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**Elimination of viruses in garlic (*Allium sativum* L.) by  
different methods**  
Bachelor thesis

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Brno 2016

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Abstract

**Elimination of viruses in garlic (*Allium sativum* L.) by different methods**

Garlic (*Allium sativum* L.), is aromatic annual herbaceous plant known for its world wide application. Overtime, myriad of viruses have infected garlic causing a serious decline in both harvest quality and quantity and big economic losses to producers. The aim of this work was to examine effect of different methods in elimination of viruses using *in vitro* culture system. Four viruses - garlic common latent virus (GCLV), leek yellow stripe virus (LYSV), onion yellow dwarf virus (OYDV) and shallot latent virus (SLV) were screened. The influence of thermotherapy of cloves followed by meristem isolation on the viruses elimination was evaluated. Moreover, meristem isolation in combination with ribavirin application was investigated in respect to its antiviral properties. Results obtained from thermotherapy treatment in combination with meristem isolation show various positive/negative virus presence among cloves of the same bulb. After chemotherapy treatment two cultivars were virus-free. The most frequent virus was OYDV and most difficult to eliminate. From our results it can be seen that more successful technique for obtaining virus-free plants was combination of meristems isolation and chemotherapy.

Key words: thermotherapy, chemotherapy, meristem isolation

Abstrakt

### **Eliminace virů u česneku kuchyňského (*Allium sativum* L.) různými metodami**

Česnek (*Allium sativum* L.), je aromatická jednoletá bylina známá po celém světě. Česnek infikuje velké množství virů, které způsobují pokles v kvalitě a množství sklizně, čímž způsobují velké ekonomické ztráty pěstitelům. Cílem této práce bylo zjistit vliv různých metod odstranění virů za použití *in vitro* technik. Čtyři viry - garlic common latent virus (GCLV), leek yellow stripe virus (LYSV), onion yellow dwarf virus (OYDV) a shallot latent virus (SLV) byly detekovány. Byl vyhodnocen vliv termoterapie s následnou izolací meristému na odstranění virů. Kromě toho, byla zkoumána izolace meristému v kombinaci s aplikací ribavirinu s ohledem na jeho antivirové vlastnosti. Po vyhodnocení termoterapie v kombinaci s izolací meristému byly prokázány různé výsledky přítomnosti viru (pozitivní / negativní) mezi stroužky pocházející ze stejné cibule. Po léčbě chemoterapií byly dvě odrůdy bez virů. Nejčastěji vyskytující se a nejhůře odstranitelný virus byl OYDV. Z výsledků je zřejmé, že neúspěšnější technika pro získání viru prostých rostlin byla kombinace izolace meristému a chemoterapie.

Klíčová slova: termoterapie, chemoterapie, izolace meristému

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# 1 INTRODUCTION

Garlic (*Allium sativum* L.), is aromatic annual herbaceous plant known for its world wide application. It is widely used as a vegetable, in traditional medicine, as a human nutrition, for its antibiotic and antiseptic effects and many other reasons and purposes. *Allium sativum* is one of the most consumed alliums which belong to the family *Alliaceae* also known as onion family.

In general, reproduction is done by underground cloves or vegetative top sets on the flower. This kind of propagation allows various pests and diseases caused by different types of fungi, bacteria and mainly viruses to damage bulbs. Overtime, myriad of viruses have infected garlic causing a serious decline in both harvest quality and quantity and big economic losses to producers. Viruses which commonly attack garlic belongs to genera's: Potyvirus - Onion yellow dwarf virus (OYDV), Leek yellow stripe virus (LYSV), Carlavirus - Garlic common latent virus (GCLV) and Shallot latent virus (SLV). Viruses as Fijivirus – Garlic dwarf reovirus and Alexivirus are also commonly found in garlic. Beside those tree main genera's of viruses, garlic can be affected by many others viruses which are not of economic importance.

Main pathway of viruses transfer is through vegetative propagation where significant role has vectors which are predominately – aphids and thrips. Moreover, viruses are accumulated within host and spread within healthy plants every production season reducing yield and bulb quality.

Due to big economic losses and reduction in quality and quantity in production of garlic it is necessary to find a certain method of yield increment and disease control. Propagation of pathogen-free garlic plants by a traditional agronomic system is expensive and difficult, since it has to be carried out in an area free of disease vectors. To overcome those problems, one of the possible pathways is viruses' elimination and further multiplication of disease-free plants in *in vitro* conditions which could be used as a solution to obtain a large number of plants in a short time, without the risk of re-infection. Through *in vitro* culture system there are possibilities for obtaining pathogen-free plants by meristem culture, followed by thermotherapy and chemotherapy treatments.



## **2 AIM OF THE RESEARCH**

The aim of this work was to examine effect of different methods in elimination of viruses using *in vitro* culture system. Four viruses GCLV, LYSV, OYDV and SLV were screened. The influence of thermotherapy of cloves followed by meristem isolation on the viruses' elimination was evaluated. Moreover, meristem isolation in combination with ribavirin application was investigated in respect to its antiviral properties.

### 3 LITERATURE REVIEW

#### 3.1 Botanical classification of garlic

*Allium sativum* L. belongs to the family *Alliaceae*, genus *Allium*. The taxonomic position of *Allium* and related genera had been a matter of controversy for long.

Takhtajan (1997) suggested the following classification which was adopted:

1. Class – *Liliopsida*,
2. Subclass – *Liliidae*,
3. Superorder – *Lilianae*,
4. Order – *Amaryllidales*,
5. Family – *Alliaceae*,
6. Subfamily – *Allioideae*,
7. Tribe – *Allieae*,

According to Block (2010) current taxonomic classification of alliums is:

1. Class – *Monocotyledons*
2. Superorder – *Liliiflorae*
3. Order – *Asparagales*
4. Family – *Alliaceae*
5. Tribe – *Alliae*
6. Genus – *Allium*

Carl Linnaeus, in 1753 described genus *Allium* and put this genus into a group *Hexandria monogynia* which was counting fifty genres and thirty species. In their work, Fritch and Keusgen (2006) confirmed the topicality of the following information obtained from different publications - contemporary classification acknowledged more than seven hundred and fifty *Allium* species (Stearn, 1992; Hanelt et al., 1992). Hanelt et al. (1992) classified genus *Allium* into 6 subgenera and 57 sections. Based on classification proposal which is established primarily on sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, genus is divided on 780 species, 15 subgenera and 56 sections (Fritsch and Friesen, 2002; Friesen et al., 2006). In 2010 Fritsch et al., reveals that genus *Allium* include more than 800 species. This

classification is based on morphological and molecular characters. Infrageneric taxonomy and evolution of genus *Allium* still remains incompletely explained (Li et al., 2010). On the Fig. 1 the phylogenetic classification according to Fritsch (2001) is represented:

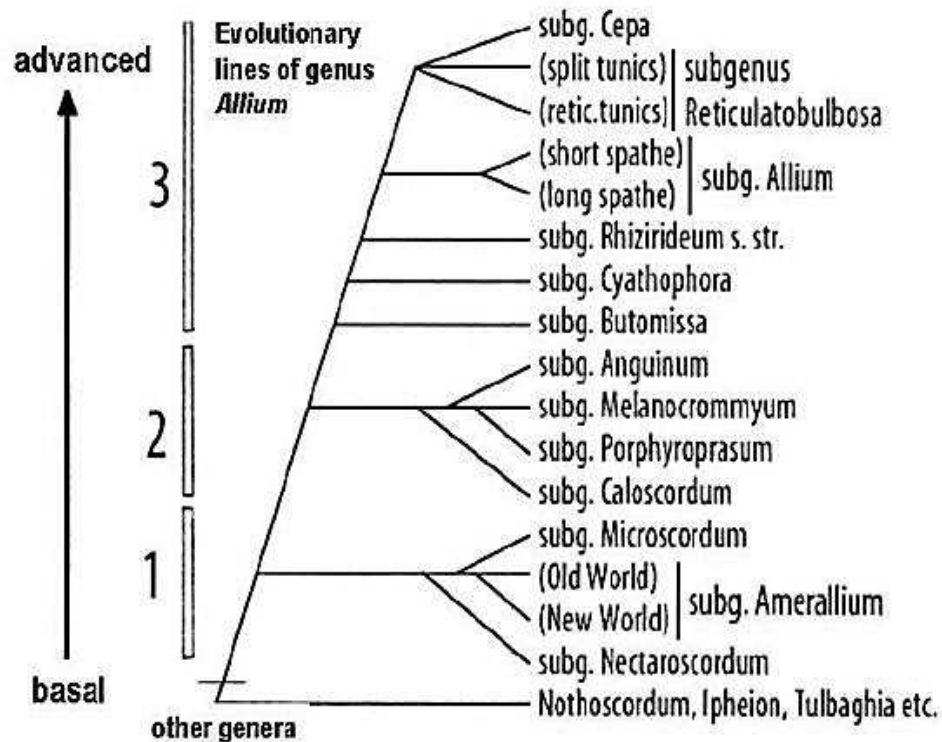


Fig.1 Phylogenetic classification of *Alliums* (Fritsch, 2001)

### 3.2 General characteristics of garlic

Cultivation of garlic and some other *Alliums* started together with the extension of the civilization and development of the human race in general. Its botanical name is *Allium sativum* where “*sativum*” means cultivated. That is because wild garlic does not exist (Block, 2010). This vegetable is an obligate apomictic (Novak et al., 1986) seed sterile plant (Novak et al., 1986; Maaß and Klaas M., 1995). Garlic was used not only for food and to add flavor to the dishes but as well it was one of the earliest documented plants used as a medicament (Block, 2010). Its chemical composition contains allicin, sulfuric compound which is mostly related with its medicinal effects (Schulz et al., 1998). According to McCollum (1976), *Allium sativum*, the same as *Allium cepa* is a diploid plant, having  $2n=16$  chromosomes.

