleic acid extraction

Subsamples of barley roots extracted from the field soil samples as described under Root microscopy above were used for DNA extraction. Total DNA was extracted from the roots initially using a modified CTAB (cetyltrimethylammonium bromide) extraction method (*Zolan* and *Pukkila*, 1986; *Mutasa* et al. 1993; *Triticarte*, 2009).

For each sample, two subsamples of approximately 50 mg of root material per mortar were ground to a fine powder using liquid nitrogen. The second type of homogenization of barley roots was by (RETSCH Mixer Mills MM 400).

- Fresh buffer working solution 500 μl (prepared according to the instructions provided by DArT Pty. Ltd. (*Triticarte*, 2009) was added to samples, mixed thoroughly, then placed to eppendorf tubes (1.5 ml) and incubated for 2 h. at 65 °C.
- After incubation period 500 μl of phenol: chloroform: isoamyl alcohol (1:24:1) was added to each sample,
- Samples were mixed carefully (vortex 10 min) and centrifuged (Hettich ZENTRIFUGEN UNIVERSAL 320R) at 9000 g for 10 min.
- The top aqueous layer (about 400 μl) was transferred to a fresh tube, to which was added (about 400 μl) chloroform: isoamyl alcohol (24:1), samples were mixed carefully (vortex 10 min) and centrifuged at 9000 g for 10 min.
4. MATERIALS AND METHODS

- The top aqueous layer (about 300 μl) was transferred to a fresh tube, to which was added (about 300 μl) of cold isopropanol.
- DNA was precipitated three times by liquid nitrogen and centrifuged to a pellet at 9000 g for 10 min.
- Pellets were washed by 70 % ethanol and centrifuged to a pellet at 9000 g for 10 min.
- Ethanol was discarded and the pellets were dried at laboratory temperature for 15 min.
- Pellets were suspended in 50 μl of TE buffer (10 mM Tris HCL, 1 mM EDTA, pH 8) containing RNase A (Invitrogen #12091-021) and stored at −20 °C before use. DNA extracts of root samples appeared discoloured brownish and had sediment present.

Another protocol of DNA extraction was optimized and used:

- Root samples were cut into small pieces and put into eppendorf tubes with 100 μl of CTAB and homogenized by using RETSCH Mixer Mills MM 400 for 90 sec at 250/min;
- 300 μl of (CTAB + mercaptoethanol) were added to each sample and homogenized again by using RETSCH Mixer Mills MM 400 for 3 min at 250/min;
- 300 μl of (CTAB + mercaptoethanol) were added to each sample with 900 μl phenol: chloroform: isoamylalcohol; all samples were shaken for 10 min and centrifuged for 10 min at 9000 g;
- Supernatant was replaced into new eppendorf tubes and the same amount of phenol: chloroform: isoamylalcohol was added; all samples were shaken for 10 min and centrifuged for 10 min at 9000 g;
- Supernatant was replaced into new eppendorf tubes and the same amount of chloroform: isoamylalcohol was added; all samples were shaken for 10 min and centrifuged for 10 min at 9000 g;
- Supernatant was replaced into new eppendorf tubes and the same amount of isopropanol was added;
- Samples were stored over night in a freeze-box; samples were centrifuged for 15 min at 9000 g;
- Supernatant were discarded and 200 μl of 70 % ethanol was added; samples centrifuged for 10 min at 9000 g;
- Ethanol was discarded and all samples were left for 15 min for drying; 20 μl TE were added to each sample.

PCR protocols
Two primer sets were employed to test the ability of the extracts to produce PCR product. The presence of *P. graminis* in the roots of plants grown in the soil samples and the positive control sample versus the absence of the vector in roots of plants in the negative control soil was verified by PCR assay. PCR method was used for DNA amplification with programme for *P. graminis* detection using the primers Psp1 5’ TAGACGCAGGTCATCAACCT 3’ and Psp2rev 5’ AGGGCTCTCGAAAGCGCAA 3’ according to Legrève et al. (2003). Steps were optimized by Department of Plant Protection in Prague, Faculty of Agrobiology, Food and Natural Resources (FAPPZ, ČZU).

For one reaction was used eppendorf tubes 0.5 ml with total volume of reaction mixture 25 µl:

- 17.35 µl dd water;
- 3 µl MgCl₂ 25mM (Promega);
- 2.5 µl 10x Taq buffer + (NH₄)₂SO₄ without MgCl₂ (Promega);
- 0.25 µl dNTP (10 mM);
- 0.4 µl Reverse primer (25 pmol) (5’ GCGTCCGTGTCACTGA 3’) + Forward primer (25 pmol) (5’ GAATTGCTTGTGCAACA 3’);
- 0.5 µl Taq DNA polymerase (Promega 5u/ µl);
- 1 µl cDNA from samples;

All amounts of previous components excepting cDNA were multiplied (according to the number of samples), then put in one eppendorf 1.5 ml (Premix). This mixture was closely vortex and separated to eppendorf tubes 0.5 ml, for each one is 24 µl, then added to each eppendorf 1 µl of cDNA (samples).

The PCR was realized using the **Thermocycler MJ research PTC-200 (Peltier Thermal Cycler)** as follows: Step by step of PCR programe Psp1:

1. 94 °C for 2 min.
2. 94 °C for 30 sec.
3. 60 °C for 1 min.
4. 72 °C for 35 sec.
5. Repeating previous steps No. 2, 3 and 4, usually 35 times.
6. 72 °C for 7 min.
7. 4 °C forever.
The annealing temperature was optimized by testing a gradient of temperatures on a set of cDNA samples in a Thermocycler MJ research PTC-200 (Peltier Thermal Cycler). After ending the thermocycler program, eppendorfe tubes were convoluted by mild centrifugation, and then the PCR products were immediately separated by gel electrophoresis.

To characterize the *P. graminis* isolates, the amplified PCR product (a DNA fragment of 472 bp) was sequenced and blasted for each of the samples that tested positive. These sequences were aligned with a known sequence (GenBank Accession No. AM259276) for *P. graminis*.

PCR protocol for the *P. graminis* types I and II-specific (Pgfwd2 5’ GGA AGG ATC ATT AGC GTT GAA T 3’ /Pxrev7 5’ GAG GCA TGC TTC CGA GGG CTC T 3’) primer set (Ward and Adams, 1998) was modified from (Mutasa et al., 1993, 1995; Mutasa-Gottgens et al., 1996). *P. graminis* types I and II are now named *P. graminis* f. sp. temperata and *P. graminis* f. sp. tepida, respectively (Legrève et al., 2002; Kanyuka et al., 2003). These probes target the ITS1 region between the nuclear small rDNA and the 5.8S rDNA (Ward and Adams, 1998). Each 20 μl reaction mixture contained 1 μl of DNA, 1X Taq reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.25 μM of primer, 0.1 μg/μl of bovine serum albumin (BSA) and 1 U of Taq polymerase (Qiagen). PCR amplification was performed according to the published protocol of (Mutasa et al., 1993, 1995; Mutasa-Gottgens et al., 1996).

