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Development of Tools for the Protection of Wheat against *Tilletia* spp.

MSc. THESIS

By

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Declaration

I declare that the Diploma Thesis **"Development of Tools for the Protection of Wheat against *Tilletia* spp."** is my own work and all the sources I cited in it are listed in the Bibliography.

Prague, 2017 Signature

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Abstract

Common bunt disease caused by the fungus *Tilletia caries* is a devastating plant pathogen of wheat. This pathogen is effectively eliminated by using synthetic fungicides in seed treatment. The residual effect of such chemicals and the rise in pathogenic resistance has led to the gradual elimination of several commercial fungicides. Furthermore these chemical fungicides are not allowed in ecological agriculture. For the purpose of discovering alternative strategies to fight *Tilletia* spp. infections, we tested the fungicidal effect of essential oils. Our selection of essential oils included; *Levandula angustifolia, Thymus vulgaris, Origanum vulgare, Eugenia caryophyllata, Pelargonium graveolens, Foeniculum vulgare* and *Rosmarinus officinales.* Essential oils are naturally occurring, volatile substances that, if present when spore germination is initiated, are capable of inhibiting such pathogen germination. For this reason we experimented the effect of these essential oils after formulating them in gelatin capsules using different kinds of biopolymers. From the tested variants, *Eugenia caryophyllata*, showed promising results in inhibiting spore germination of *Tilletia caries*. The least effective was *Foeniculum vulgare.*

Key words: essential oils, biopolymers, gelatin, *Tilletia*

Abstrakt

Choroba mazlavá sněť pšeničná, kterou způsobuje houba *Tilletia caries* je významným patogenem pšenice. Tento patogen je účinně eliminován za použití syntetických fungicidů - mořidel. Reziduální účinek těchto chemikálií a zvýšení rezistence patogenu vedlo k postupné eliminaci několika komerčních fungicidů. Kromě toho tyto chemické fungicidy nejsou používány v ekologickém zemědělství. Za účelem objevení alternativních strategiií v boji proti infekcím sněti *Tilletia* spp. jsme testovali fungicidní účinky rostlinných extraktů. Náš výběr esenciálních olejů zahrnoval: levanduli lékařskou, tymián obecný, dobromysl obecnou, hřebíčkovec kořenný, muškát vonný, fenykl obecný a rozmarýn lékařský. Esenciální oleje jsou přirozeně se vyskytující, těkavé látky, které jsou schopné inhibovat klíčení spor. Z tohoto důvodu jsme experimentovali s účinkem těchto esenciálních olejů a jejich naformulováním do želatinových kapslí za použití různých typů biopolymerů. Z testovaných variant ukázal hřebíček slibné výsledky při inhibici klíčení spór mazlavé sněti pšeničné. Nejméně účinným byl fenykl obecný.

Klíčova slova: esenciální oleje, biopolymery, želatina, *Tilletia*

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1. Introduction

This diploma thesis is about the development of new tools for the protection of wheat against fungus of the species *Tilletia* spp. Wheat is one of the most important sources of human nutrition and is widely used as animal fodder. Common bunt and smut disease caused respectively by *T. caries* and *T. controversa* deteriorate the quality of grain harvest and in severe cases renders it completely useless. This is due to infectious symptoms which include spore contamination that in turn resulted from transforming wheat kernels into a powdery mass of spores. Spore formation comes with a fishy odour caused by trimethylamin. Seed treatment against *Tilletia* spp. is successful in eliminating the pathogen. In the last few years the use of synthetic fungicides is either prohibited or used restrictively. This is because of increased pathogen resistance from repeated applications of synthetic chemicals that are usually made up of one or few active substances. Another side effect is the residual toxicity which pollutes the environment and threatens public health from direct toxic residual accumulation on wheat grains. Furthermore commercial fungicides are not used in ecological agriculture. This has led to a rise of research and at replacing synthetic chemicals by biosphere friendly and biodegradable substances.

The aim of this diploma thesis was to study the potential activity of several essential oil extracts against *Tilletia* spp. Essential oils are volatile, natural complex compounds formed by aromatic plants as secondary metabolites. Their antimycotic effect has been extensively studied in recent years for this purpose. In our experiment we identified the *Tilletia* species using PCR method and then conducted two separate experiments, one on the field and the second in small containers in greenhouse. We used different formulations to encapsulate these essential oils for optimizing their inhibiting effect against spore germination against *Tilletia* spp.

2. Hypothesis and Aim of Study

Scientific hypothesis:

Essential oils are formed of several compounds that express antimycotic inhibitory effects. This can be applied to block the compatible relationship between the plant and its pathogen.

Aim of the study:

To test such inhibitory activity of selected essential oils against the fungus *Tilletia* spp. which infect wheat kernels.

Test different formulations of essential oils encapsulation for the purpose of seed treatment. Using different essential oils in different formulations and interpreting the effects variability between the essential oils and between different formulations of each essential oil.

3. Literature Review

3.1. Tilletiales

Genus *Tilletia* was named by brothers and French mycologists Louis René Étienne and Charles Tulasne in tribute to the pioneer work of famous French biologist Mathieu Tillet on this fungus (Kochanová et al., 2004).

Bunt diseases caused by different species of the genus *Tilletia* are major problems in wheat production. Infected plants have lower nutritional values, and if presence of smut fungi exceeds limits it is forbidden to use even in feed rations for animals (Dumalasová et al., 2007).

Due to the increased use of chemical protection, yield losses caused by *Tilletia* spp. infection are lower today compared to 50 years ago (Wiese, 1987). However in ecologic agriculture, where the use of chemicals is forbidden, smut fungi can still cause considerable yield losses. (Zouhar et al., 2010).

Tilletia spp. are obligate parasites – they cannot complete their life-cycle without the presence of specific host plants (Kazda et al., 2007). This smut fungi attacks only a specific part of the host – ovary of the plant and causes bunt diseases (Trione, 1964). The main signs of infection are the absence of seeds under lemma and presence of smut fungi teliospores (Kochanová et al., 2004).

So far more than 150 species of this genus are known, and with exception of species *Erratomyces* which infects plants of the *Fabacea* family, all other species infect plants of *Poacea* family (Castlebury et al., 2005).

3.1.1. Taxonomy

Earlier studies for taxonomical classification of fungus were based on morphological characteristics of infected seeds and teliospores. Later progress with electronic microscopes and methods of molecular biology, offered more accurate categorization of different species into taxonomical orders. In several cases morphological characteristics were undistinguishable due to crossbreeding. Different methods of DNA sequence comparison such as barcoding processes provided precise information in species identification (Kochanová and Prokinová, 2004; Nilsson et al., 2006). Scientific classification of the species *Tilletia caries* is as follows (Mycobank, 2017).

Kingdom: *Fungi*
Division: *Basidiomycota*
Subdivision: *Ustilaginomycotina*
Class: *Exobasidiomycetes*
Subclass: *Exobasidiomycetidae*
Order: *Tilletiales*
Family: *Tilletiaceae*
Genus: *Tilletia*

3.1.2. Host range

Since the beginning of agriculture cereals were first cultivated in the Middle East where the process of their domestication started (Zohary and Hopf, 2000). It is suspected that *Tilletia* spp. originated in Middle East being the origin of its host. This is supported by the large number of resistant genes in cultivars that come from the Near East (Prokinová et al., 2011). Common wheat (*Triticum aestivum L*.) belongs to family *Poaceae* and is also known under names as bread wheat, common bread wheat, spring wheat, ordinary wheat or field wheat. It is one of the most important crops in the world with production over 700 million tonnes in 2014 and world harvested area being more than 200 million hectares (FAO, 2017). Primary product made from common wheat is flour, which can be utilized in number of others products ranging from bread, biscuits, pastries and cakes to pasta or noodles (Marti et al., 2016).

Tilletia caries attacks winter wheat and spring wheat. *Tilletia controversa* was not observed on spring wheat (Fuentes-Dávila et al., 2002). In Europe, *Tilletia caries* attacks several members of the wheat tribe (*Triticeae*) like rye (*Secale cereale*), *Triticum monococcum* or *Triticum dicoccon*. *Tilletia controversa* focuses more on barley (*Hordeum vulgare*) and *Triticum aestivum*. But its host spectrum also includes *Triticum dicoccon* and *Secale cereale* (Vánky, 1994).

3.1.3. Infection

The infection damages the host in many ways. Contamination during the harvest leads to progressive deterioration of yield quality and in severe cases to total uselessness for purposes of human consumption (Wiese, 1987). Such a harvest with excessive quantities of spore's isn´t suitable for use as fodder for domesticated livestock because contaminated grain causes various

health problems, such as reducing the production of milk or eggs, digestive problems and allergies (Dumalasová et al., 2007). The grain has the smell of trimethylamin, which is produced by spores (Vánky, 2002). Trimethylamin has typical fishy odour, which comes from the mass of teliospores produced by the fungus inside of the gall (Wilcoxson and Saari, 1996). The importance of smuts lies in their ability to distinctly affect the quality of the crop. Smuts can devalue the harvest to such an extent that it must be all destroyed where all costs and effort for cultivation are lost (Kochanová and Prokinová, 2004).

3.1.4. Disease cycle

After seed contamination by *T. caries* in soil, the process of teliospore germination starts. Rotational haploid cells are produced at the end of the promycelium, thus primary basidiospores are produced. Basidiospore fusion eventually creates a heterocaryon. At this lifecycle stage, infection hyphae have a parasitic character. As soon as the seed germinates, the hyphae penetrate its coleoptile. From there hyphae grow along the apical meristem but is inactive. Development of wheat kernels initiates the reproductive cycle of the pathogen. Gradually the kernel content is replaced with teliospores and seeds transform to bunts. Teliospores are dispersed on the field due to abiotic factors or during harvest. This results in contamination of neighbouring seeds on the field and eventually in storage. Spores that remain in soil serve as primary source of infection in the following season (Fischer and Holton, 1957).

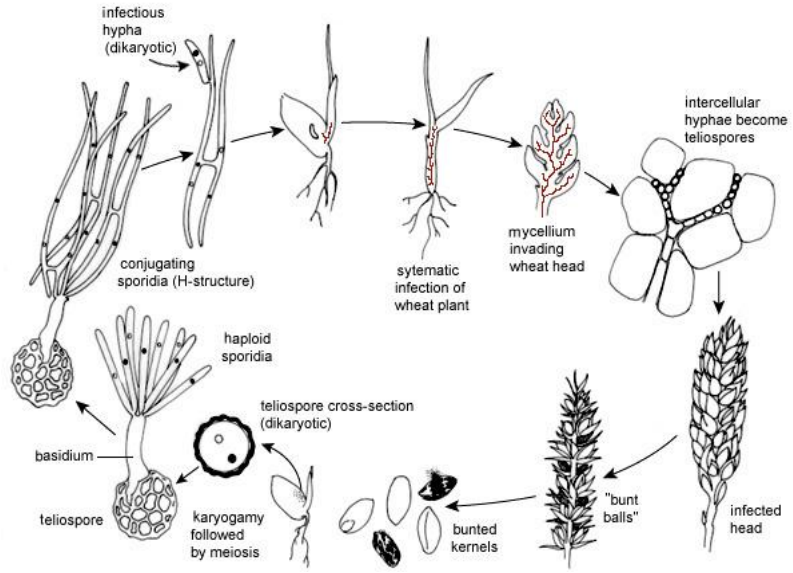


Fig. 1. Life cycle of *Tilletia* spp. (Mathre, 2017).

Winter wheat can be attacked by different kinds of *Tilletia*. First of them is *Tilletia carries*, which is one of the most spread smuts in Czech Republic. Next smut which can be found here is *Tilletia controversa*. The source of infection of *Tilletia caries* is the caryopsis with spores on its own surface while *Tilletia controversa* is causing the infection by spores in the soil surface (Polišenská, 1998). The infection by *Tilletia caries* takes place under the soil (Dumalasová et al., 2007). Cooler weather is considered ideal for spore germination which leads to the pathogenic infection of wheat. In the case of *Tilletia controversa* optimal temperature is 6°C and ideal temperature for *Tilletia caries* is around 16°C (Polišenská et al., 1998). *Tilletia caries* spores germination lasts approximately 3 days, it's preferable to higher temperatures and also needs a bit of light (Kroutil, 2006).

According to Kroutil (2011) in the Czech Republic are mainly occuring species *Tilletia caries*, *Tilletia controversa* and in small part also *Tilletia leavis*.

3.2. Tilletia caries

Tilletia caries is a pathogen of wheat and other relative grasses. It was firstly named by brothers Tulasne in 1847 and can also be known under name *Tilletia tritici*. Under common

names it is known as common bunt, stinking bunt, covered bunt, hill bunt, complete bunt, low bunt or high bunt. The diseases caused by *Tilletia caries* is known as common bunt and is present all over the world with strongest presence in temperate climate – mainly in North, Central and South Europe, central USA. They also occur in China, Iraq, Iran or India (Wilcoxson and Saari, 1996).

Teliospores have relatively thick, three-layered walls and are from light pale to reddish brown colour, with average diameter from 15-23 μm (Hess and Gardner, 1983). They have net-like, reticulate exospore with hyaline, gelatinoids sheath. Among teliospores are hyaline, smooth-surfaced, thin-walled, spore-like sterile cells with size ranging from 9.8 to 18.2 μm (Wilcoxson and Saari, 1996). Infection occurs under the soil surface, shortly after the germination of seed and prior to its emergence. Teliospores germinate on seed or occasionally in soil and produce infectious hyphae that later penetrates the coleoptile. Hyphae have to infect the apical meristems before elongation of internodes or the diseases will not develop (Wilcoxson and Saari, 1996). Detection of hyphae in the apical meristem of seedlings can predict diseases development with high accuracy (Kollmorgen and Ballinger, 1987). Disease development after infection can be influenced by environmental conditions. For example, plants with higher photoperiod have greater chance of successful smut disease (Zscheile, 1966).