### 3.2.1 Origin of Garlic

Garlic is one of the oldest known horticultural crops. There are many evidences that it was used in the Egyptian and Indian culture 5000 years ago, Babylonians 4500 years ago and in Chinese tradition over 2000 years ago. As it was mentioned before, garlic was used for centuries mainly in traditional medicine. First quotations about medical application of *Allium sativum* appeared in the Codex Ebers (1550 B.C.), an Egyptian medical papyrus (Ghalehkandi, 2014). An English name for *Allium sativum* comes from an old English word garleac, which has a meaning “spear leek”. In this old word “gar” means spear, due to garlic’s spear shaped leaves and “leak” means leek (Neeraj et al., 2014). Common for *Alliums*, is that they have Central Asian and East Asian origin (Pekárková, 2005; Etoh et al., 2001). It is well known that garlic is native to Central Asia, but what is exact country of its origin still remains questionable. From the other hand, it can be considered that garlic originates from southwest of Siberia and from there it was spread to Europe where it was naturalized (Neeraj et al., 2014). *Alliums* are native plants of temperate climate zones grown worldwide, from the Arctic Circle to tropical areas thanks to its taste characteristics and phytoncides content with bactericidal and fungicidal properties (Pekárková, 2005). Even if history of garlic cultivation is very long, little is known about early production or plant types used for its cultivation (Simon et al., 2004).

### 3.2.2 Morphology of garlic

The garlic is the herbaceous perennial plant, with foliage leaves attached to an underground stem (Fig. 2). Leaves of garlic emerge from the stem are situated on the base of the bulb which looks like the flat plate. Leaves are without stalk, V-shaped in cross section, with both solid scape and foliage. Interestingly, after the death of the stem (in case of mature bulb), the living basal plate stays on each clove, in order to carry out future growth. Inflorescence (flower cluster) stem of *Allium sativum* is without leaves. The only leaves on this plant part are those forming the inflorescence.

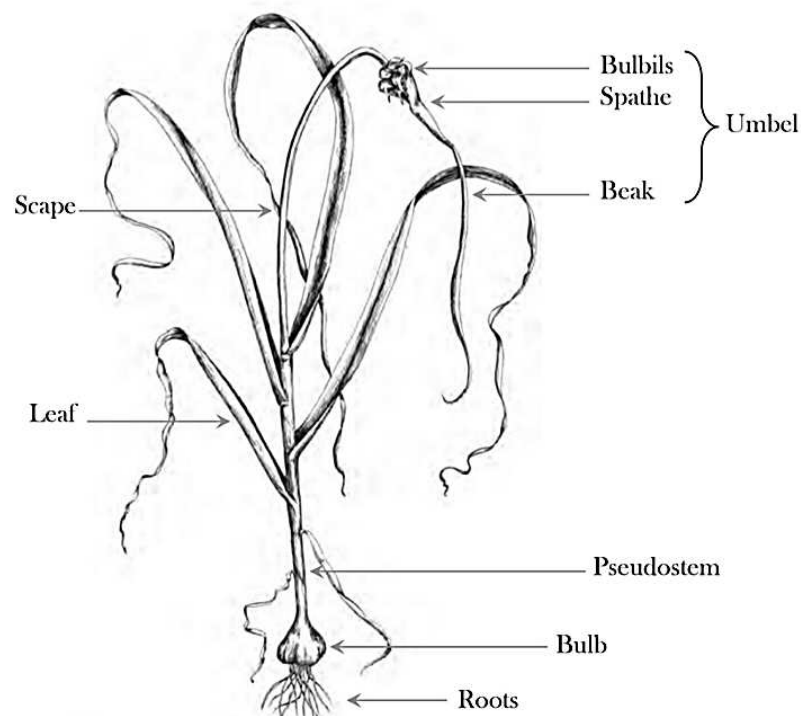


Fig. 2 Morphological appearance of garlic (<http://goo.gl/grFnPz>)

The perianth is formed from twelve parts-six anther-bearing and six petal-like. Garlic and other *Alliums* have “pseudostem” which represents aboveground stem created from leaf sheaths where each new leaf grows through the center of pseudostem having the highest position comparing to other leaves. The number of leaves and their height is variable. Adventitious roots are without root hairs and thanks to the mycorrhizal fungi and its symbiotic relationship, garlic is able to uptake needed nutrients for growth. The bulb is formed from cloves which are made from bladeless storage leaves. Typical for garlic is that cloves are protected with death leaves-sheaths but the clove is saved from drying by abscission layer. Garlic clove is consisted from protective leaf, storage leaf, sprout leaf, foliage leaf primordia, root primordia and basal plate. The inflorescence is called umbel and it is composed from small flowers-pedicels. Each of pedicels has its own flower stalk and six petals which is pink, white, off-white or purple colored, depending on cultivar. Garlic also has small bulbils which are asexual propagules and after planting those can develop in the mature bulb (Meredith, 2008). The other ways of vegetative reproduction of *Allium sativum* are by axillary bulbs, topsets and division of rhizomes as well (Kamenetsky and Rabinowitch, 2006).

### **3.2.3 Usage and importance of garlic**

After *Allium cepa*, garlic is the second most important species in the genus *Allium*. The mostly consumed part of the plant is bulb, which can be composed from few to many cloves. Beside the bulb, the other parts of the plant like fresh leaves and topsets are also used (Fritsch and Friesen, 2002). Major usage of garlic is due to its medicinal properties i.e. against bacterial infections. The first who described its antibacterial effect was Louis Pasteur. Garlic is characterized by antibacterial spectrum against gram-positive and gram-negative bacteria. It is commonly used against many pathogenic bacteria which cause diarrhea in humans but as well in animals. Garlic is showing its effect even with those bacteria strains which are resistant to antibiotics and even against toxins originated by microorganisms (Sivam, 2001). Beside antibacterial properties, garlic also has effects against many genera of fungi and viruses (Gebreyohannes and Gebreyohannes, 2013). According to Reuter et al. (1996), it also has therapeutic effects on the cardiovascular system and it is very useful as an antioxidant and antibiotic. Furthermore, it was seen that garlic has immunomodulatory, anti-inflammatory, hypoglycemic, anticancer and many other beneficial effects on human health.

### **3.2.4 Active substances in garlic**

In garlic composition it can be found around thirty three sulfuric compounds, several enzymes, amino acids and the minerals such as germanium, calcium, copper, iron, potassium, magnesium, selenium and zinc. From the group of vitamins garlic has vitamin A, B1 and C (Josling, 2005).

From all *Alliums*, garlic has the highest concentration of sulfuric compounds. Those compounds are not responsible only for garlic's odor but as well for many other medical effects (Kamenetsky et al., 2015; Świdorski et al., 2007; Randle et al., 2002). The most important and biological active enzyme in *Allium sativum* is allicin. Interestingly allicin does not exist in garlic but it appears after crushing or slicing the cloves. Conversion is done under the influence of enzyme alliinase which metabolizes alliin into allicin (an unstable product of strong garlic odor, pyruvic acid and ammonia) (Fig. 3).

