# Allelopathic compounds from Mint target the cytoskeleton from cell biology towards application as bioherbicides

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

von der KIT-Fakultät für Chemie und Biowissenschaften

des Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

von

Mohammed Mahmood Sarheed

aus

Baghdad, Iraq

Dekan: Prof. Dr. Reinhard Fischer

Referent: Prof. Dr. Peter Nick

Korreferent: Prof. Dr. Ute Schepers

Tag der mündlichen Prüfung: 17.10.2019

Die vorliegende Dissertation wurde am Botanischen Institut des Karlsruher Instituts für Technologie (KIT), Abteilung für molekulare Zellbiologie, im Zeitraum von Oktober 2015 bis Oktober 2019 angefertigt

## Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

Alle Stellen, die gemäß Wortlaut oder Inhalt aus anderen Arbeiten entnommen sind, wurden durch Angabe der Quelle als Entlehnungen kenntlich gemacht.

Diese Dissertation liegt in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vor.

Zudem erkläre ich, dass ich mich beim Anfertigen dieser Arbeit an die Regeln zur Sicherung guter wissenschaftlicher Praxis des KIT gehalten habe, einschließlich der Abgabe und Archivierung der Primärdaten, und dass die elektronische Version mit der schriftlichen übereinstimmt.

Karlsruhe, Oktober 2019

_____

Mohammed Mahmood Sarheed

## Acknowledgement

At the outset I would like to thank Prof Dr. Peter Nick for accepting me as his student and allowing me to work under his supervision. I profoundly thank him for all the patience, help and support he showered on me during the course of my studies. Without his academic as well as personal support I would not have been able to continue my studies.

I would like to kindly acknowledge Prof Dr. Ute Schepers for allowing me to work in her laboratory where I could get hands on mammalian cells.

My grates gratitude belongs to Dr. Vaidurya Sahi for helping me in ways to express my thesis and to find the best possible ways of experimental approaches

Special thanks to Dr. Beatrix Zaban who initiated me into the lab techniques and helped me to start my experiment.

Thanks to my colleagues Herr Ernst Heene, Prof Dr. Andrzej Kazmierczak University Lodz- Poland, Prof Dr. Marie Laure Fauconnier University Gembloux Belgium, Prof Dr. Sali Okur, Dr. Thomas Horn, Dr. Norbert Krauss, Fatemah, Sascha, Kevin Braun, Kai, Marc Thieme, Anna Meschkov, Vanessa Rzeznik, Donya, who have been important in the course of my studies. Their help has been important for the completion of my thesis.

I would like to place on record my sincere thanks to Dr. Reimann, Dr. Gabriele Jürges, Frau Sabine Purper and Frau Herberger for organising everything in lab and gardeners of our institute who helped me always in growing my plants.

I am grateful to KHYS for giving me the opportunity through fellowship of 4 months, to collaborate with FiBL and work there for my experiments.

I take special opportunity to thank my colleagues from FiBL Dr. Lucius Tamm, Dr. Qiyan Wang-Mullar, Mr. Hans-Jakob Schärer, Ms.Claudia Daniel.

It gives me immense pleasure to say that my stay in laboratory would not have been so nice if I did not have friends like Kinfemichael, Dr.Vaidurya Sahi, Dr.Ali Jarrar, Afaf, Islam, Eman, Hamama, Daniela, Pallavi, Preshobha, Ruipu, Peijie, Christian, Madeliene, Nasim.

I also would like to thank the Ministry of higher education and scientific research, Iraq for funding my PhD studies in Germany.

Last but not the least I would like to thank my parents, my lovely nephew Bilal, my family members and friends for having trust and patience with me. Their support and love helped me to sail through my studies.

# Table of Contents

Acknowledgement ..... IV
Abbreviations ..... VIII
Zusammenfassung ..... IX
Abstract ..... XI
1 Introduction ..... 1
1.1 Allelopathy: The battle between the plants ..... 1
1.2 Secondary metabolites in plants ..... 4
1.2.1 Monoterpene ..... 5
1.3 Mint Family ..... 7
1.4 Importance of mint species ..... 9
1.5 Effect of allelochemicals on plant growth ..... 14
1.6 Scope of this study ..... 16
2 Material and Methods ..... 19
2.1 Plant Material and Cultivation Conditions ..... 19
2.2 Rice Cultivation (for effect of menthone/isomenthone on MTs) ..... 21
2.3 *Arabidopsis thaliana* Cultivation ..... 22
2.4 Cell Culture ..... 22
2.5 Growth of HeLa cells and MTT Assay ..... 23
2.6 Phylogenetic Analysis ..... 24
2.7 Oil Extraction ..... 26
2.8 GC-MS Analysis ..... 27
2.9 Live-Cell Imaging ..... 27
2.10 Cell Mortality Assay ..... 28
2.11 Cell death estimation ..... 30
2.12 Microtubule Visualisation in Rice ..... 31
2.13 Visualisation of the Cytoskeleton in *Arabidopsis thaliana* ..... 32
2.14 Allelopathy studies ..... 33
2.15 Germination test Assay ..... 34
2.16 Influence of formulated *Mentha* oils on growth of *Convolvulus* ..... 36
2.17 Testing mint substances on apple and grape seedlings ..... 37

2.18 Germination of different seeds on the Field Soil mix with standard soil and chopped *Agastache rugosa* ..... 38
2.19 Hoechst 3342 staining of HeLa cells ..... 38
2.20 Statistical Analysis ..... 39
3 Results ..... 40
3.1 Determination of taxonomic identity ..... 40
3.2 Allelopathy effects on plant growth ..... 43
3.2.1 Screening of Germination Inhibitors ..... 43
3.2.2 Effects on Rice ..... 48
3.3 Cell Mortality ..... 51
3.3.1 Cell Mortality Assay (Menthone / Isomenthone are Cytotoxic, Depending on Microtubules) ..... 51
3.3.2 Estimation of Mortality by Cellular staining ..... 53
3.4 Effect on the cytoskeleton: ..... 62
3.4.1 BY2: Microtubules: ..... 62
3.4.2 BY2: Actin filaments: ..... 68
3.4.3 Effects on *Arabidopsis thaliana* cytoskeleton ..... 74
3.4.4 Microtubule disruption in rice ..... 76
3.5 Efficiency of compounds and plant oils as bioherbicides ..... 78
3.5.1 Effects on germination ..... 78
3.5.2 Effects on growth of *convolvulus* ..... 80
3.6 *Agastache rugosa* shoot as potential weed control ..... 83
3.7 Test of compounds as spray ..... 83
3.8 Screening of compounds for effects on viability of HeLa Cells ..... 85
4 Discussion ..... 87
4.1 Determination of taxonomic identity ..... 89
4.2 Effect of *Mentha* related oils and compounds ..... 90
4.2.1 Effect on Germination ..... 90
4.2.2 Cell death estimation ..... 91
4.2.3 Effect on the cytoskeleton ..... 93
4.3 Trial as bioherbicide (Towards application) ..... 95
4.4 Scope for cancer treatment (HeLa cells) ..... 97
4.5 Conclusion ..... 98
4.6 Outlook ..... 99
5 Appendix ..... 101
6 References ..... 115

## Abbreviations

| | |
|---|---|
| **AtTuB6** | *Arabidopsis thaliana* β-tubulin 6 |
| **FABD2** | Actin binding domain 2 |
| **MTs** | Microtubules |
| **AF** | actin filaments |
| **AO** | Acridine Orange |
| **EB** | Ethidium Bromide |
| **RFI** | Resultant fluorescence intensity |
| **TTL (OX)** | Tubulinyl-tyrosine ligase (overexpression) |
| **DMEM** | Dulbecco's Modified Eagle Medium |
| **TBS** | Tris Buffered Saline |
| **WiWa** | Winter Wheat |

## Zusammenfassung

Pflanzen entziehen sich auf natürliche Weise dem gegenseitigen Wettbewerb, indem sie andere Pflanzen durch Allelopathie unterdrücken; ein Effekt, welcher das Wachstum und die Entwicklung anderer Pflanzen beeinflusst. Frühere Untersuchungen zeigen eine starke Induktion des programmierten Zelltods (die pflanzliche Version der Apoptose) in Zellen, die mit Ölen aus Pflanzen der Gattung *Mentha* und nah verwandter Arten der Minzfamilie behandelt wurden. In der vorliegenden Studie wurde ein vergleichender Ansatz verwendet, bei dem allelopathische Effekte von ätherischen Ölen verschiedener Minzen (aus der Gattung *Mentha*, aber auch Katzenminze (*Nepeta cataria*) und Koreanische Minze (*Agastache rugosa*) mit dem Standard-Kressekeimungstest als erste Auslese getestet wurden. Durch die Verwendung von Paaren eng verwandter allelopathisch aktiver / inaktiver *Mentha*-Arten wurden zwei Kandidatenverbindungen gefunden: β-Pinen und menthon/isomenthon. Der programmierte Zelltod wird über zwei verschiedene Wege induziert: β-pinen aktiviert den programmierten Zelltod primär über Aktinfilamente, wohingegen das Ziel von menthon/isomenthon die Mikrotubuli darstellen. In der Studie wurden die Auswirkungen der verschiedenen Monoterpene und der extrahierten Öle auf BY2-Zelllinien, BY2:GF11, BY2:TUB6 und BY2 getestet. Basierend auf den beobachteten Bioaktivitäten der verschiedenen Verbindungen zeigte sich Menthon als Hauptkandidat für die allelopathische Wirkung. Um die Wirkungsweise zu verstehen, wurde der Effekt von Menthon in einer transgenen Tabak BY2-Zelllinie untersucht, die den Mikrotubuli-Marker AtTuB6 (fusioniert mit GFP) exprimiert. Hier wurde gezeigt, dass menthon/isomenthon Mikrotubuli zerstörte und Mortalität in den Zelllinien induzierte. Diese Mortalität wurde in einer anderen Tubulin-Markerlinie erhöht, in der die Mikrotubuli im Vergleich zum nicht transformierten Wildtyp leicht stabilisiert ist. Die störende Wirkung von menthon/isomenthon auf die Mikrotubuli konnte auch bei Reiswurzeln und *Arabidopsis*-Kotyledonen bestätigt werden. Diese Ergebnisse ebnen den Weg für den Einsatz neuartiger Bioherbizide, deren Wirkung spezifisch aktiv für

eine bestimmte Art ist, beispielsweise würden Unkräuter bei entsprechenden Signalen Selbstmord begehen, während Nutzpflanzen das Signal ignorieren.

Um die Wirksamkeit der Hypothese zu testen, wurde die Auswirkungen der Öle auf verschiedene Pflanzenarten untersucht. *Mentha longifolia* und ihre Verbindungen (menthon/isomenthon, Linalool) haben eine allelopathische Wirkung auf die Keimung in Kresse, *Phacelia tanacetifolia*, Winterweizen und Tomate. *Rumex obtusifolia* keimt bei Anwendung von Linalool, stirbt aber im späteren Verlauf der Entwicklung, während menthon/isomenthon eine sehr starke Hemmwirkung bei der Keimung von *Rumex obtusifolia* Samen hat. Es wurde ferner festgestellt, dass Öle, die aus *Mentha spicata* var. *crispa* und *Mentha longifolia* extrahiert wurden, das Wachstum von Pflanzen aus der Familie der Windengewächse (Convolvulaceae) stören, während *Agastache rugosa* sie stimuliert und damit die Rolle des Aktins im Prozess nahelegt. Die Studie zeigt weiterhin, dass das Sprühen von α-pinen und β-pinen das Wachstum von Apfel- und Weinpflanzen stimuliert. Die Auswirkungen von *Mentha*-ähnlichen Verbindungen auf Mikrotubuli deuten auf eine mögliche Rolle im Zellzyklus hin. Ein weiteres Anwendungsgebiet wäre ein therapeutischer Einsatz bei Krebs, zu diesem Zweck wurde der zuvor gezeigte Effekt in HeLa-Zelllinien getestet, um auch hier das Wirkungspotenzial nachzuweisen. Diese Ergebnisse zeigen, dass menthone das Wachstum von HeLa-Zellen hemmt, wodurch *Mentha*-bezogenen Verbindungen eine mögliche Rolle bei einer zukünftigen Krebsbehandlung spielen könnten.

## Abstract

Plants naturally evade competition by suppressing other plants through allelopathy which effect the growth and development of other plants. Preliminary studies showed a strong induction of programmed cell death (plant version of apoptosis) in cells treated with oils extracted from some species of *Mentha* and closely related *Mentha* plants. In the study comparative approach was used, where allelopathic effects of essential oils from different mints (including the genus *Mentha*, but also Cat Mint (*Nepeta cataria*), and Corean Mint (*Agastache rugosa*), belonging to sisters' clades within the subfamily *Nepetoideae*) using a cress germination assay as readout were tested. By using pairs of closely related active / non-active (in terms of allelopathy) *Mentha species*, two candidate compounds which suppress germination and growth: β-pinene and menthone/isomenthone were found. β-pinene activates programmed cell death using actin filaments as the prime target while in case of menthone/isomenthone the target are microtubules. In this study the effects of different compounds and the extracted oils on BY2 cell lines were tested on BY2:GF11, BY2:TuB6 and BY2. Based on the observed bioactivities of the different compounds menthone/isomenthone was found as prime candidate for the allelopathic effect and this was confirmed experimentally. In order to understand the mode of action, the effect of menthone/isomenthone in a transgenic tobacco BY2 cell line expressing the microtubule marker AtTuB6 (fused with GFP) was observed. The study showed that menthone/isomenthone, disrupted microtubules and induced mortality. This mortality was elevated in the tubulin marker line, where microtubules are mildly stabilized, as compared to the non-transformed wild type. The disruptive effect of menthone/isomenthone on microtubule could also be confirmed in rice roots and *Arabidopsis* cotyledons. The study paves the way for the use of novel bioherbicides that would be environmentally friendly. The idea is to develop a bio-herbicide, because the effect is specific for the target species example: broadleaf weeds are committing suicide, cereals ignore the signal. In order to test the efficacy of the hypothesis the effects of the oils on different plant species were studied.

*Mentha longifolia* and its compounds (menthone/isomenthone, Linalool) have an allelopathic effect on seed germination in *Cress, Phacelia tanacetifolia, Triticum aestivum* L. *var* winter wheat and *Solanum lycopersicum*. *Rumex obtusifolia* germinates on application of Linalool but dies at a later developmental stage, while menthone/isomenthone has a very strong inhibition effect on seed germination of *Rumex obtusifolia*. It was further found that oils extracted from *Mentha spicata crispa* and *Mentha longifolia* disrupts growth of convolvulus while *Agastache rugosa* stimulate it thereby suggesting the role of actin in the process because of the role of actin filament in regeneration. The study shows that spraying α-pinene and β-pinene stimulates the growth in apple and grapevine plants.

The effects of *Mentha* related compounds on microtubules suggest a possible role they might have in the cell cycle. In order to exploit this potential mode of action and see if the *Mentha* compounds could be used for therapeutic use in cancer, we tested the effects in HeLa cell lines. the results show that menthone inhibits the growth of HeLa cells. This paves the way for further studies that could emphasise the possible role these *Mentha* related compounds might have in cancer treatment.

# 1 Introduction

## 1.1 Allelopathy: The battle between the plants

Competition and cooperation go hand in hand to take forward evolution. Nature has provided to living beings means and mechanisms to survive, and a key to survival are the mechanisms an organism uses to make sure that it has enough resources. While animals mainly use movement as strategy to compete for resources, plants differ quite evidently, in this respect. Plants have developed subtle mechanisms to defend and adapt themselves against biotic and abiotic stress factors. As part of this defense, plants can suppress the growth of competing neighbors by releasing chemicals, a process called allelopathy (Lambers *et al*., 1998). Allelopathy has been known among plants since time immemorial and it has even been utilized by farmers for weed control. The molecular base of allelopathy is highly diffusible, often even volatile compounds, so called allelochemicals (**Fig. 1.1**).

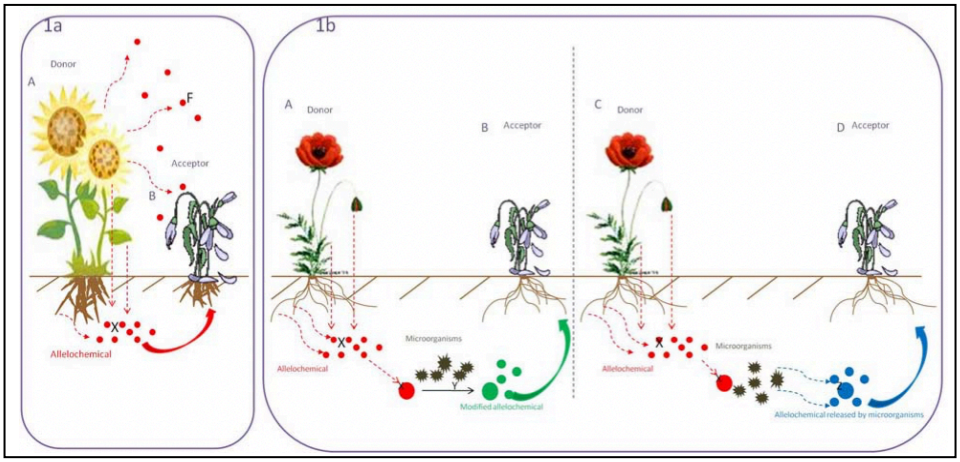



**Figure 1.1:** Plant Allelopathy. (a) Direct allelopathy: A plant releases allelochemicals which suppress the growth of other plants, (b) Indirect allelopathy: A plant releases precursors of allelochemicals which are either modified or activated by microorganisms to yield active allelochemicals which in turn affect the growth of other plants, or the allelochemical produced by the plant triggers the synthesis of allelochemicals in microorganisms, which in turn affect growth of competitors, such as weeds. The figure was taken from Dorota Soltys - Kalina *et al.,* 2013**.**

Allelochemicals can be produced in different organs and can suppress the growth of target plants by different mechanisms upon contact (and often accumulation) of the allelochemicals with the target plants (Choesin and Boerner, 1991). Plant essential oils, often with monoterpenes as primary components accumulate in different vegetative organs such as leaves, bark, wood, roots, but also in flowers or fruits, sometimes in specialized glands, sometimes in lysogenic or schizogenic oil ducts (Bruneton, 1995). In order to exert their effect, they have to be released into the environment, which is the reason, why many allelochemicals are volatiles. This leads to an interesting question that has been rarely addressed: how does the source plant prevent that it is not inhibited by its own allelochemicals? One possibility to obtain specificity would be to use compounds that activate a signaling process in the receiving plant that is not activated in the donor plant itself, due to inactivated binding of the compound.

Since allelopathy is specific, it offers interesting applications for biological pest control. In fact, plants producing essential oils have been used extensively for growth regulatorion, or herbicidal or insecticidal and other agricultural applications (Bajalan *et al*. 2013; Skrzypek *et al*. 2015; Cheng & Cheng 2015). Biocontrol by essential oils have a long history – for instance, the first food preservatives used by humans (Thompson, 1989), and already crude extracts have been found to exert efficient antifungal, antimicrobial, cytostatic and insecticidal activities (Sivropoulu *et al*., 1995). In some cases, the bioactivity has already been broken down to individual compounds. For instance, oil from *Artemisia scoparia* can inhibit germination and seedling growth of three weeds, and this allelopathic effect could be attributed to its major constituent,

β-myrcene (Singh *et al*., 2009). Components from *Eucalyptus* essential oil, such as eucalyptol, α- and β-pinene, camphene and camphor were reported to inhibit cell proliferation in roots o by interfering with organellar and nuclear DNA synthesis within the root meristem of *Brassica campestris* (Nishida *et al*., 2005). Similarly, citral was found to be a strong inhibitor of seed germination in wheat, *Amaranthus palmeri* and *Brassica nigra* (Dudai *et al*., 1999).

However, in many cases it has remained unclear, which compound or which mixture of compounds is responsible for the allelopathic effect of a given essential oil, such that the specificity of allelopathy still has remained fairly elusive.

One strategy to address the specificity of bioactive compounds is to compare related species that differ in their allelopathic effect. So called aromatic plants represent an interesting target, and are known to be emit volatile growth inhibitors (Muller *et al*. 1964; Weir *et al*., 2004). Among the aromatic plants, the Lamiaceae are of particular interest, because this family which typically produces a broad repertory of terpenoids and phenolic compounds (Wink, 2003) is extremely diverse with estimated 7000 species that often differ in their chemical characteristics and are grouped into several hundreds of genera (Harley *et al*., 2004). The pronounced chemical diversity in this group indicates that chemical signaling directed to other organisms has been an important driving force in speciation, such that there is a good chance to identify specific allelochemicals.

The differences of oil composition in these closely related species are linked with different bioactivities that are often of medical interest: The oil of spearmint (*Mentha*

*spicata* L.) is a natural antioxidant (Kanatt *et al*., 2007), while an extract from Korean Mint (*Agastache rugosa*), another member of the Lamiaceae family, shows inhibitory activity against HIV integrase, a protein responsible for integrating the viral DNA into the human genome, and therefore relevant as a possible target for selective antiviral therapy (Kim *et al*., 1999). Likewise, essential oils extracted from the mint species *Mentha pulegium* and *Mentha spicata*, containing mainly pulegone, menthone, and carvone, showed strong insecticidal activity on *Drosophila melanogaster*, whereby the oil of *Mentha spicata* was in addition mutagenic. Here, the most effective insecticidal compound was pulegone, while the genotoxic activity could be attributed to menthone. Interestingly, the strong toxicity of pulegone was suppressed in the presence of menthone, demonstrating that synergistic or antagonistic interactions between compounds of a given oil are relevant for bioactivity (Franzios *et al*., 1997). Allelopathy is attributed to two major chemical groups, plant phenolics which come out of the shikimate pathway and terpenoids which are products of the mevalonate pathway (Inderjit *et al*.,1995, 1999b; Inderjit and Mallik 2002).

## 1.2 Secondary metabolites in plants

Secondary metabolites are present in all higher plants, usually in high structural diversity and they are formed by complex interwoven pathways (**Fig. 1. 2**). In higher plants, there are two pathways for the formation of C5 terpenoid monomers, isopentenyl diphosphate: (i) the glyceraldehyde-3-phosphate/pyruvate pathway in the plastids, and (ii) the cytoplasmic acetate/mevalonate pathway (Lichtenthaler *et al*.,

1997). Biosynthesis of several mono-, sesqui- and diterpenes is regulated at the transcriptional level (Lois and West 1990 ; Facchini and Chap).

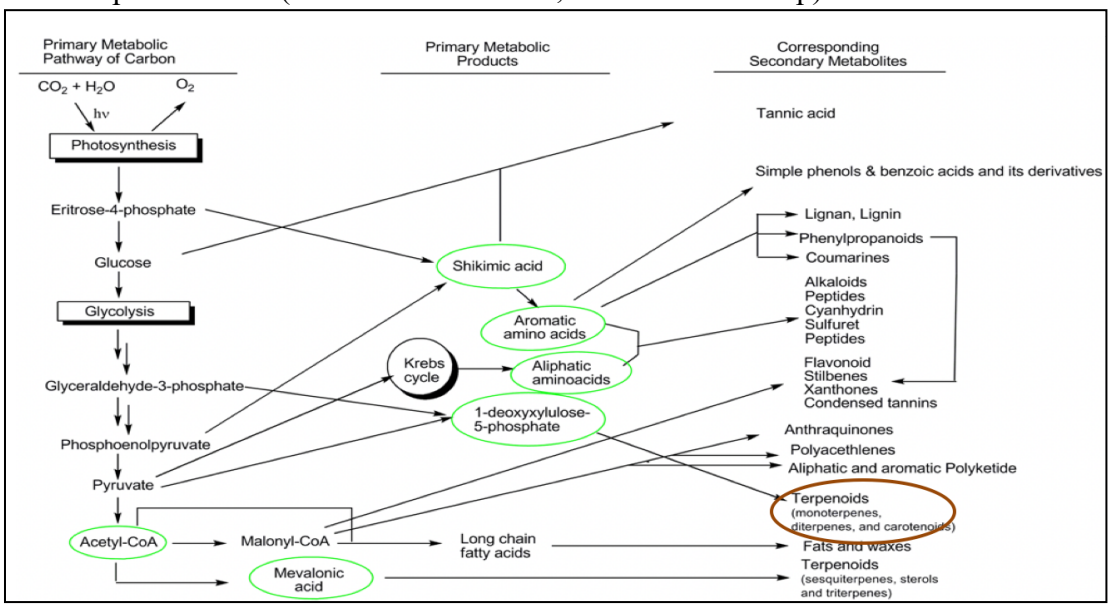



**Figure 1.2:** Interrelationship of the major biosynthetic pathways of secondary metabolites produced by plants. Monoterpenes are shown to be produced from the 1-deoxyxylulose 5-phosphate marked by red circle. Most of the volatile terpenoids produced come from this pathway. The figure was taken from Marcelo Pedrosa Gomes *et al*., 2017.