All PCR amplifications were performed in a MJ Research PTC-200 Peltier Thermal Cycler. PCR products were detected and sized using 1 % agarose in TAE (Tris/acetic acid/EDTA) buffer, stained with ethidium bromide.

PCR protocol before purification:

*Pfu* DNA polymerase is an ideal choice for a variety of techniques requiring high-fidelity DNA synthesis by the polymerase chain reaction (PCR). These applications include cloning, gene expression, and site-directed mutagenesis. Successful PCR using *Pfu* DNA polymerase is readily performed requiring only slight modifications from PCR protocols optimized with *Taq* DNA polymerase.

For 25 μl reaction were used:

- PFU buffer + MgSO₄ 5 μl;
- dNTP 0.5 μl;
- primer mix (PSP1) 0.8 μl;
- PFU polymerase 1 μl (supplier);
- DNA 2 μl;
- ddH₂O 40.7 μl.
All PCR products were separated by electrophoresis and the DNA was ready for binding.

For cloning protocol (please see the appendix Clone JET™ PCR Cloning Kit).

All samples were sent for sequencing to the company of Macrogen (Netherlands).
4.12. Transmissibility of the BaMMV by Czech isolates of *P. graminis*

Transmissibility of the BaMMV by Czech isolates of *P. graminis* was done by collecting, washing and drying the barley roots of positive plant samples which contain *P. graminis* cystosori, under room temperature for one week, then the dried barley roots cut into small pieces, approximately 2 cm, and mixed thoroughly within sand particles (large sterilized fractions). The barley roots which contain *P. graminis* cystosori were mixed thoroughly within sand without any homogenisation process. Seeds of susceptible barley cv. Florian were sown into the sand culture and irrigated (with solution fertilizer application) once a week. After 20 days (3-4 leaves stage), seedlings of barley were inoculated mechanically by homogenized sap of fresh infected BaMMV leaves. A lot of barley plants have been infected and clear symptoms appeared after 28 days from inoculation process. All barley plants left approximately 3 months (stage of ears), then were removed from the pots leaving there their roots. In the same sand pots were resowed seeds of barley cv. Florian. After one month and half and more than three months, the barley plants were tested by ELISA and by RT-PCR for occurrence of BaMMV.

Three parts (leaves, neck and roots) of the barley plants were tested for BaMMV using the above mentioned protocols.
5. RESULTS AND DISCUSSION

5.1. Inoculum source

BaMMV inocula from infected winter barley Maris Otter were mechanically transmitted to the winter barley cv. Florian. From 2008 till now the BaMMV still virulent year by year on growing barley cv. Florian plants at glasshouse conditions 12-14 °C.

BaYMV inocula which were obtained from DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German collection of microorganisms and cell cultures) were not mechanically transmitted to the barley cv. Florian plants at glasshouse condition 12-14 °C even after repeated attempts.

BaMMV is readily transmissible, while BaYMV infection rate is often very low (Adams, 2000). The reason of its difficulty in transmission by mechanical means is not well understood. Virus infection by mechanical means is affected with virus concentration, host susceptibility, stability of the virus, and environmental conditions. Several studies have been done on BaYMV focusing on these factors. Infection rates so far obtained for mechanical transmission of BaYMV was low (below 50 %) (Kashiwazaki, et al., 1989; So et al., 1997) however, if two times of inoculation were applied at 7 days interval, transmission rate was improved (Friedt, 1983).

5.2. Mechanical inoculation methods

For the efficiency between SWG and FR inoculation methods (Fig. 8) showed significant differences in the infection rates between the two methods. However, cultivar Florian showed high infection rates in SWG method than FR; thereby making SWG inoculation became faster than either by the finger rubbing methods. Existing mechanical inoculation methods were inefficient and cumbersome. Thus, in this study, the improved mechanical inoculation method using SWG (Fig. 9) was demonstrated. The SWG method was very easy in applying. The wooden stick served as a handle for easy application allowing less chance of uneven application unlike the FR method. Based on the results in Fig. 8, the SWG method was more stable and efficient than FR method. The SWG method gave stable infections and offers speedy application without jeopardizing results.

In fact, the Friedt’s (1983) finger rubbing method required two inoculations at 7 days interval for increased infection. In the improved SWG method, one time application and two
inoculations at 7 days interval were similar to generate infection in susceptible barley cv. Florian plants. The FR method required more inoculum compared with the improved method. Consequently, the SWG is efficient method; its application is easy and uses low levels of inoculum compared to other methods.

Fig. 8. Percent of BaMMV-infected plants by two methods of mechanical inoculation: A) Finger rubbing (FR), B) Stick with gauze (SWG).

The initial symptoms were irregularly distributed and elongated chlorotic streaks of varying size on the youngest emerging barley cv. Florian leaves (Fig. 10), often were associated with upward rolling of leaf margins. The streaks were most distinct on the youngest leaves, and developed into a mosaic pattern. The mosaic symptoms were occasionally associated with necrotic patches, yellow discoloration, and/or rapid death of the older leaves. The symptoms on leaves appeared normally 28 days after mechanical inoculation process.
5. RESULTS AND DISCUSSION

5.3. Means of effective mechanical transmission of BaMMV

The first mosaic symptoms appeared four weeks after the first inoculation in the single-inoculation potassium phosphate buffer with and without additives (Fig. 11). The group of plants inoculated only once with potassium phosphate buffered sap showed the first symptoms after 28 days from the first treatment and the progression of symptoms continued till 42 days and then the progression stopped (Fig. 12). The proportion of infected plants subsequently increased more rapidly in the potassium phosphate buffer with sodium diethyldithiocarbamate (Na-DIECA 0.001 M) treated plants than in the potassium phosphate buffer 0.04 M alone or potassium phosphate buffer 0.04 M + Na-DIECA 0.01 M, potassium phosphate buffer 0.04 M + PMSF 0.01 M and potassium phosphate buffer 0.04 M + PMSF 0.001 M (Fig. 11). Therewith, both concentrations of Na-DIECA 0.01 M and Na-DIECA 0.001 M were useful for the preparation of highly infective inoculation fluids for practical application (Ketta, 2010) as indicated by the results in Figure 11 (53.33 % and 73.33 % infection rates respectively). Other additive concentrations of PMSF 0.01 M did however not improve the infectivity of inocula (6.66 %), but the concentrations of PMSF 0.001 M was improved the infectivity of inocula (26.66 %); similar infection rates were obtained with the potassium phosphate buffer 0.04 M alone (6.66 %). Treated plants did usually not show severe injury as a consequence of the inoculation procedure itself. However, phenylmethylsulfonyl fluoride (PMSF 0.01 M) as an additive to the inoculum fluid regularly caused wilt of the leaf tips a few hours after the inoculation process. This did however not influence the further healthy growth of these plants.