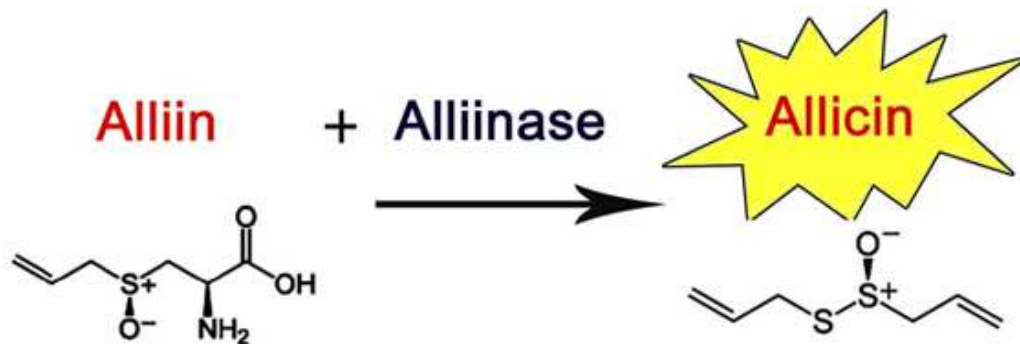


Fig. 3 Conversion of alliin to allicin <http://goo.gl/d810ut>

Alliin has bactericidal properties in relation to both gram-positive and gram-negative bacteria and it also stimulates appetite. Furthermore, it is believed to have antiparasitic properties, stabilizing intestinal flora and hypotensive activity. Concurrently, it may have irritating influence on skin and respiratory tract. It has been proven to prevent blood platelet aggregation and to decrease the level of triacyloglyceroles in blood serum. Synthetic preparations based on allicin model are used as antifungal means. On the market could be found many products with content taken from garlic, such as - garlic oil, aged garlic extract, powder garlic and many others but none of them consist allicin due to its great instability (Świdorski et al, 2007).

### 3.3 Diseases and pests attacking garlic

#### Plant disease in general

Healthy plant, with its meristematic cells has the ability to perform various functions related to plant growth, multiplication, development and survival. Functions as dividing and differentiation of the cells, absorbing water and nutrients, carrying on photosynthesis, production of seeds or other reproductive organs for survival and multiplication are of the essential meaning for the plant. In the case that plant cells are not able to perform or when they are disturbed while caring out any of these functions by any negative environmental factor or plant pathogenic microorganism's plant becomes diseased. At the beginning changes are not visible, because affliction is localized to one or a few cells, but with spreading, symptoms become more apparent. Based on measurable or visible changes caused by negative environmental factor or pathogen microorganisms, amount of the disease in the plant can be measured. Under the conception of disease, it is understood a group of visible or invisible responses of plant

cells and tissues to negative influence of pathogen or environmental factor which may lead to changes in the integrity, function or plant form and as well may cause damage or death of particular plant parts or even entire plant. Ordinary, each kind of plant can be affected by a various plant diseases. While some pathogens stick to only one variety there are those affecting even hundreds of species of the plant. According to the symptoms of cause, organs they affect or plant type, plant diseases could be divided into different groups. Very useful criteria for grouping are the type of pathogen causing the disease. This way of grouping could help to prevent possible development and spreading of the disease, but as well which control measures to use (Agrios, 2005).

### **Diseases caused by abiotic factors**

The cause of diseases could be different abiotic factors (abiotic diseases, abiotic disorders). Even if they are noninfectious, abiotic diseases can predispose the plant to those diseases caused by infectious agents. Some of those diseases could be caused by chemical injuries (by herbicides, fungicides, insecticides), mechanical injuries, soil factors (soil pH, soil structure), moisture extremes, deficiencies in available water, temperature extremes (Kennelly et al., 2012).

### **Diseases caused by biotic factors**

According to diseases in garlic caused by fungi, different authors in different world parts specified fungi species reported as pathogens which can cause a various number of symptoms. In United States and China, Dugan et al., (2007), reported occurrence of *Aspergillus niger*, *A. ochraceus*, *Botrytis porri*, *Embellisia alli*, *Fusarium oxysporum f. sp. cepae*, *F. proliferatum*, *F. verticillioides* and *Penicillium hirsutum*. In Venezuela, it was found that *Botrytis porri* occurred for the first time on *Allium sativum* causing the disease called neck rot (Cedeño et al., 2003). The appearance of *Fusarium proliferatum* on garlic was reported in different countries i.e. in Italy (Tonti et al., 2012), United States (Dugan et al., 2003), Serbia (Stanković et al., 2007), India (Sankar and Babu, 2012), Spain (De Cara et al., 2010) and in Mexico (Fuentes et al., 2013).

Many authors reported that fungi *Sclerotium cepivorum* is one of the most recognized for attacking garlic (Coventry et al., 2002; Melero-Vara, 2000; Miñambres et al., 2010; Mordue, 1976; Pinto et al., 1998; Zewide et al., 2007). Petrov (2012) found that the



main causes of mycoses and pseudo mycoses on garlic in Serbia are *Sclerotinia sclerotiorum* and *S. cepivorum* (causing white mold), *Puccinia porri* (causing rust) and *Botrytis cinerea* (causing grey mold) but as well *Fusarium oxysporum* if *sp. Cepae*, *Aspergillus niger*, *Penicillium sp.* and *Peronospora destructor*. Fungi of less importance are *Alternaria porri*, *Stemphylium vesicarium* and *Phoma terrestris*. According to Šafránková (2016) the main fungi species affecting *Allium sativum* in the Czech Republic are *Peronospora destructor*, *Fusarium oxysporum f. sp. cepae*, *Penicillium hirsutum var. hirsutum*, *Puccinia alli* and *Stromatinia cepivora*. Bacteria which are attacking garlic and causing diseases are generally gram negative microorganisms, composed from only one cell (Schwartz and Howard, 1996).

### **Pests attacking garlic**

Beside all abiotic and biotic causes of diseases there are also a various number of pests that are attacking garlic. Sapakova (2013) as pests attacking garlic named nematodes - *Ditylenchus dipsaci* and *Aphelenchoides subtenuis*; mites - *Acetia tulipae*, *Rhizoglyphus echinopus*, *Rhizoglyphus robini* and *Tyrophagus putrescentiae*; thrips - *Thrips tabaci*; wireworms (*Elateridae*) - belonging to the genres *Agriotes* and *Hemicrepidius*; butterflies - *Acrolepiopsis assectella* and *Dyspessa ulula* but also some polyphagous caterpillars - *Triodia sylvina*, *Noctua pronuba* and *Hydraecia micacea*. From aphids the most important are *Myzus ascalonicus* and *Dysaphis tulipae* but also few polyphagous species - *Aphis fabae*, *Myzus persicae*, *Myzus ornatus*, *Macrosiphum euphorbiae* and *Macrosiphum rosae*. From those polyphagous aphids attacking garlic, the most important is *Myzus persicae* which is considered as the most important vector of plant viruses worldwide. Beetles attacking *Allium sativum* are *Lilioceris lili*, *Lilioceris merdigera* and *Oprohinus suturalis*. From the group of gnats attacking garlic, Sapakova (2013) named pests - *Delia antiqua*, *Eumerus strigatus* (small narcissus fly), *Eumerus funeralis*, *Merodon equestris* (large narcissus fly), *Phytomyza gymnostoma*, *Suillia univittata* and *Suillia lurida*.

### **3.4 Plant viruses**

Plant viruses are small size, transparent nucleoproteins behaving like microorganisms but as well as chemical molecules. Those virulent pathogens infecting plants are obligate intercellular parasites, without possibility to survive and replicate without a host. The

simplest plant virus as any other virus is composed from nucleic acid, only DNA or only RNA, in which they carry genetic material, and protein protective coat (capsid) which surrounds nucleic acid. Viral genetic material can be DNA or RNA, single or double stranded. Usually, plant viruses are composed from only one protein, but there are also viruses formed from two or more.

Because of their inability to be active without living cells, viruses require vectors in order to be able to pass from one organism into another. All types of living organisms including animals, plants, fungi, and bacteria are hosts for viruses, but most viruses infect only one type of host. Viruses cause many important plant diseases and are responsible for losses in crop yield and quality in all parts of the world (Gergerich and Dolja, 2006).

#### **3.4.1 Entering a virus into a plant and transmission of plant viruses**

Plant viruses the same as other plant pathogens are believed to enter the plant because during their evolution they have acquired the capability to use the substances manufactured by their host for their own use (Agrios, 2005). The entering is possible through natural openings or damaged parts of plant (wounds) (Agrios, 2005; Schwarts and Howard, 1996).

The essential meaning for viruses is to spread from plant infected to another healthy plant and to be introduced into living cells. There is a several ways of virus transmitting:

- mechanical transmission
- vegetative, graft and dodder transmission
- transmission by pollen seeds
- insect and mite transmission
- nematode and fungal transmission.

Organisms involved in the virus transmission are called *vectors*. This type of transmission involves temporary biological interaction between vector and virus, but in many cases a particular organism will interact only with a particular virus (Stevens, 1983).