### 1.2.1 Monoterpene

Monoterpene biosynthesis and accumulation in mint is specifically localized to the glandular trichomes (Gershenzon *et al*., 1989; McCaskill *et al*., 1992), and the pathway originates in the plastids (leucoplasts) of the secretory cells of these highly specialized, nonphotosynthetic glandular structures (Turner *et al*., 2000). The monoterpene family of natural products therefore is derived from the plastidial, mevalonate-independent pathway for isoprenoid metabolism (Eisenreich *et al*., 1997; Sagner *et al*., 1998), which provides isopentenyl diphosphate (and, by isomerization, dimethylallyl diphosphate) as the universal precursors of the terpenoids (Lichtenthaler *et al*., 1997; Eisenreich *et al*., 1998; McCaskill and Croteau, 1999).

Many metabolites have been found to protect plants against viruses, bacteria, fungi, and most importantly against herbivores. Many secondary metabolites such as cyanogenic glycosides, glucosinolates, terpenes, saponins, tannins, anthraquinones, and polyacetylenes also act as allelochemicals, influencing the growth and development of neighbouring plants (Wink, 2003). For example, monoterpene limonene has shown deterrent and insecticide properties and carvone is used as sprouting inhibitors (Ibrahim *et al*., 2001, Aflatuni 2003). Essential oils are complex and highly variable mixtures of constituents that belong to two groups: terpenoids and aromatic compounds. Hydrocarbons are almost always present in monoterpenes (Bruneton, 1995). Essential oils accumulate in all types of vegetative organs: flowers (bergamot tree), leaves (mint, eucalyptus), barks (cinnamon), woods (sandalwood), roots (vetiver), rhizomes (ginger), fruits (anise), and seeds (nutmeg). Essential oils are usually associated with specialized storage in plants (Bruneton, 1995). Although essential oils are comprised of many types of compounds, the major ones are monoterpenes (Seigler, 1998). The synthesis and accumulation of essential oil structures are located near the surface, glandular trichomes (**Fig. 1.3**), secretory cavities or secretory canals of the plants (Bruneton ,1995).

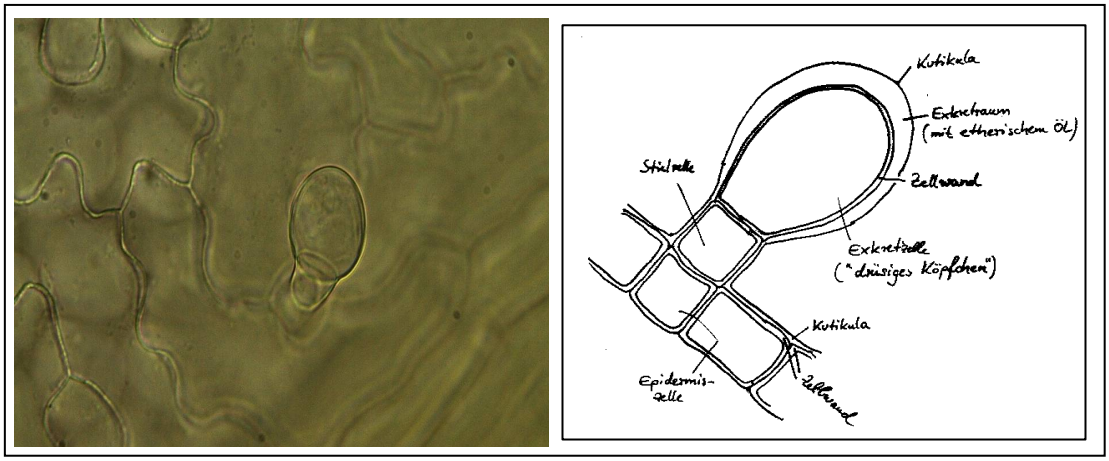



**Figure 1.3:** Microscopical image of a glandular hair of *Mentha x piperita* and a schematic drawing (A. Häser, B.Zaban) showing the different parts of the glandular hair and its neighboring cells.

The impact of environmental factors such as temperature, relative humidity, irradiance, photoperiod and cultivation practices influence the composition of essential oils. The influence of the method of extraction on oil composition and the labiality of the constituents of essential oil explains why the composition of the product obtained by steam distillation is most often different from that which is initially present in the secretary organs of the plant (Bruneton, 1995).

## 1.3 Mint Family

Lamiaceae is one of the large plant families used as a framework to evaluate the occurrence of some typical secondary metabolites (Wink, 2003). The typical secondary metabolism of Lamiaceae includes various terpenoids and phenolic compounds (Hegnauer, 1989). Lamiaceae is subdivided in to two major groupings: The Lamioideae and Nepetoideae (Bremer *et al*., 1998). In addition to essential oil, Nepetoideae produce a special “tannin”, mainly represented by the phenolic compound, rosmarinic acid. *Mentha longifolia* is one of the members of the subfamily Nepetoideae (Wink, 2003).

According to flower structure, the Labiates (Lamiaceae) form one of the largest and most highly diverse plant families with worldwide distribution, excluding the Arctic and Antarctic. The family contains around 200 genera and between 2000 and 5000 species of aromatic herbs and low shrubs (Good, 1974; Heywood, 1978, Hedge, 1992). The genus *Mentha* (Lamiaceae) is composed of 19 geographically widespread species and 13 named hybrids (Chambers & Hummer, 1994). Peppermint, *Mentha x piperita*, is native to Europe and it has become both cultivated and naturalised in the USA, India, China, the former USSR, Italy, France and Hungary. Although several of the commercially important species did originate in xeric Mediterranean environments, their developments display marked plasticity, to such a degree that their phenologies can adapt to the growing season in a range of other environments (**Fig. 1.4**). Thus, without intensive selection and breeding, a similar range of (non- chill or frost sensitive) culinary herbs can be grown profitably in Finland (Galambosi, 1989) *Mentha x piperita* has been found to be cold tolerant and thus suitable for Finnish climate conditions (Järvi *et al*., 1994).

**Figure 1.4:** Field view of horse mint (*Mentha longifolia*) growing naturally in Swiss Alps clearly shows allelopathic effects. No plants are growing around the horse mint plants (Photo Courtesy: Franz Steiner)

## 1.4 Importance of mint species

The genus *Mentha* includes 25 to 30 species that grow in the temperate regions of Eurasia, Australia and South Africa (Dorman *et al*., 2003). Spearmint is derived from a range of species, but the two most important ones are native spearmint (*Mentha spicata* L.) and Scotch spearmint (*Mentha x gracilis* Sole). In Linné's taxonomy (http://www.economicexpert. com/a/Carolus: Linnaeus.html), *Mentha spicata* (2n = 4x =48) is a tetraploid species, derived from *M. longifolia* (2n =24) and *M. suaveolens* (2n=24). *Mentha x gracilis* is a hybrid derived from *Mentha arvensis* and *Mentha spicata*. This genus comprises around 30 species of aromatic perennial herbs, distributed mostly in temperate and subtemperate regions of Eurasia, Australia, Africa, and North America (Hui *et al*., 2010; Kanatt *et al*., 2007; Dorman *et al*., 2003). Most labiates accumulate terpenes and a range of other components, mainly in the epidermal

glands of leaves, stems and reproductive structures. Adaptations that facilitate insect pollination (flower shape, pigments, secretion of nectar) are also common (Valdeyron *et al*., 1977). Among the Lamiaceae, the Mints (*Mentha*) have a long history of human use as aromatic (**Fig. 1.5**) and medical compounds.

**Figure 1.5:** The ancient romans using mints on tables to welcome guests. Mint would be served on the tables not just for flavor but also for the scent which came from the volatiles produced from mint leaves.

The essential oil of the two species differs slightly in chemical composition (Lawrence, 1992). The main components of spearmint are carvone and limonene (Tucker, 1992). Peppermint, (*Mentha x piperita* L.), which is tetraploid (2n = 72), is a sterile natural hybrid of (*Mentha aquatica* L.) (2n = 96) and *Mentha spicata* (2n = 48) and produces typical peppermint cyclic monoterpenes, menthol and menthone. Due to sterility, it is not amenable to improvement by sexual crosses. Spearmint, (*Mentha spicata*,) is either tetraploid (2n = 48) or triploid (2n = 36) and produces monoterpene carvone as the major oil component (Tucker, 1992)

Commercial use is concentrated around peppermint (*Mentha x piperita*), spearmint (*Mentha spicata*), and corn mint (*Mentha canadensis*). While for the first two species

often leaf material is used, for instance as herbal tea, corn mint is exclusively cultivated for oil production (Small, 1997, Oudhia, 2003). Main compounds in *Mentha* essential oils are monoterpenes like limonene, menthone and menthol, which are synthesized in glandular trichomes on the leaf surface (Turner *et al*., 2000). Although coming from closely related species, the essential oils from these species show characteristic differences in composition: while the economically dominating essential oil from Peppermint (*Mentha x piperita*) contains menthol and menthone as main components (Gul 1994), Corn mint (*Mentha. canadensis*) is interesting as the richest source of natural menthol (Sharma & Tyagi, 1991, Shasany *et al*., 2000), while Spearmint (*Mentha spicata*) is rich in carvone and therefore widely used as spice in several countries (Kokkini et al. 1995). From these species, corn mint is cultivated only because of oil production (Small, 1997, Oudhia, 2003). Corn mint is the richest source of natural menthol (Sharma & Tyagi, 1991, Shasany *et al*., 2000). Peppermint (*Mentha x piperita*) oil is one of the most popular and widely used essential oils, mostly because of its main compounents menthol and menthone (Gul, 1994). Carvone-scented mint plants, such as spearmint (*Mentha spicata*), are rich in carvone and are widely used as spices and cultivated in several countries (Kokkini *et al*., 1995). Peppermint oil is used for flavouring pharmaceuticals and oral preparations, such as toothpastes, dental creams, and mouth washes. It is also used as a flavouring agent in cough drops, chewing gums, confectionery and alcoholic liqueurs. It is used in medicines for internal use. The pleasant taste of it makes it an excellent gastric stimulant (Budavari *et al*., 1989, Gupta, 1991). Plant-derived natural products are extensively used as biologically active

compounds. Among them, essential oils were the first préservatives used by man (Thompson, 1989). Many of these crude mixtures have been found to have antifungal, antimicrobial, cytostatic and insecticidal activities (Sivropoulu *et al*., 1995). The essential oils extracted from the mint species (*Mentha pulegium* and *Mentha spicata*), containing mainly, pulegone, menthone, and carvone, were tested for insecticidal and genotoxic activities on Drosophila melanogaster. The essential oil of both these aromatic plants showed strong insecticidal activity, while only the oil of *Mentha spicata* exhibited a mutagenic one. Among the constituents studied, the most effective insecticide was found to be pulegone, whereas the most effective for genotoxic activity was menthone. The strong toxicity of pulegone is suppressed in the presence of menthone (Franzios *et al*., 1997). In 1984, the total world mint production came to : peppermint 2.2 Mtons, corn mint 2.1 Mtons and spearmint 1.4 Mtons (Lawrence,1985). There are some reports concerning world menthol production but new statistics concerning mint as a crude material are not available. (Clark,1998) estimated the world production of menthol at 11.8 Mtons. The producing exporting sources are estimated to be India, China and others with 5630 Mtons, 2500 Mtons, 3670 Mtons respectively. Most of the production (9400 Mtons) is from the crude oil of *Mentha arvensis* ; the vast majority of this oil comes from India. The average value of imported peppermint oil in Finland was 0.5 million € per year in 1992 and the same average was for menthol.

According to Patra and co-authors (2000), India is the leading producer and exporter of corn mint oil and its products include menthol crystals, dementholised oil, mint terpenes, etc. The cultivation of corn mint or menthol mint (*Mentha arvensis* f.

piperascens) is largely confined to northern and northwestern India. Finland imported about 8 tons of dry mint leaves, 10–20 tons of peppermint oil, and 110 tons of pure menthol in 1996 (Finnish National Board of Customs, Tullihallitus, 1996). There has been some decrease in the import of mint in the last few years because of increasing mint cultivation in Finland. There are no reliable statistics on the mint cultivation area in Finland, but recently some farmers have started mint cultivation and even some distillation with a 2000–4000 Liter capacity have been built. Monoterpenes (C10) comprise the major components of the essential oils of the mint (Lamiaceae) family, including peppermint (*Mentha x piperita*) and spearmint (*Mentha spicata*) (Lawrence, 1981). Peppermint has been developed as a model system for the study of monoterpene metabolism because of the commercial value of the essential oil, the fact that the plant is clonal and easily propagated vegetatively, and because the oil is chemically complex and the biosynthetic pathway involves essentially all of the representative reaction types of terpenoid metabolism (Croteau and Gershenzon, 1994) Although the pathway for the biosynthesis of peppermint monoterpenes is now well defined, the regulation of monoterpene metabolism in this species is only poorly understood (Gershenzon and Croteau, 1990, 1993). Both developmental and environmental factors are known to markedly influence the yield and composition of peppermint oil, with obvious consequences for the commercial production of this commodity (Burbott and Loomis, 1967; Clark and Menary, 1980); however, the means by which these variables exert regulatory control over the pathway flux to isopentenyl diphosphate and the specific steps of monoterpene metabolism are not known.

## 1.5 Effect of allelochemicals on plant growth

Allelochemicals affect the cells of target plants through a complex network of actions on different components of the cell (**Fig. 1.6**). Allelochemicals change the permeability of plasma membranes and affect the membrane proteins by interrupting their activity (Li *et al*., 2011).

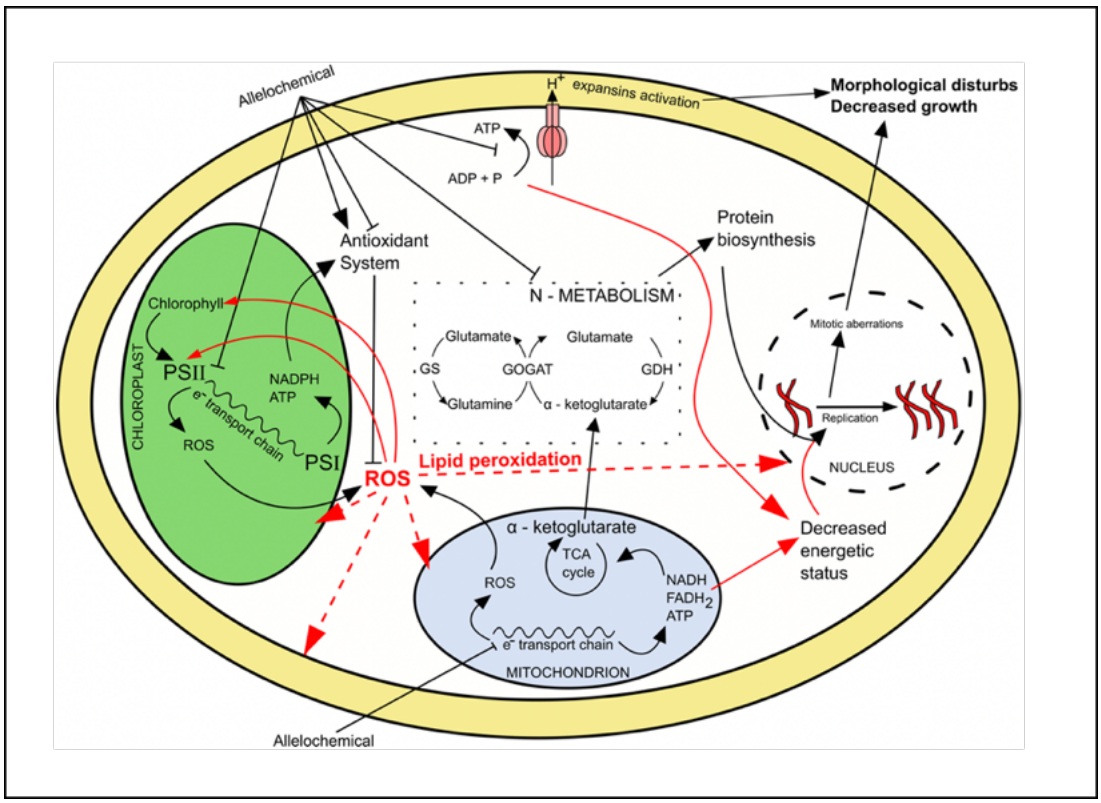



**Figure 1.6:** Mode of action of Allelochemicals, on a single cell. This figure was taken from Marcelo Pedrosa Gomes *et al*., 2017.

Plant cells have a dynamic network of cytoskeleton which not only provide stability to the cell but are also important for cell movement in general, as well as for intracellular interactions and transport mechanisms. The cytoskeleton in plants is comprised of the microtubules and the actin filaments (Quader, *et al* 1986; Zimmermann, 1965). actin filaments are built up of G-Actin monomers and the filamentous actin F-actin appears as a helical curved filament. They carry an ATP binding site (Domingues & Holmes,

2011; Mostowy & Cossart, 2012). actin filaments are important for signal transduction and also in the mechanisms involving transport in plant cells. Microtubules on the other hand are important for dividing chomosomes while cell division progresses, and they also play a role in the intracellular transport of vesicles and organelles, signal pathways and cell growth. Furthermore, they are part of the sensing system of the cell and are able to detect cues from the environment (Nick, 2008). Herbicides are known to have an effect on the microtubules. The inhibition of root growth (as well as the genotoxicity) indicates that menthone targets to microtubules. In fact, microtubules are known to be affected by important herbicide classes, such as the dinitroaniline herbicides (Anthony *et al*. 1998). Disassembly of mitotic microtubules, by either anti-microtubule drugs or mutations disrupts mitotic activity culminating in asymmetries of newly formed cell plates (Yoneda *et al*., 2005; Kawamura *et al*. 2006; Vaughn, 2006). Likewise, disassembly of cortical microtubules interferes with the ordered deposition of cellulose microfibrils, such that cell elongation is blocked. Actually, it was the loss of expansion axiality after treatment with colchicine that brought Paul Green to predict the existence of "microtubules" (Green, 1962) and, one year later, lead to the discovery of "microtubules" (Ledbetter and Porter, 1963). Disruption of plant microtubules by monoterpenes has already been shown for citral which can inhibit the expansion growth of Arabidopsis roots by disrupting cortical microtubules (Chaimovitsh *et al*., 2010), and also inhibit the proliferation of tobacco BY-2 cells accompanied with a high frequency of asymmetric cell walls (Chaimovitsh *et al*., 2012). Taxol, a compound which stabilises microtubules is able to protect BY-2 cells from citral-mediated microtubule

disruption, indicative of microtubules as primary targets of citral. Allelopathic effects of mint have been described for Horse Mint (*Mentha longifolia*,) wheat seedlings, (Bajalan *et al*., 2013) and Peppermint (*Mentha x piperita)*, sunflower seedings, (Skrzypek *et al*., 2015).

## 1.6 Scope of this study

From the moment humanity developed the ability to utilise agriculture as a constant food source, people are fighting against the negative effect on yield by the uncontrollable growth of unusable weeds. These weeds decrease the productivity of the growing zones by competition for nutrients and space. (Putham & DeFrank, 1983).

For facing this problem, modern industry elaborated different synthetic herbicides, which are showing a high effect in controlling the weed population, but they also come with various side effects like reduction of soil and water quality, herbicide residues and unwanted effects on non-target organisms. It has also been reported, that weeds are already developing resistance against specific synthetic herbicides (Beckie and Morrison, 1993; Yongquan *et al*., 2003). Especially the rising number of resistant weeds are forcing for the need of new bioactive components with new modes of action. Development of resistance against herbicides demands new ways to control weeds and one way to do so is exploitation of already existing mechanisms in nature such as allelopathy. In this context, we might need to go a step backwards and take a look into our history. Already ancient civilisations like the romans and the greek searched for potent biologically active components, which are able to control the weed population

in defined areas. They learned very fast, that essential oils can be utilised as natural herbicides/pesticides (Yang & Tang, 1988). This ability of plants, to influence the growth of another plant by delivery of chemical compounds, has been termed allelopathy by Hans Molisch in 1937 (Patni et al., 2018). In modern times, natural components of plants are used in a broad spectrum for medicinal purposes. In fact, about 80% of pharmaceuticals are generated by natural products and analogs derived from them. But in case the usage of these plants and their components as a tool for weed control has not been in the focus of research yet and only a handful of natural components has been identified as herbicidal products (Petroski RJ and Stanley DW, 2009; Dayan *et a*l., 2012). A promising candidate for potent allelochemical effects is plants from the species *Mentha*, which are member of the Lamiaceae family. *Mentha* plants are known to grow in separated colonies, without tolerance for other plants to grow on their area. This leads directly to the suggestion of allelopathic active compounds contained by *Mentha* plants. Although *Mentha* is highly used in pharmaceuticals, research in the field of allelopathic effects is rare (Islam & Kato-Noguchi, 2013).

In the study, I, therefore used a comparative approach, where we tested allelopathic effects of essential oils from different Mints (including the genus *Mentha*, but also Cat Mint (*Nepeta cataria*), and Corean Mint (*Agastache rugosa*), belonging to sisters clades within the *Mentheae*) using the standard cress germination assay as readout. Based on the observation between bioactivity and chemical components, we identified menthone/isomenthone as prime candidate for the allelopathic effect and confirmed this

experimentally. In order to understand the mode of action, we followed the effect of menthone/isomenthone in a transgenic tobacco BY-2 cell lines (expressing the microtubule marker AtTuB6 in fusion with GFP and actin labelled with GFP in GF11 lines). The study could show that menthone/isomenthone, disrupted microtubules and induced mortality. This mortality was elevated in the tubulin marker line, where microtubules are mildly stabilized, as compared to the non-transformed wild type. The results showed specificity of extracted oils in context to cytoskeleton (Microtubules and actin filaments). To further confirm the mode of action we used *Arabidopsis thaliana* plants with GFP labelled tubulin and actin to study the effects of the extracted oils from the *Mentha* plants. This is also significant because *Arabidopsis thaliana* is a dicotyledonous weed. The allelopathic effect of menthone/isomenthone and the microtubule disruption could also be confirmed in rice roots using immunofluorescence. The study paves way for the use of novel bioherbicides that would be environment friendly. The extractions of *Mentha* spp and the standard compounds which are present in the extracts show a very specific mode of action on MTs. As we know, MTs are key to cell cycle and thereby connected to cancer biology. In order to test this hypothesis, HeLa cells line was used to see the effects of *Mentha* related compounds (menthone,etc.). HeLa cells are a model cell system to study cancer (Tan *et al.,* 2015). The preliminary results show that menthone treated HeLa cells clearly, have more mortality. This paves way for further studies.

# 2 Material and Methods

## 2.1 Plant Material and Cultivation Conditions

Different accessions from the genus *Mentha*, along with related taxa (**Table 1**) were raised at the Botanical Garden of the Karlsruhe Institute of Technology in glass houses for the purpose of oil extraction. To stimulate the formation of essential oils, the plants were exposed to sunlight till they start to flower. Plants of different species were grown in separate pots (**Fig 2.1**).