Fig. 9. Inoculation method stick with gauze (SWG)

Fig. 10. Symptoms of BaMMV infected winter barley cv. Florian leaf (right) and non-infected leaf (left).
5. RESULTS AND DISCUSSION

Fig. 11. Final frequencies of infected plants of cv. Florian six weeks after inoculation with sap containing the various additives after single mechanical inoculation with: water (control), potassium phosphate buffer 0.04 M pH 7 (A), additive PMSF 0.01 M pH 8.3 (B), additive PMSF 0.001 M pH 7.6 (C), additive Na-DIECA 0.01 M pH 8.1 (D), additive Na-DIECA 0.001 M pH 7.4 (E).

Obtained data were statistically analyzed by SAS program, T tests (LSD) for variable: Y1, Alpha= 0.05.

Control: 0.0, (A): 6.67b, (B): 6.67b, (C): 26.67b, (D): 53.33a and (E): 73.33a. Means with the same letter are not significantly different.

It could be demonstrated, that BaMMV-infection via mechanical transmission is feasible. The salient point is the addition of reducing additives of polyphenoloxidase to the potassium phosphate buffers during the homogenization process of BaMMV-infected leaves. This is in full agreement with the earlier findings, that different phosphates can increase the efficiency of mechanical inoculation of many viruses spectacularly (Matthews, 1981; Schmelzer, 1980). The percentage of infected plants increased over the time after inoculation. This indicated that BaMMV needs a long incubation period (Fig. 12). For practical application of mechanical inoculation technique, the production of large and unique quantities of inoculum is still a major problem. Chemical additives to the inoculation buffer increased infection, through chelating and inhibiting polyphenoloxidase as Na-DIECA. This is in full agreement with previous work (Gibbs and Harrison, 1980; Friedt, 1983; Kuntze and Bauer, 2000). Mechanical inoculation was especially more enhanced by the addition of Na-DIECA 0.001 M at pH 7.4 than Na-DIECA 0.01 M at pH 8.3 to the inoculation buffer, which may
directly help stabilize the nucleocapsid structure of BaMMV through inhibiting polyphenoloxidase activity (Goodwin, et al., 1984).

Fig. 12. Progression of the frequency of BaMMV-infected cv. Florian plants (i.e., appearance of visible symptoms) after single mechanical inoculation with: water (control), potassium phosphate buffer 0.04 M pH 7 (A), additive PMSF 0.01 M pH 8.3 (B), additive PMSF 0.001 M pH 7.6 (C), additive Na-DIECA 0.01 M pH 8.1 (D), additive Na-DIECA 0.001 M 7.4 (E).

Phenolic materials may cause serious difficulties in the isolation and preservation of viruses. Several methods have been used more or less successfully to minimize the effects of phenols on plant viruses during isolation. Cysteine or sodium sulphite added to the extraction medium both probably act by inhibiting the phenol oxidase and by combining with the quinine (Pierpoint, 1966). Polyphenoloxidase is a copper-containing enzyme. Two chelating agents with specificity for copper, diethylthiocarbamate and potassium ethyl xanthate have been used to obtain infectious preparations of several viruses (e.g., Prunus necrotic ring spot Ilavirus; Barnett and Fulton, 1971). Materials that compete with the virus for phenols have sometimes been used. For example, Brunt and Knten (1963) used various soluble proteins and hide powder to obtain infective preparations of swollen shoot virus from cocoa leaves. Synthetic polymers containing the amide link required for complex formation with tannins have been used effectively to bind these materials. The most important of these is polyvinyl pyrrolidone (PVP). On the other hand, Takanashi et al., (1967) indicated that low infectivity of BaYMV and WYMV by mechanical inoculation was due to the action of polyphenol oxidase. High infection rates were attained in addition of potassium cyanide (KCN) in extraction buffer. While in another study, addition of phenylmethylsulfonyl fluoride (PMSF)
was found to increase infectivity in mechanical inoculation of BaYMV-2 (Kuntze et al., 2000). Consequently, in an effort to increase BaYMV transmission by mechanical means, Jonson et al., (2007) are reporting effects of age of source plants grown at varying temperatures. Also, effects of enzyme inhibitors such as KCN and PMSF added in the inoculum were further confirmed.

From the results of first experiment we observed, that the chemical additive Na-DIECA in concentrations 0.01 M and 0.001 M was more effective during the mechanical inoculation than other chemical additives. For the previous reason we designed the second experiment of mechanical inoculation with potassium phosphate buffers 0.04 M and potassium phosphate buffers 0.04 M with additives (Na-DIECA 0.001 M and Na-DIECA 0.01 M) in different pH values (Table 5).

The obtained results confirmed, that the chemical additive Na-DIECA 0.001 M with pH value 8 was the most effective substance for mechanical inoculation of BaMMV to susceptible barley plants cv. Florian (Fig. 13).

Fig. 13. Percentage of infected barley plants cv. Florian four weeks after inoculation with sap containing the various additives after single mechanical inoculation with: Phosphate buffer 0.04 M, Na-DIECA 0.01 M, Na-DIECA 0.001 M and negative control in different pH values (6.5, 7, 7.5 and 8).

Explanation why the effectivity of pH is different when Na DIECA is added (buffer alone: pH 7 is the best, Na DIECA 0,001 M pH 7 is the worst) is not well understood.
5. RESULTS AND DISCUSSION

5.4. Monitoring of BaMMV and BaYMV in barley in the Czech Republic

All of seventeen barley plant samples, planted into soil samples collected separately from different localities of cereal production (barley) during April 2008 and the fifty-six tested barley plant samples, planted into soil samples collected separately from different localities of sugar beet production during 1989 and 1999 were infected neither by BaMMV nor by BaYMV. All plant samples were tested by DAS-ELISA using positive and negative control (ADGEN Phytodiagnostics - Protocol 1) and by RT-PCR assay using primers, reverse primer (5' GCGTCCGTTGCAACTGA 3') and forward primer (5' GAATTGCTTGTGCAACA 3') according to Hariri et al., (2000).

Rather: So, despite of the presence of both viruses in neighbouring countries we have not succeded to find them in Czech Republic. On the other hand the number of samples we tested is not too high and such attempts should be repeated.

5.5. Long-term preservation of BaMMV-infected tissues

All of barley cv. Florian plants of this experiment which were mechanically inoculated with four treatments of BaMMV-infected tissues 13 months preserved (Lyophilisation, freezing, glycerol and CaCl₂) compared with negative and positive control were tested by ELISA after four weeks from the inoculation proces. From the obtained results, the preservation method with CaCl₂ was the only working way for BaMMV-infected tissues preservation for a long-term (Table 6).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Infected plants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
</tr>
<tr>
<td>Fresh infected leaves</td>
<td>16.6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
</tr>
<tr>
<td>Freezing</td>
<td>0</td>
</tr>
<tr>
<td>Lyophilization</td>
<td>0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Table 6. The percentage of infected barley plants cv. Florian after mechanical inoculation with four treatments of BaMMV-infected tissues 13 months preserved (Lyophilisation, freezing, glycerol and CaCl₂) compared with negative (only grinding buffer) and positive control (fresh BaMMV-infected plants).
This experiment was repeated once 14 months preservation of the same source. The obtained results were similar to the experiment results of 13 months preservation. Preservation method with CaCl₂ was the only working way for BaMMV-infected tissues preservation for a long-term (13 and 14 months).