#### **3.4.2 Symptoms of plant viruses**

In plants, viruses are causing a various number of symptoms, or plants remaining symptomless (Fig.4). That is of course depending on type of virus, amount of virus in

plant, plant age and other. Typical signs that plant is infected by viruses are chlorotic spots, mosaics, necrosis, changes in plant structure, reduction of yield etc. Those symptoms also may be caused not by virus but some other pathogenic organism or even abiotic factor as it was mentioned before.



Fig. 4 Symptoms of plant viruses on plants (<http://goo.gl/xJjb2L>; <http://goo.gl/lxTf8V>; <http://goo.gl/1JpDs0>)

### 3.4.3 Common viruses attacking garlic

Due to the vegetative method of reproduction *Allium sativum* is one of the most vulnerable vegetable crop attacked by large number of plant viruses. There are several economically significant viruses belonging to the genus *Potyvirus* which is one of the most important virus genus attacking *Allium sativum* and all other *Alliums* (Bagi et al., 2010; Dovas et al., 2001; Klukáčková et al., 2007). From the viruses belonging to this genus in garlic could be found: Onion yellow dwarf virus (OYDV), Leek yellow stripe virus (LYSV), but also viruses belonging to genus *Carlavirus* as Garlic common latent virus (GCLV) and Shallot latent virus (SLV) (Kudělková, 2010). From *Fijiviruses* appearing in garlic the most important is Garlic dwarf reovirus (GDR) while viruses from genus *Alexivirus* can also be found (Klukáčková et al., 2007; Dovas et al., 2001; Bagi et al., 2010). Those pathogenic viruses may occur in all areas where garlic is cultivated. This plant is usually attacked by a mixture of viruses at once (Kudělková, 2010; Klukáčková et al., 2007; Dovas et al., 2001).

#### Potyvirus

Potyvirus is an aphid-transmitted genus of viruses attacking plants, belonging to the virus family *Potyviridae*. All the members of *Potyviridae* are positive-strand RNA viruses

(López-Moya et al., 2009). Genus got the name because of its prototypical member Potato Virus Y and it is the largest genus of this family (Danci et al., 2009). The members of the Potyvirus genus have non-enveloped rod shaped flexuous particles 680-900 nm long and 1113 nm in diameter, helix pitch 3.4-3.5 nm, encapsidating a genome of about 9.7 kb with multiple copies of a single protein species of 30-47 kDa (Danci et al., 2009). From all *Allium* viruses, the Onion yellow dwarf virus (OYDV) and Leek yellow stripe virus (LYSV) belonging to this genus are the best studied (Klukáčková et al., 2007).

### **Onion yellow dwarf virus (OYDV)**

Onion yellow dwarf virus (OYDV) is an economically important plant virus causing a diseases in all the members of genus *Allium*, including *Allium sativum* (Koch and Salomon, 1994). Virus is discovered in 1960 and has virion particles with dimensions of 750 to 775 x 14 to 16 nm. Heat inactivation point (TIP) is 60-65°C, point where the virus is still detectable in the subsequent plant juice (DEP) is from  $1 \times 10^{-2}$  to  $1 \times 10^{-4}$  and ability to stay alive in *in vitro* condition (LIV) is two to three days (Bos et al., 1978; Šutić et al., 1999). Onion yellow dwarf virus may decrease expecting yield in annual crops for 25 % and potential yield loses varying from 50 % up to 75 % (Šutić et al., 1999). On the diseased plants symptoms are easy visible (Schwarz et al., 1996), but usually the symptoms may be similar to symptoms in the case of physiological disorders (Šutić et al., 1999). Leaves of those plants are wilted and drooped with pale green and yellow stripes (Fig. 5). The most vulnerable are stands of seedlings because viroses occur right in the second year of growing. Spreading of infectious agents by seeds or soil is unknown (Schwarz et al., 1996). The virus is named yellow dwarf since it is causing crinkling of flower stems and leaves which are chlorotic with narrow stripes. OYDV can be transmitted vegetatively or by aphids. There is around sixty aphid species transmitting this virus. Some of the most important are *Aphis fabae* (black bean aphid), *Aphis craccivora* (cowpea aphid), *Acrythosiphon pisum* (pea aphid), *Hyalopterus pruni* (mealy plum aphid), *Myzus ascalonicus* (shallot aphid), *M. cerasi* (black cherry aphid), *M. persicae* (green peach aphid), *Rhopalosiphum maidis* (corn leaf aphid) and *R. padi* (bird cherry-oat aphid) (Šutić et al., 1999).



Fig. 5 Symptoms of Onion yellow dwarf virus on garlic (<https://goo.gl/n3ULc0>)

### **Leek yellow stripe virus (LYSV)**

Leek yellow stripe virus on *Allium sativum* was first described by Walkey et al. (1987) in England, affecting mostly leek but also other *Alliums*. This virus may cause reduction of yield up to 50 % (Diekman, 1997). Symptoms are appearing in form of chlorotic spots firstly from the base of leaf going to apex (Fig.6). Yellow stripes are starting to grow and then whole leaf becomes chlorotic. Stems of those plants are weak and lighter and usually plant dies during the winter or after overwintering. Those symptoms are visible only in September when plant are already fully infected and then after overwintering LYSV continues with infecting other plants (Šutić et al., 1999). In garlic symptoms are in form of lighter and darker green stripes on the younger leaves then changing to yellow color in case of basal and central leaves (Diekman, 1997). This virus is transmitted mostly by aphids in non-persistent manner or by vegetative propagation (Diekman, 1997; Šutić et al., 1999). Virion particles are going in length of 815 to 820 nm and are flexuous (Šutić et al., 1999). Heat inactivation point (TIP) is 50-60°C, point where the virus is still detectable in the subsequent plant juice, (DEP) is from  $1 \times 10^{-2}$  to  $1 \times 10^{-3}$  and ability to stay alive in *in vitro* condition (LIV) is three to four days. Garlic, shallot and onion are essential hosts to Leek yellow stripe virus (Šutić et al., 1999).



Fig. 6 Symptoms of Leek yellow stripe virus (<http://goo.gl/JgUXd3>, <http://goo.gl/U9WXpd>)

### **Carlavirus**

This group of viruses was first proposed in 1971, as the “Carnation latent virus group” but during the years changed the name several times. In 1975 it was renamed in Carlavirus Group, in 1995 in *Carlavirus*, in 2005 was placed into a plant virus family *Flexiviridae* (named *Flexiviridae* because all the members of this group are having flexuous virions) and finally in 2009 it was placed in the *Betaflexiviridae* (Adams et al, 2004; ICTV, 2015). This genus of viruses is currently having 52 members and the family belongs to order *Tymovirales* (ICTV, 2015). Carlaviruses are having filaments of length about 610–700nm, 12–15nm in diameter which are flexuous. Genome of this plant virus group is ssRNA 7.4–7.9kb in size and including six open reading frames (ORFs) while symmetry is helical. Genus has a one type of coat protein subunits of size 31–36kDa. Carlaviruses are transmitted mechanically or by aphids (non-persistent) or they can be seed born e.g. *Pea streak virus* and *Red clover vein mosaic virus* (Adams et al., 2004; Foster, 1992).

### **Garlic common latent virus (GarCLV)**

This plant virus is an aphid-borne (Adams et al., 2004; Van Dijk, 1993) but as well transmitted by vegetative propagation (Barg et al., 1994; 1997; Van Dijk, 1993). It was

first discovered in Argentina (Conci et al., 2003) which is on the second place in the world for exporting the garlic (Burba, 2008). According to Van Dijk (1993) this virus is one of the most common viruses affecting *Allium sativum*. Garlic common latent virus has an intraspecific diversity which can be proven by phylogenetic and recombination analyses established on gene of capsid protein (CP) (Torricono et al., 2010). Virion filamentous particles are flexuous around 650 nm long (Diekmann, 1997; Adams et al., 2004). In infected garlic plants, alone any symptoms are not visible but in mixture with OYDV and LYS yellowing and mosaic is appearing. According to Pramesh et al. (2013), molecular characteristics of Garlic common latent virus are still not very well researched.