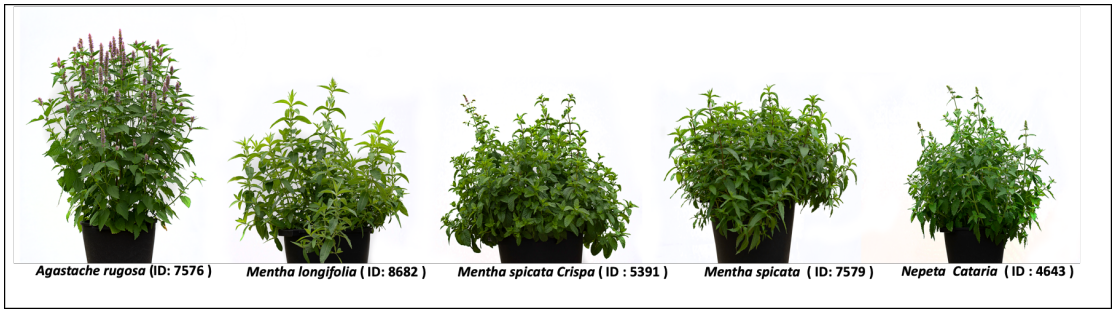



**Figure 2.1:** Plants from Lamiaceae family from *Mentha* and closely related genus were chosen based on the phylogenetic analyses and grown in pots at the Botanical garden of KIT, Karlsruhe Germany.

**Table 1:** Accessions used in the current study maintained as living specimens in the Botanical Garden of the Karlsruhe Institute of Technology under the indicated voucher ID. Scientific taxonomy follows the plant list (www.theplantlist.org). All *psbA-trnH igs* sequences from these accessions were deposited in GenBank under the given ID and identity verified by comparing these accessions with curated sequences from GenBank.

| KIT ID | Declared taxonomy | Vernacular names | Source | GenBank ID for *psbA-trnH igs* | Identity |
|---|---|---|---|---|---|
| 7579 | *Mentha spicata* L. | Spear Mint | BG KIT | MH753576 | *M. spec.* |
| 5391 | *Mentha spicata* L. *var. crispa* | Curly Mint | BG KIT | MH753570 | *M. spicata* L. |
| 8680 | *Mentha aquatica* L. | Water Mint | WEL 3/451 | MH753578 | *M. aquatica* L. |
| 8681 | *Mentha arvensis* L. | Corn Mint | WEL 3/437 | MH753577 | *M. spec.* |
| 8682 | *Mentha longifolia* (L.) L. | Horse Mint | WEL 3/72 | MH753572 | *M. longifolia* (L.) L. |
| 5393 | *Mentha x piperita* L. | Pepper Mint | BG KIT | MH753571 | *M. x piperita* (L.) |
| 3638 | *Mentha suaveolens* Ehrh. | Apple Mint | BG Vakratot | MH753574 | *M. spec.* |
| 4643 | *Nepeta cataria* L. | Catnip | BG KIT | MH753573 | *N. cataria* L. |
| 4639 | *Melissa officinalis* L: | Common Balm | BG KIT | MH781964 | *M. officinalis* L: |
| 7576 | *Agastache rugosa* (Fisch. & C.A.Mey.) Kuntze | Korean Mint | BG KIT | MH753575 | *A. rugosa* (Fisch. & C.A.Mey.) Kuntze |

Based on our phylogenetic analysis we selected 5 species for our experiments (Fig 2.1).

## 2.2 Rice Cultivation (for effect of menthone/isomenthone on MTs)

*Oryza sativa* L. *ssp. indica var.* 'Sathi', *Oryza sativa* L. ssp. *japonica* cv. Dongjin, and a tubulinyl-tyrosine ligase overexpression line in the Dongjin background TTL(ox) mutant were cultivated on a sterilised floating mesh inside a sterilised Plexiglass box (9.5 cm length, 9.5cm width, 5.5 cm height) containing 100 ml of distilled water in the dark at 26°C. Twelve seeds of each genotype were sown equidistantly and treated with 50 µl menthone (Carl Roth, Karlsruhe, containing around 20% isomenthone) placed in an open reaction tube in the canter of the mesh (**Fig. 2.2**). The cultivation boxes were placed in a cardboard box which was lined with black cover to prevent exposure to stray light. The cardboard box was kept in a cultivation room at 26°C for 5 days. The experiment was repeated in three biological replications.

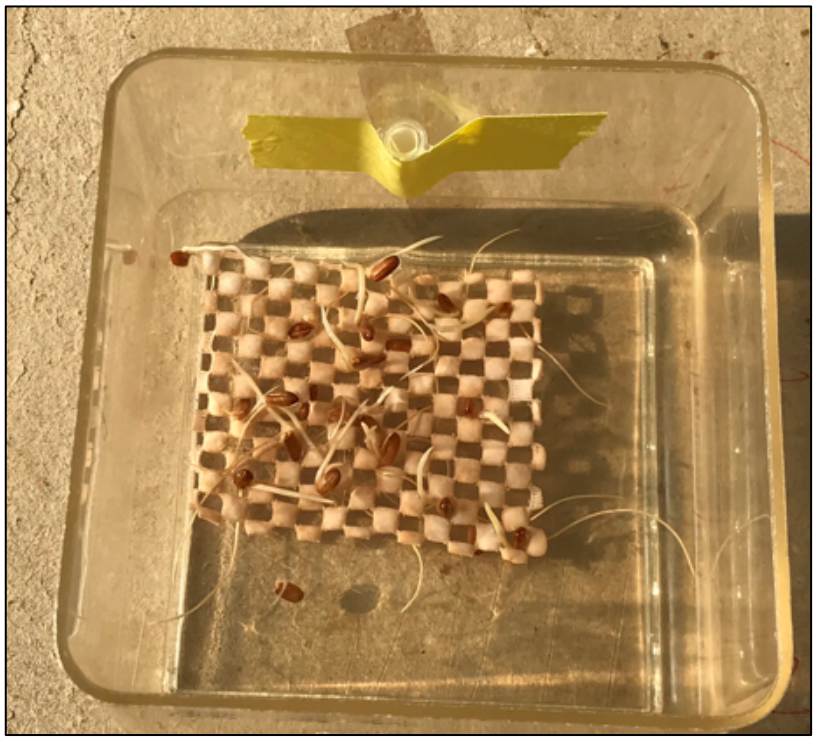


**Figure 2.2:** Setup of the Gerda boxes with menthone/isomenthone and the seedlings on the floating mesh. Seeds were grown on the floating mesh. For treatment, 50µl of menthone/isomenthone was soaked in filter paper and placed in an Eppendorf tube.

## 2.3 *Arabidopsis thaliana* Cultivation

In order to test the effect of oils extracted from the Lamiaceae plants and the compounds on cytoskeleton in vivo, *A.thaliana* lines with GFP labelled actin and tubulin were grown. The line GFP TuB6 overexpressed an N-terminal fusion of β-tubulin 6 from *A. thaliana* N-terminally fused with GFP under a Cauliflower Mosaic Virus (CaMV) 35S promoter generated in the Col-0 background kindly provided by Dr. Kateřina Schwarzerová, Institute of Plant Physiology, Charles University, Prague. The line GFP-FABD overexpressed a N-terminal fusion of the actin-binding domain 2 of AthFIMBRIN driven by the 35S-promoter of (CaMV) generated in the same background (Voigt *et al*., 2005). The seeds were grown on moist filter paper in a petri dish at 25°C for 5 days in the dark.

## 2.4 Cell Culture

Different strains of tobacco BY-2 (*Nicotiana tabacum* L. cv Bright Yellow-2) cell lines (Nagata *et al.,* 1992) were used for this study. Cells were subcultured in Murashige-Skoog medium at weekly intervals, by inoculating 1.5 ml of stationary culture cells into 30 ml of fresh new medium. The cells were cultivated at 26°C in the dark under constant shaking as described previously (Maisch and Nick 2007). To follow microtubule responses *in vivo*, the strain BY2-TuB6-GFP, expressing β-tubulin (AtTUB6) fused to GFP under the control of the constitutive Cauliflower Mosaic Virus 35S promotor was used (Hohenberger *et al.,* 2011), while the responses of actin filaments were followed using the strain GF11, expressing the second actin-binding

domain of fimbrin (AtFIM1) in fusion with GFP, also under a CaMV-35S promoter (Sano et al., 2005).

## 2.5 Growth of HeLa cells and MTT Assay

To quantify the cytotoxicity of the compounds *in vitro* MTT assays were performed. The MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent can be reduced to blue-purple formazan by the mitochondrial enzymes of living cells. The amount of the resulting formazan can be determined photometrically and correlates with the cell viability, since this reaction can only take place in metabolically active cells. Therefore, HeLa cells were cultivated with various concentrations of the extracted Mentha compounds and the cell viability was monitored after 72 h of incubation.

HeLa cells were seeded in the 96 well plates at the density of $1x10^4$ cells/well in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. After 24 h of incubation at 37°C under 5% $CO_2$, the medium was removed and the cells were treated with various concentrations of the compounds and incubated for 72 h at 37°C and 5% $CO_2$. The negative control was untreated, the medium was exchanged, and the cells were treated in the same way as the other samples. Thereafter, 15 µl of the MTT reagent were given in each well. For a positive control, Triton X-100 (1%) was added in some wells before treating them with the MTT reagent. After 3 h of incubation the cells were lysed using the Stop Solution to release the blue-purple formazan. The cell viability was determined by measuring the absorbance of the resulting formazan at 595 nm using a multiwell plate reader.

## 2.6 Phylogenetic Analysis

Fresh leaves of reference plants (~60 mg) were used for DNA extraction, the DNA was isolated using the Invisorb® Spin Plant Mini Kit (Stratec biomedical AG) following the protocol of the producer. The weighed plant material was shock-frozen in liquid nitrogen and homogenised in reaction tubes with steel beads (Qiagen TissueLyser) for two minutes. The isolated DNA was evaluated by spectrophotometry (Nanodrop, Peqlab) for quality and quantity, and DNA concentration was diluted to 50 ng/µl as template for genomic PCR. A reaction volume of 30 µl containing 20.4 µl nuclease free water (Lonza, Biozym), 1-fold Thermopol Buffer (New England Biolabs), 1 mg/ml bovine serum albumin, 200 µM dNTPs (New England Biolabs), 0.2 µM of forward and reverse primers (**Table 2**), 100-150 ng DNA template, and 3 units of Taq polymerase (New England Biolabs) was used to amplify the marker sequences. The psbA-trnH *intergenic spacer* region was amplified in a thermal cycler by initial denaturation at 95°C for 2 min; followed by 33 cycles at 94°C for 1 min, annealing at 56°C for 30 s, and elongation at 68°C for 45 s; ending with an extension of 68°C for 5 min. The PCR amplicons were evaluated by agarose gel electrophoresis using NEEO ultra-quality agarose (Carl Roth, Karlsruhe, Germany). The DNA was visualised using SYBR safe (Invitrogen, Thermo Fisher Scientific Germany) under blue light excitation. A 100-bp size standard (New England Biolabs) was used to determine the fragment size of the products. The amplicons were purified for sequencing using the MSB® Spin PCR apace kit (Stratec). Sequencing was conducted by Macrogen Europe (Netherlands) or GATC (Germany), and the quality of the obtained sequences was tested by Finch TV

Version 1.4.0 (https://digitalworldbiology.com/FinchTV). The marker region was sequenced from two directions to get a robust result. The resulting two sequences were merged for each accession by aligning the forward read with the reverse complemented backward read. For the sequence alignment and the phylogenetic analysis the program MEGA7 (Version 7.0.14) with the integrated tree explorer was used. The Sequences were aligned using the Muscle algorithm of MEGA7. Alignments were trimmed to the first nucleotide downstream of the forward primer and the nucleotide preceding the reverse primer. With the same software, the evolutionary relationships were inferred by using the neighbor-joining algorithm with a bootstrap value that was based on 1000 replicates. The species *Melissa officinalis* was chosen as an outgroup. Additional psbA-trnH *intergenic spacer* sequences for the use in molecular phylogeny were obtained from GenBank using a BLAST tool in combination with the Taxonomy View tool.

**Table 2:** oligonucleotide primers used for genetic identification of the tested accessions.

| Name | 5' → 3' sequence | Target | Reference |
|---|---|---|---|
| *psbA*$^{u}$ | GTTATGCATGAACGTAATGCTC | psbA-trnH intergenic spacer | Sang *et al*., (1997) |
| *trnH*$^{u}$ | CGCGCATGGTGGATTCACAATCC | | Tate and Simpson (2003) |

## 2.7 Oil Extraction

To test the effect of essential oils on living plant cells and plants, the oil was extracted by water-steam distillation **(Fig. 2.3**). For each plant species, 30-50 g of fresh leaf material were harvested, frozen in liquid nitrogen and mildly ground to break up the oil cavities of the glandular hairs. The frozen plant particles and a stirrer bar were added to the still pot, which was then filled until the level of water reached the edge of the heating mantle. The distillation apparatus was filled with distilled water and the water cooling system was connected in a way that enables water flow in the opposite direction of the still. The oil was now distilled by heating the plant material for about two hours at 100°C.The extracted oil was collected in a graded burette, and the oil phase measured. To improve yield, the tube was washed with a small quantity of n-hexane. Afterward the distillate was sealed in a glass vial and stored at 4°C till experimental use.


**Figure 2.3:** Steam Distillation unit for extraction of oil from *Mentha* plants. The plants were weighed (1), heated in a round bottom flask (2), and the oil was extracted using the distillation unit (3) and finally the oil was collected (4).

## 2.8 GC-MS Analysis

The essential oils were analysed by GC-MS in the lab of Prof. Dr. W. Boland at the Max Planck Institute for Chemical Ecology Jena. For GC-MS, a TRACE MS (Thermo-Finnigan) device equipped with a ZB5 column (15 m, 0.25 mm I.D, 0.25 µm film thickness) was used with a 10-m guard column (Phenomenex, Aschaffenburg, Germany). Mass spectra were collected using the electron impact (EI) mode at 70 eV, 33-450 m/z. Volatiles were eluted under programmed conditions: 40°C (2 min isotherm), followed by heating at 10°C $min^{-1}$ to 220°C, followed by 30°C $min^{-1}$ to 280°C, using helium (1.5 ml $min^{-1}$) as carrier gas. The GC injector (split ratio 1:7), transfer line and ion source were set at 220°C, 280°C, and 200°C, respectively. For comparison, a GC-MS ITQ (Ion Trap Instrument, Thermo) Rtx200 MS column was used with the same program as described above. The ZB 5 column was used for separation of co-eluted compounds, while the more sensitive GC-MS ISQ (Thermo, Quadrupol) was targeted to the more polar compounds that are not too low in concentration.

## 2.9 Live-Cell Imaging

After three days of cultivation, about 1 ml of cell suspension (BY2-TuB6-GFP line and GF11 line) was transferred to a tube and diluted with cultivation medium to appropriate density allowing for observation. In order to see the effects, menthone/isomenthone, linalool, limonene α-pinene and β-pinene as well as oils isolated from *Agustache rogusa*, *Mentha longifolia, Mentha spicata*, *Mentha spicata crispa*, *Nepeta cataria* as well as *Leptospermum citratum* and pure compounds corresponding to *L.citratum*, Geraniol

and β-citronellol were tested. Slides for microscopy were mounted as follows: 20 µl of cell suspension were spread on the slide. Close to the cells, but without touching them, 0.5 µl of oil or compound was added on both sides, before a 40 x 24 mm coverslip was placed on the slide. There was no direct contact between oil/compound and suspension, such that the action of the compounds in the essential oils had to occur through the gas phase. The cells were observed under the Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000), using a 20x air, and a 63x oil immersion objective. The cells were observed under differential interference contrast (DIC), and after excitation with the blue line (488 nm) of an Ar-Kr laser (Zeiss, Jena, Germany) to detect the GFP signal. For each oil and each cell line, random cells were chosen and observed for 30 minutes.

### 2.10 Cell Mortality Assay

To test the effect of limonene, menthone/isomenthone and menthol on the overall viability of the cells (from WT, BY2-TuB6-GFP line and GF11 line), the Evans Blue Dye Exclusion Assay (Gaff and Okong' O-Ogola, 1971) was used (**Fig. 2.4**). Viable cells exclude the dye from their cytoplasm and remain unstained, while dead less, due to loss of membrane integrity take up the dye. The effect of the compounds was assessed in mitotically active cells (day 3 after subcultivation) in sampling aliquots of 500µl suspension transferred to a reaction tube and incubated with 1µl of n-hexane and ethanol ( corresponding to 0.2% V/V n-hexane and 0.2% V/V ethanol as solvent controls in tobacco BY-2 cells.), and 1µl of compounds (limonene,

menthone/isomenthone and menthol), for either 15, or 30 min. After incubation, the suspension was transferred to a custom-made cell filter tube (Nick *et al*., 2000). Different staining and rinsing times were adjusted before deciding on a protocol of staining for 5 min in 2.5 % Evans Blue, before rinsing the cells in BY-2 medium three times for two minutes each. Subsequently, 20 µl of the suspension were transferred into a hematocytometer slide (Fuchs-Rosenthal) and scored under the brightfield microscope. The percentage of observed cells that were unstained was used as readout for viability. Data represent three independent replications with a population of 600 individual cells per replica. To check the specificity in time of menthone/isomenthone in the BY2 cell lines, three volumes of menthone/isomenthone (0.25, 0.5 and 1µl corresponding to 0.05, 0.1, and 0.2% V/V) were used.

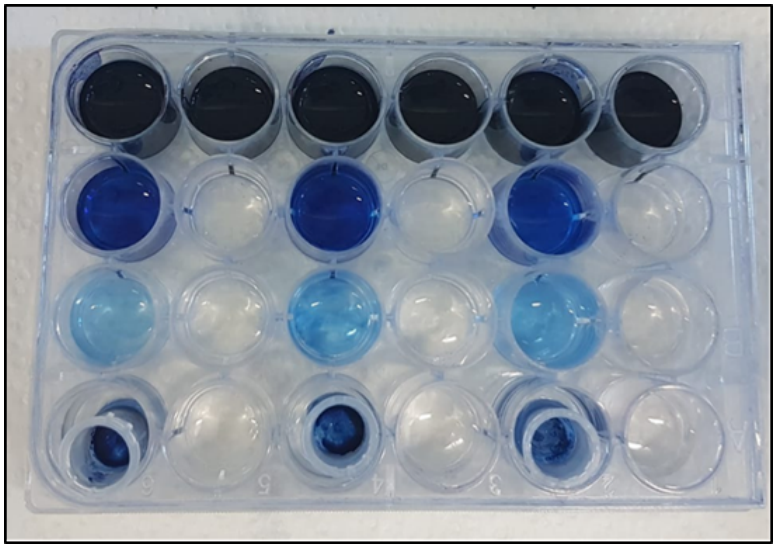


**Figure 2.4:** Experimental setup for Evans Blue Assay to determine the mortality of cells. The cells were stained for 15 minutes and 30 minutes and were subsequently washed to remove extra stain.

## 2.11 Cell death estimation

Fresh 200 µl of 3-d-old suspension cultures of BY2 cells were placed in Eppendorf-like tubes with appropriate amount (0.1, 0.5 and 1.0µl) of one of the pure compounds namely menthone/isomenthone, linalool, limonene α-pinene and β-pinene as well as oils isolated from *Agustache rogusa, Mentha longifolia, Mentha spicata, Mentha spicata crispa, Nepeta cataria* as well as *Leptospermum citratum* and pure compounds corresponding to *L.citratum*, Geraniol and β-citronellol. The cells with the compounds or oils were incubated in closed Eppendorf-like tubes in dark culture room on shaker (IKA®KS 260 basic) with 150 rpm for 15 min. Then 50µl of sample were stained with the same amount of staining mixture (10µg.ml−1 AO, orange acridine, and 10µg·ml$^{-1}$ EB, ethidium bromide, at PHB (0.1M Na·phosphate buffer at pH 7.4) for 5min (Pietrowska *et al*., 2015). After that 100 l of PHB was added to samples and 50µl of suspension cells was placed on slide glass and covered with coverslips. Photos were taken under blue light of fluorescence microscope (Diaplan, Litz) using the camera (Leica DFC 500) and software (Leica Aplication Suite, ver.4) and used to measurement. According to this method the changing colour of chromatin, from green to red, it was allowed to distinguish living, dying and dead cells after the measurement of resultant fluorescence intensity (RFI) of green AO, migrating into the nuclei via cell membrane which did not change its permeability or integrity, and red EB, permeating to dying or dead cells in which cell membrane permeability or integrity was changed. The RFI values increased with the changing colour from green to red. Measurements of RFI have been done using ScnImage (Byczkowska *et*

*al*.,2013). About 250–300 nuclei in each of two experimental series were analysed. Based on the RFI values the cell death was grouped into categories. The cells were divided based on fluorescence of dying cells in two ranges from 36 to 40 and 41 to 45 RFI values, the lower ones are for I step and the second one are for the second, it is important for understanding whether the process is slight or strong , i.e. have chance to go to dead phase.

### 2.12 Microtubule Visualisation in Rice

Microtubules were visualised by immunofluorescence staining in rice roots after stabilisation with 12.5 μM taxol (Sigma) for 1hour at 25°C according to (Nick *et al*.1994) with minor modifications. The specimen was fixed in a solution of 3.7% paraformaldehyde (PFA) in microtubule-stabilising buffer (50 mM PIPES, 5 mM EGTA, 1 mM $MgSO_4$, 1% v/v glycerol, 0.25% v/v Triton-X100, $dH_2O$, pH 6.9). Paraformaldehyde (PFA) is cross-linking amino groups of basic amino-acid residues producing an imine. Pairs of imines in neighboring amino acids can then form a methylene bridge, such that proteins are connected, by that conserving the status of the cell. After fixation for 60 min, the specimen was hand-sectioned using a sharp scalpel, followed by washing with MSB three times for five minutes to wash out the residual formaldehyde. To prevent unspecific binding of the antibodies, the samples were incubated for 20 minutes at 25°C in blocking solution (5% bovine serum albumine in Tris Buffered Saline, TBS). Unbound blocker again washed out three times for five minutes with TBS, before a monoclonal antibody against α-tubulin (DM1A, Sigma Aldrich, Neu-Ulm) diluted 1:200 in TBS, was added and incubated at

4°C overnight. Next morning, unbound primary antibody was washed out three times for five minutes with TBS. Subsequently, as secondary antibody a polyclonal anti-mouse IgG conjugated with TRITC (Sigma-Aldrich, Neu-Ulm, Germany) diluted 1:20 in TBS was added to the specimen. The use of a polyclonal antibody allows binding to different epitopes of the primary antibody leading to signal amplification. After one hour of incubation at 37°C in a humid chamber (to prevent that the sample dries out), in a last washing step the unbound secondary antibodies were removed out by washing three times for five minutes with TBS. Signals were observed by spinning disc microscopy as described above.

### 2.13 Visualisation of the Cytoskeleton in *Arabidopsis thaliana*

The effect of oil extracted from *A. rugosa* along with menthone/isomenthone were tested on cotyledons of *Arabidopsis thaliana* where either microtubules, or actin filaments were labelled with GFP.

The line GFP TuB6 overexpressed a N-terminal fusion of β-tubulin 6 from *A. thaliana* N-terminally fused with GFP under a Cauliflower Mosaic Virus (CaMV) 35S promoter generated in the Col-0 background kindly provided by Dr. Kateřina Schwarzerová, Institut of Plant Physiology, Charles University, Prague. The line GFP-FABD overexpressed a N-terminal fusion of the actin-binding domain 2 of AthFIMBRIN driven by the 35S-promoter of (CaMV) generated in the same background (Voigt *et al*., 2005). The seeds were grown on moist filter paper in a petri dish at 25°C for 5 days in the dark. To assess the effect on the cytoskeleton, 50 µl of either essential oil extracted

from *A. rugosa*, or pure menthone/isomenthone were transferred onto a microscopic glass slide. The seedlings were placed directly into the respective liquid, covered by a coverslip, and then the sample was immediately observed by spinning disc microscopy. As negative control, plants were placed in distilled water instead.