5.6. DAS-ELISA protocol (PAb)

As experimental material plants of the highly BaMMV-infected winter barley cv. Florian were used. Homogenization method of plant materials by the machine (RETSCH Mixer Mills MM 400) was much easier and quicker than other used methods of homogenization.

Sensitivity of ELISA

Detection sensitivity using the classical ELISA method was reported (Fig. 14). BaMMV was detected using ELISA in the three greatest concentrations ($10$, $10^{-1}$ and $10^{-2}$). A sample was considered positive for BaMMV when absorbance at 405 nm was more than three times the mean of the negative control (Sutula et al., 1986).

![Fig. 14. Sensitivity the ELISA assays for BaMMV detection, using dilution series of an extract on a German reference isolate (DSMZ). Histogram shows the evolution of the optical density (O.D) measured after 120 min at a wavelength of 405 nm in ELISA for the 7 first dilutions realized ($1\rightarrow10^{-7}$). N: negative control and P: positive control.](image-url)
5.7. RNA extraction and RT-PCR method

Although the using of mortar and pestle with liquid nitrogen for homogenization of plant materials for RNA extraction is a labour intensive and it takes much time, it was the best method for homogenization of plant material.

All tested RNA extraction methods, the isolation of RNA using phenol-chloroform isolation, isolation with GeneJET RNA kit and magnetic isolation were used for the detection of BaMMV in the infected leaves of barley, were on the bases of RT-PCR analysis to be reliable. The presence of BaMMV was confirmed in all samples analyzed.

Purity of RNA expressed as an \( \frac{A_{260}}{A_{280}} \) coefficient ranged between 2.02 - 2.06 with the isolation method GeneJET RNA kit and isolation by magnetic method, while the method of phenol-chloroform isolation values ranged between 1.59 – 1.65 (Table 7).

<table>
<thead>
<tr>
<th>Isolation of total RNA by phenol chloroform</th>
<th>Isolation of total RNA by GeneJET kit</th>
<th>Isolation of total RNA by magnetic method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration ng/µl</td>
<td>( \frac{A_{260}}{A_{280}} )</td>
<td>Concentration ng/µl</td>
</tr>
<tr>
<td>Mean of 7 samples</td>
<td>103</td>
<td>1.61</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>±22.30</td>
<td>±0.03</td>
</tr>
</tbody>
</table>

Table 7. Purity and concentration of total RNA extracted from BaMMV-infected barley plants by three of total RNA isolation: phenol-chloroform, isolation with GeneJET RNA kit and magnetic isolation.

In terms of the time consumption and total number of steps during the process of total RNA isolation, magnetic method was evaluated as the optimal one for the isolation of total RNA.

Total number of steps during the process of total RNA isolation by phenol-chloroform method was 18 steps, while in isolation with GeneJET RNA kit was 16 steps and only 8 steps in magnetic isolation. In terms of the time consumption by phenol-chloroform method was
100 min., while in isolation with GeneJET RNA kit was 40 min., and only 15 min. in magnetic isolation.

The separated fragments of cDNA on the gel were so sharp and clear with the extraction method by magnetic isolation in comparison with other methods of extraction (Fig. 15).

![Image of gel with DNA fragments]

**Fig. 15.** Comparison of sharpness and clearness of BaMMV DNA separated fragments 445 pb of two samples: A) GeneJET kit; B) Phenol chloroform extraction method and C) Isolation by magnetic method, M: Marker (MassRuler™ DNA Ladder, Low Range, ready-to-use (#SM0383) (Fermentas)

### 5.8. Monitoring of *P. graminis*

**Field observations**

When observed in the field 3 months after sowing, symptomatic barley plants were short (20 cm high). The leaf chlorosis did not occur in stripes or mosaic patterns and resembled nitrogen deficiency (Grundon, 1987). In comparison, asymptomatic plants were taller (50 cm high). Symptomatic plants occurred singly and in small irregular patches which appeared more prevalent towards one corner of the field and in lines probably corresponding with tractor wheel tracks. Plant density appeared less in affected patches than in healthier areas. These plants had somewhat similar leaf chlorosis to that of symptomatic plants further
Tuittert and Hofmeester (1992) found that soils with compaction layers retard both drainage and root growth and enhance infection of sugar beet with *P. betae*. The wetter microenvironment under such soil conditions after rainfall or irrigation events would also provide better conditions for *Polymyxa* zoospores to move and find roots to enter resulting in greater numbers of plasmodia, zoosporangia and sporosori in the roots.

**Root microscopy**

The first report of *P. graminis* occurrence in the Czech Republic was recorded by (Ryšánek et al., 2009). This was an observation of sporosori in the roots of barley plants, planted into soil samples collected from different localities of sugar beet production. In this work we reported the identification of *P. graminis* in the roots of barley plants, based on microscope observations of characteristic sporosori in unstained roots and on PCR amplification of *P. graminis* (Ward et al., 1994; Ward and Adams, 1998; Legrèве et al., 2003). The protist was identified as *P. graminis* on the basis of morphology of resting spores (cystosori) and sporangia and the size of individual cystosori (4 to 5 μm in diameter) according to (Thouvenel et al., 1980).

Photomicrographs of *P. graminis* sporosori which were obtained from barley roots are shown in Fig. 16 A–C. The appearance of the sporosori corresponds with the descriptions and photomicrographs of *P. graminis* in wheat given by (Ledingham, 1939; Barr, 1979). The roots without staining had a moderate infection on the scale of Legrèvé et al., (2000) with *P. graminis* seen as a few groups of sporosori in several root pieces including heavy infection in some parts (Fig. 16 A). Some of the sporosori appeared discoid (Fig. 16 C) as described by Karling (1968) and Littlefield et al., (1998) for mature sporosori rather than spheroid or cylindrical.
Fig. 16. Photomicrographs of sporosori of *P. graminis* in roots of barley cv. Florian from baitplants grown in soil from the same site (near Královéhradecký Region). Roots cleared and observed at magnifications up to x100 by bright field microscopy. (A) Higher magnification of the root. (B) Multiple sporosori in a single cell from a bait-plant. (C) Discoid shaped sporosori from a bait-plant.

This part of work documented the occurrence of *P. graminis* in a cereal crop in the Czech Republic for the first time as shown by microscopy of characteristic sporosori in the roots. Furthermore, barley bait-plants using soil from the site produced similar sporosori in the roots of barley cv. Florian grown in the glasshouse at 22-25 °C. The only other organisms that produce morphologically similar sporosori belong to *Ligniera* spp. (Braselton, 1995) Fig. 17.