### **Shallot latent virus (SLV)**

This virus is described by Bos et al., (1978) in Netherlands as a new virus attacking shallots without causing symptoms and also detected in *Allium cepa* and *A. porrum* which are natural hosts. SLV has filamentous particles c. 650 nm long, straight or lightly curved and it is transmitted by aphids (*Myzus ascalonicus* and probably *Aphis fabae*) in the non-persistent manner and mechanically. Beside that it is very widespread in shallots but also attacking garlic and other *Alliums* (Bos et al., 1978). In shallots and garlic, SLV is mostly transmitted vegetative. There are no reports that it can be transmitted by seeds (Van Dijk, 1993). *Chenopodium amaranticolor* and *C. quinoa* are reported as testing plants in which symptoms appears in form of small necrotic lesions on older leaves or chlorotic lesions on young leaves (Šutić et al., 1999; Bos et al., 1978). Plant used for virus propagation is *Allium porrum* (Bos et al., 1978).

### **3.5 Virus detection methods**

In order to detect and suppress plant diseases on time, before they cause losses in quantity, quality or both, it is very important to identify with certainty, pathogen which causes the disease.

Usage of molecular techniques, especially of polymerase chain reaction (PCR), made a big difference in plant pathogen diagnostic and detection. With knowing of genome structure of those organisms the new way of its diagnostic emerged. Because of high sensibility, specificity and speed, molecular methods are widely used, especially for identification and detection of those pathogens for which is impossible or very hard to be grown *in vitro* or those with the inability to be grown in a short period of time. Diagnosis

based only on visible symptoms is very inaccurate and unreliable (Ivanović et al., 2004; Webster et al., 2004). Some viral diseases cause the same symptoms or the symptoms may be caused by other influence or the infected plant may stay symptomless. Because of those reasons, the diagnosis based on symptomatology has to be followed by identification of pathogen in the laboratory with the usage of microscope, selective nutrient media, biological and physiological tests and serological tests (Ivanović et al., 2004). With development of methods related to isolation and growing of pathogen on nutrient medium or test-plants, accurate pathogen diagnostic is ensured (Ivanović et al., 2004).

For the detection of plant viruses conventional method is biotest which is very reliable but it is also very time consuming and conditional on a number of factors. The other method is immunoelectron microscopy but even if is very sensitive and can detect viruses in small amounts it is not commonly used because it is expensive so it finds a place in fundamental research. The most used are serological techniques which are based on virus coat protein and other virus coded proteins. Among them DAS-ELISA is the most used. Comparing to biotest, with DAS-ELISA the results are obtained in a shorter amount of time (Krstić and Tošić, 1994).

The “dot-blot” hybridization is a method for detection of biomolecules and proteins and as well for protein analyzation, detection and identification. It is used in molecular biology for the plant virus detection (Ivanović et al., 2004; Loebenstein et al., 1997; Pallás et al., 1998; Pokorný personal communication, 2016). Moreover, PCR technique used in molecular biology is based on identification of the nucleic acid segments specific to the given organism (Pokorný personal communication, 2016; Ivanović et al., 2004). In cases when is needed to detect more different pathogens at the same time, the Multiplex PCR is used (Ivanović et al., 2004). For the reason of not having direct method developed in order to control diseases caused by viruses, the early and correct detection is essential in a fight against those infectious agents (Pallás et al., 1998).

### **3.6 Virus elimination methods in garlic**

#### **Thermoterapy**

This method is based on placing the cloves or whole infected garlic bulbs under the different temperatures after what the garlic virus free material may be obtained. According to Senula et al., (1999), the most effective temperature for approaching good



results is 36 °C or higher. On that temperature is believed that multiplication of virus in infected cells is reduced or even completely stopped but the meristematic cells are continuing with multiplication and that can lead to obtaining of virus free material.

### **Chemotherapy**

This method is based on the usage of chemicals which are affecting nucleic acid of virus which is after not able to multiply. Among growers this method is not very favorable because used chemicals may cause mutations in the plant genetic material (Pokorný personal communication, 2016). The chemicals are applied to media (Senula et al., 1999) or on garlic embryos (Ramírez-Malagón et al., 2006). Chemotherapy is very successful in virus elimination but there is also a risk that by this practice is possible to damage the tissue.

Both, thermo - and chemotherapy methods are usually combined with meristem culture technique. Meristem is isolated from cloves which have been subjected to the high temperature, specific number of days or embedded to the nutritional media which was used for growth with antivirotics applied. The temperature and number of days in thermo-therapy is varying and it is dependent to variety, plant cultivar and the type of virus. As well the amount and the type of chemicals chosen are also varying. According to some authors, the combination of chemo- and thermo-therapy is leading to elimination of viruses (Cassells and Long, 1980; Senula et al., 1999; Szyndel et al., 2003).

## **4 MATERIALS AND METHODS**

### **4.1 Laboratory**

Experiments were performed in the tissue culture laboratory at Faculty of Agronomy, Department of Plant biology. Laboratory is divided on four parts: unsterile part for washing glassware (here is placed autoclave and cabinet with chemicals which are every day used); the second part of the laboratory is also unsterile and used for media preparations (here are placed pH meters, refrigerator with different solutions, microwave, analytical balance, vortex, heating magnetic stirrer etc.); third part is sterile part where five flow boxes are placed; last part is the cultivation room where after being transferred on nutritional media explants are left to grow.

### **4.2 Plant material**

Plant material was obtained from Seed Company SEMO a.s. Smržice in order to test if there are possible virus infection and if yes to cure the explants and multiply virus-free plants on nutritional media. For experimental purposes four different cultivars (cv.) were used i.e. Václav, Štěpán, Karel IV and Ivan (Fig. 7). Evaluation and performance of ELISA test for virus detection was done in Výzkumný Ústav Bramborářský Havlíčkův Brod S.r.o. Virus detection was performed in Havlíčkův Brod due to economical reason and lacking of ELISA kit for certain viruses at Mendel University.

Plant cultivars used in this work are:

#### **Cv. Václav**

Cultivar Václav is a medium late broadleaf hard neck (harvested around 25<sup>th</sup> July). Medium size bulb with cream-like color peel dotted with brown brindle. Cloves are of medium size, 8-10 pieces, peel is light brownish. Due to late sprouting it shows very high resistance to frosting. Leaves are wide medium-high, blue-green, does not suffer from yellowing tips. Good health conditions in the stand and long-term shelf life.

### **Cv. Ivan**

This cultivar is not licensed yet. It is a medium late broadleaf violet hard neck garlic cultivar. The leaves are upright, with no bending ends, no yellowing tips. The massive stature, good health, bulb medium to large, slightly flat, peel yellowish white with a distinctive purple veining. Bulb contains 6-7 bigger cloves. Medium late variety - harvest is roughly in the first half of July (approximately 5-7 days earlier than Karel IV).

### **Cv. Štěpán**

It is medium late variety of broadleaf soft neck garlic. Bulbs are large; weighting 80 -100 g consisted of eight to ten large cloves protected with light brown peel. Peel enclosing a bulb are white with fine light brown veining. Characteristics of this variety are distinctive, delicate flavor, wide leaves, very good health condition and long-term shelf life. Harvesting time is around 10<sup>th</sup> of July.

### **Cv. Karel IV**

This cultivar is a medium late broadleaf hard neck with a large bulbs weighing around 90-110 g. Bulb is white with peel having purple veins, composed from five to six relatively big cloves. Each clove is enclosed in purple-brown peel. Leaf is big, wide, upright and in the end slightly bent. Strong garlic flavor is one of characteristics of this cultivar. Karel IV has a high yield, steadily in both wet and dry harvesting seasons. It is characterized by very good health condition and long-term shelf life. Harvesting time is around 20<sup>th</sup> of July.



Fig. 7 Cultivars Václav, Štěpán, Karel IV and Ivan used in this research

### 4.3 Preparation and sterilization of plant material

Before work, flow box were sterilized for 15 minutes with UV lamp and afterwards cleaned with 70% ethanol. Bulbs of cv. Václav, Štěpán, Karel IV. and Ivan were peeled and split into individual cloves and selected by size to standardize the *in vitro* development. All cloves which had wounds or signs of decay were removed. Cloves in beaker were washed with tap water and soap. Furthermore, beaker with cloves was filled with sterilization agent 0.2 % of sodium hypochlorite solution and left for 13 minutes, as previously explained by Kudělková (2010). After sterilization, the solution was decanted and the cloves were rinsed three times with sterile distilled water in the flow box. Isolated meristems comprised of first set of primordial leaves were transferred to Petri plates (Fig. 8).



Fig. 8 Meristem isolation

### 4.4 Media preparation

The basic culture media used to induce shoot growth consisted of mineral salts of MS with full-strength vitamins (Murashige and Skoog, 1962) (Tab. 1), with addition of 3% sucrose, 0.7% agar, naphthalene acetic acid  $0.25 \text{ mg.L}^{-1}$ , 2-isopentenyladenine  $0.5 \text{ mg.L}^{-1}$  and with/without  $30 \text{ mg.L}^{-1}$  ribavirin. pH 5.8 was adjusted before autoclaving.