### 2.14 Allelopathy studies

In order to probe for potential allelopathic activity of the different species of *Mentha* and other Lamiaceae plants, the different accessions were either planted isolated in individual pots as control, or in combination with other accessions in same pots. A further experiment addressed allelopathic interaction between rice (using the *O. sativa ssp. japonica* variety Dongjin as well as the *O. sativa ssp. Indica* landrace Sathi), 5 plants of each accession were raised in separate pots as control. In parallel, each of the two rice accessions was cultivated with *A. rugosa* in a joint pot, respectively (**Fig. 2.5**). To assess the effect of co-cultivation, after two months the plants were measured for height and inflorescence length (as readout for fitness).

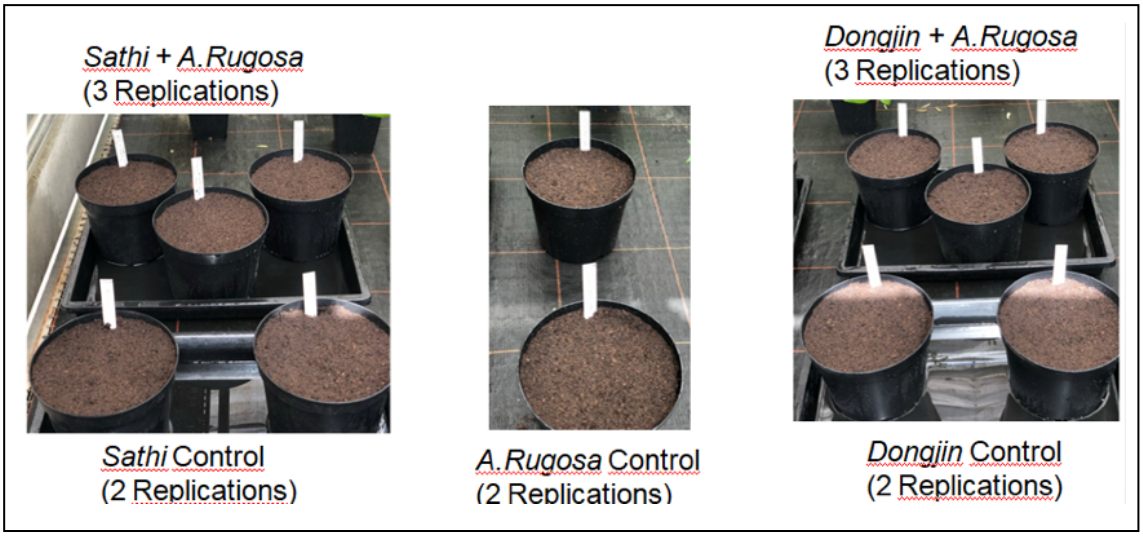



**Figure 2.5:** Cocultivation of rice and *Agastache rugosa* plants for studying allopathic effects. In setup the plants were grown in same pot, and also to see effects from shoot and root one setup the plants were separated and also control plants were grown completely separate.

## 2.15 Germination test Assay

In order to quantify allelopathic activity of essential oils, or candidate compounds identified therein, the standard cress germination test (International Seed Testing Association) was performed in Plexiglass boxes (30 mm x 20 mm x 15 mm) that were covered and sealed. The bottom of the boxes were covered with humidified absorbent papers (No. 914446/4; Hartmann, Heidenheim/Brenz,FRG) Hundred seeds of *Lepidium sativum* (var. 'Glatt', FloraSelf Co.) per box (30 mm x 20 mm x 15 mm) were sown equidistantly, and a glass slide with filter paper loaded with a defined volume (0.1, 1, or 10 µl corresponding to 0.01, 0.1, or 1 ppm) of the respective compound or the respective solvent was placed in the center of the box. After the preparation, the box was sealed with Parafilm, and stored in an incubator with a temperature of 27 °C in the dark for two days. After two days, the germinated seedlings were counted, and hypocotyl and root length were measured. Each experiment was replicated three times.

To further see the effect of volatile oil extracted from *M.longifolia* and its compounds (menthone/isomenthone, Linalool, Limonene, α-Pinene, and β-Pinene) on germination of cress plants, germination tests were performed in plastic boxes with covers (**Fig 2.6**). The bottoms of the boxes were covered with humidified absorbent papers. 100 seeds of *L.sativum*, *Phacelia tanacetifolia*, and 50 seeds of wheat (cultivar Winter Wheat 'WiWa') and tomato (cv. 'Marmande') were sown per box (Height: 4cm, Width at bottom: 9cm, width at top: 10cm, length at bottom: 11.5cm, length at top: 12.5cm). For release of the volatile substances, a glass slide with filter paper carrying the respective

compounds was placed in the center of the box and the box was thereafter closed with Parafilm to prevent loss of volatile compounds. The compounds were tested at a volume of, 1 µl and 10 µl of oil or water (control). Each combination was tested three times replicated three times. Seeds were incubated in the dark at 25°C. After two (wheat, cress) to five (tomato) days, the number of germinated seeds was counted. In case of *Rumex obtusifolia* , the seeds were collected from plants in the fields around FiBL, Frick, Switzerland, and the seed germination boxes were incubated two weeks under light with a temperature of 25 °C with three different compounds that had been isolated and identified from *Mentha* essential oils (menthone/isomenthone, linalool, limonene) and control with only water .

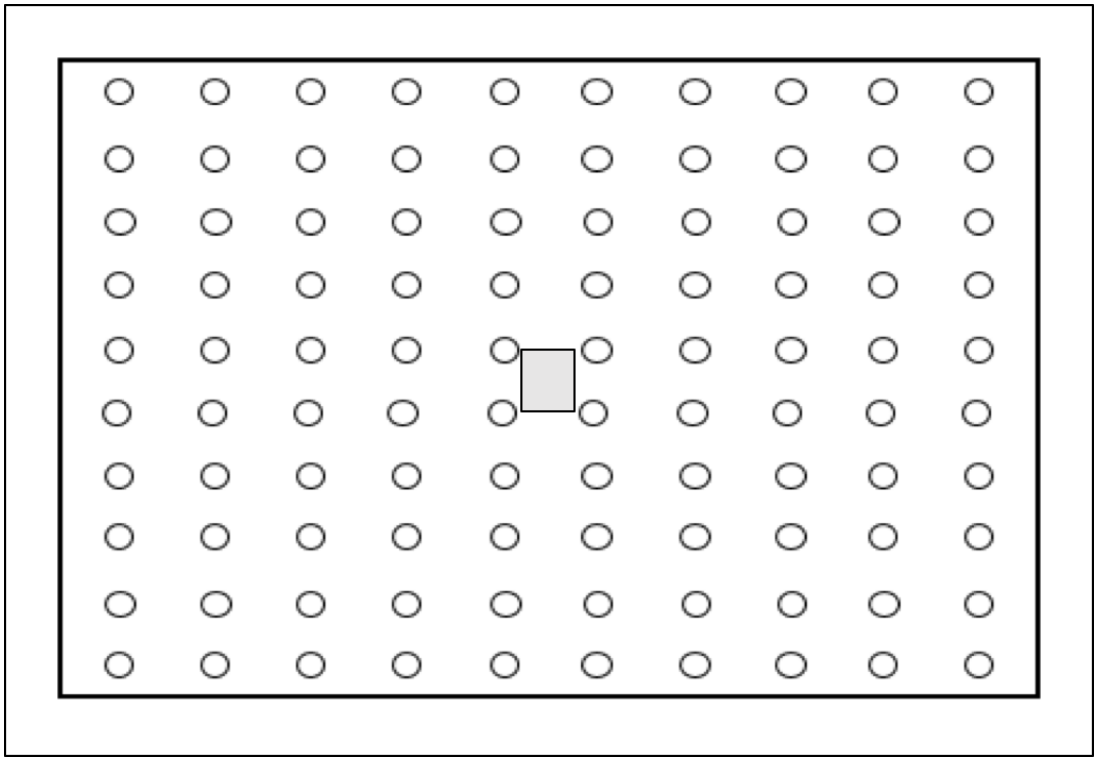


**Figure 2.6**: Germination array. Setup of the germination array as shown here had seeds (Circle) placed on a moist tissue paper and a piece of tissue paper with the oil/compound was placed with a coverslip in the center (Grey square). Circles = Seeds, grey area = coverslip and applied oil

## 2.16 Influence of formulated *Mentha* oils on growth of *Convolvulus*

We did this experiment to see the effect of *Mentha* oils and their compounds on the growth of the root weed *Convulvulus* Acker-Winde *(Convolvulus arvensis)*. *Mentha* oils (*M.spicata crispa , M. longifolia and A. rugosa*) and compounds isolated thereof (menthone/isomenthone, linalool, limonene) were embedded in a slow release carrier formulation (proprietary knowledge by Ms. Claudia Daniel). The substances/oils were added to the carrier material in two different amounts, in order to receive two concentrations of formulated substances/oils, i.e. 50µl in 2 g of formulation and 100µl in 2 g of formulated material. Pieces (length 3-4 cm) of the runner roots / rhizomes of *Convolvulus* (*Convolvulus arvensis,* rhizomes collected locally from heavily infested fields) were placed in the upper 2 cm of pots filled with standard field soil (potting soil) (**Fig. 2.7**). They were either covered directly with the formulated substances/oils ("Spaghetti-formulation") and then with additional field soil (application directly in close vicinity of the rhizome pieces) or the rhizomes were first covered with a layer of soil, then with the formulated substances/oils and then with additional soil (application 'top', formulated substance separated from rhizome pieces). Two grams of the formulation (i.e. 50 µl substance/oil) was used per pot, 9 pots served as replicates for each application and each substance. The effect of the pure substances and the mint oils on the growth of *Convolvulus* plants was measured (shoot length (cm), number of leaves and fresh weight (g) after two weeks, when most volatiles were expected to have been released from the formulation. A second measurement was done after one month,

in order to check for the durability of the effect, respectively to evaluate if the rhizomes have started to re-grow. The experiment was repeated twice.

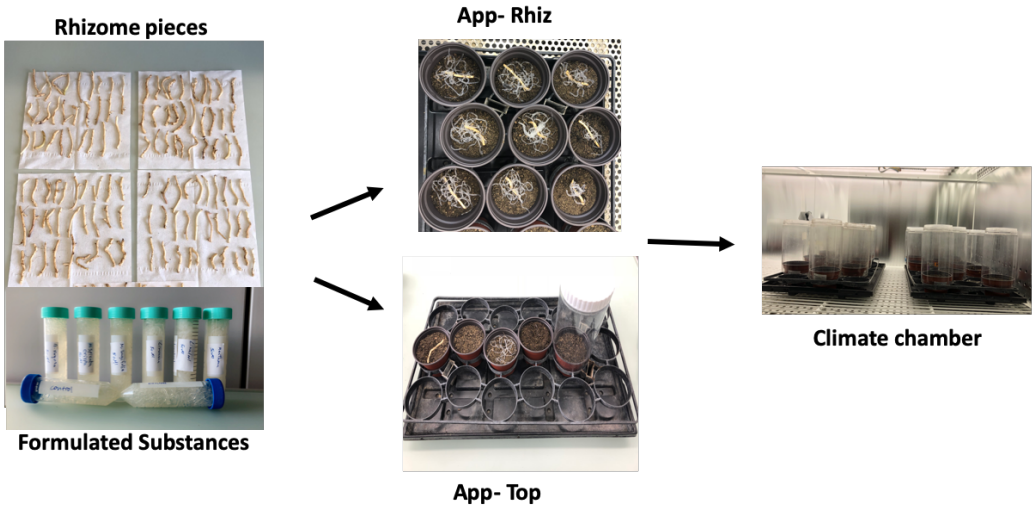



**Figure 2.7:** *Convolvulus* assay was performed to see the specificity of compounds based on rhizome pieces of *convolvulus* where the material was placed in two ways. The treated rhizomes were grown in growth chambers. App-Top: Application on top of the soil, App-Rhiz: Application directly in contact with rhizome.

## 2.17 Testing mint substances on apple and grape seedlings

Mint oil components (α-pinene, β-pinene, linalool) were tested for growth suppression of apple and grape seedlings as described above for *Convolvulus*. For these experiments, we used seedlings of apple (cv. Jonagold) and grapevine (cv. Chasselas) to spray them with three different compounds of mint oils (α-pinene, β-pinene, linalool) in two different concentrations (0.5% v/v and 5% v/v) besides spraying or Emulsogen El 360 as solvent controls. To assess the effect, images of the seedlings were recorded at the time of spraying, and 24 h later, (**Fig. 2.8**) while after one week the increase in fresh weight was determined

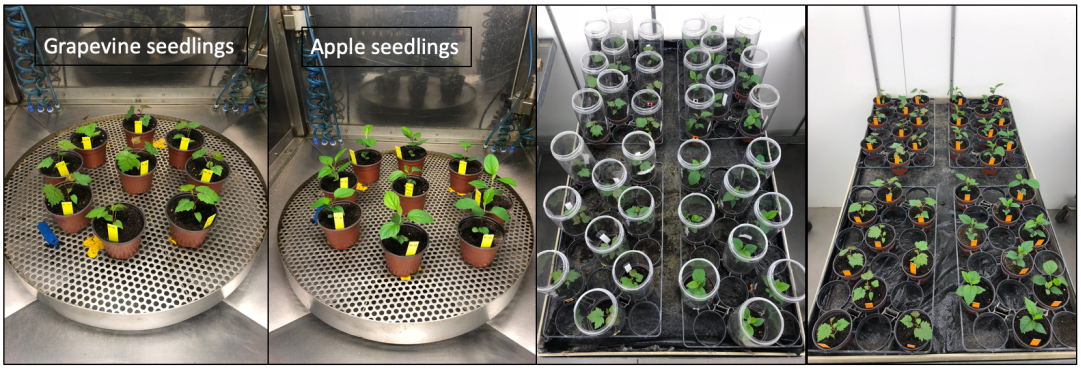



**Figure 2.8:** Screening of effects of α-pinene, β-pinene on grapevine and apple seedlings. Plants were grown in pots and each biological replication had 9 plants per treatment. After spraying of the respective compound, the plants were covered with a plastic cover for 24 hours and then observed.

## 2.18 Germination of different seeds on the Field Soil mix with standard soil and chopped *Agastache rugosa*

Fresh plant material of *A. rugosa* was chopped at the flowering stage and 5 g of this chopped material was added per pot and mixed with soil. Next, seeds of different plant species (Cress 15 seeds, Tomato 5 seeds, WiWa 5 seeds, *Phacelia tanacetifolia* 5 seeds and *Rumex* also 5 seeds) were added to the pots. the pots were 9 for each Rep beside control for all species. After one week the measurements were taken (fresh weight. (g) / pot).

## 2.19 Hoechst 3342 staining of HeLa cells

HeLa were seeded in 8 Well ibidi µ-slides at the density of 20 000 cells/well. After 6 h of incubation at 37°C, 5% $CO_2$, the medium was removed, and the cells were treated with 0.15 % v/v and 0.3 % v/v menthone. The control was treated with DMEM (Dulbecco's Modified Eagle Medium). After 24 h, the cells were analyzed by

confocal microscopy (Leica TCS SPE (DM2500)) ($\lambda_{exc}$ = 405 nm, $\lambda_{em}$ = 410-510 nm). In cells which were treated with 0,15 % v/v menthone we could observed more multinuclear cells and apoptotic bodies, compared with the control. At a concentration of 0,3 % v/v menthone most cells were dead.

## 2.20 Statistical Analysis

All experiments were conducted in three replications. In order to check the standard error, Microsoft Excel program was used. Significance was done by doing a t-test. For the results of germination test, the statistical analyses were performed with multivariate Anova followed by Duncan's test using **IBM SPSS Statistics 22 software**. $P \leq 0.05$* was considered statistically significant. Results were expressed as mean ± SE of three independent replicates.

# 3 Results

## 3.1 Determination of taxonomic identity

In order to authenticate the plants used in the study, we used the psbA-trnH *intergenic spacer*, a plastidic marker. The sequences obtained from the amplified regions were aligned with their curated homologues recovered from GenBank using a BLAST search and the Taxonomy View tool. Only curated sequences from sources, where the identity of the plant had been determined, were used for reference (i.e. sequences from commercial products were excluded, since they are often of doubtful identity). Based on the aligned sequences, a phylogenetic tree was constructed and bootstrapped using the neighbor-joining algorithm (**Fig. 3.1A**). The tree clearly shows that the sequences from *Mentha* species clustered separately from sequences obtained from the other tested genera within the Lamiaceae family (*Agastache*, *Nepeta*, *Melissa* that all formed well supported separate clades). This *Mentha* clade differentiated into four subclades: *M. arvensis*, *M. spicata* were clearly distinct with sufficient bootstrap support (99% for both clades against the other *Mentha* accessions, 87% for the separation between *M. arvensis* and *M. spicata*). Also *M. suaveolens* differentiated into a separate clade, albeit with lower bootstrap support (65%). The largest group comprised *M. longifolia* and *M. x piperita*, without any differentiation between these two species. Our analysis also revealed that some plants had been wrongly assigned. From the study it can be clearly seen that MH753577, declared as *Mentha arvensis*, MH753576, declared as *Mentha spicata*, and MH753574, declared as *Mentha suaveolens* cluster with *Mentha longifolia* and *Mentha x piperita*. These plants, which had obviously been mis-

identified, were then annotated as *Mentha spec.* (**Table 1**).We can clearly see the differences in the morphology of the leaves based on the glandular hairs and trichomes on the surface (**Fig. 3.1 B and C, Suppl. SF 2, 3 and 4**). There are visibly longer hairs on the surface of *M. longifolia* leaves when compared to *Agastache rugosa* leaves. Also it can be clearly seen that the adaxial side of the leaves have visibly lesser trichomes and hairs when compared to the abaxial side.

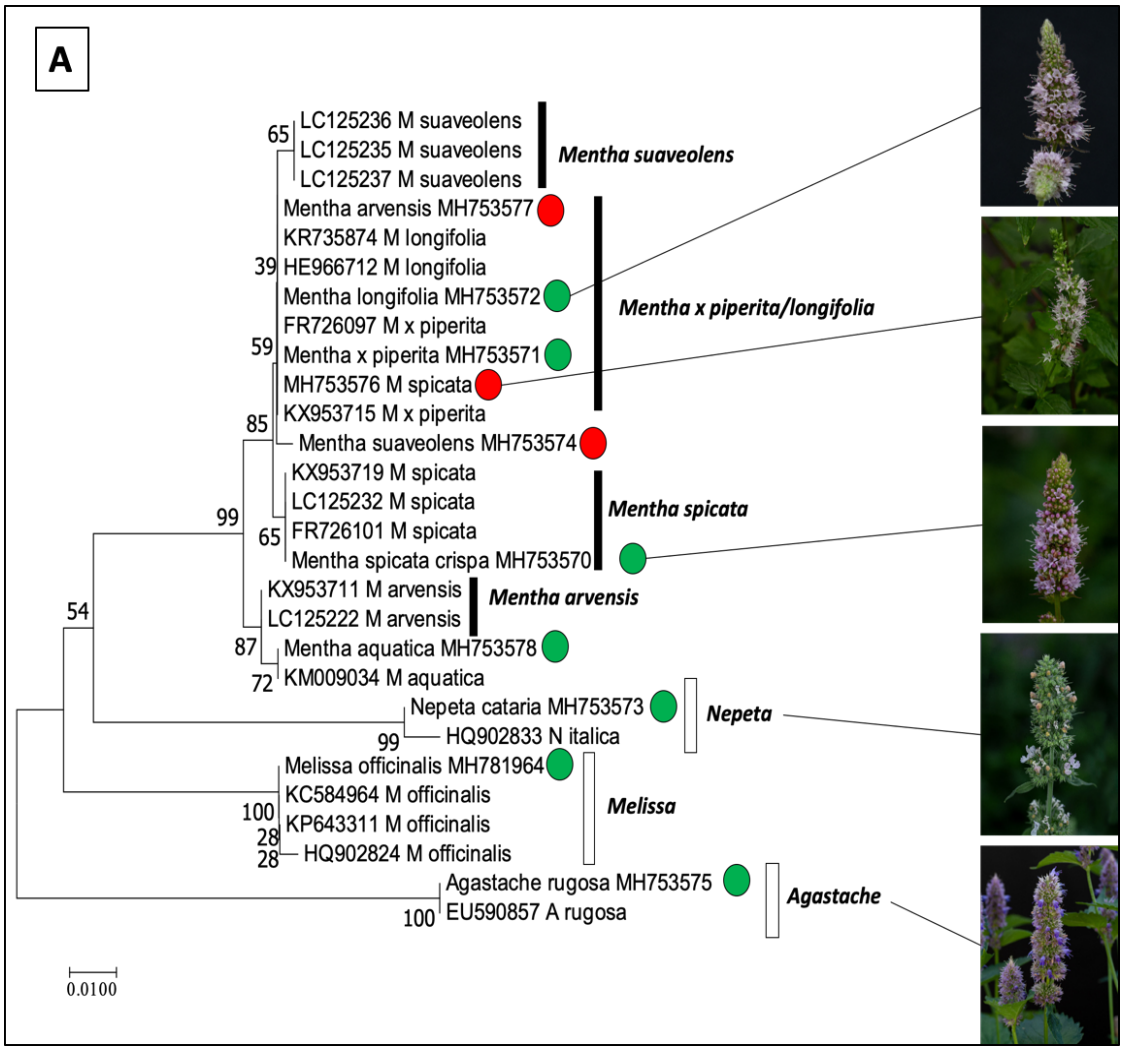



**Figure 3.1A:** Phylogenetic tree based on psbA trnH marker. An Evolutionary relationship of 28 sequences for the psbA-trnH marker from different specieis of *Mentha* along with *Melissa* as outgroup is shown using the Neighbour-Joining algorithm. The green dots represent the correct identity based on sequesnces. The red dots show the incoherence between sequences and the names annotated to them.

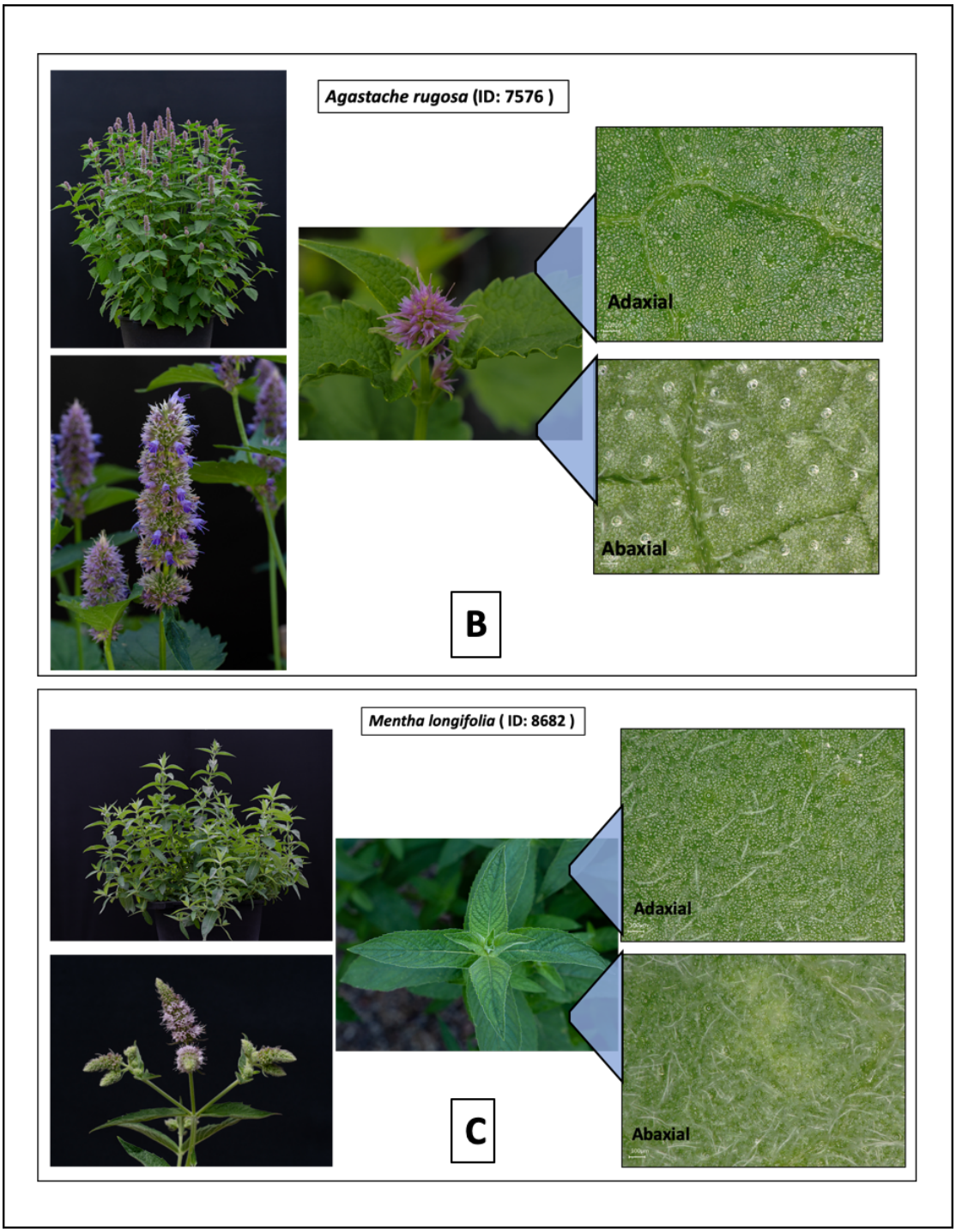



**Figure 3.1B, C:** Morphological details of *Agastache rugosa* (B) and *Mentha longifolia* (C) showing the overview of the plants with leaves and inflorescence. The adaxial and abaxial side of the leaves were observed to see differences in the glandular hairs and trichomes. Images for the magnified view of the leaves was taken using the Keycene microscope.