Fig. 17. Sporosori of *Ligniera* spp. in the roots of barley cv. Florian in the different shape compared with rounded sporosori of *P. graminis*. Roots cleared and observed at magnifications up to x100 by bright field microscopy.
Soil collection

Cystosori of *P. graminis* were observed in the roots of barley plants cv. Florian grown in 2 of the 17 soil samples which were collected during April 2008 (Table 8) and 17 of the 46 soil samples which were collected in 1999 from different localities in Czech Republic (Table 9) and 3 of the 10 soil samples which were collected in 1988-1989 from other different localities (Table 10). This is the first confirmation about cystosori surviving in soils up to 20 years and occurrence of *P. graminis* in the Czech Republic (Ryšánek *et al.*, 2009).

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Locality</th>
<th>Polymyxa graminis on barley</th>
<th>Sample No.</th>
<th>Locality</th>
<th>Polymyxa graminis on barley</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Přešťovice</td>
<td>-</td>
<td>11</td>
<td>Borovnice</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Nová Dobev</td>
<td>-</td>
<td>12</td>
<td>Radim vpravo</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Pišek</td>
<td>-</td>
<td>13</td>
<td>Holín</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Dobšice</td>
<td>-</td>
<td>14</td>
<td>Prachov</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Vráž u Písk</td>
<td>-</td>
<td>15</td>
<td>Sobotka</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Čerhonice</td>
<td>-</td>
<td>16</td>
<td>Obrubce</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Mirovice</td>
<td>+</td>
<td>17</td>
<td>Kněžmost</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Výzkumná stanice Trutnov</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Inokulace - Plachká</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Dolní Kalná - Slemeno</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Presence of *P. graminis* in soil samples collected from different localities of cereal production (barley) in April 2008.

The presence of *P. graminis* in the roots of plants grown in the soil samples and the positive control sample versus the absence of the vector in roots of plants in the negative control soil was verified by PCR assay.
Table 9. Presence of *P. graminis* in soil samples collected from different localities of sugar beet production in 1999.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Locality</th>
<th><em>Polymyxa graminis</em> on barley</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Troubky-směr Přerov</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Kojetín-naproti železáren</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Želátovice-Kozlovice</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Stříbrnice</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Slatinice</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Rataje-u sílnice</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Loučany</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Vrbouce</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Polkovec</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Luková</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Rataje-Sedliště</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Miroslav</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Těšetice</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Měrovice</td>
<td>++</td>
</tr>
<tr>
<td>15</td>
<td>Rataje-zadní díl</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>Bříství</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Troubelice</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Šelešovice-Jarohněvice</td>
<td>++</td>
</tr>
<tr>
<td>19</td>
<td>Topolany</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Měrovice-Dostál</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Krňovice-Dostál</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Tištín za prefou</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Troubky-směr Vlkoš</td>
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<tr>
<td>24</td>
<td>Břežany střed</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>Kojetín-u železáren</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>Troubelice</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>Těšice</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>Rataje-Podsedky</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>Hradická</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 10. Presence of *P. graminis* in soil samples collected from different localities of sugar beet production in 1989.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Locality</th>
<th><em>Polymyxa graminis</em> on barley</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>Želátovice</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>Kojetín</td>
<td>-</td>
</tr>
<tr>
<td>35</td>
<td>Popice</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>Neslovice</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>Zíželice</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>Vyškov</td>
<td>+</td>
</tr>
<tr>
<td>60</td>
<td>Vajany</td>
<td>+</td>
</tr>
<tr>
<td>61</td>
<td>Kroupek</td>
<td>-</td>
</tr>
<tr>
<td>74</td>
<td>Počáply</td>
<td>+</td>
</tr>
<tr>
<td>99</td>
<td>Chorušice</td>
<td>-</td>
</tr>
</tbody>
</table>
Surviving of *P. graminis* sporosori at least 20 years in soil samples collected when *P. betae* was monitoting, probably the same requirements as *P. betae*. That is probably why it was found in small part of freshly collected samples which were taken from higher elevation where pH is probably lower.

**DNA extraction and PCR method**

The first method of DNA extraction was not optimal for obtaining the DNA of *P. graminis* cystosori. The reason was by the way the homogenization process. Homogenization by using mortar and pestles with liquid nitrogen was unsuitable for obtaining the DNA of *P. graminis* cystosori. In addition the time consumption was very long and the extraction by this way was very labour intensive. The second method of extraction and homogenization of barley roots material by using RETSCH Mixer Mills MM 400 was more suitable for obtaining the DNA of *P. graminis* cystosori and the time consumption was shorter in comparison with the first method of extraction and homogenization.

The presence of *P. graminis* in the roots of plants grown in the soil samples and the positive control sample versus the absence of the vector in roots of plants in the negative control soil was verified by PCR assay with DNA extracts and the Psp1 and Psp2rev primers according to Legrève *et al.* (2003). To characterize the *P. graminis* isolates, the amplified PCR product of a DNA fragment of 472 bp (Fig. 18 A) was ligated, cloned (Fig. 18 B), sequenced, and blasted for each of the samples that tested positive. These sequences were aligned with a known sequence (GenBank Accession No. AM259276) for *P. graminis*.

---

**Fig. 18.** A) PCR products of DNA fragments of 472 bp obtained from *P. graminis* cystosori. M: Marker (MassRuler™ DNA Ladder, Low Range, ready-to-use (#SM0383) (Fermentas), B) PCR products of DNA fragments for sequencing.
The sequences from *P. graminis* on barley were 100% homologous to the published sequence of *P. graminis* f. sp. *temperata*. To our knowledge, this is the first report of *P. graminis* f. sp. *temperata* in the Czech Republic (Ketta *et al.*, 2011a).

The sequence was submitted to the National Centre for Biotechnology Information (NCBI) GenBank Accession No. HQ378505.1.

**LOCUS** HQ378505 472 bp DNA linear INV 27-OCT-2010

**DEFINITION** Polymyxa graminis f. sp. temperata 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence.

**ACCESSION** HQ378505

**SOURCE** Polymyxa graminis f. sp. temperata

**ORGANISM** Polymyxa graminis f. sp. temperata Eukaryota; Rhizaria; Cercozoa; Plasmodiophorida; *Plasmodiophoridae*; Polymyxa.