The germination rate of the teliospores occurs over wide range of temperatures, most rapid growth is between 18-20 °C but most uniform is between 14-16 °C. Better germination percentage is during neutral to slightly acidic pH and soil type is not a critical factor for successful germination (Baylis, 1958).

Common bunt occurs on both spring and winter planted wheat and under favorable conditions and on plants that are not chemically protected, may infect more than 70 % of spikes. Symptoms of infection may not be apparent until after heading, when begins sporulation. Immature infected spikes are usually darker green, while mature infected spikes are lighter in colour. Usually spikes have a near normal appearance. Other symptoms are failure to extrude anthers, production of nonviable pollen, lengthened rachises, partially smutted spikes and kernels and a fishy odour caused by chemical compound called trimethylamine (Wilcoxson and Saari, 1996).

3.3. Tilletia controversa

For many years, *Tilletia controversa* was considered as *Tilletia caries*. First description as singular species was by Young (1935) in 1935. It can also be known as *Tilletia contraversa* or *Tilletia brevifaciens*. Under common names it is known as dwarf bunt, short smut, stunt smut, stubble smut, or TCK smut. Disease caused by this pathogen is known as dwarf bunt. *Tilletia controversa* can be found all over Europe, North Africa and Middle East (Prokinová et al., 2011). It occurs on fall-planted wheat, and usually in areas with snow cover, which provides favourable conditions for teliospore germination (Wilcoxson and Saari, 1996).

Teliospores of *Tilletia controversa* are from yellowish brown to reddish brown, globose to sub globose with hyaline gelatinoids sheath of thickness from 1.5 to 5.5 µm and of average diameter from 19-24 µm. Exospore is usually with polygonal reticulations. Sterile cells are globose with average diameter from 9-22 µm, with smooth walls and sometimes encased in a hyaline gelatinous sheath. Process of germination is similar as in case of common bunt (Wilcoxson and Saari, 1996). As was stated above, *Tilletia controversa* is limited to areas with snow cover. Snow insulates the ground and provides stable low temperatures and high humidity that is needed for successful germination and growth. According to Baylis (1958), teliospores of *Tilletia controversa* are stimulated by low levels of light, and no germination occurs in darkness. Similarly as *Tilletia caries*, dwarf bunt teliospores prefer pH neutral to slightly acidic and soil type is again not critical for development of infection (Warmbrunn, 1952).

Main source of inoculum is teliospores that are deposited in soil from a previously infected crop, or teliospores brought by wind. Infection begins when hyphae penetrates seedlings after teliospore germinate at or near soil surface. Success of infection depends long period of stable, low temperature and moisture, provided by snow cover. This reason together with spring dormancy of teliospores, is why dwarf bunt infects only winter sown wheat (Wilcoxson and Saari, 1996). Symptoms of dwarf bunt infection can be seen firstly as faint yellow spots and/or streaks. Other symptoms are: culms are usually shortened, anthers fail to extrude and the pollen is not viable. *Diseased* spike has characteristic appearance due to hyphal growth and spore formation that spreads the lemma and palea apart (Wilcoxson and Saari, 1996).

3.4. Resistance

The development of bunt-resistant cultivars can be one of the best methods to control the disease. In USA some dwarf bunt resistant cultivars have been successfully developed and in areas of southern Idaho and northern Utah the dwarf bunt has been reduced to trace levels (Wilcoxson and Saari, 1996). Some of the resistant wheat cultivars produced in the Czech Republic is Globus and Bill. Váňová et al. (2006) described resistance of cultivar Bill not only to common bunt but also to dwarf bunt. In the Czech Republic, there is testing of winter wheat cultivars since 1988, although irregularly. These tests are carried out by Research Institute of Crop Production in Prague-Ruzyně (Dumalasová and Bartoš, 2010).

A study done by Prokinová et al. (2011) tested the sensitivity of cultivars used in the Czech Republic towards *Tilletia caries* and *Tilletia controversa*. *Tilletia caries*: their research showed that none of the tested cultivars can be put into category resistant or very low sensitivity. In category low sensitivity belonged cultivars Alana, Stela, Banquet, Baroko, Bill, Brea and Versailles. Into category as very sensitive belonged cultivar Eurofit. *Tilletia controversa*: due to different used methodology there are various results depending on timing of study. From results of years 2008 to 2010, seven cultivars can be put into resistant category and only one to very sensitive. From result of year 2011 none of the cultivars can be put either to resistant or very sensitive category. As relatively resistant seemed to be cultivars Darwin, Radúza and Eurofit.

3.5. Management and control strategy

Basic control strategy how to protect crop should be prevention. That means using seeds that are controlled and pathogen free and subsequent health observation and control of growing crops (Kroutil, 2006). Another possibility of prevention is using crop rotation, where wheat should not be planted after wheat or other relative plants from the *Poaceae* family, due to the increased risk of teliospores survival in soil (Kochanová and Prokinová, 2004).

3.5.1. Chemical control

Chemical seed treatment is the most widely used control for bunts and smuts (Mamluk, 1992). To avoid the infection, it is necessary to use suitable chemical seed treatment, otherwise the grain is irreversibly damaged and it’s unsuitable to use in food industry (Benzinger et al.,

2003). In case of common bunt, we can divide fungicides at two groups: chemicals that are effective controlling the infection from both the seed borne and soil borne inoculum and those that are effective only at control of only seed borne inoculum. First group contains chemicals like carboxin, etaconazole, hexachlorbenzene, thiabendazole, triadimefon, triadiminol and pentachloronitrobenzene. Second group contains chemicals like benomyl, chloroneb, fuberidazole, maneb, pyrocarbolid and TCMTB (Wilcoxson and Saari, 1996). Quality of chemical seed treatments is influenced mainly by: occurrence of pathogen, concentration of fungicide, technique used for application and quality of distribution of fungicide (Chadová, 2007). Chemical control for dwarf bunt is more complicated. Fungicides have not been widely used due to several reasons like cost, phytotoxicity or dependence on late seeding and as it was mentioned earlier, availability of dwarf bunt resistant cultivars (Wilcoxson and Saari, 1996).

Chemicals can also be divided by effect in plant – systematic and contact. Systematic chemical penetrate the plant and are distributed over the whole plant. Their effect is longer however there is possibility of development of resistance. Contact chemicals do not penetrate the plant and are effective only on surface of plant in the place of application.

As was mentioned earlier, ecologic agriculture does not allow chemical controls, instead alternative methods are used: for example Tillecur powder from yellow mustard, applied as a suspension of surface of seeds (Barroga-Matanguihan et al., 2011).

3.5.2. Biological control

There is possibility of biological control on *Tilletia caries*. As was proved by Kollmorgen and Jones (1975), species *Streptomyces* and *Bacillus* can suppress in in vitro conditions the germination of *Tilletia caries* teliospores. The authors also found out that species *Bacillus* decreases the occurence of infection also in field conditions. Biological control has since then progress and new species are introduced specially in ecological agriculture.

3.5.3. Agrotechnical control

Occurrence of common bunt may be reduced by shallow seeding. For more significant reduction of occurrence, shallow seeding into warm soil can be utilized (Gaudet and Puchalski, 1990). In contrast, dwarf bunt occurrence can be reduced by deep sowing, or by very early or late

planting. This is done to avoid most susceptible plant stages. However these practices can lead to lower yields and can cause plants to be more susceptible to other diseases (Wilcoxson and Saari, 1996).

3.6. Essential oils

Essential oils are volatile, natural complex compounds formed by aromatic plants as secondary metabolites which are responsible for their characteristic aroma (Bakkali et al., 2008). They are constituted by terpenoid hydrocarbons, oxygenated terpenes and sesquiterpenes . In nature, essential oils play an important role in the protection of the plants as antivirals, antibacterials, antifungals, insecticides and also against herbivores by reducing their appetite for such plants. They also served as a mean of attraction for some insects to disperse pollens and seeds (Bakkali et al., 2008). For humans, essential oils can be used in cosmetic industry as ingredients for fragrances, decorative cosmetic or flavouring. In food industry it is used as aromas and flavours, in pharmaceutical industry as components of medicine and as antibacterials or antimicrobials.

The first systematic investigation of constituents from essential oils was done by French chemist Jean Baptiste André Dumas in 1833, analysed some hydrocarbons and oxygen as well as sulphur and nitrogen containing constituents. Most important studies were performed by German chemist Otto Wallach when realized that several terpenes described under different names according to their botanical sources were often chemical identical. He then tries to isolate these oil constituents and began study their basic properties. Wallach wrote around 180 articles and his work is summarized in his book Terpene und Campher. For his work was in 1910 honoured with the Nobel Prize for Chemistry “in recognition of his outstanding research in organic chemistry and especially in the field of alicyclic compounds.” (Baser and Buchbauer, 2010).

Essential oils are very complex natural mixtures that can contain up to 60 components at different concentrations. They are usually characterized by two or three components which have higher concentrations (20-70 %) that the rest of components which are present in trace amounts. Generally these two or three components determine the biological properties of the essential oils (Pichersky et al., 2006). The components include two main groups of biosynthetical origin. First groups are terpens and terpenoids and second group are aromatic and aliphatic constituents

(Croteau et al., 2000). Terpens are made from combinations of several 5-carbon-base (C_5) units called isoprene (2-methylbutadiene). The main terpenes are monoterpenes (C_{10}) and sesquiterpenes (C_{15}). A terpene containing oxygen is called a terpenoid. Monoterpenes constituted around 90 % of the essential oils (Bakkali et al., 2006). Aromatic compounds are derived from phenylpropane and occur less frequently than the terpenes. Aromatic compounds comprise of aldehydes, alcohols, phenols, methoxy derivates or methylene dioxy compounds. Main plant sources for these compounds are for example anise, cinnamon, fennel, nutmeg or tarragon (Bakkali et al., 2006).

3.7. Production of essential oils

Important notice is that produced essential oils used in agriculture, industry etc. is not identical with essential oils in plants, meaning that chemical composition of distilled essential oils is different of those presented in oil cells of plants (Baser and Buchbauer, 2010).

Currently there three main methods of commercial extraction of essential oils: expression, hydro distillation or steam distillation and dry distillation.

3.7.1. Extraction

It is probably oldest method for extraction of essential oils. Nowadays it is used exclusively for extraction of essential oils from plants of *Rutaceae* Family. It is prefered method due to thermal instability of the main constituents of the essential oils (Schmidt, 2010). Before industrialization of extraction, several methods were used. For example cold expression, is expression at ambient temperatures without involvement of extra heat. Today main methods can be classified into four categories: sfumatrici method, Pellatrici method, FMC whole fruit process and Brown oil extractors (Arnodou, 1991).

Sfumatrici method used machines with two parts: fixed part and moveable part. Fruit is cut in half, and flesh is removed. Peel is squeezed and squeezed-out oils is rinsed away with water. Oils is separated from water and collected by decantation. Since the epicarp can contain organic acids like citric acid etc. it is soaked in lime solution in order to neutralize the acids. There exists alteration of sfumatrici method called special sfumatrici method in which the peel is soaked in lime solution for 24 hours before pressing (Baser and Buchbauer, 2010).

Pellatricci method consists of two steps. In first step fruit is pushed through slowly turning Archimedean screw with spikes. These will bruise the oil cells in the epicarp and initiate flow of the oil, which is rinsed away with water. Fruit is then carried into a fast rotating, spiked, roller carpet where is again bruised to achieve maximum oil yield. This process is then followed by centrifugation, filtration and wintering (Baser and Buchbauer, 2010).

The Brown process is used mainly in the Unites States of America and in countries of South America. This process uses machine similar as in Pellatricci method. In machine is present numerous spike rollers used for bruising of peel to reach oils cells. Fruit is submerged in water for easier transportation. As the fruit travels through the machine, water with oils is removed and passed through fine sieve to rid away of all solid particles. Emulsion is then centrifuged (Baser and Buchbauer, 2010).

FMC whole fruit process is most frequently used method of expression. It is estimated that in the USA more than 50 % of producers uses this method and is preferred method in countries like Brazil or Argentina. Advantage of this method is used machinery, when fruit juice and essential oils are produced simultaneously without the two coming in contact with each other. The fruit is carried into a fixed cup with identical moveable cup placed above it. Moveable cup is then lowered and at the same time, circular knife cuts a hole into the bottom of fruit. Pressure is applied and fruit juice exit through the cut hole while at the same time oil is squeezed out of the surface of the peel. Oil is then rinsed away with water and oil-water emulsion is centrifuged (Baser and Buchbauer, 2010).