## 3.2 Allelopathy effects on plant growth

### 3.2.1 Screening of Germination Inhibitors

In order to identify candidate compounds responsible for the bioactivity of the plant oils, the cress standard bioassay for seed germination was used, followed by an analysis of the GC profiles of the extracted plant oils. Based on preparatory studies, 0.1 ppm of essential oil extracted from different accessions, were employed, since they produced a substantial inhibition for some oils (**Fig. 3.2 A**). The strongest inhibition of seed germination was observed for the oils from *M. spicata,* in fact just confirmed as *M. spec.* (83.3%)*, M. spicata crispa* (84.7%)*, M.longifolia* (81.3%)*, A. rugosa* (79.6%) and *N cataria* (72.6%), while the oil from *M. suaveolens* (just confirmed as *M. spec.*), *M. x piperita*, and *Melissa officinalis* inhibited cress germination only mildly. As positive control, we used two essential oils obtained from two species designated as Lemon Myrtle (*Leptospermum citratum* and *Backhousia citriodora*), because these are rich in citral, a compound with known allelopathic activity (Chaimovitch *et al*., 2010). As expected, also these positive controls exerted a stringent inhibition. In order to get insight into the chemical compounds responsible for this bioactivity, the oils from these ten accessions were analysed by GC, and many peaks identified and measured using commercially available standards (**Suppl. Data S1**). These profiles were then compared with the observed bioactivity. To quantify the average bioactivity for a given compound, a bioactivity score was defined as

$$B = \frac{1}{n} \left( \sum a(i) * g(i) \right)$$

With n number of accessions (10), a(i) relative peak area of the respective compound for accession i, and g(i) the germination inhibition observed by the essential oil of accession i. This bioactivity score increased with the relative abundance of the respective compound in the oil, and with the bioactivity of the essential oil. If an essential would be composed only of one compound and this compound would give full inhibition, a bioactivity score of 10000 would result. The largest scores obtained in this assay were around 1000 (**Suppl. Data S1**). From this analysis, three candidates emerged (**Fig. 3.2 C**): menthone/isomenthone (since both compounds interconvert easily, they were pooled) with a score of 522, β-citral (isomeric with α-citral with a score of 247) with a score of 604, and carvone with a score of 1262. Citral has already been described as allelopathic compound with anti-microtubular activity (Chaimovitch *et al*., 2010), and, thus, served as a positive control for the feasibility of the approach, carvone, while being the most active compound, was mainly found in an accession of unclear identity (*M. spicata*, which in fact clustered with *M. x piperita*). We focused therefore on the third compound, menthone/isomenthone, which were most abundant in *M.longifolia* and in *A. rugosa*. The comparison of GC profiles for the oils from *M.longifolia* and *A. rugosa* (**Fig. 3.2 B**) show in both cases abundance of menthone and isomenthone, but also clear differences in the chemical speciation: while the overall content of menthone and isomenthone was comparable in both species (around 30% relative peak area), *M. longifolia* harbours menthol as main component (more than 50% relative peak area), which is barely detectable in the essential oil from *A.rugosa*.

Instead, *A. rugosa* oil contains significant amounts of the menthone precursors limonene and pulegone.

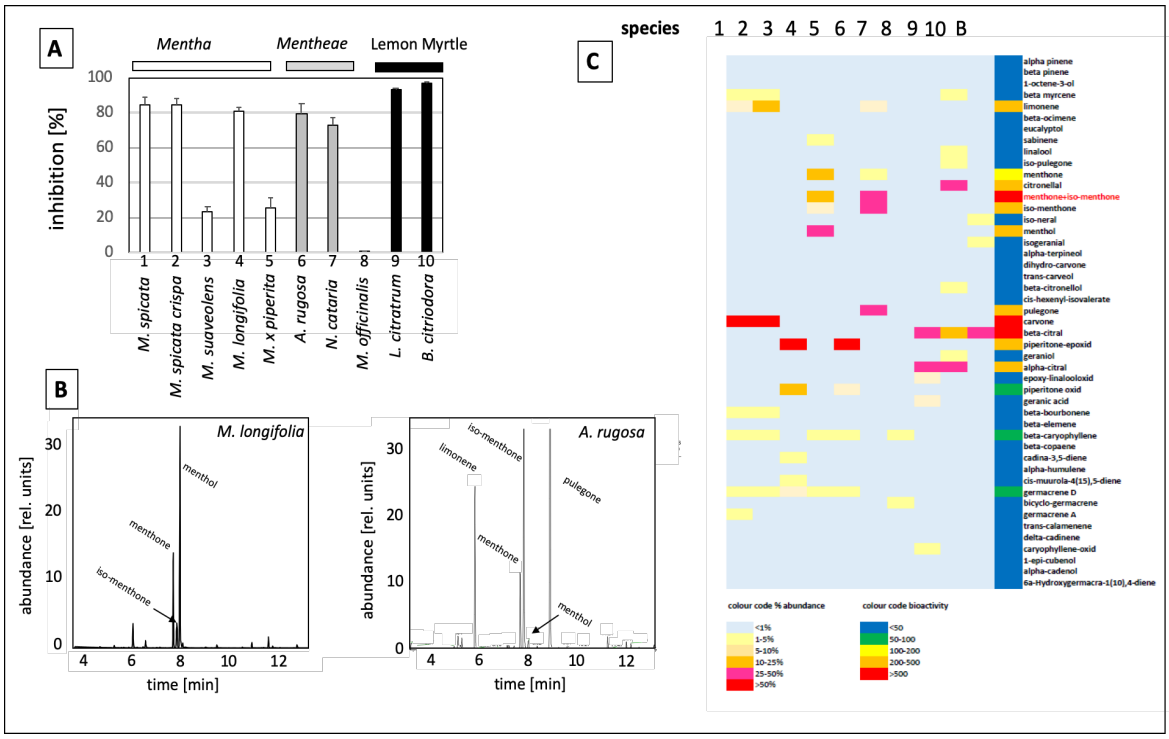



**Figure 3.2: Activity Guided Fractionation. (A)** Inhibition of germination by essential oils extracted from plants of Lamiaceae. Inhibition (%) of cress seeds after 48 hours incubation in darkness at 27 °C in under treatment of Mentha and other Lamiaceae oils is shown. Lemon Myrtle is shown as a positive control. In each case, the inhibition was tested in three biological replications. **(B)** To get a profile of the essential oils a GC-MS a TRACE MS (Thermo-Finnigan) device was used. The peaks **(B)** clearly show the specificity of pulegone and limonene in *A.rugosa* oils. The bioactivity and the abundance of compounds as shown in the heat map **(C)** shows the high bioactivity of menthone/isomenthone and also their high presence in *A rugosa* and *M longifolia.*

Based on the abundance of compounds and the high bioactivity score of menthone, we determined dose response relationships for the inhibition caused by menthone/isomenthone, menthol as their derivative, limonene as their precursor, and linalool as chemically similar monoterpene with differing structure features (**Fig. 3.3 A, B**). In order to exclude the possibility of effects of solvents, the defined volume (0.1, 1, or 10 µl corresponding to 0.01, 0.1, or 1 ppm) of n-hexane and ethanol were used as

solvent controls. Both had only negligible effect on the germination of seeds. A dose dependent effect on germination was observed when 0.01, 0.1, and 1 ppm of menthone/isomenthone, menthol, linalool and limonene were used (**Fig. 3.3 A**). The strongest effect can be seen with menthone/isomenthone, where even a low concentration of 0.01ppm inhibits the germination by about 70% which goes up to almost 99% at a concentration of 1 ppm. The least effects are observed for limonene, where even with a high concentration of

1 ppm the inhibition is just 20%. Menthol and linalool produced a similar pattern, where a dose dependent gradual increase can be seen. The shape of the curves differed. For concentrations up to 0.1 ppm, both, menthol and linalool inhibited only mildly (between 30 and 40% as compared to 80% for menthone/isomenthone). Only at the highest concentration, 1 ppm, they reached to the level seen for menthone/isomenthone. Thus, menthone/isomenthone emerged as most promising candidate for the inhibitory effect of *M. longifolia*, and *A. rugosa* essential oils, while their structurally similar precursor limonene, as well as their structurally similar derivative menthol, was much less active.

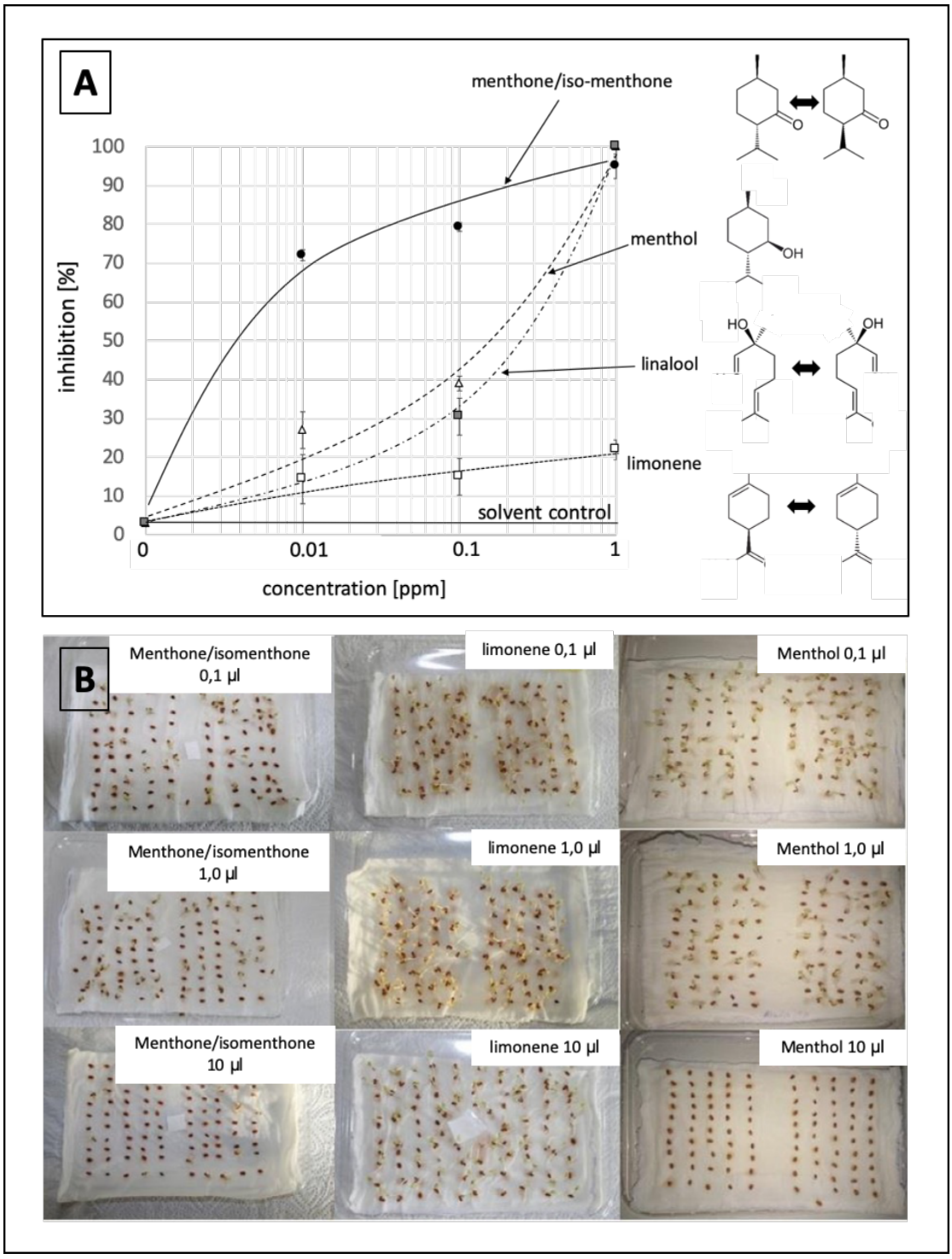



**Figure 3.3: (A)** Comparison of inhibitory effects of compounds specific to essential oils from Lamiaceae on cress **(B)**. The inhibiotion % of germinated cress seeds after 48 hours incubation in darkness at 27 °C in presence three concentrations of menthone/isomenthone, menthol, linalool and limonene were tested. A control with solvent was also carried out. In each case, three different concentrations (0.01 ppm, 0.1 ppm and 1ppm) were tested as biological triplicates.

### 3.2.2 Effects on Rice

In order to see allelopathic effects of *A.rugosa* on rice species, the height of all plants was measured after cultivation of rice in presence of *A. rugosa* for 2 months. Also, since we know that rice, too, possesses allelopathic properties, the inflorescence length and inflorescence number of *A.rugosa* plants was measured.

#### 3.2.2.1 Plant Height

Differences in the height in the control groups and the cocultivated groups of all plants were studied (**Fig. 3.4**). While the growth of Dongjin seems not to be repressed in a significant way, Sathi even boosts its growth on cocultivation with *A.rugosa*. On the other hand *A.rugosa* is not affected by Dongjin, but in cocultivation with Sathi its growth is affected. The results do not show a significant difference in the final height of Dongjin plants, while Sathi plants cocultivated with *A.rugosa* grow higher than control plants. Both results are not expected in this case since *A.rugosa* should act as an repressing factor for both plants. The observation that Dongjin is not affected by *A.rugosa* speaks for an overall resistance to its allelopathic effects. Sathi seems to benefit from the cocultivation. On the other hand the *A.rugosa* plants are also not affected by Dongjin like expected. But cocultivated with Sathi, *A.ugosa* has shorter height. This suggests an allelopathic effect of Sathi represses the *A.rugosa* plant, while itself is getting an advantage.

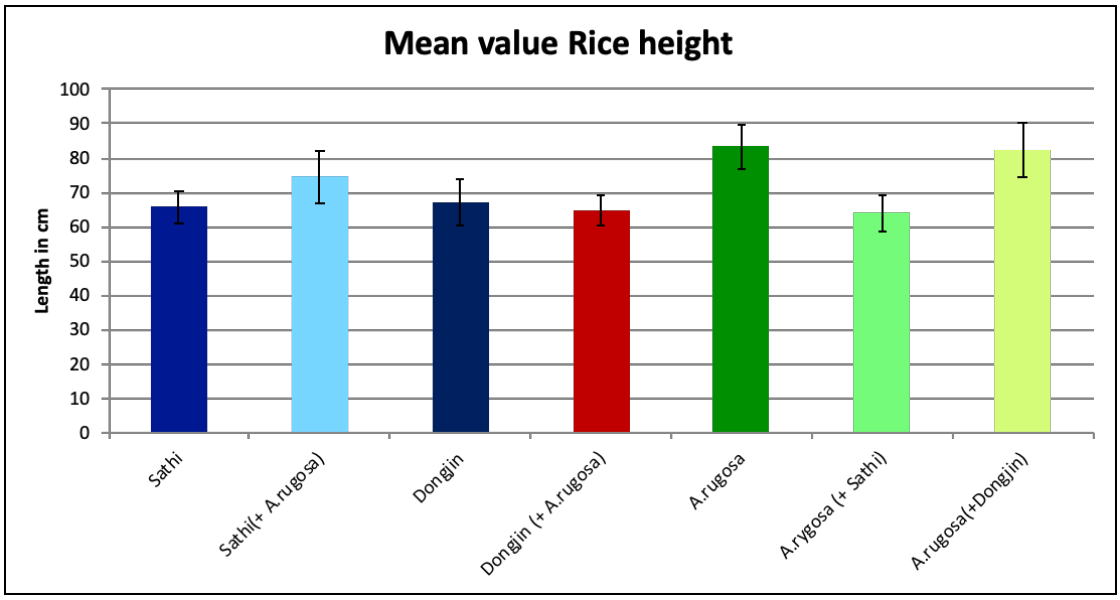



**Figure 3.4:** Height of plants after cocultivation. Rice (*Sathi* and *Dongjin*) were cocultivated with *Agastache rugosa* and the height of rice and *A. rugosa* plants was measured.

### 3.1.1.1 *Agastache rugosa* inflorescence

Since *A.rugosa* seems to be affected by Sathi, a closer look onto *A.rugosa* was obvious. In the next experiment the length and the total number of the inflorescence buds of *A.rugosa* was measured (**Fig. 3.5**).The diagrams show a massive repression in inflorescence growth of *A.rugosa* when cocultivated with Sathi, while a cocultivation with Dongjin does not seem to have an impact.

The results show a drastic decrease of *A.rugosa* inflorescence length if cocultivated with Sathi. This reenforces the hypthesis of an allelopathic effect of Sathi to *A.rugosa*. If *A.rugosa* is cocultivated with Dongjin, there is no significant difference observable to *A.rugosa* control groups.

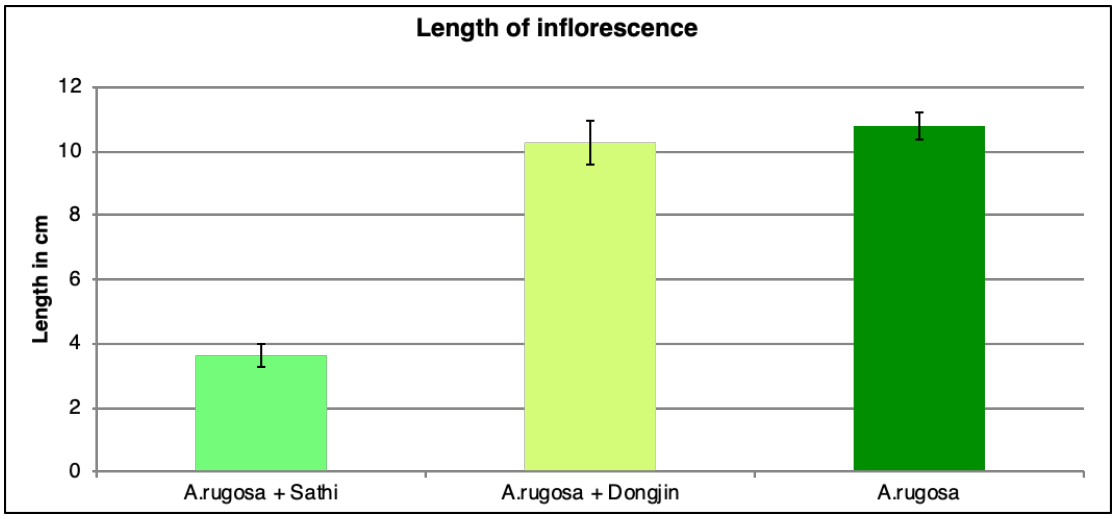



**Figure 3.5:** Length of *A. rugosa* inflorescence. The length of the inflorescence on the top of each branch was measured to see effects of *Sathi* on the *A. rugosa* growth and development.

Also in this part of the experiment, the results show a repression of inflorescence bud (**Fig 3.6**) production if *A.rugosa* is cocultivated with *Sathi*. This suggests that not only the growth of the inflorescence of *A.rugosa* is affected by *Sathi*, but even the production of them is repressed. *A.rugosa* plants cocultivated with Dongjin do not show any effects on the number of buds like expected.

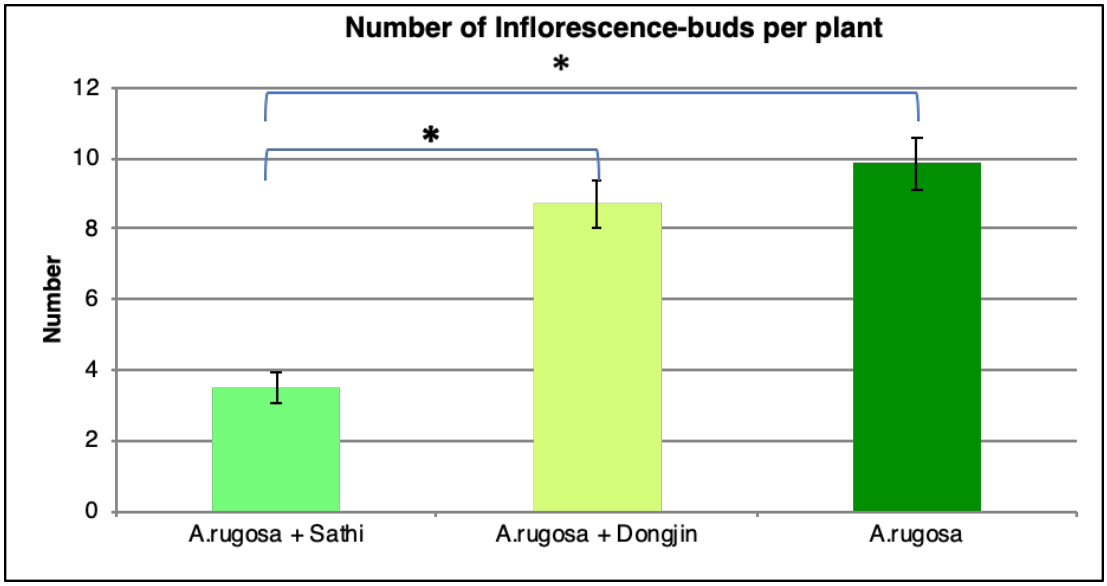



**Figure 3.6:** Total number of inflorescence buds of *A.rugosa* plants. The picture shows the difference of the number of buds produced by *A.rugosa* while in cocultivation with Dongjin or Sathi. A significant repression of bud production is observable in *A.rugosa* groups cocultivated with Sathi. A cocultivation with Dongjin does not create an effect.