#### Media preparation procedure

Preparation of one liter media was done by dividing solutions into two parts. For first part beaker was filled with 500 ml of distilled water where 4.4 g/l of MS mineral salts were added. After adding mineral salts pH was adjusted to 5.8 with HCl/KOH 0.1/1M solution. In this solution 30g/l of sucrose was dissolved. In other beaker with 500 ml of distilled water 7g/l agar was dissolved in microwave while first beaker with rest of the

medium was also heated for one minute. Afterwards, solutions were merged, stirred and put in autoclave for sterilization (121°C, 100 kPa, 20 min) (Fig. 9). After autoclaving when medium cooled off at about 50 °C all thermolabile components i.e. naphthalene acetic acid, 2-isopentenyladenine and ribavirin were sterilized separately by usage of syringe and filter (Milipore with size of pores 0.22 µm). After mixing all components, the 40 ml of medium was put into sterilized Magenta boxes.

Table 1. Basal culture medium according to Murashige and Skoog (1962)

| Inorganic salts (mg/l)                |      | Inorganic salts (mg/l)                               |       | Vitamins etc. (mg/l) |     |
|---------------------------------------|------|--|-------|----------------------|-----|
| NH <sub>4</sub> NO <sub>3</sub>       | 1650 | H <sub>3</sub> BO <sub>3</sub>                       | 6.2   | Nicotinic acid       | 0.5 |
| KNO <sub>3</sub>                      | 1900 | MnSO <sub>4</sub> · 4H <sub>2</sub> O                | 22.3  | Pyridoxine-HCl       | 0.5 |
| CaCl <sub>2</sub> · 2H <sub>2</sub> O | 440  | ZnSO <sub>4</sub> · 4H <sub>2</sub> O                | 8.6   | Thiamine . HCl       | 0.1 |
| MgSO <sub>3</sub> · 7H <sub>2</sub> O | 370  | KI   | 0.83  | Myoinositol          | 100 |
| KH <sub>2</sub> PO <sub>4</sub>       | 170  | Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O | 0.25  | Glycine              | 2   |
| Na <sub>2</sub> -EDTA                 | 37.3 | CuSO <sub>4</sub> · 5H <sub>2</sub> O                | 0.025 |                      |     |
| FeSO <sub>4</sub> · 7H <sub>2</sub> O | 27.8 | CoCl <sub>2</sub> · 6H <sub>2</sub> O                | 0.025 |                      |     |

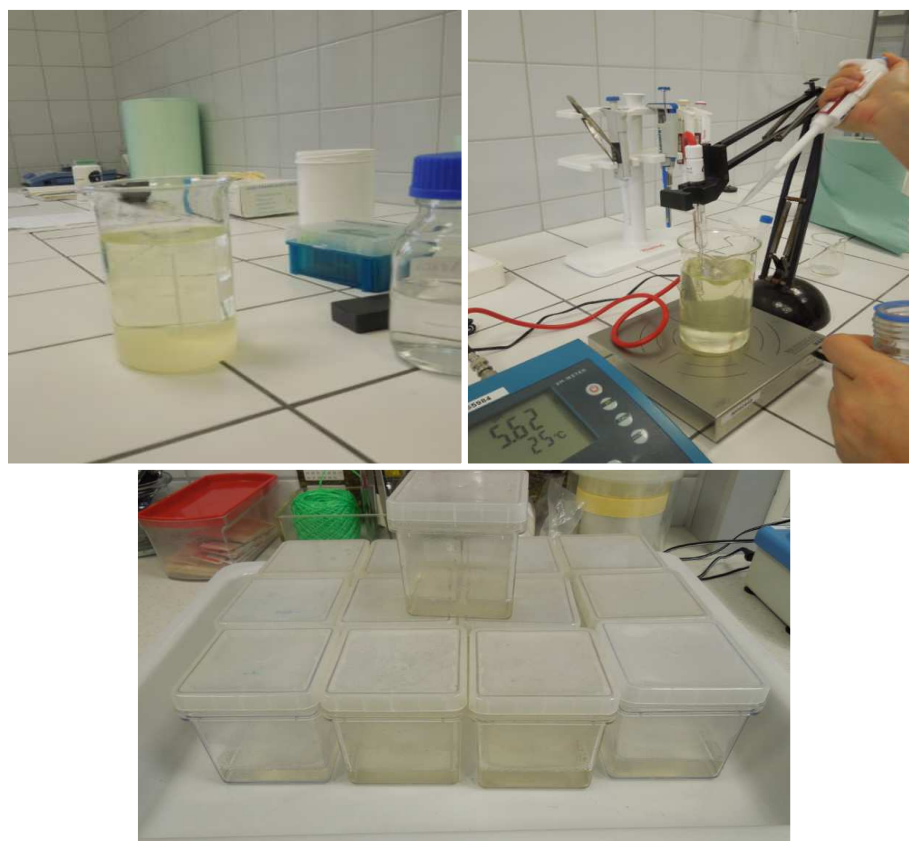


Fig. 9 Media preparation procedure

## 4.5 Thermoterapy

Groups of 10 cloves per cultivar were put in trays and kept under 38 °C for 10, 20 and 35 days. For thermotherapy treatments laboratory sterilization oven with air circulation was used (Fig. 10).



Fig. 10 Laboratory sterilization oven

In order to produce virus-free plants from all cloves subjected to thermotherapy treatment meristems were isolated and put on prepared medium without ribavirin. The isolated meristems were cultivated at 23 °C, photoperiod light 16h and 8h night for one month. After that pair of leaves per cultivar was cut with scissors and sent for ELISA analysis.

## 4.6 Chemoterapy

After obtaining results from thermotherapy treatments which were positive on virus appearance, it was decided to perform chemoterapy treatments with usage of 30 mg.L<sup>-1</sup> ribavirin, as previously published by Kudělková (2010). Isolated meristem were left to grow on medium with ribavirin for one month in cultivation room at 23 °C, photoperiod light 16h and 8h night. Ribavirin is a guanosine (ribonucleic) analog used in chemotherapy treatments to stop viral RNA synthesis and viral mRNA capping (Carter and Saunders, 2007).

#### **4.7 ELISA test (Enzyme-linked immunosorbent assay)**

For detection and quantification of viruses in plants are described different methods and techniques.

From all methods used nowadays, the most favorable is ELISA test, due to its sensitivity for virus detection and the fact that it is relatively inexpensive (Dovas et al., 2001). It is used not only for qualification but also for virus quantification (Kranz and Rotem, 1988). ELISA, the same as other serological methods is based on the antigenic properties of the virus coat protein. It is a biochemical technique used in medicine and plant pathology and also in various industries, to detect antibody or an antigen in a sample. In general, it consists of virus detection by utilizing antibodies ability to recognize a protein substance (usually the virus coat protein) or antigen associated with the virus of interest (Webster et al., 2004; Kranz and Rotem, 1988; Kaya and Gokdogan, 2015). May be used for testing several plants to one virus using one well per plant sample, or one plant can be tested for more than one virus on a single plate with different antibodies coated to each well (Webster et al., 2004). The detection is based on visual methods which are based on color changes coming as a result of interaction between the substrate and the immobilized enzyme (Fang and Ramasamy, 2015). In plant virology is commonly used method called DAS-ELISA (Double antibody sandwich ELISA) based on double reaction antibody – antigen (Dovas et al., 2001; Conci et al., 2003).

## 5 RESULTS AND DISCUSSION

In order to produce virus-free plants thermotherapy, meristem tip culture and chemotherapy in different temperature/time combinations were used as a treatment. All tested cultivars were infected with two or more viruses in control samples. Furthermore, since cloves subjected to thermotherapy treatment was used for meristem isolation it was noticed appearance of viruses in second part of experiments (days 20 and 35).

In the control samples, cv. Štěpán was infected only with GCLV virus (Fig.11). After 10 days of thermotherapy treatment, viruses GCLV, OYDV and SLV were detected while after 20 and 35 days of thermotherapy it was infected only with OYDV virus.