3.7.2. Steam or water distillation

Steam or water distillation is the most frequently used method for the extraction of essential oils from plants. The principle is that the release of the essential oils form the oil cells is done by bursting of the oil cells walls due to increased pressure of the heat induced expansion of oil cell contents. The steam then acts as a carrier of the essential oil molecules. The hydrodistillation (water distillation) works on principle that two immiscible liquids (oil and water) form two separate phases. Then the total vapour pressure of that system is equal to the sum of the partial vapour pressures of the two pure liquids. The simplest method of hydro distillation is immersion of the plant into boiling water. The plant material soaks up the water

and the oil in the oil cells diffuses through the cell walls by osmosis. Once is the oil out of the oil cells, it is vaporized and carried away by steam. The design of steam/water distillation plants evolve form simple like „false bottom apparatus“ into more modern ones consisting of biomass container with cooling systems and oil separator with high capacity steam generator. The biomass container looks like a cylindrical vertical tan with steam pipes at the bottom of the container, often accompanied by sieve like plates. The outlet for the steam with oil is usually in the container lid. The steam is then passed through the cooling system (for example cold water condenser). The condensate liquid is then separated into essential oil and water by centrifugation or oil separator like Florentine flask. Nowadays many alterations of this method exist. Some raw materials like hard grains need to be comminuted before steam/water distillation (Schmidt, 2010).

3.7.3. Dry distillation

Dry distillation is used only rarely and in some very special cases. Dry distillation involves heating in the absence of aerial oxygen in closed vessel preventing combustion. For example birch tar from wood exudate of *Betula pendula* Roth. or cade oil from the wood of *Juniperus exycedrus L.* are made this way. However as was discovered both oils contain phenols some of which are proved carcinogens. (Baser and Buchbauer, 2010)

For the best quality of essential oils, sometimes rectification is required. This involves redistillation of the crude oil in order to remove undesirable impurities such as small amounts of constituents or volatile compounds producing undesirable odour. Rectification is usually carried out by redistillation under vacuum to avoid overheating and subsequent decomposition of oil constituents. In commonly rectified oils belong eucalyptus, clove, mint, turpentine or peppermint.

3.8. Lavandula angustifolia

Lavandula angustifolia, also known under common name as English lavender belong to the family *Lamiaceae* and is most widely cultivated species of this family. It is known for its wide variety of therapeutic and cosmetic purposes and has been used as such for centuries. There documented evidence that lavender was used by ancient Greek and Romans (Cavanagh and

Wilkinson, 2002). *Lavandula angustifolia* is native to Mediterranean in countries Italy, Andorra, Spain and France. This aromatic shrub can grow up to 2 meters.

Essential oil from lavender is usually produced by steam distillation from both the flower heads and foliage. However the chemical composition differs greatly and the most sweet and aromatic oil is from the flowers (McGimpsey and Porter, 1999). Commercially lavender essential oils is most widely used in fragrance industry including soaps, colognes, perfumes, skin lotions and other cosmetic (Pauli, 2006). Recently lavender is used in form of aromatherapy as a relaxant (Lis-Balchin et al., 1998). As was reported by Buchbauer et al. (1991), lavender essential oil has several therapeutic effects such as sedative, spasmolytic, antiviral and antibacterial. Several studies also reported that essential oil from *Lavandula angustifolia* has acaricidal activity (Perrucci et al., 1996).

3.8.1. Chemical composition of lavender essential oils

According to aroma analysis performed by An et al. (2001), essential oil from *Lavandula angustifolia* have lower levels of camphor (<2 %) then other species of *Lamiaceae* family such as *Lavandula stoechas* and *Lavandula lanata*. While lower on camphor, *L. angustifolia* has higher levels of terpenes and sesquiterpenes.

3.9. Rosmarinus officinalis

Rosmarinus officinalis also known under common name as rosemary belong to family *Lamiaceae*. It is woody, perennial herb with small, evergreen leaves and white, pink, purple or blue flowers. It is native to Mediterranean area. Rosemary has wide variety of uses. It is used in culinary world as a flavouring, essential oil from *Rosmarinus officinalis* is used in perfumes, incense or soaps. In India it is used in traditional medicine for help with respiratory disorders. Rosemary has been cultivated since ancient days and is closely associated with love, marriage, birth and death (Al-Sereiti et al., 1999).

3.9.1. Chemical composition of rosemary essential oils

Essential oil from *Rosmarinus officinalis* is produced by steam distillation and is colourless or pale yellow. Main constituents are Piperitone (24 %), Linalool (15 %) and α-Pipene

(15%). Other notably constituents are 1,8-Cineole (8 %), Camphor (5 %) and Borneol (4%) (Gachkar et al., 2007).

Essential oil from *Rosmarinus officinalis* has antimicrobial effect as was reported by Iacobellis et al. (2005). According to study by Miresmailli et al. (2006) it has also acaricidal effect.

3.10. Foeniculum vulgare

Foeniculum vulgare also known under common name as Fennel is an annual, biennial or perennial (depending on variety) belonging to family *Apiaceae* and native to Mediterranean area (Özbek et al., 2004). *Foeniculum vulgare* is an aromatic herb with fruits of greenish or yellowish brown in color. Each fruit weighs between 6 to 7 mg and is about 6 mm long (Warrier et al., 1978). Fennel is mainly used in culinary, where dried, aromatic fruits are utilized in preparation for flavouring of bread and pastry, candies and alcoholic liqueurs. Fennel is also used in cosmetic and medicinal products (Farrell, 1988). In medicine it is used for dyspeptic complaints like mild, spasmodic gastric-intestinal complaints, bloating and flatulence. It can also be used for treating of catarrh of the upper respiratory tract (Czygane, 1989). Essential oil from *Foeniculum vulgare* is used in treating of pediatric colic and some respiratory disorders, due to its antispasmodic effect (Reynolds, 1982). Özbek et al. (2003) also reported that essential oil has potent hepatoprotective action against CCl_4- induced acute liver injury. Oktay et al. (2003) reported antioxidant activity of *Foeniculum vulgare* seed extracts. According to Kaur (2009) fennel also show antibacterial effects.

3.10.1. Chemical composition of fennel essential oils

According to study done by Özbek et al. (2004) main components of essential oil from *Foeniculum vulgare* are (*E*)-anethole (75 %) followed by limonene (11 %) and methyl chavicol (5 %). However chemical composition depends on variety of fennel, when in essential oil from sweet fennel the fenchone content can be as high as 20 %, whereas in bitter types its content does not exceed 5 % (Bernath et al., 1996).

3.11. Pelargonium graveolens

Pelargonium graveolens can be also known under common names rose geranium, sweet scented geranium, old fashion rose geranium and rose-scent geranium and belongs to *Geraniceae* family. This fairly uncommon Pelargonium species is native to South Tropical Africa mainly Mozambique and Zimbabwe and to Cape Province in South Africa. It is an erect, branched shrub reaching up to 1.3 m and spreading up to 1 m. The leaves are strongly rose scented (Peterson et al., 2006). Aerial parts of *Pelargonium graveolens* are used in folk medicine of Iran and in the world as a food and tea additive. It can also be used for treating of some gastrointestinal, topical, dental and cardiovascular disorders (Ghannadi et al., 2012). Essential oil for *Pelargonium graveolens* can be used in perfumery and cosmetic products. Historically geranium essential oil has been used for treatment of dysentery, hemorrhoids, and inflammation or heavy menstrual flows. It has also be reported that essential oil is extremely useful for reducing pain due to post-herpetic neuralgia following shingles (Greenway et al., 2003). Several studies also reported antioxidant, anticancer, antifungal and antibacterial effects of essential from geranium (Ghannadi et al., 2012; Fayed, 2009). Currently most used method for extraction of essential oil is steam distillation. However it has been reported as highly inefficient due to large losses caused by thermal degradation (Higley and Higley, 1998).

3.11.1. Chemical composition of geranium essential oils

According to study done Boukhatem et al. (2013) main constituents of essential oil are citronellol (30 %), citronellyl formate (9 %) and geraniol (8%).

3.12. Eugenia caryophyllata

Eugenia caryophyllata also known under common name clove belongs to family *Myrtaceae* and is native to Indonesia and cultivated in Tanzania, Malaysia, Brazil, Sri Lanka, Madagascar or India. It is an evergreen plant reaching up to 20 cm of height and has spear shaped leaves and coniferous yellow flowers (Öztürk and Özbek, 2005). Dried clove can be used in cuisine as flavouring, cigarettes or to make fragrance pomander. It has been also used in traditional medicine and is said to have antiseptic, analgesic and anesthetic effects (Chaieb et al., 2007a). Essential oil made from *Eugenia caryophyllata* is colourless and most commonly

produced by steam distillation (Öztürk and Özbek, 2005). Several studies reported various biological activity of essential oil from clove. Antibacterial activity was demonstrated by Burt and Reinders (2003), antiviral efficacy by Kurokawa et al. (1998), antifungal activity by Velluti et al. (2004) and also acaricidal activity by (Kim et al., 2004).

3.12.1. Chemical composition of clove essential oils

According to study by Öztürk and Özbek (2005) main constituens are β-caryophyllen (45 %), and eugenol (44 %). Other constiutents are α-humulen (3.5 %), eugenyl acetate (1.3 %) and α-copaen (1 %).

3.13. Origanum vulgare

Origanum vulgare also known under common name Oregano (Janssen, 1999) is a popular medicinal plant flowering from July to September. It can be found in lowlands as well as in mountainous regions because its freeze proof ability. It is very adaptable in this way (Haragsim, 2008). It originates from the Mediterranean, but also can be found in warm areas of Europe. It's very aromatic so it must be separated from other plants during drying (Milos et al., 2000).

The oil takes principal part in cosmetic products such as soaps or hair products (Bodlák, 2005). One of its main components is thymol, which is used for the medical purposes against cough or breathing difficulties (Haragsim, 2008). It is also used for the manufacture of perfumes and the treatment of inflammation due to its anti-inflammatory and disinfecting effects. It relieves anorexia, indigestion and affects the nervous system (Milos et al., 2000).

3.13.1. Chemical composition of oregano essential oils

The basic components are carvacrol and thymol with content of about 0.4%. They have antioxidant effects and are also part of anticancer drugs because of their anti-mutagenic effects (Ipek et al., 2005). Zodrow et al. (2012) reported, that carvacrol prevents the formation of biofilm on the surface of polymers. De Souza et al. (2010) is also describing that both of components have antibacterial effects for example against *Staphylococus aureus*. The essential

oil of oregano has the ability to disrupt bacterial cytoplasmatic membranes and cell walls (De Oliviera et al., 2010).

3.14. Thymus vulgaris

Thymus vulgaris (thyme), locally known as "zaatar", a member of the *Lamiaceae* family, is a pleasant aromatic herb and sub shrub, which grows in several regions in the world (Davis, 1982). It is spread in a localized way in Southern Europe, North Africa and the Mediterranean zone. Its existence does not exceed directly boarding regions to the Mediterranean. Several karyological studies held the same results that Thymus vulgaris has a number of chromosomes of 2n = 30 (Jalas, 1948; Stahl-Biskup and Saez, 2002; Maksimovic et al. 2008). It is commonly known that the composition of the essential oils determine the specific aroma of plants and flavour of condiments (Martins et al., 1999).

Agricultural factors affect quantity and quality in thyme; spacing and harvesting schedule are very effective factors in this area. Shalby and Razin (1992) cultivated thyme in rows 60 cm apart with inter-row spacing of 15, 30 and 45 cm. They stated that the wider the spacing, the greater were the yields of essential oil per plant. Thus close spacing increased the yield of herbage and oil per unit area, but in close spacing the plant could be subject to fungal infections where ideal microclimates for fungi and bacterial infections could form. Harvesting time is also an important factor that influences oil yield. From June to August, plants start to develop pink to white and violet flowers; (McGimpsey et al., 1994) experiments proved most oils produced from flowering plants had oil content at its highest levels at that time. A report on thyme grown in northern Italy indicated that phenol content at full flowering varied from year to year (Piccaglia and Maroti, 1991).

3.14.1. Chemical composition of thyme essential oils

The main chemical composition in thyme is in its essential oils, which include thymol, carvacrol, borneol, linalool, myrcene, p-cymene and other flavonoids. Other oils in the plant are tannin, saponins and triterpenic acids. Furthermore Thymus vulgaris shows a polymorphic variation in monoterpene production, the presence of intraspecific chemotype variation being common in the genus Thymus. Each of the six chemotypes, geraniol (G), α-terpineol (A),

thuyanol-4 (U), linalool (L), carvacrol (C), and thymol (T), is named after its dominant monoterpene (Shabnum and Wagay, 2011).

3.15. Cytotoxicity

Essential oils seem to have no specific cellular targets due to large number of constituents (Carson et al., 2002). They act as typical lipophilic and pass through the cell wall and cytoplasmic membrane where they can disrupt the structure of layers of polysaccharides, fatty acids and phospholipids (Knobloch et al., 1989). In eukaryotic cells, essential oils can provoke depolarization of the mitochondrial membranes by decreasing membrane potential and affecting ionic Ca^{2+} cycling along with other ionic channels and can reduce the pH gradient affecting the proton pump and the ATP pool (Richter and Schlegel, 1993). They change the fluidity of membranes, causing them to be abnormally permeable which leads to leakage of radicals, cytochrome C, calcium ions and proteins. Permeabilization of the outer and inner mitochondrial membranes can lead to cell death by apoptosis and necrosis (Yoon et al., 2000).

Cytotoxic properties are of great importance in the application of essential oils against certain human and animal pathogens or parasites and also for preservation of agricultural products. Essential oils are effective against large variety of organisms including bacteria, fungi, virus, protozoa, parasites, larvae, worms and insects (Basile et al., 2006; Duschatzky et al., 2005; Hammer et al., 2002; Rim and Jee, 2006).