## 3.3 Cell Mortality

### 3.3.1 Cell Mortality Assay (Menthone / Isomenthone are Cytotoxic, Depending on Microtubules)

To get insight into the mode of action of the bioactive candidate compounds identified in the essential oils, we probed for the potential cytotoxicity of menthone / isomenthone (the commercially available product was a racemate of around 80% menthone and 20% isomenthone, its precursor limonene, and its derivative menthol (**Fig 3.7A**) along with 1µl (corresponding to 0.2% v/v) n-hexane, and 1µl (corresponding to 0.2% v/v) ethanol as solvent controls in tobacco BY-2 cells. Mortality was scored using the Evans Blue Dye Exclusion test in a time-course experiment, testing mortality at 15 or 30 minutes after addition of 1µl (corresponding to 0.2% v/v) of the respective compound (**Fig 3.7 B**). To test a potential impact of the cytoskeleton, in addition with the non-transformed BY-2 cells (WT), two cytoskeletal marker lines were tested: the actin marker line GF11, where actin is labelled and mildly stabilised by overexpression of the actin binding domain 2 of fimbrin with GFP (FABD2-GFP), and the microtubule marker line TuB6, where microtubules are labelled and mildly stabilised by overexpression of *Arabidopsis thaliana* β-tubulin 6 fused to GFP (TuB6-GFP). In non-transformed BY-2 cells (**Fig 3.7 B**, top), menthone/isomenthone induced a rapid increase of mortality, already after 15 min, more than 90% of the cells were dead. In contrast, limonene was mostly ineffective with only 20% mortality reached after 30 min, menthol exerted intermediate cytotoxity, which developed slower (at 15 min, only 25% of the cells were killed, while at 30 min, mortality had increased over 60%), and the solvent control did not produce

any significant mortality. The time course was almost identical in the actin-marker line (**Fig 3.7 B**, center). For the microtubule-marker line, the response to limonene was basically unaltered, and the response to menthone / isomenthone, which was already saturated in the non-transformed WT, was still close to saturation. A clear difference was observed for the response to menthol, however (**Fig 3.7 B**, bottom): here, the amplitude of the response was significantly amplified by around the half to reach almost 90% mortality after 30 min. Since the concentration of menthone /isomenthone in the time-course experiment was close to saturation, we conducted then a dose-response study with lower concentrations (**Fig 3.7 C**). This experiment revealed clearly that the sensitivity of the microtubule marker line was elevated, because saturation was reached at around 4-fold lower concentrations of menthone/isomenthone as compared to the wild type. Interestingly, also the actin marker line was somewhat more sensitive than the non-transformed WT, albeit not to the same extent. The fact that structurally closely related compounds differ in cytotoxicity, and that the effects of menthone/isomenthone and menthol are enhanced in cells, where microtubules have been mildly stabilised argues for a specific activity of menthone/isomenthone. We therefore, followed this further focussing on microtubules as potential target.

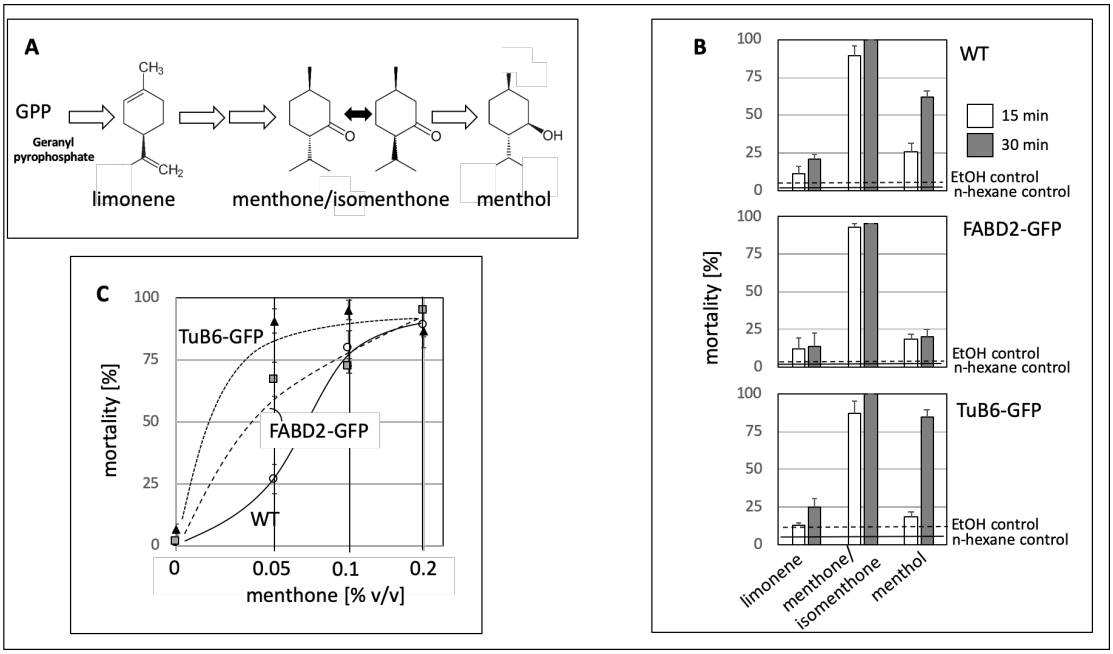



**Figure 3.7:** Structure-function relationship for the cytotoxicity of limonene, menthone/isomenthone, and menthol. A position of the tested compounds in the biosynthetic pathway for menthol. B time course of cytotoxicity in non-transformed BY-2 cells (WT), in cells overexpressing actin-binding domain 2 of fimbrin (actin), and in cells overexpressing TuB6 (MTs). The values observed for the solvent controls with EtOH after 30 min are represented by the dashed lines, the values observed for the solvent controls with n- hexane after 30 min by solid lines. C dose response of cytotoxicity over the volume of menthone/isomenthone in the assay for the three cell lines scored after 15 min. Data represent mean value and standard errors from three independent experiments scoring individual cells per data point

### 3.3.2 Estimation of Mortality by Cellular staining

Double staining of nuclei with Acridine Orange (AO) and Ethidium Bromide (EB) based on their different abilities to permeate via cell membranes allowed detection of cell death because AO permeated intact cells and emitted green fluorescence as a result of intercalation in the double-stranded DNA, while increasing changes in cell membrane allowed EB to intercalate into nuclear DNA and gradually, by red color, EB masks green color of AO. Thus, the computerized measurement of increasing Resultant fluorescence intensity (RFI) values of both fluorochromes allows to count the number

of living, dying and dead cells. The results of measurement showed that in 0.1µl suspensions about 92 % cells were living (**Fig. 3.8 A-E**) but rest of them were dead.

After treatment with 0.1, 0.5 and 1.0µl of menthone/isomenthone the living cells were not detected, but the I-step-dying ones appeared (**Fig. 3.8 A**). Their number in 0.1µl was about 73%, but in 0.5 and 1.0µl in both cases was lower by about 95% compared to 0.1µl. While the number of II-step-dying ones in 0.1µl was about 25% and was similar to the 1.0µl but in 0.5µl the number of cells was more than two times greater compared to these series. Whereas number of dead cells in the 0.1µl was similar to 0µl, but in the 0.5 and 1.0 µl the number of these cells was greater three and six greater respectively than in 0µl. After treatment with 0.1, 0.5 and 1.0µl of linalool (**Fig. 3.8 B**) the living cells were detected only in 0.1µl but their number was not statistically significant. The number of the I- and II-step-dying cells in 0.1µl was about 75% and 25% and was similar to these in 0.5 µl but in 1.0µl their number was lower and greater respectively by 15%. Dead cells in 0.1, 0.5 and 1.0µl were not detected.

After treatment with 0.1, 0.5 and 1.0µl of limonene (**Fig. 3.8 C**) the number of the living cells were lower respectively by about 25%, 50% and 60% compared to 0µl. The number of I-step-dying ones in 0.1µl was about 25% but in 0.5 and 1.0µl number of these cells was greater respectively by about 25% and 30%. In these series the number of the II-step-dying cells were about 1-3% and differences were not statistically important.

Treatment with 0.1, 0.5 and 1.0µl of oil from *A. rogusa* (**Fig. 3.8 D**) leaded to decrease the number of leaving cells. Their number was lower respectively by about 20%, 40% and 80% compared to 0µl. The number of the I-step-dying ones in 0.1µl was about 30% but in 0.5 and 1.0µl number of these cells was greater respectively by about 20% and 30%. The II-step-dying cells were detected only in 1.0µl but their number was about 1% and differences were not statistically important.

Treatment with 0.1, 0.5 and 1.0µl of oil from *M. longifolia* (**Fig. 3.8 E**) leaded to decrease the number of leaving cells. Their number was lower respectively by about 20%, 50% and 90% compared to 0µl. The number of I-step-dying ones in 0.1µl was about 20% but in 0.5 and 1.0µl number of these cells in both cases was greater by about 40%. The number of the II-step-dying cells number was about 1% and differences were not statistically important.

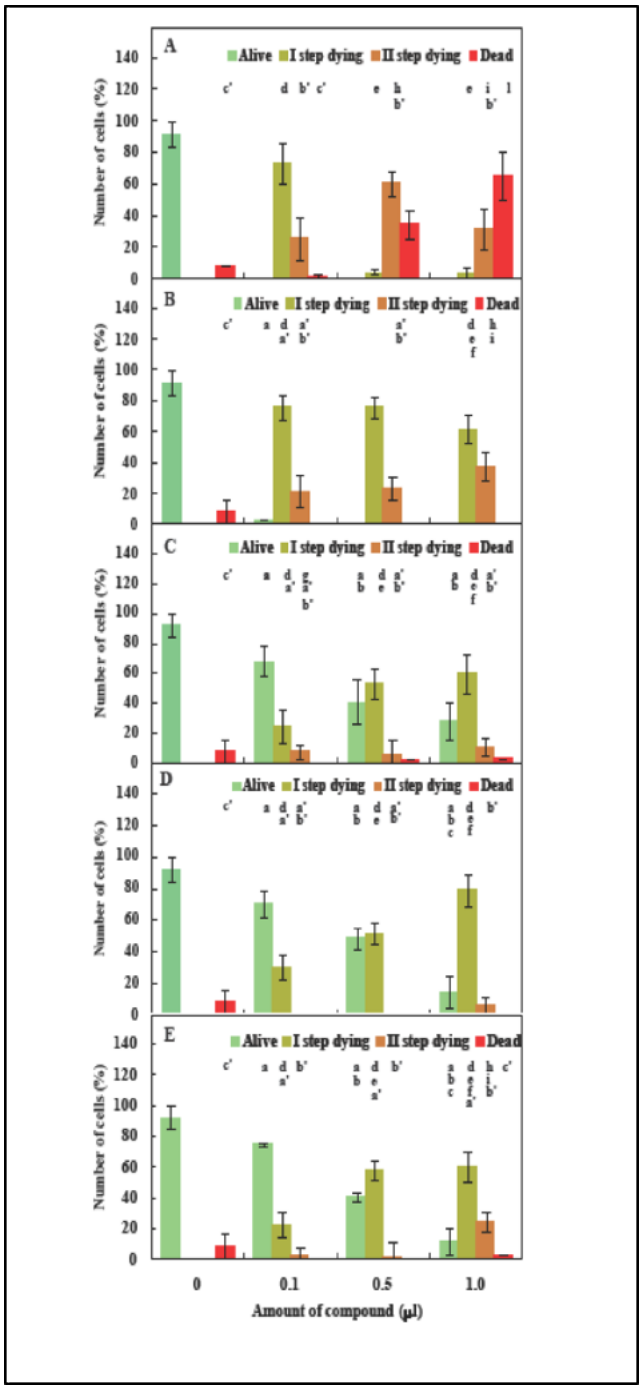



**Figure 3.8**: Numbers (%) of alive, dying (at the first – I and the second – II, step) and dead of 3-d-old BY-2 cells treated for 15 min with menthone/isomenthone (A), linalool (B), limonene (C) as well as oil isolated from *Agastache rugosa* (D) and from *Mentha longifolia* (E), estimated based on measurement of resultant fluorescence intensity after double staining with ethidium bromide and orange acridine. Bars indicated SE from two experiments repeated three times each. Letters a,b,c and d,e,f and g,h,i as well as j,k,l indicated important differences between results of numbers respectively of alive, dying (at the first – I and the second – II, step) and dead cells between 0 and 0.1, 0.5 or 1.0µl. Letters a',b' and c' indicated important differences between results of numbers respectively of alive and dying (at the first – I and the second – II, step) and dead cells among the series.

The results of measurement showed that in 0µl suspensions of BY-2 cells about 92 % cells were living (**Fig. 3.9 A-E**) but rest of them were at the I-step of dying.

After treatment with 0.1 and 0.5µl of oil from *M. spicata* the numbers of alive cells were similar to the respective cells in control. In 1.0µl their number decreased more than 5 times. The numbers of I-, II-step dying and dead cells in 0.1 and 0.5µl series were about 1-10% and differences were not significant. In the 1µl series the numbers of II-step and dead cells were about 50% and 20% and were greater by about four times compared to respective cells in 0.1 and 0.5µl series as well as three and two times compared to alive and I-step dying cells respectively within this series (**Fig. 3.9 A**).

Treatment with 0.1 µl of oil from *M. spicata crispa* lead to decrease in the number of alive cells by about four times compared to control series. In the 0.5 and 1.0µl series this kind of cells disappeared but the number of the I-step dying cells in 0.1 and 0.5µl series were similar and was 10 times greater compared to respective cells in control series. In the 1µl series the most numerous group of cells was the fraction of II-step dying cells which was two times greater compared to respective cells in 0.5µl series as well as compared to numbers of I-step dying and dead cells within the series

(**Fig. 3.9 B**).

After treatment with 0.1µl of oil from *N. cataria* the number of alive and I-step dying cells was similar to control, but the number of existing dead cell was not statistically important. In the 0.5µl series the most numerous group of cells was also the fraction of alive cells but their number was 25% lower to the respective cells in control series. The differences of numbers of I- and II-step of dying and dead cells were not statistically

significant within this series, but their numbers were about four times lower compared to alive cells. In the 0.5µl series the numbers of I- and II-step of dying and dead cells were similar but were about four times greater compared to the number of alive cells within this series (**Fig. 3.9 C**).

Treatment with 0.1µl of *α*-pinene oil did not induced cell death process, and thus almost all cells were alive. Their numbers were about two times lower compared to number of alive cells in Ctrl series. In the 0.5 and 1.0µl series the most numerous group of cells was the fraction of I-step dying cells, numbers of them were similar and 10 times greater compared to the number of alive cells respectively in the Ctrl and 0.5-1.0µl series. Additionally, in the 1.0µl series the fraction of II-step dying cells appeared, but their number was similar to alive cells compared to 0.1-0.5µl series and 10 times lower compared to the I-step dying cells within 1.0µl series (**Fig. 3.9 D**).

Treatment with 0.1, 0.5 and 1.0µl of *α*-pinene oil (**Fig. 3.9 E**) lead to a decrease in the number of alive cells. Their numbers were lower respectively by about 20%, 25% and 30% compared to control cells. But between the series the differences numbers of this cells were not statistically significant. The number of I-step-dying ones in 0.1, 0.5 and 1.0 µl series was greater by about two, three and four times compared to the respective control cells. The fraction of II-step of dying cells detected in 0.1 and 0.5µl series were either non-significant or not detected in 1.0µl series (**Fig. 3.9 D**). The results of measurement showed that in 0µl suspensions of BY2 cells about 92 % cells were living (**Fig. 3.9 A-E**) but rest of them were at the I-step of dying.

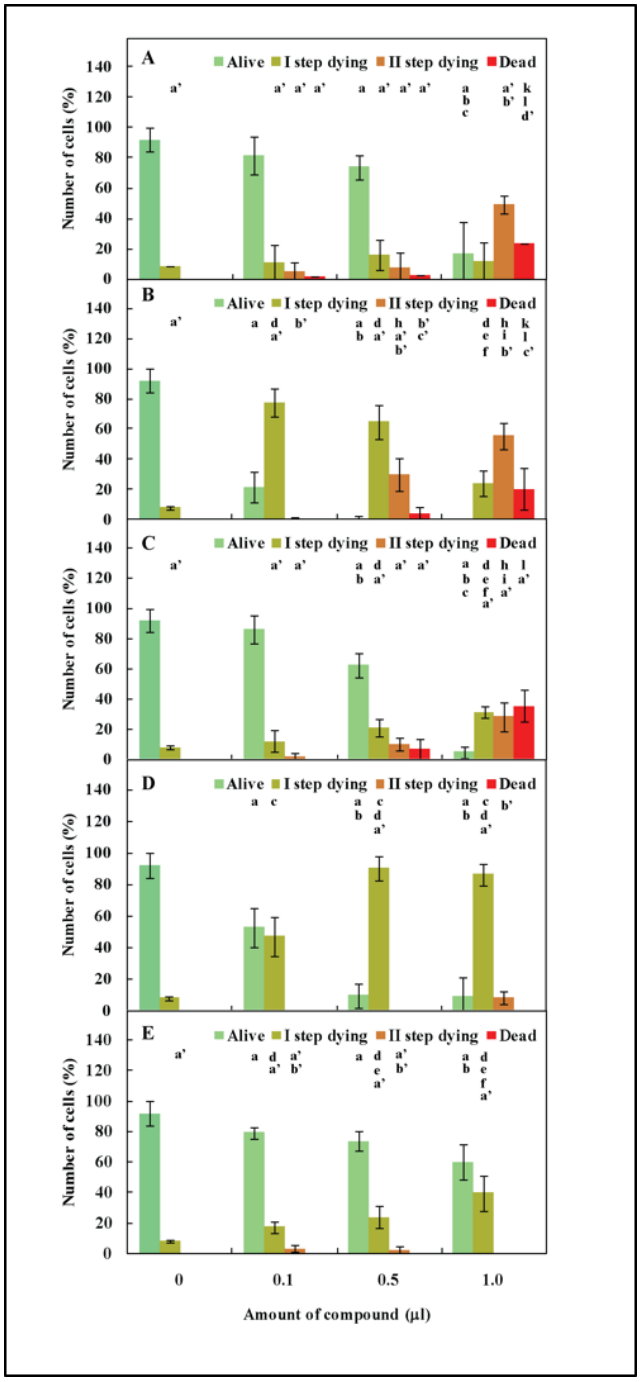



**Figure 3.9:** Numbers (%) of alive, dying (at the first – I and the second – II, step) and dead of 3-d-old BY-2 cells treated for 15 min with oil isolated from *Mentha spicata* (A), *Mentha spicata crispa* (B), *Nepeta cataria* (C) as well as pure oils of *α*-pinene (D) and β-pinene (E), estimated based on measurement of resultant fluorescence intensity after double staining with ethidium bromide and orange acridine. Bars indicated SE from two experiments repeated three times each. Letters a,b,c and d,e,f and g,h,i as well as j,k,l indicated important statistical differences at P<0,05 between results of numbers respectively of alive, dying (at the first – I and the second – II, step) and dead cells between 0 and 0.1, 0.5 or 1.0µl. Letters a',b',c'and d' indicated important differences between results of numbers respectively of alive and dying (at the first – I and the second – II step) and dead cells as well as dead and II-step dying cells within the series.

After treatment with 0.1, 0.5 and 1.0µl of oil from *Leptospermum citratum* the fraction of alive cells were not detected. The number of cell of fraction of I-step dying cells in 0.1µl series was similar to the number of alive cells and 10 times greater compared to the respective cells within control series, but in 0.5 and 1.0µl series their numbers were similar to each other and one and two times greater compared respectively to Ctrl and 0.1µl series. The numbers of cells of fraction of II-step dying cells in the 0.5 and 1.0µl series were similar to each other and were five times greater compared to respective cells in 0.1µl series. Additionally, in 0.1, 0.5 and 1.0µl series the fraction of dead cells was detected. Their numbers in 0.5 and 1.0µl series were about 50% and were about 10 times greater than in 0.1µl series (**Fig.3.10 A**).

After treatment with 0.1, 0.5 and 1.0µl of geraniol oil, the number of alive cells were not detected. In 0.5 and 1.0µl series numbers of the I- and II-step of dying cells were about 35% and 65% respectively. In 1.0µl the number of these cells were about two times lower compared to the earlier mentioned series. Additionally, fraction of dead cells appeared in this series and their number was to the numbers of II-step dying cells within this series (**Fig. 3.10 B**).

After treatment with 0.1, 0.5 and 1.0µl of β-citronellol oil the fraction of alive cells disappeared. The number of I- and II-step dying cells in 0.1 and 0.5µl was similar to each other and were about 70% and 25%, respectively. The number of existing dead cell was not statistically significant in these series but in 1.0µl series its fraction was the greatest and the number was similar to alive cells in control series (**Fig. 3.10 C**).

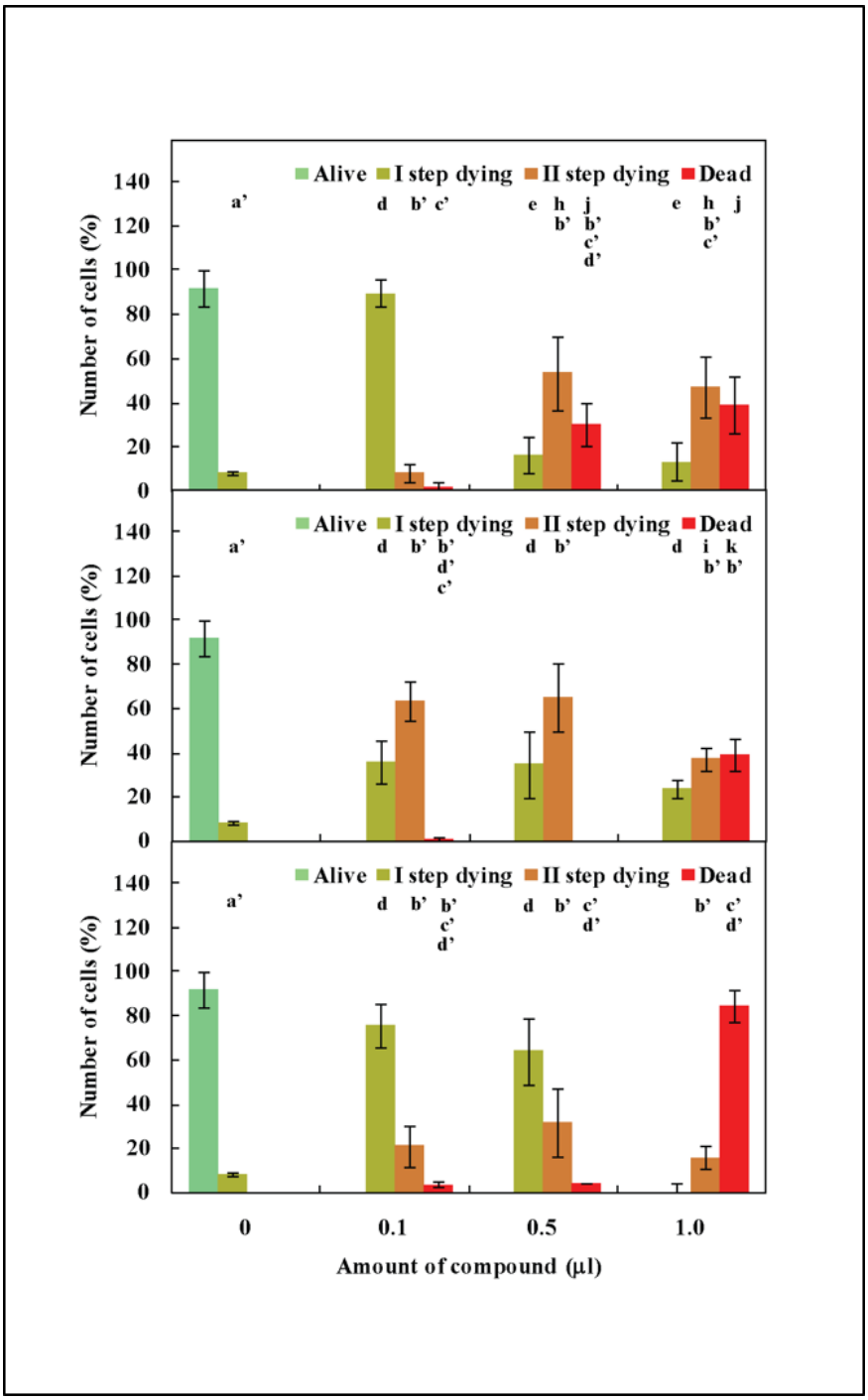



**Figure 3.10:** Numbers (%) of alive, dying (at the first – I and the second – II, step) and dead of 3-d-old BY-2 cells treated for 15 min with oil isolated from *L. citratum* (A) as well as pure oils of geraniol (B) and β-citronellol (C), estimated based on measurement of resultant fluorescence intensity after double staining with ethidium bromide and orange acridine. Bars indicated SE from two experiments repeated three times each. Letters a,b,c and d,e,f and g,h,i as well as j,k,l indicated important statistical differences at P<0,05 between results of numbers respectively of alive, dying (at the first – I and the second – II, step) and dead cells between 0 and 0.1, 0.5 or 1.0µl. Letters a',b',c'and d'indicated important differences between results of numbers respectively of alive and dying (at the first – I and the second – II step) and dead cells as well as dead and II-step dying cells within the series.