**REFERENCE** 1 (bases 1 to 472)

**AUTHORS** Zouhar, M., Mazakova, J., Ketta, H. and Rysanek, P.

**TITLE** First occurrence of *Polymyxa graminis* in the Czech Republic

**JOURNAL** Unpublished

**REFERENCE** 2 (bases 1 to 472)

**AUTHORS** Zouhar, M., Mazakova, J., Ketta, H. and Rysanek, P.

**TITLE** Direct Submission

**JOURNAL** Submitted (06-OCT-2010) Department of Plant Protection, Czech University of Life Sciences, Kamycka 129, Prague 16521, Czech Republic

**FEATURES** Location/Qualifiers

**source** 1..472

/organism="Polymyxa graminis f. sp. temperata"

/mol_type="genomic DNA"

/db_xref="taxon: 380679"

/PCR_primers="fwd_name: Psp1, fwd_seq: tagacgcaggtcatcaacct, rev_name: Psp2rev, rev_seq: agggctctcgaaagcgcaa"

(note="forma_specialis: temperata"

/rRNA 1..210

/product="18S ribosomal RNA"

/misc RNA 211..342

/product="internal transcribed spacer 1"

/rRNA 343..>472

/product="5.8S ribosomal RNA"
5. RESULTS AND DISCUSSION

Molecular diversity of isolates of Polymyxa species have been studied using sequencing of transcribed regions of ribosomal RNA genes (nuclear ribosomal DNA, rDNA). Fragments of nuclear 18S, 5.8S and internal transcribed spacers (ITS1 and ITS2) rDNA regions have been analysed (Legrève et al., 2002; Morales et al., 1999; Ward and Adams, 1998; Ward et al., 1994). In these studies, the two Polymyxa species can be clearly distinguished, and there are several subgroups of P. graminis recognized. These distinct rDNA sequence types appear (to some extent at least) to be related to the host range, temperature requirements and geographical origin, and this has prompted Legrève et al., (2002) to propose classifying them as different formae speciales. The variation observed is far greater than would generally be expected between isolates of the same species; sequence differences of more than 20% across the two ITS regions can be seen between some isolates. Ribosomal DNA sequences have also been used to develop Polymyxa- and P. graminis-specific PCR tests that allow P. graminis subgroup determination by amplicon size or RFLP analysis (Morales et al., 1999; Ward and Adams, 1998; Ward et al., 1994).
5.9. Transmissibility of the BaMMV by Czech isolates of *P. graminis*

The obtained results from the experiments of the sand culture (Fig. 19) indicated that, all tested barley cv. Florian roots were parasitized by *P. graminis*, but were not infected by BaMMV after one and half month of growing. The chosen plants were tested by ELISA and RT-PCR.

Later after more than three months and half we obtained clear symptoms of BaMMV with the seedlings of barley cv. Florian. The results confirmed that the Czech isolates of *P. graminis* are able to transmit the BaMMV (Ketta *et al.*, 2011b). This information is essential for the growers of winter barley as in this case both viruses could easily spread in the country.

Fig. 19. Sand culture for growing of barley cv. Florian.
6. CONCLUSION

BaMMV inocula from infected winter barley Maris Otter were mechanically transmitted to the winter barley cv. Florian. From 2008 till now the BaMMV is still virulent year by year on growing barley cv. Florian plants at glasshouse condition 12-14 °C. BaYMV inocula which were obtained from DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German collection of microorganisms and cell cultures) were not mechanically transmitted to the barley cv. Florian plants at glasshouse conditions 12-14 °C.

For the efficiency between SWG and FR inoculation methods, the results showed significant differences in the infection rates between the two methods. Cultivar Florian showed higher infection rates in SWG method than FR; thereby making SWG inoculation became faster than either by the finger rubbing methods.

The initial symptoms were irregularly distributed and elongated chlorotic streaks of varying size on the youngest emerging barley cv. Florian leaves often were associated with upward rolling of leaf margins. The streaks were most distinct on the youngest leaves, and developed into a mosaic pattern. The mosaic symptoms were occasionally associated with necrotic patches, yellow discoloration, and/or rapid death of the older leaves. The symptoms on leaves appeared normally 28 days after mechanical inoculation process.

The proportion of infected plants subsequently increased more rapidly in the potassium phosphate buffer with sodium diethyldithiocarbamate (Na-DIECA 0.001 M) treated plants than in the potassium phosphate buffer 0.04 M alone or potassium phosphate buffer 0.04 M + Na-DIECA 0.01 M, potassium phosphate buffer 0.04 M + PMSF 0.01 M and potassium phosphate buffer 0.04 M + PMSF 0.001 M. Therewith, both concentrations of Na-DIECA 0.01 M and Na-DIECA 0.001 M were useful for the preparation of highly infective inoculation fluids for practical application as indicated by the results in Figure 11 (53.33 % and 73.33 % infection rates respectively). Other additive concentrations of PMSF 0.01 M did however not improve the infectivity of inocula (6.66 %), but the concentrations of PMSF 0.001 M was improved the infectivity of inocula (26.66 %). From the results of first experiment we observed, that the chemical additives Na-DIECA 0.01 M and Na-DIECA 0.001 M were more effective during the mechanical inoculation than other chemical additives. For the previous reason we designed the second experiment of mechanical inoculation with potassium phosphate buffers 0.04 M and potassium phosphate buffers 0.04 M with additives (Na-DIECA 0.001 M and Na-DIECA 0.01 M) in different pH values (6.5, 7, 7.5 and 8). The
obtained results confirmed, that the chemical additive Na-DIECA 0.001 M with pH value 8 was the most effective chemical substance for mechanical inoculation of BaMMV to susceptible barley plants cv. Florian.

For the monitoring of BaMMV and BaYMV in some localities in the Czech Republic, seventeen soil samples, which collected separately from different localities of cereal production (barley) during April 2008 using a digging fork were placed separately and planted to barley in pots in a greenhouse at 12-14 °C and fifty-six tested barley plant samples, planted into soil samples collected from different localities of sugar beet production during 1989 and 1999 were infected neither by BaMMV nor BaYMV.

From the obtained results, the preservation method with CaCl$_2$ was the only working way for BaMMV-infected tissues preservation for a long-term (13 and 14 months). Homogenization method of plant materials by the machine (RETSCH Mixer Mills MM 400) was much easier and quicker than other used methods of homogenization for the reason of BaMMV detection by ELISA. Detection sensitivity using the classical ELISA method reported that BaMMV was detected using ELISA in the three greatest concentrations. A sample was considered positive for BaMMV when absorbance at 405 nm was more than three times the mean of the negative control.

Although the using of mortar and pestle with liquid nitrogen for homogenization of plant materials for RNA extraction is a labor intensive and it takes much time, it was the best method for homogenization of plant material. All tested RNA extraction methods, the isolation of RNA using phenol-chloroform isolation, isolation with GeneJET RNA kit and magnetic isolation were used for the detection of BaMMV in the infected leaves of barley, were on the bases of RT-PCR analysis to be reliable. The presence of BaMMV was confirmed in all samples analyzed. Purity of RNA expressed as an A$_{260}$/A$_{280}$ coefficient ranged between 2.02 - 2.06 with the isolation method GeneJET RNA kit and isolation by magnetic method, while the method of phenol-chloroform isolation values ranged between 1.59 – 1.65. In terms of the time consumption and total number of steps during the process of total RNA isolation, magnetic method was evaluated as the optimal one for the isolation of total RNA.