3.16. Essential oil application

Essential oils have many different kinds of biological effects. It inhibits and reduces growth and spread of microorganisms such as fungus, bacteria and viruses. It is also used as an antioxidant mainly in the food industry, in medicine as an aphrodisiac, narcotic or sedative and for its healing effect as an analgetic and antibiotic. Antimicrobial substances contained in oils prohibit the multiplication of microorganisms. Amount of antibacterial substances and the efficiency of the oil depends on the chemical composition, plant species, cultivation method and storage and finally on the method of extraction (Deans and Ritchie, 1987).

Essential oils have recently become more important because of bacterial resistance to antibiotics. Research of treatment of bacterial diseases continues to find new ways to prevent

infectious diseases. Some of the essential oils qualities are important for their treatment. An example would be the ability to support and to stimulate the immune system and induce the reaction to infection. Oils with this capability include for example lavender oil. (Davisová, 2005).

It is easier for bacteria to make resistance against antibiotics than essential oils because of their chemical composition. Essential oils are consisted of several components while antibiotics include one molecular unit (Solórzano-Santos and Marinda-Novales, 2012).

Viruses have developed immunity against the medicament as well as bacteria. There hasn´t been found any way of treatment for many kinds of viral diseases. That is why people are searching for new ways of treatment. One of the natural ways are essential oils that contain many antiviral substances (Berger, 2007). Essential oils have the ability to prevent replication and reproduction of viruses and its spread to other cells (Basser, 2010).

Essential oils have many different qualities in medical aspects. It is functioning as analgesics, antiseptics, sedatives, narcotics and it has also anti-inflammatory and pain soothing effects (Janča and Zentrich, 1994). The oils help against the pain thanks to the analgesic effect because essential oils contain monoterpenes up to 90% (Berger, 2007). Examples of analgesics oils are *Mentha spicata* and *Levandula angustifolia* which is stronger (Guimaraes et al., 2013).

Essential oils are used for production of antiseptic solutions which prevent microbial infections, inflammations and sepsis (Farrer-Hallsová, 2007). They are applied to the skin and can be also gargled in case of oral inflammation or dental pain. Typical example is tea tree oil mainly used for oral inflammation (Poth, 2000) or *Syzigium aromaticum* which is used for toothache (Tilia, 2003).

Antioxidant effect come with high importance and are in the food production chain as organic substances that relieve the oxidation process in organism and its damage (Basser, 2010). Essential oils are one of the natural kinds of antioxidants, because it mainly consists of phenolic substances which act as antioxidants (Berger, 2007).

Some species of essential oils can be used as ingredients in food production and storage because of their antipathogenic effect. These kinds of oils aren´t considered dangerous for human use. They have natural preservative effect and insure to stop the contamination by microorganisms (Razzaghi et al., 2009).

Mentha spicatas essential oil is good example of the antioxidant. It is used in food

industry for its ability to prevent the oxidation of lipids in meat which helps to extend the time of the durability (Djenane et al., 2012). Another type of antioxidant essential oil is *Rosmarinus officinalis* also known for its antibacterial quality (Ojeda-Sana et al., 2013).

3.17. Biopolymers

Chemical pesticides are primarily used for controlling plant pathogens around the world; these agricultural micro-organisms are considered economically important as they promote decay on a variety of agricultural crops. Also, the effect of such micro-organisms occurs during both the growing season and postharvest. The continuous use of chemical pesticides raised public awareness over their potentially toxic effects and the development of resistance in pest populations (Casida and Quistad, 1998; Carson, 1962; Houetocet et al., 1995). This invoked emphasis on environmental friendly technologies as an alternative to the detrimental synthetic pesticides (Ben-Yeohshua and Mercier, 2005; El Ghaouth and Wilson, 1995; Rabea et al., 2003).

Consequently, various nonchemical treatments have been developed to reduce pathogens, but each of these treatments has its limitations and in turn affects its commercial applicability. Biopolymers, on one hand, showed satisfactory results as safe natural alternatives against plant pathogens with negligible effects on the environment and human health (Muzzarelli, 1983)

In the food industry synthetic materials are now being replaced by biodegradable and environmental friendly edible films and coatings for the preservation and protection of food (Tharanathan, 2003). These advantages over synthetic materials led to increasing studies on the antimicrobial and antioxidant properties of edible films. Thus, they have the ability to prevent the growth of pathogenic and spoilage microorganisms with their lag-phase extension and their growth rate reduction (Quintavalla and Vicini, 2002).

Several authors indicated the capabilities of edible films, such as to retard moisture and oxygen by reducing water vapour permeability, improving aromas and solute transport through a variety of additives that include antioxidants, antimicrobials, colorants, flavours , and nutrients (Pranoto et al., 2005a; Botsoglou et al., 2002).

Such biopolymers can be formulated with essential oils for their isolating effect. Essential oils for example are natural volatile compounds with various biological properties, they can be extracted from aromatic plants and include antimicrobial effects. Essential oils show antibacterial

activity (Alves-Silva et al., 2013; Burt, 2004), but rare studies evaluated their antifungal effect (Alves-Silva et al., 2013; Avila-Sosa et al., 2012; Perdones et al., 2012; Perdones, Vargas et al., 2014; Rosello et al., 2015; Saggiorato et al., 2012; Sanchez-Gonzalez et al., 2010). Also, EOs have been noted to reduce oxygen permeability of several polymer films in agreement with their oxygen scavenging activity (Bonilla et al., 2013). EOs affect microbial cell walls via different mechanisms, including interactions with their cellular phospholipid bilayer, the disruption of enzyme systems and changing the genetic material of bacteria.

Encapsulation of essential oils in polymeric materials is extensively studied in order to have their antimicrobial advantage and to reduce their drawback (Beyki et al., 2014; Wen et al., 2016; Biddeci et al., 2016). The addition of EOs into a polymer matrix reduces their strong flavor, and in turn enhances its use as food preservatives; but this may also modulate the diffusion of the antimicrobials into the product (Ruiz-Navajas et al., 2013). Such biodegradable coatings are considered an interesting alternative to conventional plastic packaging, which is why several biopolymers are used for eco-friendly food packaging (Azeredo, 2009).

3.17.1 Arabic gum

Gum Arabic is a natural gum which is obtained from stems and branches of Acacia species in the form of dry and hard nodules about 10-50 mm in diameter, ranging from colourless to brown. Gums are also extractable from land plants (e.g. locust bean, guar) or marine plants (e.g. carrageenan, alginate), from microorganisms (e.g. xanthan, gellan, pullulan) or animal source (e.g. chitosan) (Azzaoui et al., 2015).

Gum is the most common polysaccharide used in various ways in the food industry as stabilizing, thickening agent and emulsifier, mainly in the food industry (e.g. in soft drinks syrup, gummy candies and marshmallows), but also in the textile, pottery, lithography, cosmetics and pharmaceutical industries. Gums are classified according to their source of extraction, chemical structure and physical characteristic. Gum Arabic has high water solubility, good emulsifying properties and relatively low viscosity, even in fairly concentrated solutions (Verbeken et al., 2003).

Edible coatings based on natural products (gum arabic) can provide an additional protection for fresh fruit and vegetables and can be complementary to low temperature,

controlled atmosphere and hypobaric storage techniques (Baldwin et al., 1995). Appropriate forms of an edible coating may provide an excellent barrier against gaseous exchange and water loss which are detrimental to postharvest quality. When is used as an edible coating, gum arabic also showed positive results and significantly overdue ripening of cold-stored apples (Ali et al., 2013).

Study of Ali et al. (2013) shows that gum arabic, as a preservative material, could delay the ripening process by inhibiting the respiration rate and ethylene production in tomato fruit, where was gum arabic solution used on Tomato (*S. lycopersicum L.* var. Money Maker) fruit. Fruit were dipped in each concentration ofgumarabic coating solution (5, 10, 15 and 20%) for 2–3 min and it was assured that the coating solution was applied uniformly on the whole surface while control fruit were dipped in purified water only.

3.17.2 Chitosan

Chitosan, a cationic polysaccharide is the deacetylated form of chitin. Chitosan is a polysaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine. It is made by treating the chitin shells of shrimp and other crustaceans with an alkaline substance. The source of the chitin from which is chitosan obtained is crabs shell, crayfish, mussels, but also fungi and insects elytra. Chitosan is one of the polysaccharides chemically (Yuan et al., 2016).

Chitosan edible films and coatings show great promise for their application in food preservation and also are promising systems to be used as essential oil carriers. Incorporation of essential oils significantly increased the antioxidant, antibacterial and antifungal efficacy of chitosan films. Essential oil incorporated films and coatings also showed greater effectiveness against postharvest fungi and foodborne bacteria in food systems beside pure films and coatings. Chitosan coatings incorporated with essential oils were more effective in maintaining fruit and vegetable quality, and controlling their postharvest decay during storage and shelf life than pure chitosan coatings. (McHugh, 2000; Yuan et al., 2016).

Chitosan films and coatings are promising systems to be used as active ingredient carriers. In terms of active ingredients that can be incorporated into films and coatings, essential oils have received much attention as having potential biological activity. Many essential oils,

such as clove, oregano, thyme, nutmeg, basil, mustard or cinnamon are gaining popularity due to their volatile nature, which facilitates the use of small concentrations that are safe for consumption (Sivakumar and Bautista-Banos, 2014).

3.17.3 Alginate

Alginate is a unique polysaccharide derived from marine brown algae and known for its colloidal properties and its ability to form gels or insoluble polymers after reacting with with multivalent metal cations such as calcium (Montero-Calderón et al., 2008; Rojas-Graü et al., 2007).

One approach to inhibit browning reactions on cut surfaces of fruits and vegetables is to exclude oxygen, add antioxidants, or inhibiting the activity of the responsible enzymes. An important alternative to reduce the deterioration of fresh fruits can be edible coatings. This semipermeable barrier provided by edible coatings can reduce moisture and solute migration, respiration and oxidative reaction rates, gas exchange, suppress physiological disorders of fresh-cut fruits, ultimately extending shelf life (Robles-Sánchez, 2013).

As mentioned earlier, antimicrobial agents like essential oils, colorants, flavors, nutrients and spices can be added to edible polysaccharide-based coatings (Pranoto et al., 2005b; Rojas-Graü et al., 2009); an important example of these polysaccharide-based coatings is alginate.

3.18. Methods currently used in agriculture for plant disease detection.

Monitoring plant health and detecting pathogen early are essential to reduce disease spread with the least damage to crop production and to facilitate effective management practices (Yang et al., 2013). Techniques for Detection and identification of plant diseases can be broadly classified into direct and indirect methods. The direct disease identification methods include molecular and serological methods that could be used for analysing large numbers of samples. The traditional disease identification by visual assessment of plant symptoms (leaves become red or yellow and twigs stay soft) has been aided by advances in technology such as direct microscopic observation of pathogens and their in vitro manipulation. These methods are Laboratory-based techniques such as polymerase chain reaction (PCR) which is based on specific deoxyribose nucleic acid (DNA) sequences of the pathogen,enzyme-linked

immunosorbent assay (ELISA), in turn based on proteins produced by the pathogen (Prithiviraj et al., 2004; Das, 2004; Li et al., 2006; Saponari et al., 2008; Ruiz-Ruiz et al., 2009; Yvon et al., 2009). The sensitivity of the molecular techniques refers to the minimum amount of microorganism that can be detected in the sample (López et al., 2003). Using the following methods Immunofluorescence (IF), fluorescence in-situ hybridization (FISH), flow cytometry (FCM) and gas chromatography-mass spectrometry (GC-MS), we are able directly to detect the disease causing pathogens such as bacteria, fungi and viruses to provide rapid, accurate, and reliable detection of plant diseases in early stages for economic, production, and agricultural benefits (Alarcon et al., 1990; Caruso et al., 2002). On the other hand, indirect methods include thermography, fluorescence imaging and hyperspectral techniques and are used to identify the plant diseases through various parameters such as morphological change, transpiration rate change, temperature change and volatile organic compounds released by infected plants. (Fang and Ramasamy, 2015).

3.19. Polymerase Chain Reaction

In the year 1993, Kary Mullisreceived was awarded the Nobel Prize and the Japan Prize for the development of monoclonal antibodies and amplification of nucleic acid sequences, respectively, using the technology of polymerase chain reaction (PCR) (Bartlett and Stirling, 2003).

PCR is a powerful technique which caused a veritable revolution in biological research, PCR was initially used as a highly applicable method for detecting diseases caused by bacteria and viruses, now rapidly it has become one of the most widely used techniques involving diagnoses and genetic improvements for plants and animals because it is quick (completed within 2 to 3 h); specificity (DNA probes can be designed to amplify nucleic acids from the desired genus, species, subspecies, race, etc.); sensitivity (single copies of nucleic acids can be detected after amplification), inexpensive and relatively simple (Cai et al., 2014).

PCR is based on the fidelity of DNA hybridization, replication purification and amplification before performing the gel electrophoresis (Grothues and Rudolph, 1991). Commercial kits specifically designed to extract nucleic acids from different types of plant material are widely used.

One of the key discoveries underlying the PCR technique was a thermostable DNA polymerase enzyme (Taq polymerase), which takes part in the replication of the cellular genetic material and that was isolated from *Thermus aquaticus*, a bacterium that grows in hot springs (Mullis, 1990). This polymerase can withstand the heating and cooling cycles needed for PCR, allowing the synthesis of complementary DNA sequences, as described in the next paragraph. PCR requires 4 primary components: the thermostable DNA polymerase enzyme, nucleotide triphosphates (which serve as building blocks for the creation of DNA and consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G).), sample DNA to be amplified, and gene-specific primers, as a small fragment is connected to one of the DNA strands in the specific site chosen to start the synthesis. The source of sample DNA can be either genomic DNA, isolated from cells or tissues, or DNA obtained from RNA samples through reverse transcription (RT). Primers are short, sequence-specific oligonucleotides that are generated via chemical synthesis to be complimentary to a chosen DNA sequence of any gene of interest.