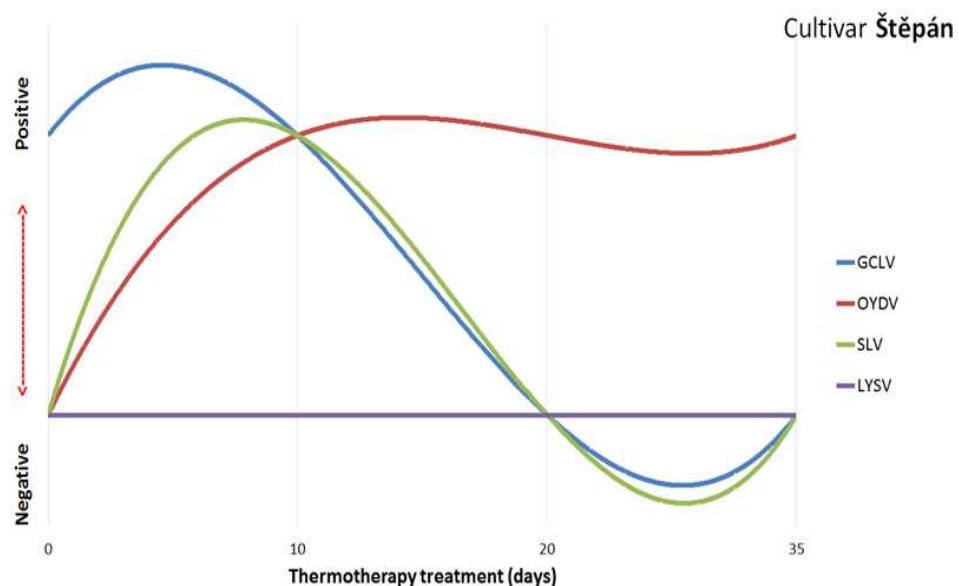


Fig. 11 Appearance of viruses in cultivar Štěpán during 35 days of thermotherapy

In the beginning of experiments cv. Václav was infected with GCLV, OYDV and SLV viruses in control samples and in the end it was affected with all four viruses (Fig.12).



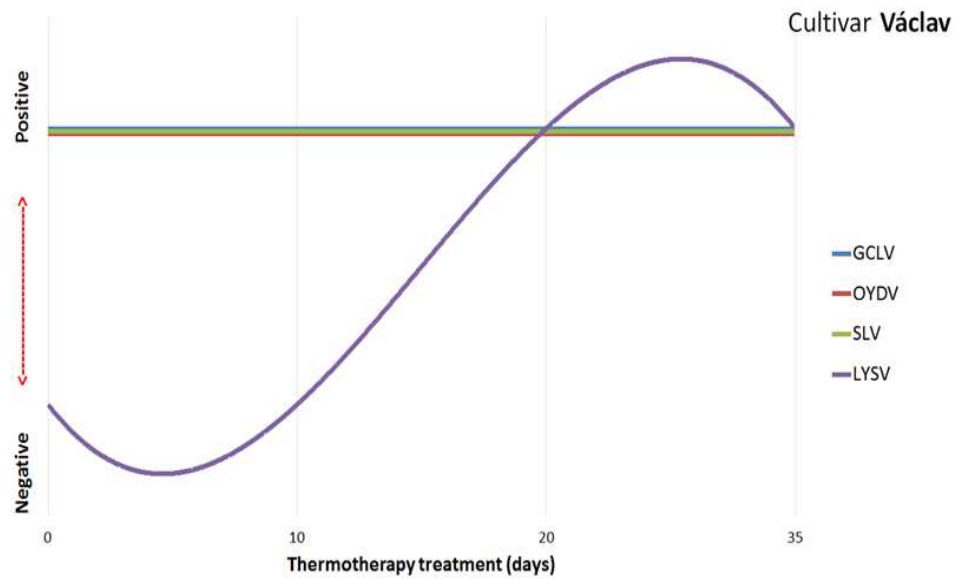


Fig. 12 Appearance of viruses in cultivar Václav during 35 days of thermotherapy

Similar results were obtained with samples of cv. Ivan, where control was infected with OYDV, SLV and LYSV, but after 10 and 20 days of thermotherapy virus GCLV appears (Fig.13).

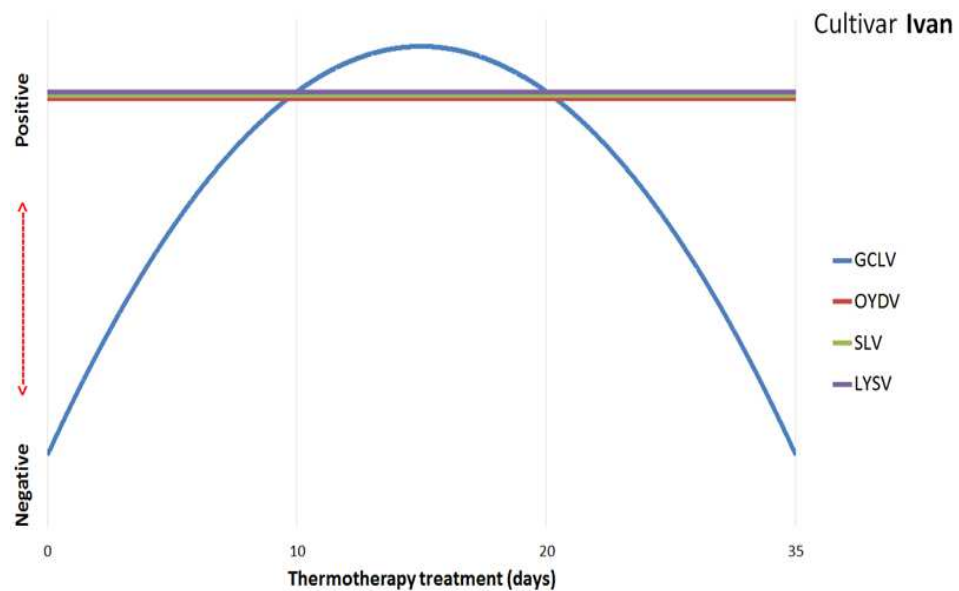


Fig. 13 Appearance of viruses in cultivar Ivan during 35 days of thermotherapy

In the control samples, cultivar Karel IV, was infected with 3 viruses OYDV, SLV and LYSV. After 35 days of thermotherapy treatments, eradication of GCLV and LYSV viruses was obtained (Fig. 14).

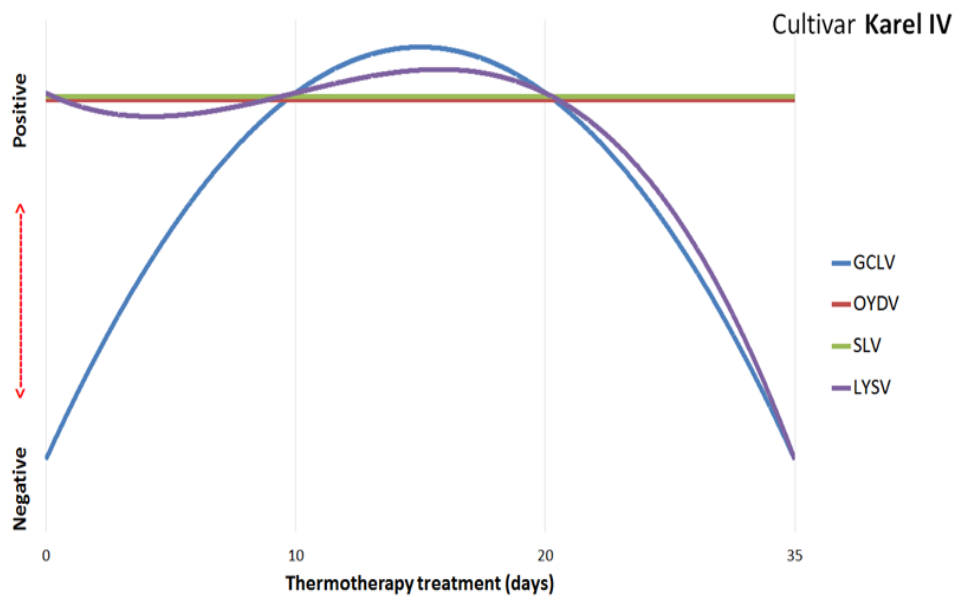


Fig. 14 Appearance of viruses in cultivar Karel IV during 35 days of thermotherapy

Since, virus-free plants were not obtained after thermotherapy treatments, further experiments were performed with addition of ribavirin to growth media. In the end of chemotherapy treatments in combination with isolated meristems virus-free plants were obtained in cv. Štěpán and Karel IV while cv. Václav was still infected with OYDV and cv. Ivan with LYSV, OYDV, respectively (Fig.15).