### 3.4 Effect on the cytoskeleton:

To further understand the mode of action of the compounds and the oils extracted from the plants, we used BY2 cells, *Arabidopsis thaliana* and rice plants to see cellular effects.

#### 3.4.1 BY2: Microtubules:

The effects of oils and compounds on BY2: TuB6 cell lines. Three days old BY-2 cell lines were used to see the effect of *mentha* related oils and compounds on the microtubules. In the experimental setup there were two sets of control one with

n-hexane (as it is used for dilution) (**Fig. 3.11 A, B**) and the other using Ethanol (**Fig. 3.12 A, B**) because ethanol was used as a solvent for Menthol. *A.rugosa* oil (**Fig. 3.11 C,D**) completely disrupts the microtubules after 30 mins while in case of the cells treated with the oil of *M.longifolia* (**Fig. 3.11 E, F**) *and Nepeta cataria*, (**Fig. 3.11 G, H**) the disruption of microtubules was not observed.

Cells treated with limonene (**Fig. 3.12 C, D**) show disruption of Microtubules after 5 minutes of treatment which increases after 30 minutes. Cells treated with menthone /isomenthone (**Fig. 3.12 E, F**) show start of disruption of microtubules after 5 minutes and the microtubules are visibly disrupted after 30 minutes. There is no visible effect of menthol (**Fig. 3.12G, H**) on the microtubules. The oils from *Mentha spicata* (**Fig. 3.13 A, B**) and *Mentha spicata crispa* (**Fig 3.13 C, D**) show disruption of microtubules after 30 minutes of treatment. Cells treated with $\alpha$- pinene (**Fig. 3.13 E, F**) and β-pinene (**Fig. 3.13 G, H**) after 30 minutes of treatment shows a mild disruption of MTs.

After treatment with oil of *L.citratum* (**Fig. 3.14 A,B**) the MTs get disrupted. Similarly, treatment with β-citrenellol (**Fig. 3.14E, F**) leads to disruption of MTs completely after 30 minutes. Geraniol has slight effect on the MTs after 5 minutes (**Fig. 3.14 C, D**). The strongest effect on microtubules when compared among all compounds and control treatments is in the cells treated with linalool. In case of microtubules, linalool leads to a complete break-down of the network. At the beginning a network with many fine microtubules can be observed. After 30 min no single microtubule is left. Additionally, many vesicle-like structures can be noticed in the cells (**Fig. 3.19 A, B**)

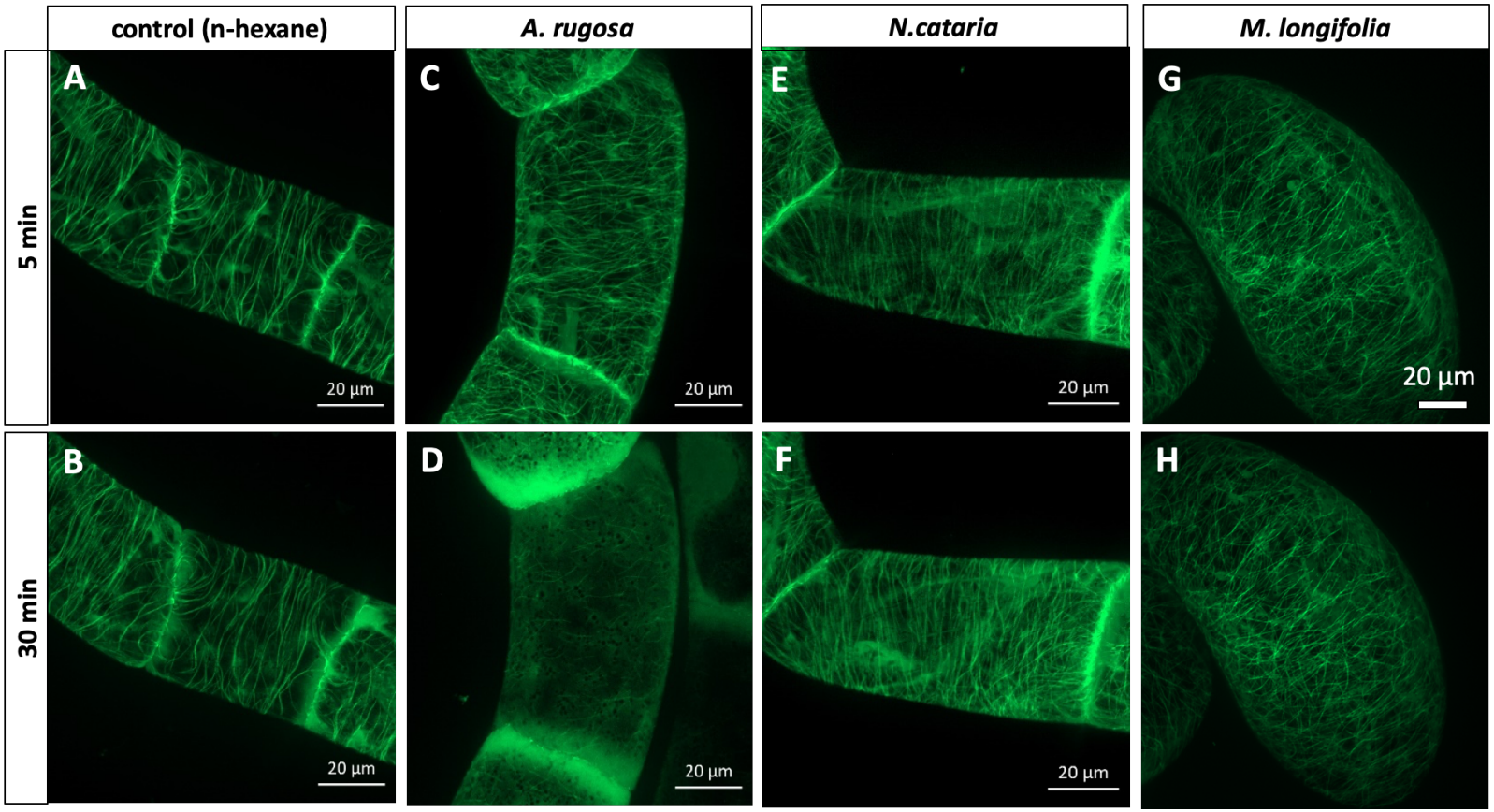



**Figure 3.11:** Live cell imaging of BY2: Tub6 cells. In order to see the effects of the different essential oils of plants from Mint family, cells were incubated with 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from *A. rugosa* (C and D) *N. cataria* (E and F) and *M. longifolia* (G and H) were compared after 5minutes (A, C, E, G) and 30 minutes (B, D, F, H) with control cells (A and B). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000)

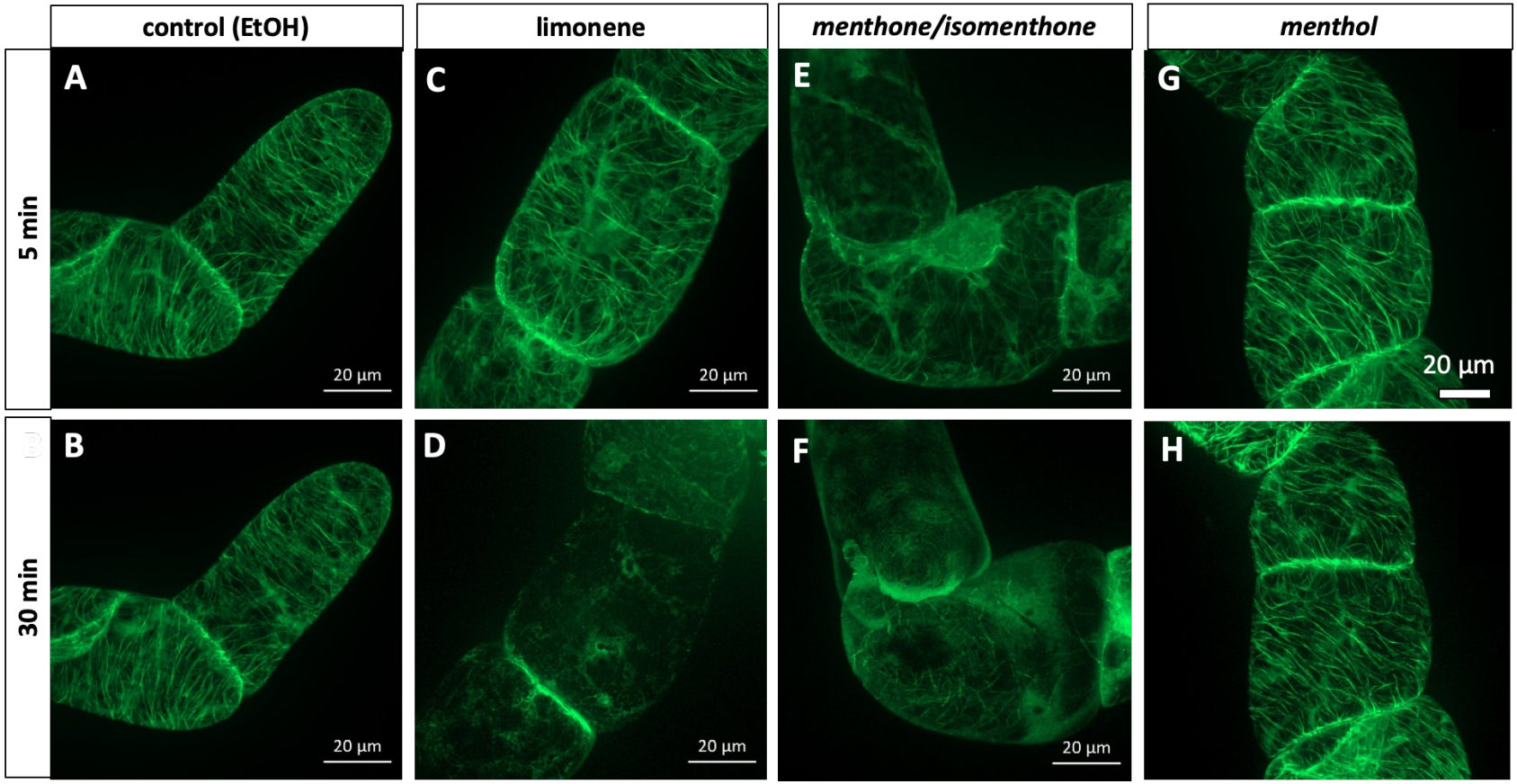



**Figure 3.12:** Live cell imaging of BY2: Tub6 cells. In order to see the effects of the different compounds of the essential oils of plants from Mint family. Cells were incubated with 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from limonene (C and D) menthone/isomenthone (E and F) and menthol (G and H) were compared after 5minutes (A, C, E, G) and 30 minutes (B, D, F, H) with control cells treated with ethanol(A and B). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000)

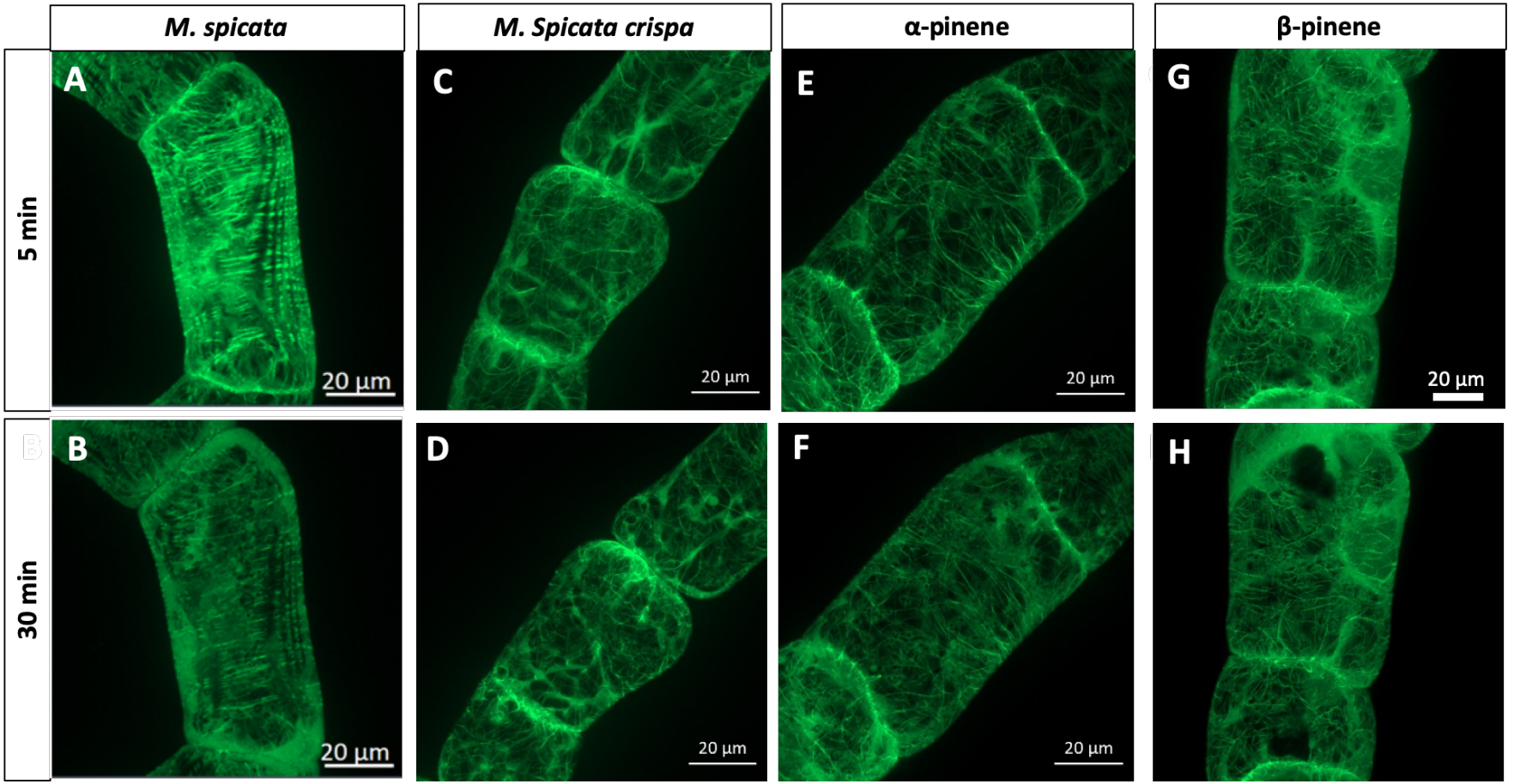



**Figure 3.13:** Live cell imaging of BY2: Tub6 cells. In order to see the effects of the different essential oils of plants from Mint family, cells were incubated with 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from *M. spicata* (A and B) *M. spicata crispa* (C and D) and *α*-pinene (E and F) and β- pinene (G and H). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000)

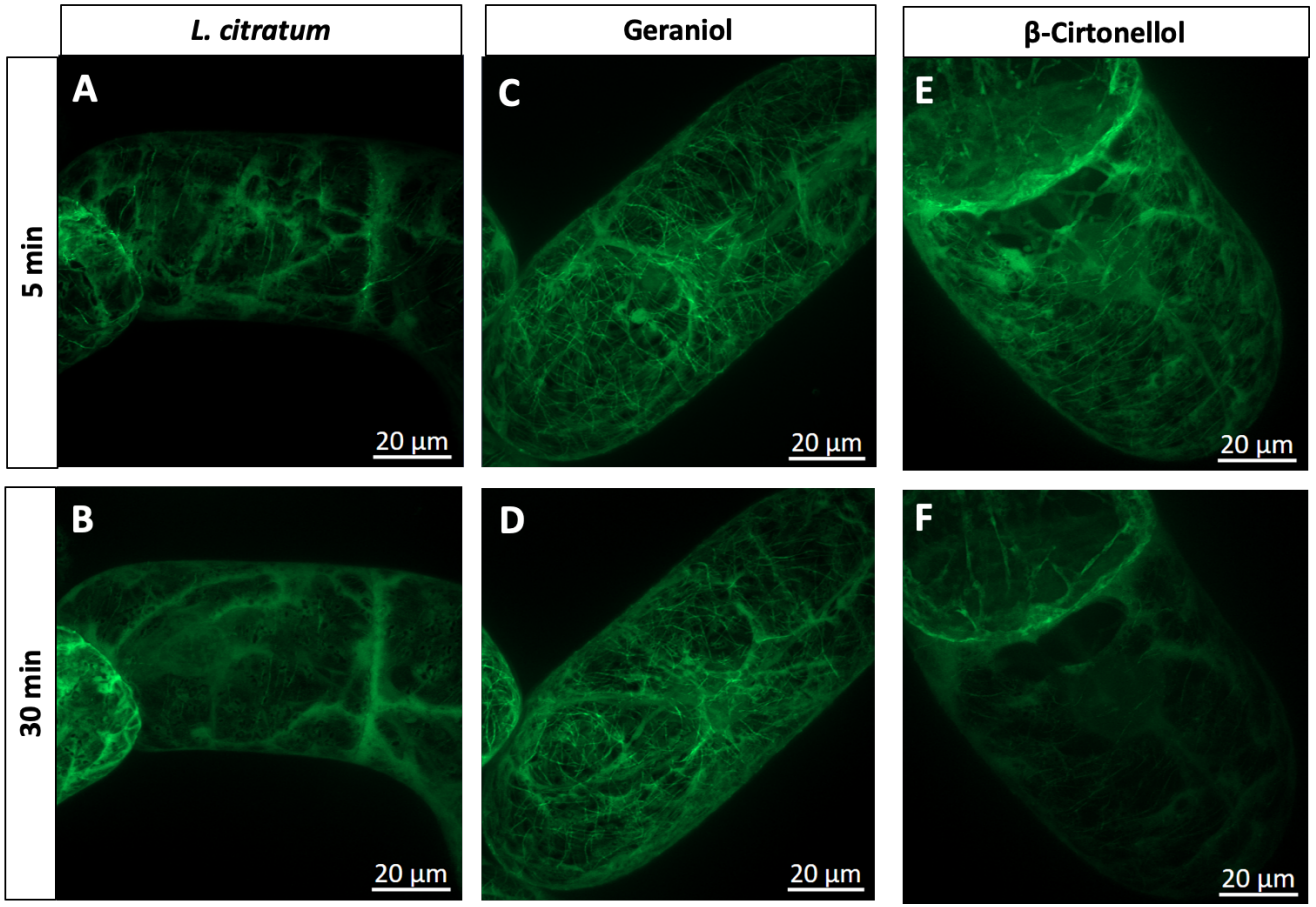



**Figure 3.14:** Live cell imaging of BY2: Tub6 to see the effects essential oils of *L.citratum* and its constituent compounds. Cells were incubated with 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from *L.citratum* (A and B) Geraniol (C and D) and β-Citronellol (E and F). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000)

### 3.4.2 BY2: actin filaments:

In order to see the specificity of the mode of action of the oils and compounds, three days old BY2:GF11 cell lines were used to see the effect of *mentha* related oils and compounds on the actin filaments. In the experimental setup there were two sets of control one with n-hexane (as it is used for dilution) (**Fig. 3.15 A, B**) and the other using Ethanol (**Fig. 3.16 A, B**) because ethanol was used as a solvent for Menthol. Cells treated with oils of *A. rugosa* (**Fig. 3.15 C, D**) and *Nepata cataria* (**Fig. 3.15 E, F**) showed no visible effect after 5 minutes. However, in case of treatment after 30 minutes There was some disruption of actin filaments. The effect of *M.longifolia* oil (**Fig. 3.15 G, H**) is very significant and just after 5 minutes of treatment the actin filaments get disrupted.

The effect of limonene on actin filaments can be seen even after 5 minutes (**Fig. 3.16 C, D**) and after 30 minutes the filaments are disrupted. In case of the cells, actin disruption is not visible when treated with 1µl menthone/isomenthone (**Fig. 3.16 E, F**) even after 30 minutes. Treatment of cells with menthol (**Fig 3.16 G, H**) did not affect the actin filaments.

Cells treated with oils of *M.spicata* (**Fig. 3.17 A, B**)and *M. spicata crispa* (**Fig. 3.17 C,D**) showed no visible effect after 5 minutes. However, in case of treatment after 30 minutes in case of *M. spicata* there was some disruption of actin filaments while it was completely disrupted in *M. spicata crispa*. β-pinene(**Fig. 3.17 G, H**) disrupts the actin filaments strongly after 30 minutes of treatment while *α*- pinene (**Fig. 3.17 E, F**) has

no effect. After treatment with oil of *L.citratum* (**Fig. 3.18 A, B**) the actin filaments get disrupted completely. Similarly, treatment with Geraniol (**Fig. 3.18 C, D**) leads to disruption of AFs. It is clearly visible that the oil and geraniol lead to vesicle like structures. β-citrenellol has no effect or mild effects on the AFs (**Fig. 3.18 E, F**). In case of cells treated with linalool there effect is clearly visible as the cells show actin disruption after 30 minutes. However, the effects of linalool are not as strong in actin as on microtubule. (**Fig. 3.19 A', B'**)

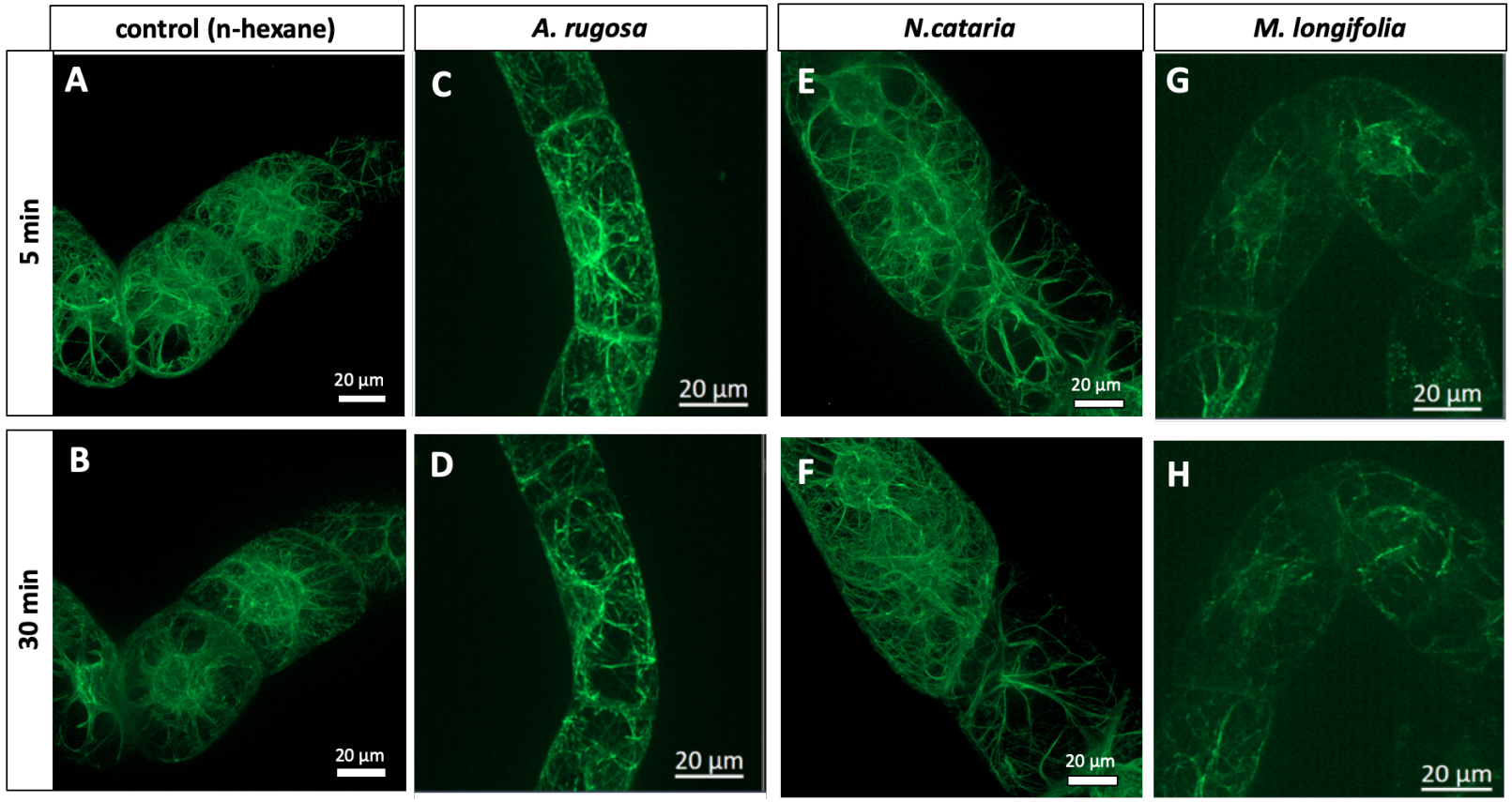



**Figure 3.15:** Live cell imaging of BY2: GF11 cells. In order to see the effects of the different essential oils of plants from Mint family, cells were incubated with 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from *A. rugosa* (C and D) *N. cataria* (E and F) and *M.longifolia* (G and H) were compared after 5minutes (A, C, E, G) and 30 minutes (B, D, F, H) with control cells (A and B). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000)