PCR is composed of repeating cycles of 3 consecutive steps (denaturation, annealing, and extension) that require distinct temperature conditions. Each step is devoted to a specific process, ultimately leading to the generation of more copies of the chosen gene. This reaction is accomplished through the use of a thermocycler, an apparatus that holds the samples in a heating block, where rapid and controlled changes in temperatures are performed in the different phases of the amplification process (Novais et al., 2014).

The first PCR step is separation of the double-stranded DNA (dsDNA) by heating the sample at high temperatures (from 90 - 97 degrees Celsius). In the second step, the sample is cooled, which allows the primers annealing to the DNA template strands to prime extension. The temperature of this step is determined by using several physicochemical variables of the chosen primers (Wittwer et al., 1997). In the third step, the mixture is heated to 72°C, which is the optimal temperature for the activity of Taq DNA polymerase enzyme. The polymerase catalyzes builds two new strands of DNA using the primers as a starting point and uses the nucleotide triphosphates present in the mix to generate the sequence-specific complementary strand, in a process called elongation. Repetition of these 3 steps results in doubling of the copy number with each cycle (copy number = 2n, where n is the cycle number). The generation of PCR products, therefore, follows an exponential pattern and reaches a plateau after approximately 30 to 40 cycles; each cycle doubles the number of copies of the desired DNA strand. After 25-30 cycles,

whoever is performing the PCR process on a sample of DNA will have plenty of copies of the original DNA sample (Palmer et al., 2003).

Different types of PCR techniques are described such as reverse-transcription PCR (RT-PCR) have also been used for plant pathogen identification due to their high sensitivity (Cai et al., 2014). Multiplex PCR was proposed to enable simultaneous detection of different DNA or RNA by running a single reaction (James, 1999; Williams et al., 1999). Real-time PCR platforms have also been used for on-site, rapid diagnosis of plant diseases based on the bacterial, fungal and viral nucleic acids (Lievens et al., 2006; Schaad and Frederick, 2002). This technique allows the accurate quantification of the target pathogen, by interpolating the quantity measured to a standard curve with known amounts of target copies (Garrido et al., 2009). The advantages of real-time PCR are a high throughput method for the analysis of a large number of samples due to the use of a platebased system which permits the analysis of 96 or 384 samples at the same time and the capability to perform multiplex detection of two or more pathogens in the same reaction. Once the PCR is completed the products of the reaction (the amplified DNA fragments) are analyzed, visualized by gel electrophoresis.

3.20. Agarose gel electrophoresis

Is the easiest method of visualizing and analysing the PCR product, which detected in the form of a bright band, with ethidium bromide, a compound that fluoresces once bound to dsDNA. This so-called end-point detection is semi quantitative at best for several reasons: ethidium bromide lacks sensitivity, the end point levels of product from the same sample vary from PCR run to run, and there is a limited dynamic range for precise quantification of the product based on densitometry analysis of the brightness of the band (Wong and Medrano, 2005; Valasek and Repa, 2005).

4. Materials and Methods

Certified winter and spring wheat seeds were provided by the department of plant protection. The variety Bohemia was used for winter wheat on the field experiment. The variety Anabel was used as spring wheat for the container experiment in the glass house.

The experiment was divided in to three parts. Teliospores of *Tilletia* fungus were obtained from infected plants which were used as control variants from previous experiments done by the department of plant protection. First polymerase chain reaction was used to identify the exact species we worked with. After identification of *T. caries*, glass house experiment was conducted in containers. Final part was the field experiment which took place on the experimental and demonstration field of the Czech University of Life Sciences in Prague - Suchdol.

Essential oils used in the experiment were purchased from Sallus (Prague, Czech Republic).

Essential oils were chosen according to their effect obtained from previous *in vitro* and *in vivo* experiments and from former experience of the department of plant protection.

List of the seven essential oils:

- *Thymus vulgaris* - TV
- *Origanum vulgare* - OR
- *Eugenia caryophyllata* - EUG
- *Pelargonium graveolens* - PG
- *Foeniculum vulgare* - FV
- *Rosmarinus officinales* - RO
- *Levandula angustifolia* – LO

Preparation of E.O. and Seed Treatment

By using laboratory seed treating equipment the oils were coated by gelatine capsules. 11.4 g of wheat seeds inoculated by *Tilletia* were used for one dose. In each treatment 200 seeds were mixed with 1 ml of a 5% emulsified essential oil and one spoon of approximately 2 g of sepiret (aiding component of white colour with a powdery form used to increase seed coating).

Container experiment

The container experiment was conducted on the following essential oils; *Thymus vulgaris, Origanum vulgare* and *Eugenia caryophyllata*. First seeds were inoculated with *T. caries* spores as intended source of infection. Each essential oil was mixed in four different mechanisms using homogenization mixing equipment such as Ultra Turrax. Other variants included mixing with polysaccharides to form a viscous gum or a gelatine capsule. 4 variants for 3 essential oils after 5 repetitions were sown into 60 small containers. 6 additional containers were used to sow the control variants.

Field experiment

Sowing the experiment was on 30.10.2015. Parcels (2 times 1 m each) were used to sow each variant with 400 seeds sowed in an area of 1 m^2. Spore inoculum obtained from previous experiments was used. Preparing the inoculum, 18 g of spores was introduced to 900 g of seeds. Seed treatment as in choice of essential oils, added substances and mixing mechanisms, was decided based on obtained experience from previous experiments. The field was prepared and sowed manually. After ripening, wheat spikes were also manually harvested.

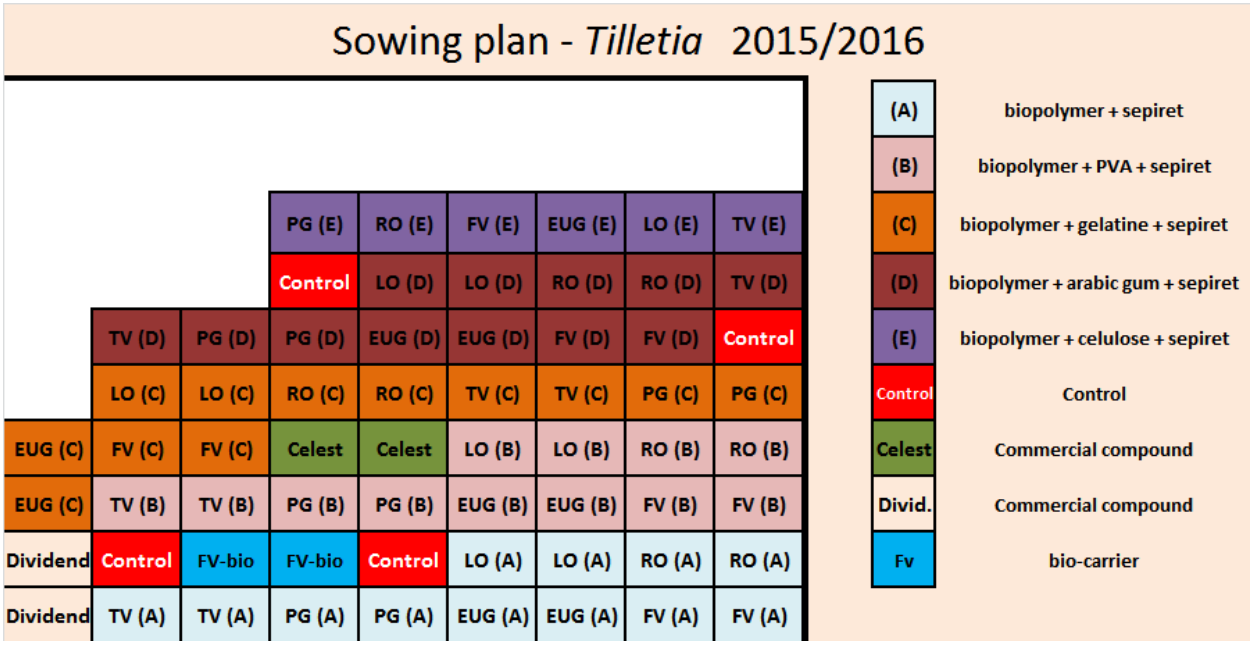


Fig. 2. Sowing plan

Detection of *T. caries* from seed samples

Samples of *Tilletia* spores where analyzed using the PCR assay. Spores obtained from harvested ears from an artificially infected crop which was sown at the experimental and demonstration field of the Czech University of Life Sciences - Suchdol. We manually removed infected bunt seeds from ears and crushed them through a filtering net to remove debris in order to collect teliospores for DNA extraction.

Working procedure

We performed the following protocol to achieve DNA isolation and purification. Pestle and mortar used to pulverize the material, were thoroughly chilled with liquid nitrogen. 700 µl of CTAB buffer with mercaptoethanol were immediately pipetted in order to avoid continues chilling. The resulting homogenate material pipetted into 20 ml microtube and incubated in a thermostat with occasional reverse stirring for 1 hour at 60 ° C. Following the incubation, 350 µl of phenol and 350 µl of Chloroform - isoamyl alcohol mixture in the ratio (24:1) added to the micro tube. The contents of the micro tube were vortexed for 10 minutes at 3500 rpm. Then centrifuged at 10 000 x g for 10 minutes to separate the mixture fazes. The upper aqueous faze was carefully pipetted into new 20 ml microtube. 700 µl of chloroform with isopropanol was pipetted in to the resulting microtube. The microtube was vortexed for 10 min. followed by 10 min. centrifugation on same speeds as mentioned before. Again the upper aqueous faze was carefully pipetted into 1.5 ml eppendorf. An equivalent amount of iced isopropanol pipetted followed by manual mixing in a rotational manner. Eppendorf was left inside freezer during the night. The following day, the frozen mixture in the Eppendorf was centrifuged for 10 minutes. Then all the supernatant removed by pipetting. The formed DNA pellet washed with 500 µl of 70% ethanol and centrifuged for 10 minutes. Washing the pellet with ethanol and its centrifugation was repeated twice. The pellet was left around 20 min. to dry. As last part of DNA isolation for preparing the template for PCR assay, the pellet was dissolved in 150 µl TE buffer with pH 8 in a micro test-tube for safe DNA storage in freezer. This was performed on 10 different samples to identify *Tilletia* species. This method of genomic DNA extraction using CTAB buffer insured a high quantity and stable yield of pure isolated DNA. Zouhar et al. (2010) proved the following method to have best results in the detection of *Tilletia caries*.

Chemicals used

- 1% CTAB - cetyltrimethylammonium bromide
- β-mercaptoethanol
- Phenol
- Chloroform
- Isoamyl alcohol
- Isopropanol
- 70% Ethanol
- TE buffer (pH 8)

Preparing the PCR sample

We used conventional PCR to detect and identify the fungal species. Zouhar et al. (2010) designed and tested 10 different PCR primer sets from which the author originated the following primer pair, TillIGS2A_F 5'-TAGCGACCCGACCCGACCAG-3' and TillIGS2A_R 5'-CCCTCACGTTCCACGAGGG-3'. The primer pair enabled the detection of *Tilletia caries* and *Tilletia controversa* on a specific fragment of about 122 bp in length.

dd H_2O	Solution sum added to 25 µl	For 10+1 samples 190.85 µl
PCR buffer	2.5 µl	27.5 µl
$MgCl_2$ (25 mM)	3 µl	33 µl
dNTP	0.25 µl	2.75 µl
Primer mix	0.4 µl	4.4 µl
DNA isolate	1 µl	11 µl
Polymerase	0.5 µl	5.5 µl

Tab. 1. PCR constituents

Isolated DNA sample was removed second day from freezer, vortexed then briefly centrifuged. The PCR reaction started after inserting the micro test-tubes in to the thermo-cycler. During the PCR reaction temperature reached 95 °C for a period of 5 min. where DNA

denaturation started. After that 35x consecutive repetitions of the following: “Denaturation at 94 °C for 1 min., Annealing at 63 °C for 30 s, Polymerization at 72 °C for 45 s.” The last step was the final polymerization at 72 °C for 4 minutes. After this automated process the samples were ready for DNA molecule visualization.

Electrophoresis

Isolated genome DNA samples were put through electrophoreses. Heated 1% agarose gel was dissolved in 1 x Tris-Borate-EDTA buffer and mixed with a 1 mg/ml ethidium bromide solution. The gel mixture at the time with an approximate temperature on 60 °C was poured into electrophoreses chamber. A comb with 12 teeth was inserted to create sample wells and was left around 30 min. until the gel solidified. After which the comb was removed and the solidified gel slab was inserted into the electrophoresis tank and 1 x TBE buffer added. 1 µl dye solution to stain the samples for enhanced visibility was mixed with 5 µl of DNA sample. For each sample the mixed PCR product with loading dye were pipetted into each well in the gel. The process was carried out at 5 V. for approximately 1 hour during which the DNA moves towards the anode. When the electrophoresis was complete, the stained molecule fragments in the gel were observed under a UV trans-illuminator. An autoradiogram was obtained by picturing the stained molecules illustrating their migration along the gel.