The separated fragments of cDNA on the gel were so sharp and clear with the extraction method by magnetic isolation in comparison with other methods of extraction.

Cystosori of *P. graminis* were observed in the roots of barley plants cv. Florian grown in 2 of the 17 soil samples which were collected from different localities of cereal production (barley) during April 2008 and 17 of the 46 soil samples which were collected in 1999 from different localities of sugar beet production in Czech Republic and 3 of the 10 soil samples
which were collected from different localities of sugar beet production in 1988-1989 from other different localities. This is the first confirmation about cystosori surviving in soils up to 20 years and occurrence of *P. graminis* in the Czech Republic.

The method of extraction and homogenization of barley roots material by using RETSCH Mixer Mills MM 400 was more suitable for obtaining the DNA of *P. graminis* cystosri and the time consumption was shorter in comparison with the first method of extraction and homogenization. To characterize the *P. graminis* isolates, the amplified PCR product (a DNA fragment of 472 bp) was sequenced and blasted for each of the samples that tested positive. These sequences were aligned with a known sequence (GenBank Accession No. AM259276) for *P. graminis*. The sequences from *P. graminis* on barley were 100 % homologous to the published sequence of *P. graminis* f. sp. *temperata*. To our knowledge, this is the first report of *P. graminis* f. sp. *temperata* in the Czech Republic.

The obtained results from the experiments of the sand culture indicated that all tested barley cv. Florian roots were parasitized by *P. graminis*, but were not infected by BaMMV after one and half month of growing. The chosen plants were tested by ELISA and RT-PCR. Later after more than three and half months we obtained clear symptoms of BaMMV with the seedlings of barley cv. Florian. The results confirmed that the Czech isolates of *P. graminis* are able to transmit the BaMMV.
7. REFERENCES


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Fig. 1 hand homogenizer (Bioreba)

Fig. 2 RETSCH Mixer Mills MM 400

Fig. 3 Marker-MassRulerTM DNA Ladder, Low Range, ready-to-use (#SM0383) (Fermentas).

Fig. 4 A) NanoDrop2000; B) NanoDrop2000 Pipette-Product page and C) UV-Vis-Column.

Fig. 5 A) The machine (InnuPure® C12) for magnetic isolation of RNA and B) The rank for samples.

CloneJET PCR CLONING KIT
Fig. 1 hand homogenizer (Bioreba)

Fig. 2 RETSCH Mixer Mills MM 400

Fig. 3 Marker-MassRuler™ DNA Ladder, Low Range, ready-to-use (#SM0383) (Fermentas).
Fig. 4 A) NanoDrop2000; B) NanoDrop2000 Pipette-Product page and C) UV-Vis-Column.

Fig. 5 A) The machine (InnuPure® C12) for magnetic isolation of RNA and B) The rank for samples.
CloneJET™ PCR Cloning Kit

CLONING PROTOCOLS

Blunt-End Cloning Protocol

- For cloning blunt-end PCR products generated by proofreading DNA polymerases, such as Pfu DNA polymerase. (If the DNA end structure of the PCR products is not specified by the supplier of the DNA polymerase, follow the Sticky-End Cloning Protocol on page 6).
- For cloning of blunt-end DNA fragments generated by restriction enzyme digestion. Gel-purify the DNA fragment prior to ligation and use in a 3:1 molar ratio with pJET1.2/blunt (see Table 1).

1. Set up the ligation reaction on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Reaction Buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>Non-purified PCR product or purified PCR product/other blunt-end DNA fragment</td>
<td>1 µl</td>
</tr>
<tr>
<td>pJET1.2/blunt Cloning Vector (50 ng/µl)</td>
<td>0.15 pmol ends</td>
</tr>
<tr>
<td>Water, nuclease-free</td>
<td>up to 19 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Total volume: 20 µl

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the ligation mixture at room temperature (22°C) for 5 min.
   
   Note. For PCR products >3 kb, ligation can be prolonged to 30 min. Ligation times longer than 30 min are not recommended and may decrease cloning efficiency.

3. Use the ligation mixture directly for transformation (see page 7 for Transformation).
   
   Note. Keep the ligation mixture at -20°C if transformation is postponed. Thaw on ice and mix carefully before transformation.
Sticky-End Cloning Protocol

- For cloning PCR products with 3'-da overhangs generated by TagDNA polymerase, DreamTaq DNA polymerase or enzyme mixtures containing TagDNA polymerase.
- For cloning PCR products when DNA end structure of the generated PCR products is not specified by the supplier of the DNA polymerase.
- For cloning DNA fragments with 5' or 3'-overhangs generated by restriction enzyme digestion. Gel-purify the DNA fragment prior to ligation and use in a 3:1 molar ratio with pJET1.2/blunt (see Table 1).

**Note.** The DNA Blunting Enzyme is a proprietary thermostable DNA polymerase with proofreading activity. It will remove 3'-overhangs and fill in 5'-overhangs. Nucleotides for the blunting reaction are included in the reaction buffer.

1. Set up the blunting reaction on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Reaction Buffer</td>
<td>10 μl</td>
</tr>
<tr>
<td>Non-purified PCR product or purified PCR product/other sticky-end DNA fragment</td>
<td>1 μl (0.15 pmol ends)</td>
</tr>
<tr>
<td>Water, nuclease-free</td>
<td>to 17 μl</td>
</tr>
<tr>
<td>DNA Blunting Enzyme</td>
<td>1 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>18 μl</strong></td>
</tr>
</tbody>
</table>

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the mixture at 70°C for 5 min. Chill on ice.

3. Set up the ligation reaction on ice. Add the following to the blunting reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJET1.2/blunt Cloning Vector (50 ng/μl)</td>
<td>1 μl (0.05 pmol ends)</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20 μl</strong></td>
</tr>
</tbody>
</table>

Vortex briefly and centrifuge for 3-5 s to collect drops.

4. Incubate the ligation mixture at room temperature (22°C) for 5 min.

**Note.** For PCR products >3 kb, ligation can be prolonged to 30 min. Ligation times longer than 30 min are not recommended and may decrease cloning efficiency.

5. Use the ligation mixture directly for transformation (see page 7 for Transformation).

**Note.** Keep the ligation mixture at -20°C if transformation is postponed. Thaw on ice and mix carefully before transformation.
Transformation

- The CloneJET™ PCR Cloning Kit is compatible with all common E.coli laboratory strains. Transformation of competent E.coli cells with the ligation mixture can be performed using different transformation methods (Table 2 and 3).

- The number of transformants on the plates directly depends on the transformation efficiency of the competent cells.

- For successful cloning, competent E.coli cells should have an efficiency of at least 1x10⁶ cfu/μg supercoiled plasmid DNA. To check the efficiency, prepare a control transformation with 0.1 ng of a supercoiled vector DNA, e.g., pUC19 DNA, #SD0061 (Table 2).