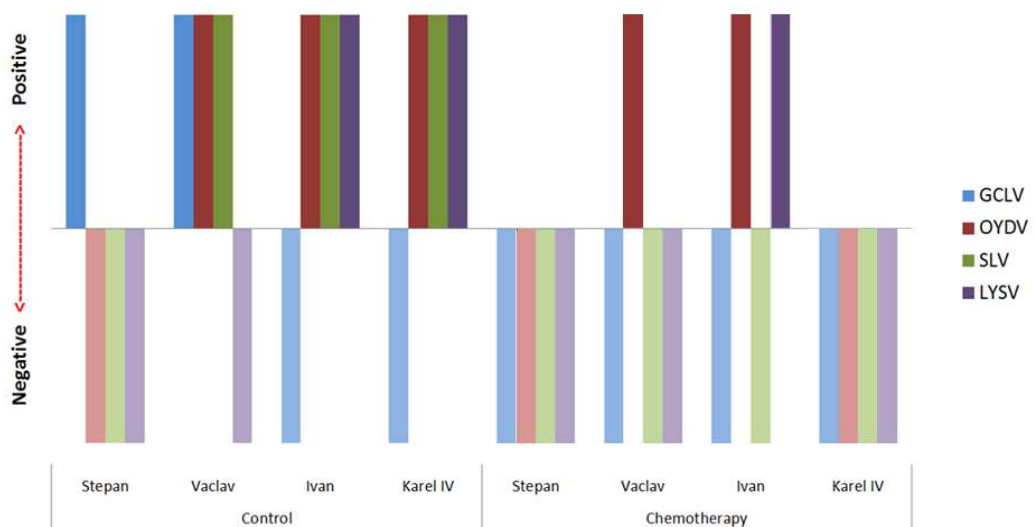


Fig. 15 Appearance of viruses in all cultivars in chemotherapy treatments

After obtaining virus free plants in cv. Štěpán and Karel IV, plantlets were grown *in vitro* on nutritioral media for multiplication purposes (Fig. 16).



Fig. 16 Plantlets of virus free cv. grown *in vitro* for multiplication purposes

Results obtained from thermotherapy treatment in combination with meristem isolation show various positive/negative virus presence among cloves of the same bulb. Since starting material - cloves which undergone thermotherapy during different sampling days, was used for meristem isolation, variations in appearance and disappearance of viruses during ELISA test were observed. This can confirm that among cloves from the same bulb different combinations of viruses can be found. Conci et al. (2010) published the similar results where in 6% from all cloves tested by ELISA test, positive and negative cloves were found in the same bulb. Furthermore, the tests of virus concentration in relation to the layers of each bulb revealed important differences. Moreover, presence of potyvirus in single garlic cloves from the same bulb, and in five single leaves excised from commercial field-grown individual plants was studied using ELISA (Ramírez-Malagón et al., 2006). It was observed that some cloves of the same bulb were infected with potyvirus but some others were potyvirus-free. However, this positive reaction detected in individual leaves ranged from 20 to 60% for both cultivars tested. These results also demonstrate that potyviruses are not uniformly distributed in all organs of an infected garlic plant.

Senula et al. (2000) tested different treatments for OYDV, LYSV, GCLV, SLV and MbFV elimination in garlic. Hot air treatment of bulbils at 36 °C for six weeks did not affect plant regeneration but virus elimination was significantly increased. From the other hand, in chemotherapy treatment plant regeneration was decreased but virus elimination was increased with addition of ribavirin to nutritional media. Moreover, the highest virus elimination was observed when thermos- and chemotherapy were combined. Walkey et al., (1987) tested thermotherapy treatment in combination with meristem tip culture isolation. Both shallot and garlic were infected with OYDV, SLV and a second potyvirus. It was observed that when infected parent plants were subjected to thermotherapy (38 °C) prior to tissue culture the percentage of virus-free garlic plants regenerated, increased up to 85%.

Furthermore, garlic shoot tip culture associated with dry heat thermotherapy (cloves exposed to 37 °C for 35 days) were essential for recovering virus free plants of the cv. Amarante (Torres et al., 2000). Cloves which were subjected to thermotherapy treatments (37 °C) had higher regeneration rate - 70% of the explants developed *in vitro* and produced plants and 77 % of those plants were virus-free. However, the percentage of regeneration decreased to 20 % as the temperature increased up to 40°C. However 90 % of those plants were virus free, leading to a final index of 18 % virus free plants out of treated cloves. In preliminary tests, it was noticed that only combination of thermotherapy and meristem isolation could lead to viruses' eradication since separately done thermotherapy and meristem isolation were 100 % infected with viruses. Bruna (1997) tested different temperatures (38 °C) during different time periods (48, 54, 60, 67 and 75) for obtaining virus free plants. This was compared to garlic cloves preconditioned at 30 °C for seven days before being exposed to 38 °C. The percentage of virus free plants obtained increased within 38 % for 48 days to 100 % to 75 days exposed but plant regeneration decreased. Ramírez-Malagón et al. (2006) treated two infected garlic cultivars by potyviruses with thermotherapy - at 32 °C for one week, 36 °C for two weeks, and 38 °C for three weeks. This treatment was found to affect survival of explants and 36.5% cloves from one and 26.8% from the other cultivars were recovered after the usage of this method. Nevertheless, after testing by ELISA test obtained results showed that 63% of the cloves from the first cultivar were potyvirus free and from the second cultivar 70.9 %.

Chemotherapy treatments are commonly used for eradication of viruses. Ramírez-Malagón et al. (2006) applied 205 µM ribavirin solutions to nutritional media and obtained that the explants (cloves) survived, but only an average of 27.0–34.8% were virus free. After meristematic dissection, 41.7% explants of first and 34.2% of the second cultivar were alive. From both cultivars, around 64% of explants were potyvirus free. Krajíčková (2012) tested meristem isolation and combination of chemotherapy with meristem isolation for elimination of viruses in 10 genotypes. For the chemotherapy to the MS media was added ribavirin in the amount of 50 mg/L. It was concluded that for the elimination of GCLV and SLV viruses is better to use a combination of chemotherapy with meristem isolation while for elimination of the virus LYSV is better to use only apical meristem isolation. Furthermore, OYDV was the hardest virus for elimination. Kudělková et al., (2014) reported that for elimination of virus GCLV in three garlic cultivars, the best method is usage of chemotherapy with ribavirin in amount of 25 mg/L. Soliman et al., (2011) tested methods of electrotherapy, thermotherapy, chemotherapy or meristematic dissection followed by *in vitro* culture in order to eliminate virus OYDV from *Allium sativum*. He combined methods of electro- and chemotherapy (15 mA/10 min + 20 mg L<sup>-1</sup> virazol) and obtained the best results. According to him, the 85% of the plantlets that survived were OYDV virus free. Sidaros et al., (2004) for obtaining the virus free garlic plants isolated meristems 1, 3 and 5 mm big and cultivated them on the media with ribavirin in amount of 50 mg/L. The best results were achieved with chemotherapy combined with isolated meristem in size of 3mm.

Methods, such as meristem isolation, chemo- and thermotherapy may be used in obtaining virus free plants not only in garlic but many other virus infected plants. Cassels and Long (1982), used the method of chemotherapy by virazole to eliminate potato viruses X, Y, S and M. Cieślińska (2007) for the elimination of viruses in *Prunus sp.* trees used the techniques of thermotherapy and chemotherapy *in vitro* while Deogratias et al., (1989) used the same methods for virus elimination in sweet cherries. Those two methods are successful in obtaining healthy plants without viruses in sand pear (Hu et al., 2012), banana (Kabir Shiragi et al., 2008) and apple (Hu et al., 2015). Griffiths et al., (1990) used ribavirin for the chemotherapy treatment for obtaining the virus free potato plants. According to Balamuralikrishnan et al. (2002) combination of chemotherapy and

meristem culture is useful in elimination of sugarcane mosaic virus, while eliminating of viruses in apricot may be done by thermotherapy (Křižan and Ondrušiková, 2008).

## 6 CONCLUSIONS

Results obtained from thermotherapy treatment in combination with meristem isolation show various positive/negative virus presence among cloves of the same bulb. In the end of thermotherapy treatment the lowest number of viruses was observed in cv. Štěpán and Karel IV. After one month of chemotherapy treatment cv. Štěpán and Karel IV was virus-free while cv. Václav was infected with one virus (OYDV) and cv. Ivan with two viruses (OYDV and LYSV). The most frequent virus was OYDV and most difficult to eliminate.

From our results it can be seen that more successful technique for obtaining virus-free plants was combination of meristems isolation and chemotherapy.

## 7 LITERATURE

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## **8 LIST OF ABBREVIATIONS**

GCLV - garlic common latent virus

LYSV - leek yellow stripe virus

OYDV - onion yellow dwarf virus

SLV - shallot latent virus

ITS - internal transcribed spacer

GDR - Garlic dwarf reovirus

TIP - Heat inactivation point

DEP - Detectable in the subsequent plant juice

LIV - alive in in vitro condition

PCR - Polymerase chain reaction

ELISA – Enzyme-linked immunosorbent assay

MS - Murashige and Skoog medium

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