5. Results

5.1 Species determination

Before introducing the inoculum to the seeds we used conventional PCR and specific primers for the detection of *Tilletia* spp. The species we worked with was determined to be *Tilletia caries.* In the following Electrophoregram, PCR products of amplificated DNA samples isolated from the inoculum migrated along specific *Tilletia* primers. 9 out of 10 samples were positive for *T. caries*. The negative sample occurred due an error either during isolating the DNA or during its preparation for PCR and electrophoreses. Figure 3 shows these results.

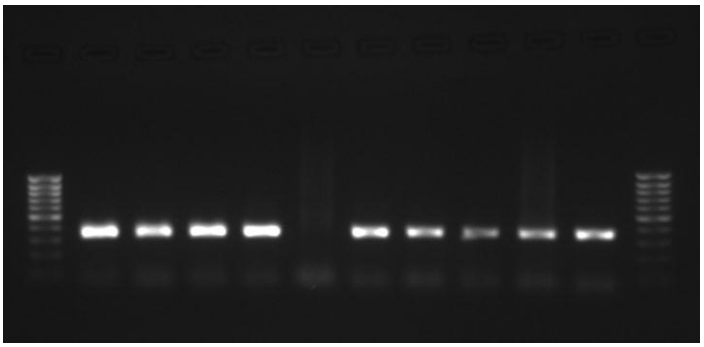
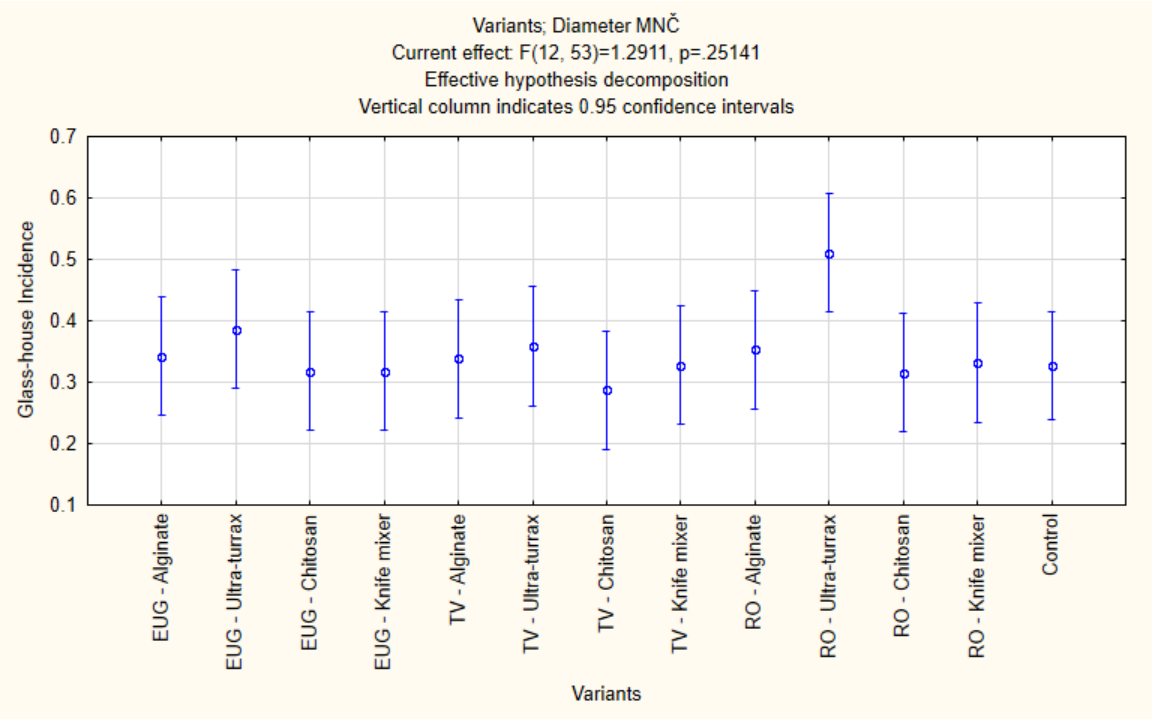


Fig. 3. Electrophoregram of PCR after DNA amplification from *Tilletia* inoculum

5.2 Effect of seed treatment on *Tilletia caries* - Container experiment

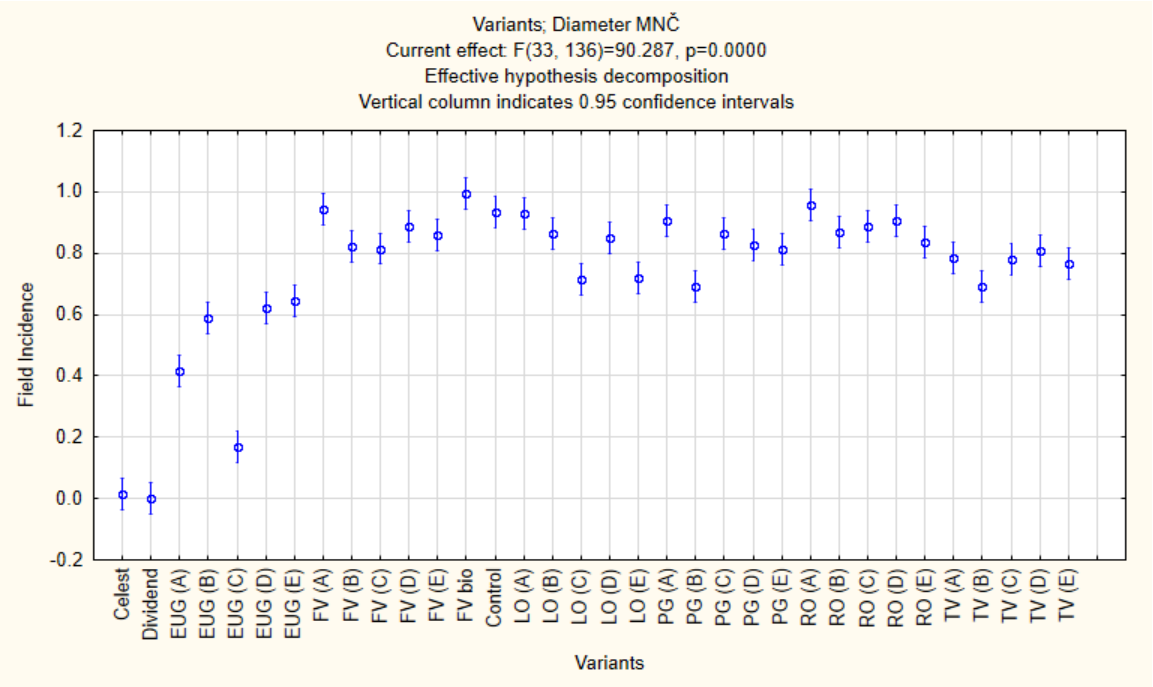
Graph 1. shows three tested essential oils in four different formulations using biopolymers like Alginate and Chitosan or by different mixing mechanisms. Essential oils in different formulations are shown on the x-axis and the incidence of the infection on the y-axis. The experiment was conducted in small containers in a controlled environment to further test the antimycotic effect of *Eugenia caryophyllata* - EUG, *Thymus vulgaris* - TV and *Origanum vulgare* - RO. Statistically significant difference is shown between *T. vulgaris* formulated by chitosan and *O. vulgare* formulated using ultra-turrax mixing mechanism.



Graph 1. Seed treatment effects of E. oils in different formulations on *T. caries* - Container experiment

5.3 Effect of seed treatment on *Tilletia caries* - Field experiment

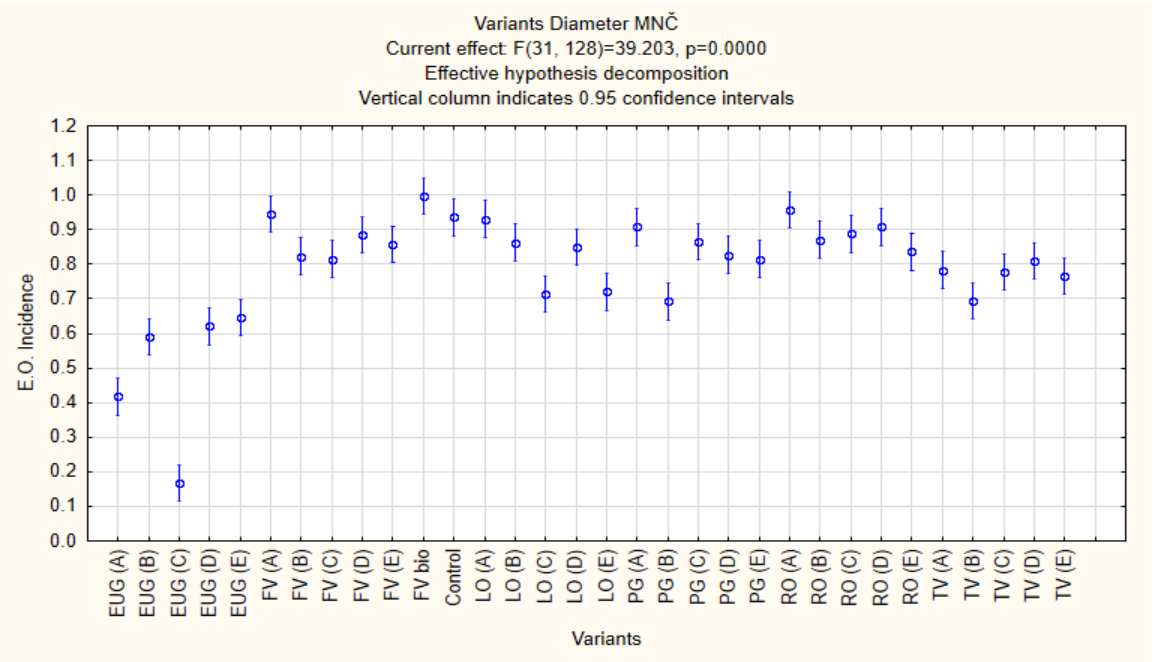
In this diploma thesis we carried out trails on the antimycotic activity of 7 essential oils against *T. caries.* The outcome of the experiment was analyzed using program STATISTICA 12. We obtained significant statistical differences between test and control variants. Essential oil extracts from *Eugenia caryophyllata* effectively inhibited spore germination of the fungus. On the other hand, extracts from *Levandula angustifolia, Pelargonium graveolens* and *Thymus vulgaris* did not display affirmative results. Finally extracts from *Rosmarinus officinalis* and *Foeniculum vulgare* respectively showed the weakest inhibitory effect.



Graph 2. Seed treatment effect of E. oils in different formulations on *T. caries* - Field experiment

Graph 2. shows the results of *Tilletia caries* infection occurrence on the y-axis and on the x-axis the tested variants where wheat was treated respectively with; two commercial fungicides (Celest, Dividend), *Eugenia caryophyllata* - EUG, *Foeniculum vulgare* - FV, control variants, *Levandula angustifolia* - LO, *Pelargonium graveolens* - PG, *Rosmarinus officinalis* - RO and *Thymus vulgaris* - TV. Each of the essential oils had 5 repetitions in different formulations (A, B, C, D and E) except *Foeniculum vulgare* which was further formulated with a mixture of bio-carriers.

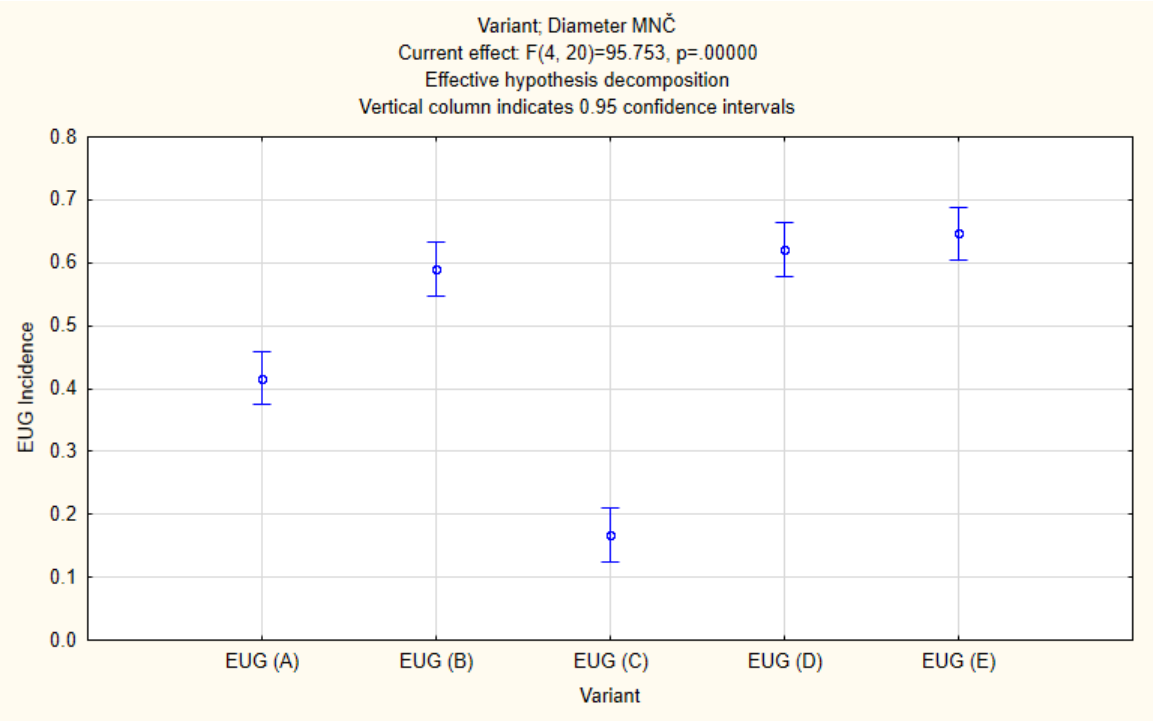
In graph 3. the incidence of the infection is shown for the essential oils and the control variants without commercial fungicides. The significant statistical difference between *Eugenia caryophyllata* and control variants is clear. *Foeniculum vulgare* formulated by bio-carriers was slightly worse than the control variant although no significant statistical difference can be observed.