Table 2. Evaluation of transformation efficiency of competent cells (control transformation).

<table>
<thead>
<tr>
<th>Transformation method</th>
<th>Number of transformants per μg of supercoiled plasmid DNA</th>
<th>Amount of pUC19 DNA for control transformation (to yield ~1000 colonies per plate)</th>
<th>Volume of competent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransformAid™ Bacterial Transformation Kit</td>
<td>~1x10⁷</td>
<td>0.1 ng</td>
<td>50 μl</td>
</tr>
<tr>
<td>(#K2710)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium chloride transformation</td>
<td>~1x10⁶</td>
<td>1 ng</td>
<td>50 μl</td>
</tr>
<tr>
<td>Electro-transformation</td>
<td>~1x10⁶</td>
<td>0.01 ng</td>
<td>40 μl</td>
</tr>
</tbody>
</table>

* XL1-Blue, ER2267, ER1727 E. coli strains are the best strains for transformation with TransformAid™ Bacterial Transformation Kit. DH10B, DH5α and TOP10 strains are not efficient with TransformAid™ but are recommended for calcium chloride transformation or electroporation.

- For transformation of the ligation mixture, refer to Table 3.

Table 3. Recommendations for transformation of ligation mixture.

<table>
<thead>
<tr>
<th>Transformation method</th>
<th>Treatment of the ligation mixture before transformation</th>
<th>Volume of the ligation mixture for transformation</th>
<th>Volume of competent cells for transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransformAid™ Bacterial Transformation Kit (#K2710)</td>
<td>Not necessary</td>
<td>≤2.5 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>Calcium Chloride Transformation</td>
<td>Not necessary</td>
<td>≤5 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>Electro-transformation</td>
<td>Spin column (GeneJET™ PCR Purification Kit, #K0701) or chloroform extraction (see protocol on p. 11)</td>
<td>1 μl of purified ligation mixture</td>
<td>40 μl</td>
</tr>
</tbody>
</table>
Analysis of recombinant clones

Analyze 4-6 colonies for the presence and orientation of the DNA insert using one of the following methods:

Colony PCR

Use the following protocol for colony screening by PCR if the cloned PCR fragment is shorter than 3kb. For longer inserts, perform restriction analysis.

1. Prepare enough PCR master mix for the number of colonies analyzed plus one extra. For each 20 μl reaction, mix the following reagents:

<table>
<thead>
<tr>
<th>Component</th>
<th>Using Taq DNA Polymerase</th>
<th>Using 2X PCR Master Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Taq buffer</td>
<td>2.0 μl</td>
<td>–</td>
</tr>
<tr>
<td>dNTP mix, 2 mM each</td>
<td>2.0 μl</td>
<td>–</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1.2 μl</td>
<td>–</td>
</tr>
<tr>
<td>pJET1.2 Forward Sequencing Primer, 10 μM</td>
<td>0.4 μl</td>
<td>0.4 μl</td>
</tr>
<tr>
<td>pJET1.2 Reverse Sequencing Primer, 10 μM</td>
<td>0.4 μl</td>
<td>0.4 μl</td>
</tr>
<tr>
<td>Water, nuclease-free</td>
<td>13.9 μl</td>
<td>9.2 μl</td>
</tr>
</tbody>
</table>

*Tag DNA Polymerase 5 u/μl, #EP0401 or DreamTaq™ Green DNA Polymerase, #EP0711*

<table>
<thead>
<tr>
<th>PCR Master Mix (2X), #K0171 or DreamTaq™ Green PCR Master Mix (2X), #K1081</th>
<th>–</th>
<th>10 μl</th>
</tr>
</thead>
</table>

| Total volume | 20 μl | 20 μl |

2. Mix well. Aliquot 20 μl of the mix into the PCR tubes on ice.

3. Pick an individual colony and resuspend in 20 μl of the PCR master mix.

4. Perform PCR: 95°C, 3 min; 94°C, 30 s, 60°C, 30 s, 72°C 1 min/kb; 25 cycles.

5. Analyze on an agarose gel for the presence of the PCR product.

Note

Due to considerable amount of recircularised vector plated on the surface of plate, colony PCR may give some false negative results. Prior to clone analysis propagate short strikes of individual colonies on ampicillin plates. Then use small amount of each for colony PCR.

Restriction analysis

Isolate plasmid DNA from an overnight bacterial culture. To speed up the process and to assure the quality of purified plasmid DNA, use the GeneJET™ Plasmid Miniprep Kit (#K0503). To digest DNA from recombinant clones in just 5 minutes, use FastDigest® restriction enzymes.

Sequencing

Use the pJET1.2 Forward Sequencing Primer or pJET1.2 Reverse Sequencing Primer supplied with the kit to sequence the cloned insert. See page 11 for primer sequences.
CONTROL CLONING EXPERIMENT

The control reaction should be used to verify the efficiency of the blunting and ligation steps. The 976 bp control PCR product (nucleotide sequence is available at www.fermentas.com) has been generated with Tag DNA polymerase, which adds extra nucleotides to the 3’-end. Therefore, the Sticky-End Protocol must be followed.

1. Set up the blunting reaction on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Reaction Buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>Control PCR Product (24 ng/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Water, nuclease-free</td>
<td>5 µl</td>
</tr>
<tr>
<td>DNA Blunting Enzyme</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>18 µl</td>
</tr>
</tbody>
</table>

Vortex briefly and centrifuge for 3-5 s to collect drops.

2. Incubate the mixture at 70°C for 5 min. Chill on ice.

3. Set up the ligation reaction on ice. Add the following to the blunting reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJET1.2/blunt Cloning Vector (50 ng/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Vortex briefly and centrifuge for 3-5 s to collect drops.

4. Incubate the ligation mixture at room temperature (22°C) for 5 min.

5. Use the ligation mixture directly for transformation (see page 7 for Transformation). Keep the ligation mixture at -20°C if transformation is postponed. Thaw on ice and mix carefully before transformation.

   Analyze colonies by colony PCR (see page 8). At least 9 of 10 analyzed colonies should contain recombinant plasmid with the 976 bp insert.

   The number of transformants depends on the transformation efficiency of the *E. coli* cells. Verify the transformation efficiency by transforming supercoiled plasmid, e.g., pUC19 DNA (#SD0061) in parallel. Refer to page 7 Table 2 for correct control transformations.
MAP AND FEATURES OF pJET1.2/blunt CLONING VECTOR

The pJET1.2/blunt cloning vector has been linearized with Eco32I (EcoRV) (GenBank/EMBL Accession number EF894056). The blunt ends of the vector contain 5'-phosphoryl groups. The nucleotide sequence of pJET1.2/blunt is available at www.fermentas.com.

![Diagram of pJET1.2/blunt Vector Map]

Fig. 1. pJET1.2/blunt Vector Map.