Graph 3. Seed treatment with E.O. in different formulations with control variants of *Tilletia caries*

Graph 4. Shows the statistical difference of *Tilletia caries* infection occurrence in wheat treated by the essential oil of *E. caryophyllata* with different formulations. In these formulations we used several biopolymers to encapsulate the seeds with the essential oil. The exact formulation is a part of an ongoing study and repeated experimentation that will be eventually patented. These results are further shown using Tukey test in table 2. This test, also called Tukey's Honest Significant Difference test shows that the obtained results are in overall statistically significant.

Formulations A and C showed significant statistical differences from each other and from other formulations. These two formulations will be repeated in future experiments to further optimize their effect.

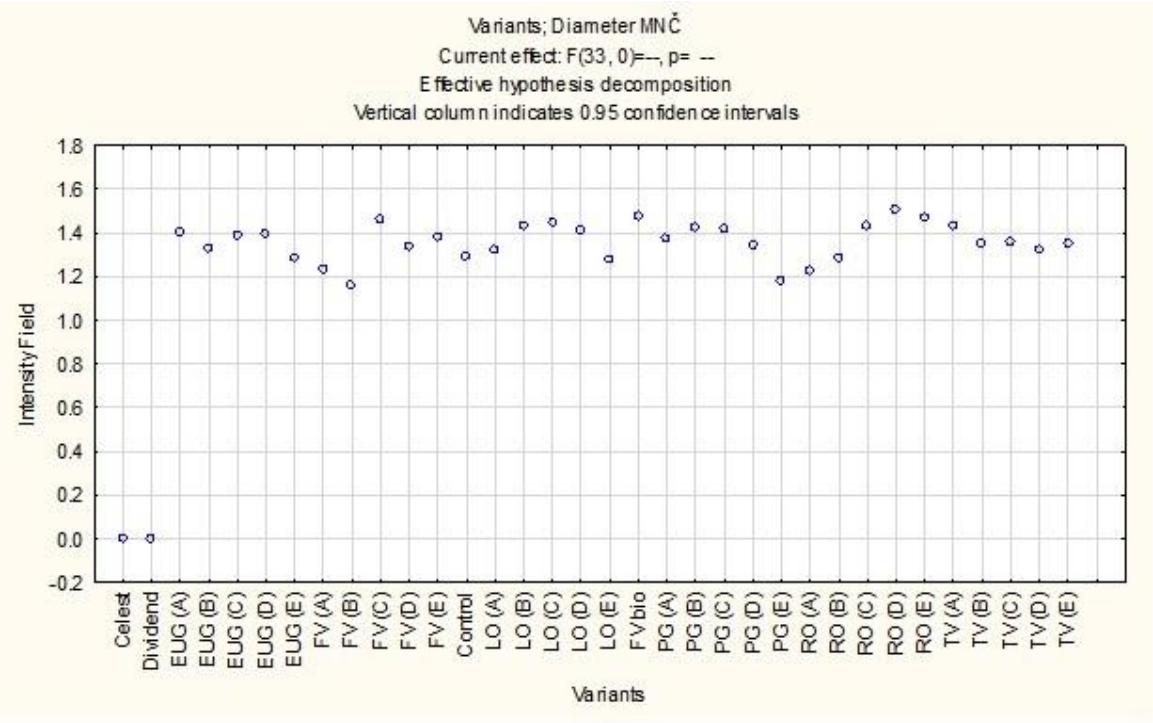


Graph 4. Seed treatment with *Eugenia caryophyllata* in 5 formulations against *Tilletia caries*

N. cells	Tukey HSD test; variable EUG Incidence (Table) Approximate probability for post hoc tests Error: between groups. PČ = .00211, sv = 20.000 Variants	{1} .41675	{2} .58997	{3} .16665	{4} .62074	{5} .64610
1	EUG (A)		0.000187	0.000132	0.000136	0.000132
2	EUG (B)	0.000187		0.000132	0.824340	0.332761
3	EUG (C)	0.000132	0.000132		0.000132	0.000132
4	EUG (D)	0.000136	0.824340	0.000132		0.903314
5	EUG (E)	0.000132	0.332761	0.000132	0.903314	

Tab. 2. Tukey test for *Eugenia caryophyllata*

Graph number 5 shows the intensity of the infection on the y-axis. All the variants which were tested on the field are shown on the x-axis. To count the intensity we randomly chose 10 infected spikes from each variant. Seeds were removed from each spike; infected seeds were separated from healthy seeds and counted. Statistically significant differences are shown between individual variants.



Graph 5. Infection intensity of the variants from the field experiment

6. Discussion

As discussed earlier, synthetic fungicides are undesirable and problematic, either on the human health or in the development of fungal resistance. As a result, efforts were focused on developing environmental friendly fungicides such as essential oils as an alternative.

Tries were carried out in the antimycotic effect of essential oil extracts from clove (*Eugenia caryophyllata*) against 53 pathogenic yeasts. The extract was isolated by hydro-distillation and its chemical composition evaluated using gas chromatography-mass spectrometry (GC – MS). Both methods are listed in the review above. *E. caryophyllata*, showed a remarkable effect against all examined strains of the pathogenic yeasts belonging to *Candida* species (Chaieb et al., 2007b).

In this diploma thesis our experiments also showed promising results of *E. caryophyllata* where it's essential oil extract inhibited the germination of *Tilletia caries* spores. Significant statistical differences between EUG treated variants and control variants demonstrate this favourable result. The variant where essential oil of *E.* caryophyllata was formulated with biopolymers and encapsulated in gelatin capsules offered best results. This variant was far closer to results that chemical fungicides achieved. The results of this experiment show promising potential of EUG oil extract against *Tilletia caries* and offer a purposeful need to carry on similar experiments in order to reach ideal patterns of essential oil formulations.

In another study, López et al. (2005) conducted an experiment on six essential oils including *E. caryophyllata* and rosmary (*Rosmarinus officinales*) to determine their fungicidal activity against *Penicillium islandicum* and *Aspergillus flavus*. *E. caryophyllata* resulted in strongest inhibition where *R. officinales* was relatively weaker in inhibiting these saprotrophic and pathogenic food-borne fungus of the *Poaceae* family. Similarly in our experiment, *R. officinales* was one of the weak variants tested in the field. This may be due to incompatibility of the formulation with the essential oil, which may have resulted in quicker evaporation of the oil. The effect of *R. officinales* would have been more functional in inhibiting spore germination and mycelia growth if the essential oil was present at the time of seed germination.

Da Silva et al. (2015) analyzed the chemical composition of *Rosmarinus officinalis* essential oil by nuclear magnetic resonance and by gas chromatography– mass spectrometry, and showed that the major compounds were 1.8 cineole (52.2%), camphor (15.2%) and α-pinene

(12.4%). This essential oil was tested on *Fusarium verticillioides,* where the mycelia growth was noticeably reduced mainly due to the 1.8 cineole component of *R. officinales* (Jalali-Heravi et al., 2011; Jiang et al., 2011). After the application of essential oil, the cell wall ruptured and cytoplasm leaked; such microscopic morphological effects are significant and show the effectiveness of rosemary extract as a fungicide. In general, the hydrophobic nature of essential oils causes leakage of cellular material and cellular components loss; and this was well documented during the disruption of the *F. verticillioides* cell wall, loss of membrane integrity, and eventually the blockage of cell growth after the application of the oil (Da Silva et al., 2015). Angioni et al. (2004) obtained the extract of *Rosmarinus officinales* using steam/hydro distillation and studied its antifungal and antimicrobial activity. The essential oil extract was also weak in this study but it showed a promising induction outcome on the fungal growth of *Fusarium graminearum*.

Kujund et al. (2003) demonstrated that most of *Foeniculum vulgare* essential oils; camphor, bifonazol and fenchone possess very strong antimycotic activity. They were highly effective against *Phomopsis helianthi, Trichophyton mentagrophytes* and *Cladosporium cladosporioide*. But it was not as effective against highly resistant species including *Penicillium funiculosum* and *Penicillium ochrochloron.* In our experiment *F. vulgare* showed no significant statistical differences from the control variants in all the formulations. Being the weakest of tested variants does not mean that the components of fennel essential oils lack antifungal effects.

According to Kujund et al. (2003) the chemical composition of essential oils showed differences in their activities, but results proved that all investigated oils accounted their antifungal activity. Furthermore the authors recommended using fennel oil in food industry for its strong activity as an antifungal against *Aspergillus* species and associated mycotoxin contamination, in addition against dermatophytes causing fungal infections of the hair, nails and skin.

In a similar experiment essential oils acquired from *Foeniculum vulgare* demonstrated antioxidant capacities, similar to some reference antioxidants; but also showed antibacterial activities against several bacterial genera including plant and food pathogens (Ruberto et al., 2000). Also, the same study denoted that *F. vulgare* presented a high and broad degree of inhibition; for example against *Clostridium perfringens, Brevibacterium linens, Staphylococcus aureus and Leuconostoc Cremoris.*

In one study on *Levandula angustifolia*, Zabka et al. (2014) demonstrated the efficiency of twenty essential oils based on the abundance of predominant active substances towards several fungus species such as *Alternaria alternata*, *Stachybotrys chartarum*, *Cladosporium cladosporioides* and *Aspergillus niger*. The essential oils tested in this study showed varying extent of efficacy in terms of achieved inhibition and overall spectrum of pathogens inhibited. Among these oils, *L. angustifolia* provided greater than 50 % inhibition for all pathogenic fungi, except for *A. niger* which is considered the most resistant. The authors also tested the effectiveness of *Origanum vulgare* and *Thymus vulgaris* which achieved up to 100% inhibition levels for all pathogenic fungi.

In the results above, statistical differences between *L. angustifolia* and the control variants are clear in 2 out of the 5 formulations used for seed treatment against *T. cries.* As for *T. vulgaris,* 4 out of 5 variants presented significant statistical differences. Both essential oils were formulated with same components and similarly encapsulated in gelatine capsules in each of the five variants. This can lead us to deduce that there is an incompatible inhibitory effect between different major compounds contained in each essential oil against the fungus *T. caries*. Nonetheless this does not present *T. vulgaris* as more effective than *L. angustifolia* in a general spectrum where each of the mentioned oils is more or less effective according to the pathogen it is used to inhibit.

The fungus species *A. niger*, required the highest minimum inhibition concentrations; thus showing high resistance to the essential oils. Meanwhile, the species *Oreganum vulgare*, and *Thymus vulgaris* recorded high antifungal efficacy, since they showed the lowest minimum inhibition concentrations (Zabka et al., 2014).

In another study, Bouzenna and Krichen (2013) showed that thymol from *T. vulgaris* essential oils remarkably inhibited mycelia development; relating this effect to the interaction with ergosterol. Over the past years, several essential oils, especially those from species of *Thymus* along with their phenolic components have been explored for their antimicrobial characteristics against different bacteria, protozoans, and fungi; yet there is still little information on the mechanisms of essential oils of *T. vulgaris* (Sienkiewicz et al., 2012; Santoro et al., 2007; Pina-Vaz et al., 2004).

Thyme oil displayed a wide fungi-toxic spectrum inhibiting mycelia growth of various food infecting fungi. It suppressed mycelia growth of *Fusarium oxysporum*, *Aspergillus flavus*,

Cladosporium herbarum, Curvularia lunata, A. fumigatus, A. niger, A. terreus, Alternaria alternata and Botryodiploidia theobromae (Kumar et al., 2008).

However, in the reported study, the essential oil of *T. vulgaris* did not completely inhibit the growth of *A. niger* and *C. herbarum*. On the other hand it is well noted that Kumar et al. (2008) recommended the use of thyme oil as a botanical preservative for controlling bio-deterioration of food products during storage.

Rota et al. (2008) studied the antimicrobial activity of different essential oils from three *Thymus* species against microorganism pathogens. The effectiveness results where variable between the oils denoting the highest for *T. hyemalis* followed by *T. zygis* and *T. vulgaris*; but all of the aforementioned species demonstrated strong and similar growth inhibition activity.

Bouzenna and Krichen (2013) identified the chemical composition of the essential oil from *Pelargonium graveolens*, and among its 15 compounds, citronellol (35%) and geraniol (28.8%) dominated the major fraction; these in turn are oxygenated monoterpenes. In addition, *P. graveolens* exhibited insecticidal effects against adult instars of *Rhysopertha dominica* and antifungal activity against *Rhizoctonia Solani*.

In our results *P. graveolens* showed significant statistical differences in one formulation when compared with the control variants. Such a result shows that this essential oil could establish better results in enhanced formulations. For example further experiments could be conducted with several concentrations to further test its antifungal effects against *Tilletia caries*. The prospective activity of essential oil extracts from geranium can include a wider pathogen spectrum.

Nematicidal properties of *Pelargonium graveolens* L. haven't been yet reported; meanwhile in the presented study analysis of its major components showed the presence of citronellol 41.3%, geraniol 9.9 % and linalool 12.7 % mainly. These components were responsible for 100 % nematode mortality at 2000 µl/L of essential oil; nevertheless essential oils to be used against nematodes should have all of the mentioned constituents combined, elsewise a reduction to 85 % mortality will occur (Leela et al., 1992).

Viuda-Martos et al. (2007) determined the antifungal potential related to essential oils from oregano (*Origanum vulgare*), clove (*Eugenia caryophyllata*), and thyme (*Thymus vulgaris*); two food spoilage moulds where selected, *Aspergillus flavus* and *Aspergillus niger*. Although all

analysed essential oils presented inhibitory effects, oregano exhibited the highest efficacy for inhibiting mould growth, followed by clove and thyme. These antifungal agents are highly suitable because of their natural origin, broad antifungal spectrum, and extremely low risk that a resistance will develop by the pathogens towards the mixture of components in the oils. Since the antifungal mixture provides a diversity of mechanisms; all these together make essential oils from these species suitable for consumers.

In our glass-house experiment we tested essential oil extracts from *Origanum vulgare* for its antifungal prospective. This essential oil proved to be most effective in inhibiting spore germination of *Tilletia caries*. Based on this result further study of its antimycotic activity against *T. caries* and other *Tilletia* spp. could be beneficial.

In another study, essential oils of oregano (*Origanum syriacum*) and fennel (*Foeniculum vulgare*) were demonstrated to be used as alternatives to synthetic fungicides in soil amendment; their antifungal effects were tested against *Sclerotinia sclerotiorum* (Soylu et al., 2007). The inhibition effects of essential oils were pronounced on the morphological structures of sclerotia and hyphae and significantly reduced sclerotial viability. Soylu et al. (2007) concluded that although fennel was more inhibitory than oregano oil to *S. sclerotiorum*, both can be used as bio fungicides and enhance crops production rates

Kordali et al. (2008) was able to validate the effectiveness of oils from *Origanum acutidens* as a fungicide, insecticide and herbicide; the study was able to quantify the major aromatic monoterpene constituents of which carvacrol was the most important.

Carvacrol and thymol inhibited mycotic growth of seventeen tested phytopathogenic fungi, and showed higher antifungal effects than benomyl, a commercial fungicide. Carvacrol and thymol completely suppressed seed germination and growth of *Chenopodium album*, *Rumex crispus*, and *Amaranthus retroflexus*; their effect exceeded that of the commercial herbicide, 2,4-D, isooctyl ester. Kordali et al. (2008) highlighted the importance of using alternative biodegradable herbicides, pesticides, and insecticides; as the oils used showed efficient toxicity on two pests, *S. granarius* and *T. confusum.*

7. Conclusion

Essential oil extracts have promising antifungal activity against *Tilletia caries* spore germination. We observed such inhibitory effects between tested essential oil formulations. In some cases, these formulations were compatible with the essential oil and enabled its persistence on the treated seeds. *Eugenia caryophyllata* showed significant statistical differences when compared with control variants. *E. caryophyllata* has been proved to be effective against other pathogens as well as mentioned above. We conclude that this experiment could be further studied and optimized to reach more satisfactory results with other essential oils against the fungus *Tilletia caries* respectively against *Tilletia* spp.

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9. List of figures Fig.

10. List of tables

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12. Appendix

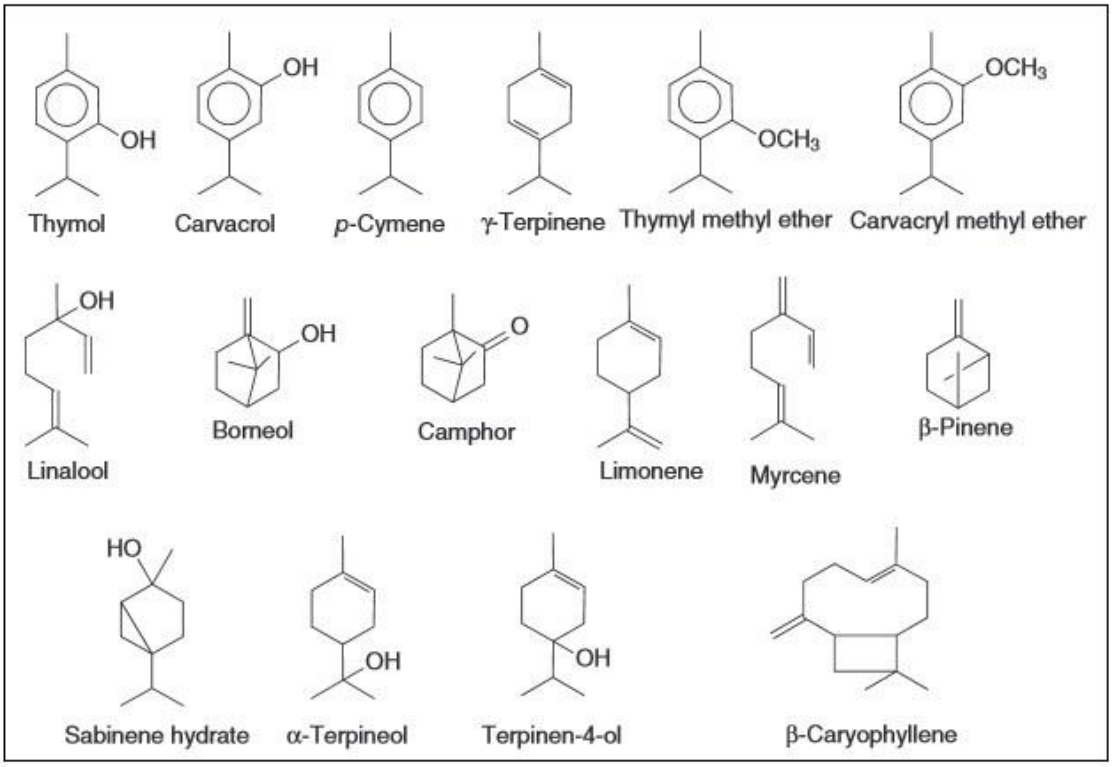

Fig. 4. Terpenes in essential oil (Stahl-Biskup and Venskutonis 2012).

Fig. 5. Essential oils formulated with biopolymers.

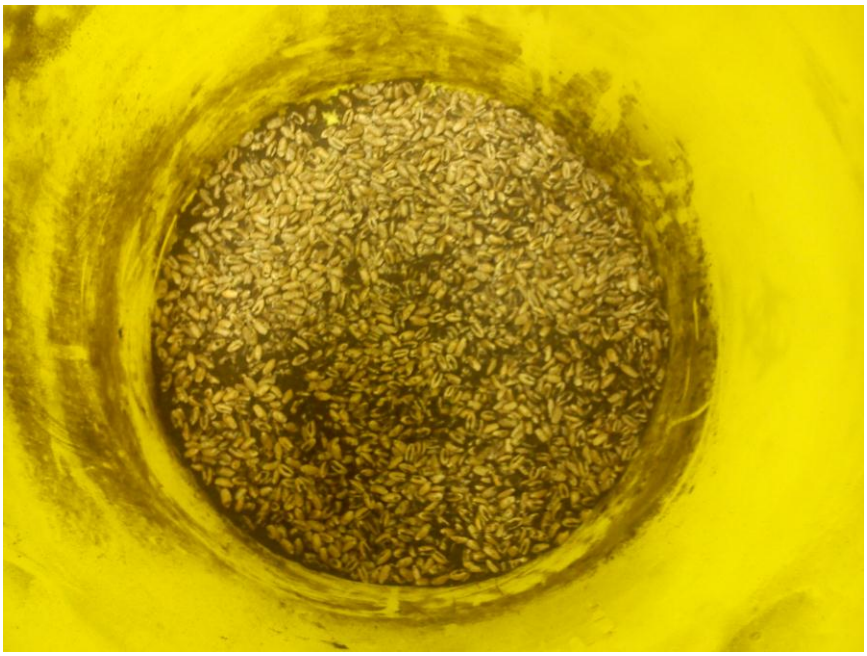

Fig. 6. Mixing wheat seeds with *Tilletia caries* spores.

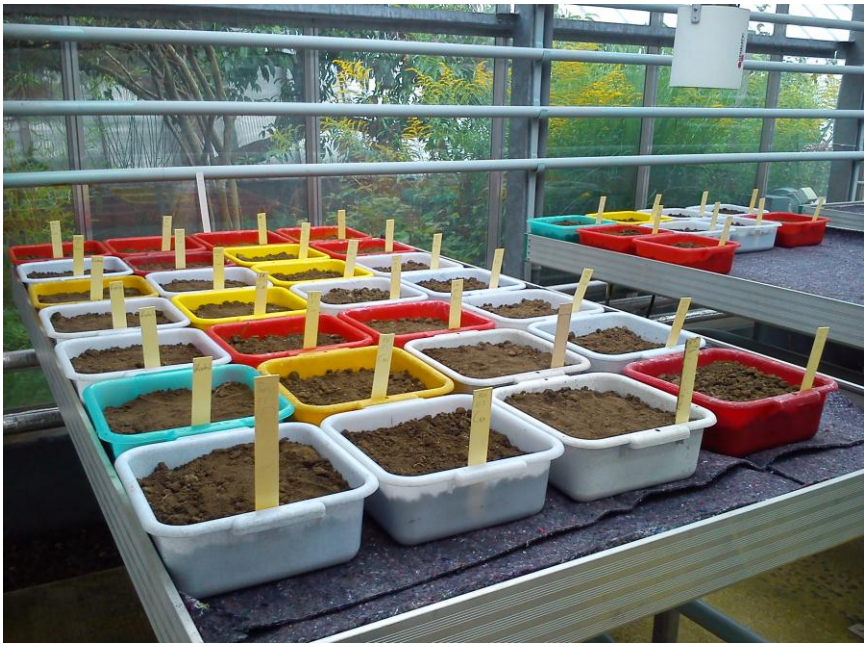

Fig. 7. Glass house experiment after sowing.

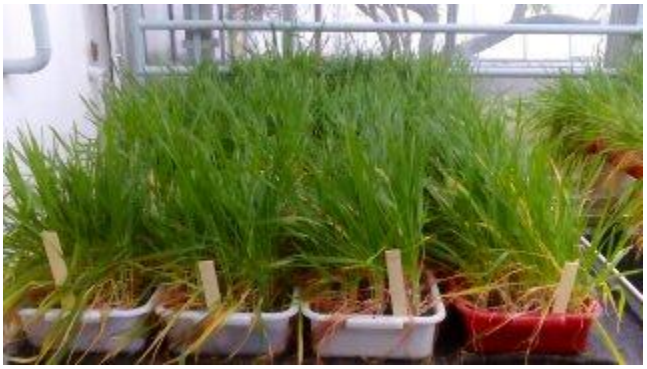

Fig. 8. Start of wheat stem elongation phase in containers.

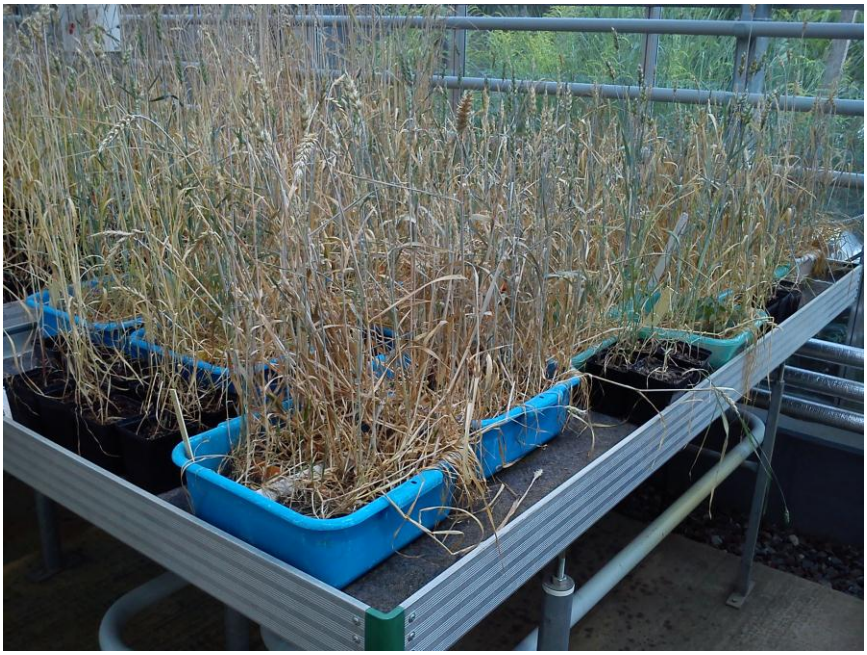

Fig. 9. Ripening phase of wheat in container experiment.

Fig. 10. Infected ear in the container experiment.

Fig. 11. Sowing the field experiment.

Fig. 12. Preparing the field after sowing.

Fig. 13. Ripened wheat spikes in the field.

Fig. 14. Harvesting the field experiment.

Fig. 15. Five parts of one variant ready for separation and counting.

Fig. 16. Separated infected spikes on the left and healthy spikes on the right ready for counting.

Fig. 17. Upper healthy spike in comparison with bottom infected spike.

Fig. 18. Seeds of an infected spike removed, separated and counted.

Fig. 19. Healthy seeds.

Fig. 20. Infected seeds.

Fig. 21. Powdery mass of fungal spores released from infected bunts.

Fig. 22. Different colours, sizes and shapes of infected bunts.