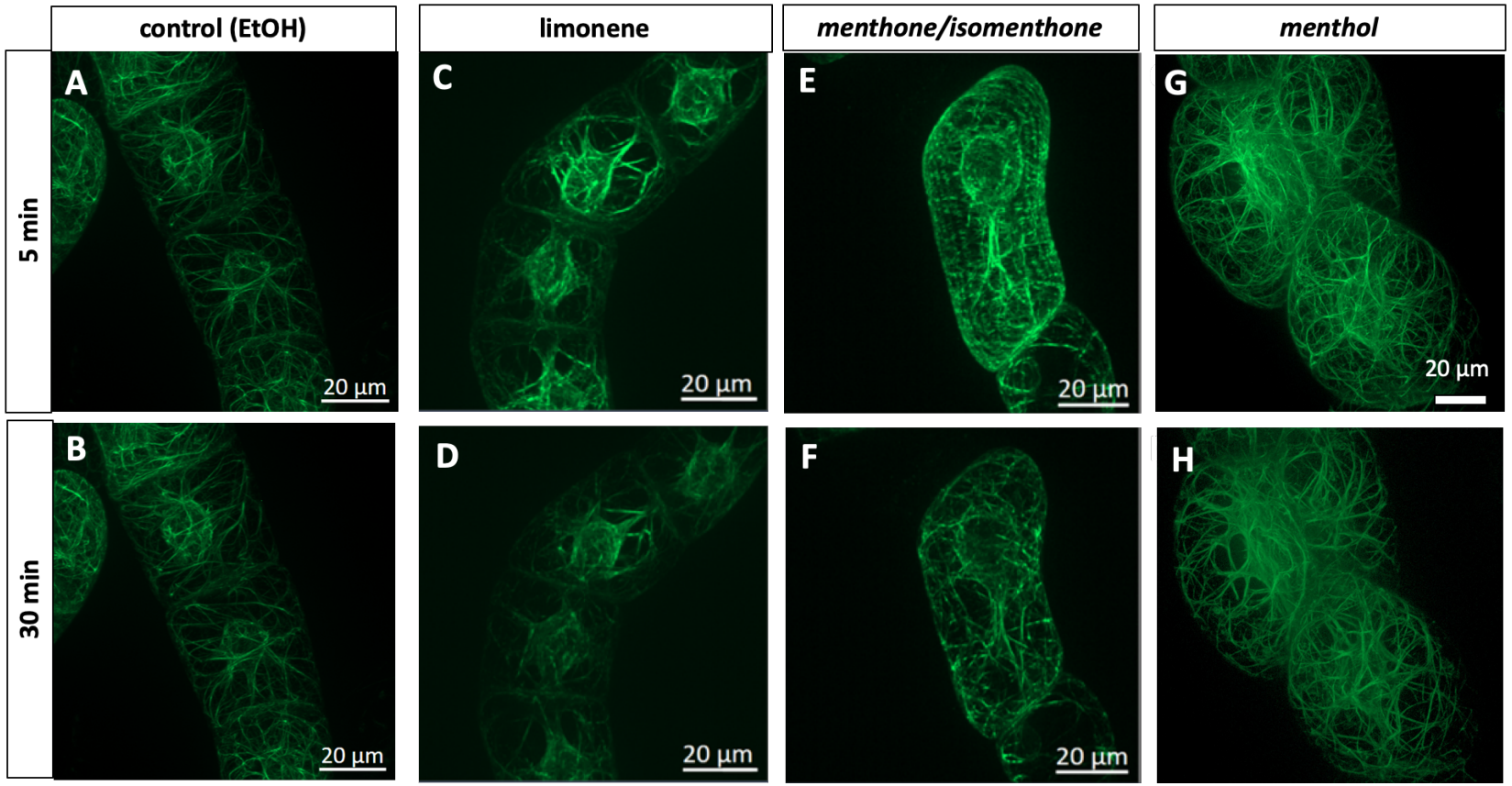



**Figure 3.16:** Live cell imaging of BY2: GF11 cells. In order to see the effects of the different compounds of the essential oils of plants from Mint family. Cells were incubated with 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from limonene (C and D) menthone/isomenthone (E and F) and menthol (G and H) were compared after 5minutes (A, C, E, G) and 30 minutes (B, D, F, H) with control cells treated with ethanol(A and B). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000)

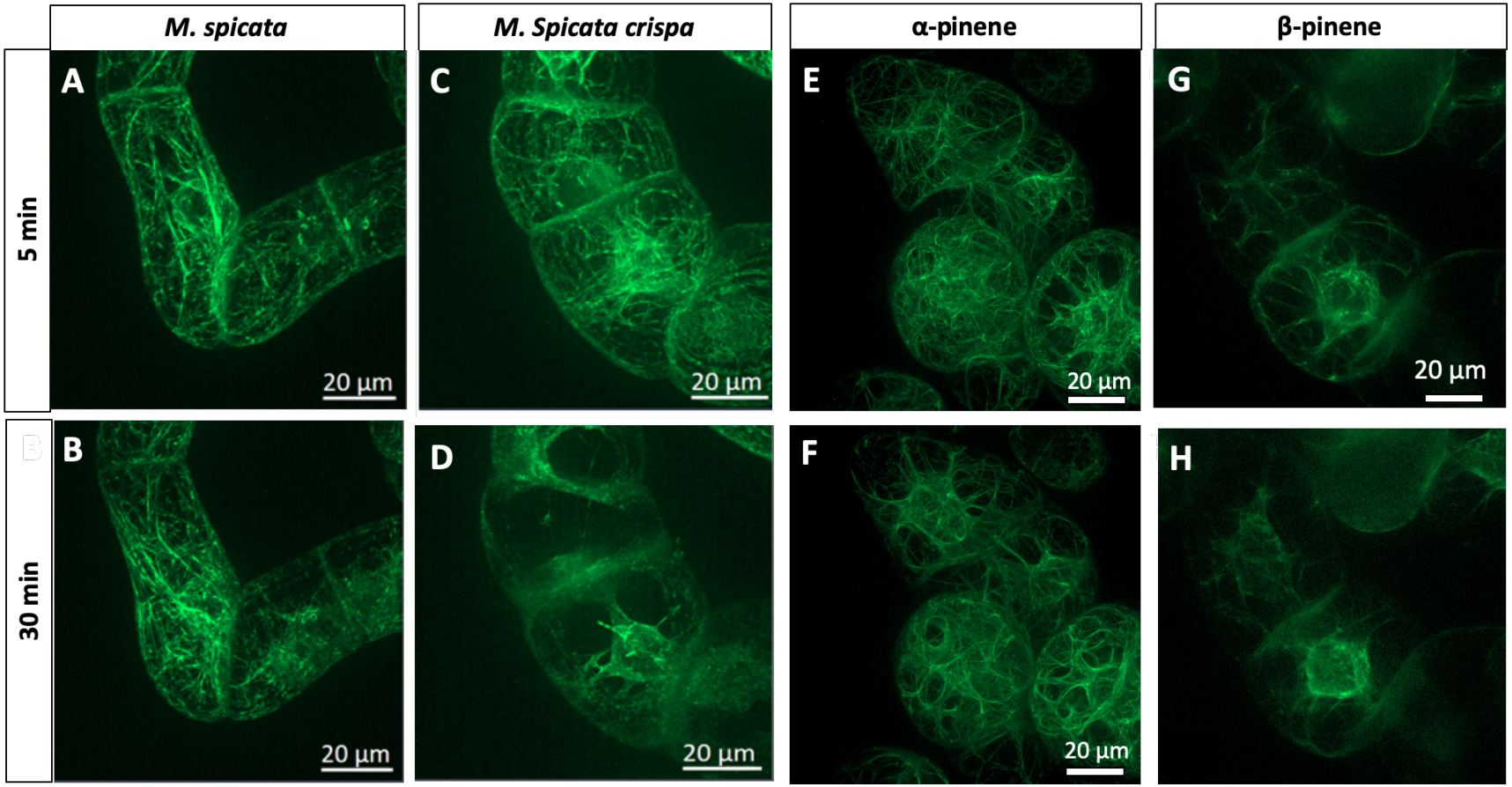



**Figure 3.17:** Live cell imaging of BY2: GF11 cells. In order to see the effects of the different essential oils of plants from Mint family, cells were incubated with 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from *M.spicata* (A and B) *M.spicata crispa* (C and D) and α-pinene (E and F) and β-pinene (G and H). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000).

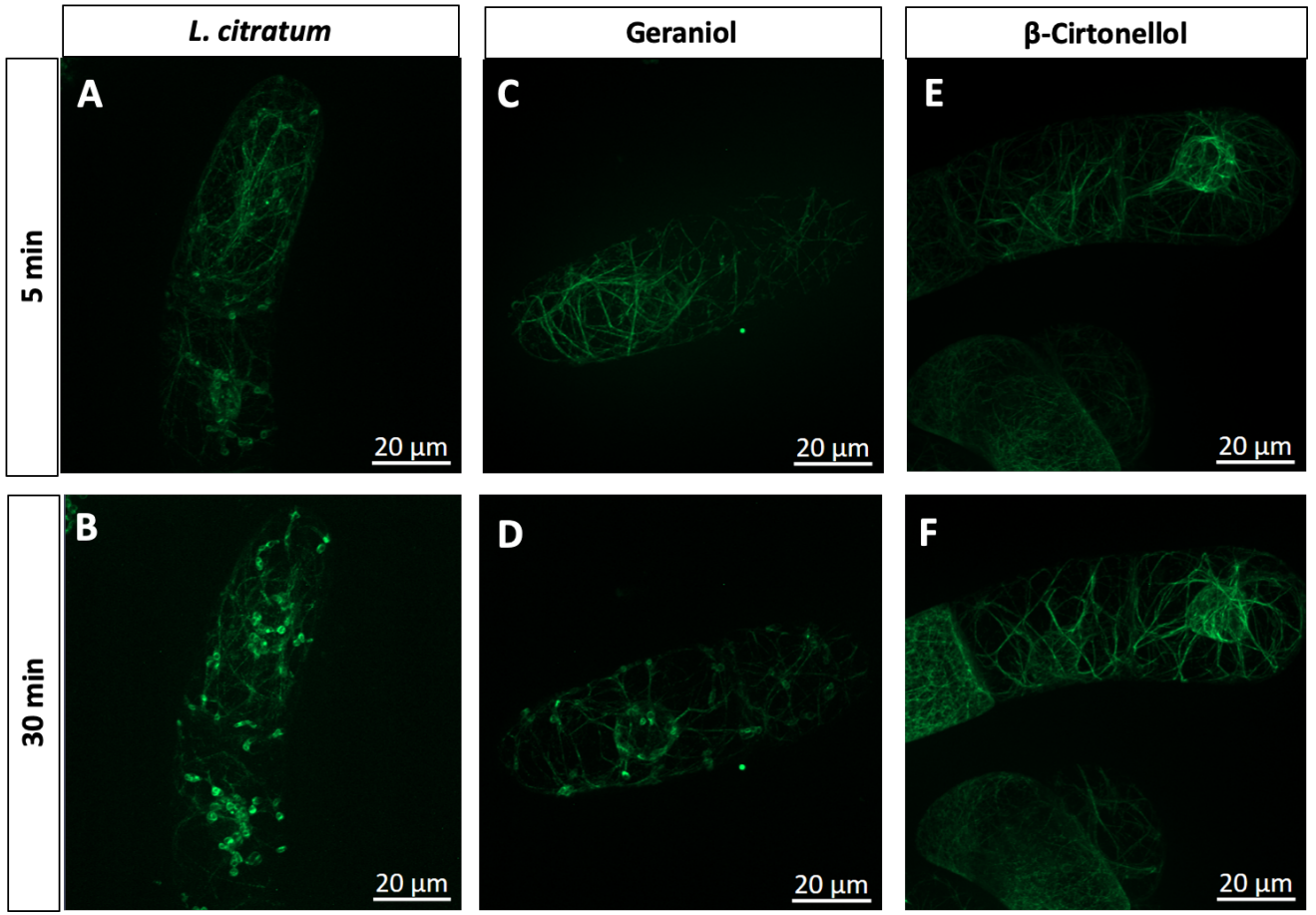



**Figure 3.18:** Live cell imaging of BY2: GF11 to see the effects essential oils of *L.citratum* and its constituent compounds. Cells were incubated with the 1µl of oils or compounds and observed under spinning disk microscope. The effects of oils from *L.citratum* (A and B) Geraniol (C and D) and β-Citronellol (E and F). The pictures were taken using Zeiss Cell Observer Spinning Disc (Zeiss, Jena, Germany) equipped with a cooled digital CCD camera (AxioCam MRm), and a spinning-disc device (YOKOGAWA CSU-X1 5000)

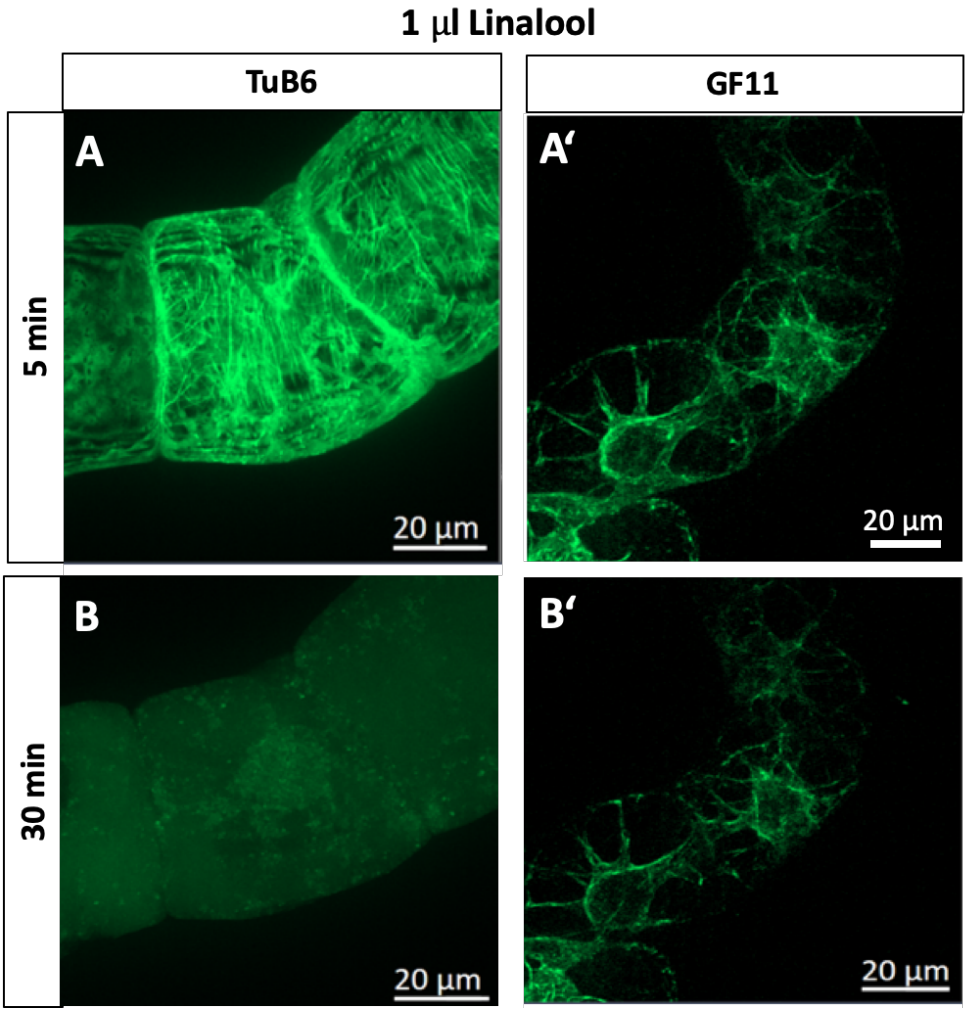



**Figure 3.19:** Live cell imaging of BY2: TuB6(A, B) and GF11(A', B') cell lines. In order to see the effects of Linalool on MTs and actin filaments cells were incubated with 1µl linalool and observed under spinning disk microscope after 5 and 30 mins.

### 3.4.3 Effects on *Arabidopsis thaliana* cytoskeleton

For the first experiment essential oil from *A.rugosa* was extracted via steam distillation as described before. To be able to observe the effect of the *A.rugosa* extract to microtubules in planta, an *A.thaliana* mutant containing GFP labelled tubulin was exposed to 50 µl of the essential oil and observed immediately after application by an Axiobserver Z1 confocal microscope. To get a view on the whole plant, pictures of different plant parts were observed. Control plants were placed in 50µl distilled water. The results (**Fig. 3.13, 3.14 A, B, E, F**) show in control plants well visible microtubules,

with the typical mesh-like structure. After exposure to the essential oil from *A. rugosa*, they start to disintegrate immediately. A difference in effectiveness within the different parts of the plant is also observable. While in cells of the cotyledon the microtubules seems to be able to resist longer to the effect of the essential oil, the further one travels towards the root the more the microtubules are depolymerized. On treatment with menthone/isomenthone, the microtubules show similar effects (**Fig. 3.14 C, D**) as when exposed to oil from *A. rugosa* plants (**Fig. 3.13 C, D**).

In case of *A.thaliana* plants where FABD is labelled with GFP, application of menthone/isomenthone completely disrupts the actin filaments (**Fig. 3.14 G, H**). However we observed that the application of *A.rugosa* oil had no effect on the actin filaments (**Fig. 3.13 G, H**).

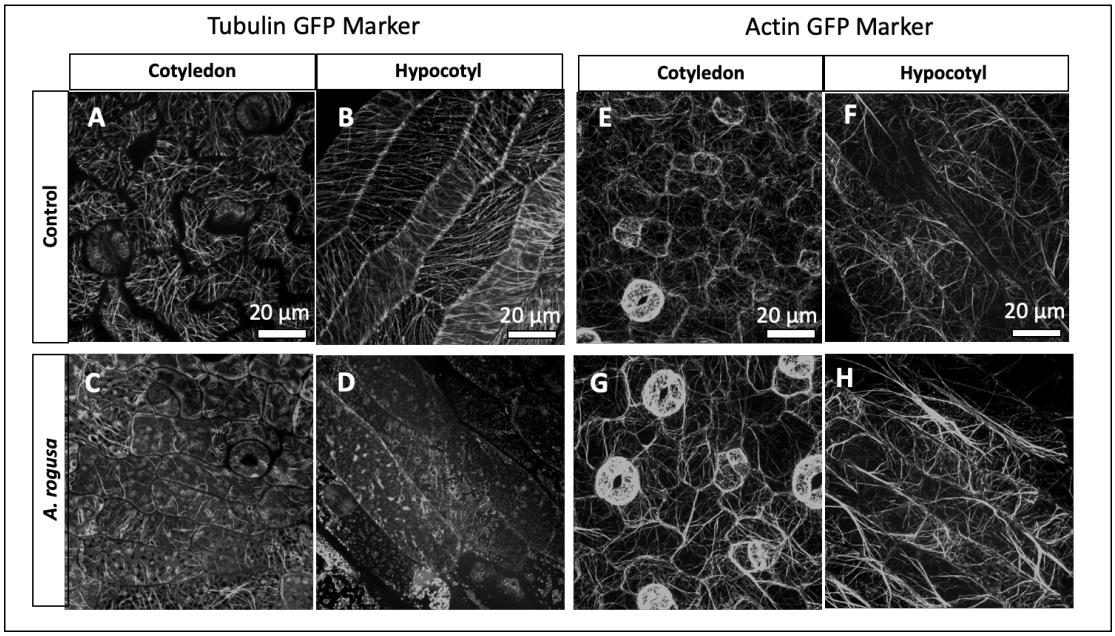



**Figure 3.20:** Effect of *A.rugosa* oil on cytoskeleton. Microtubules of cotyledon (A) and hypocotyl (B) get disrupted after treatment with oil (C, D). actin filaments of cotyledon (E) and hypocotyl (F) have no effect after treatment with oil (G, H).

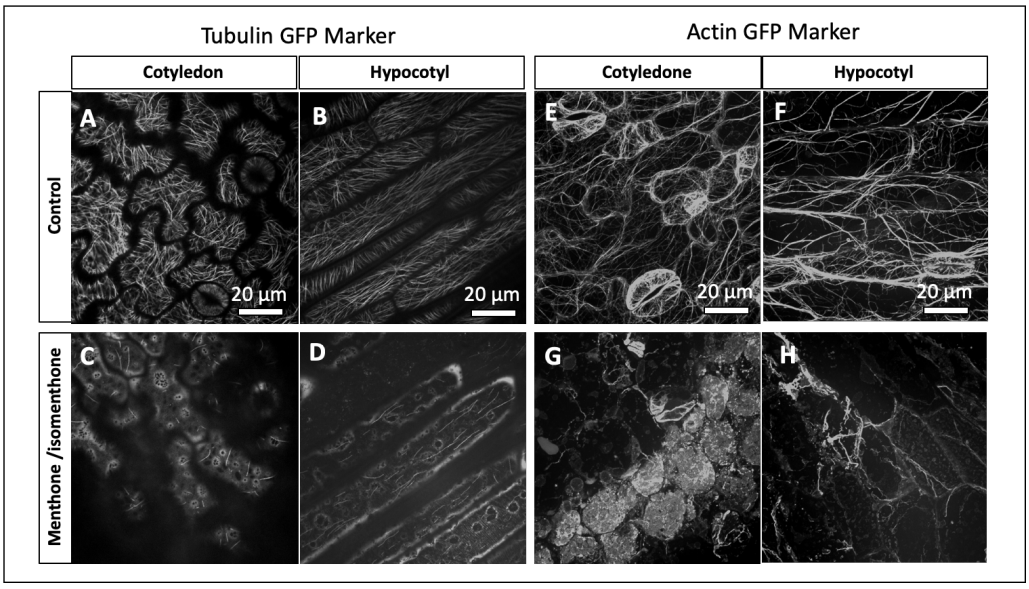



**Figure 3.21:** Effect of menthone/isomenthone on cytoskeleton. Microtubules of cotyledon (A) and hypocotyl (B) get disrupted after treatment with menthone (C, D). actin filaments of cotyledon (E) and hypocotyl (F) are also disrupted after treatment with menthone (G, H).

### 3.4.4 Microtubule disruption in rice

Immunofluorescence of rice roots treated with menthone/isomenthone clearly shows disruption of microtubules in both Dongjin and TTL(ox) (**Fig. 3.22 D and E**) when compared to microtubules of the control roots (**Fig. 3.22 A and B**). Surprisingly the microtubules of Sathi roots (**Fig. 3.22 C**) after treatment with menthone/isomenthone look more bundled rather than being disrupted (**Fig. 3.22 F**). The results show that exposure of rice seedlings to menthone/isomenthone has detrimental effects on the microtubules. The microtubules in the roots of Dongjin and TTL (ox) lines clearly show disruption of microtubules. Whereas in case of roots of Sathi, the microtubules become more bundled on exposure to menthone/isomenthone, when compared to the control roots.

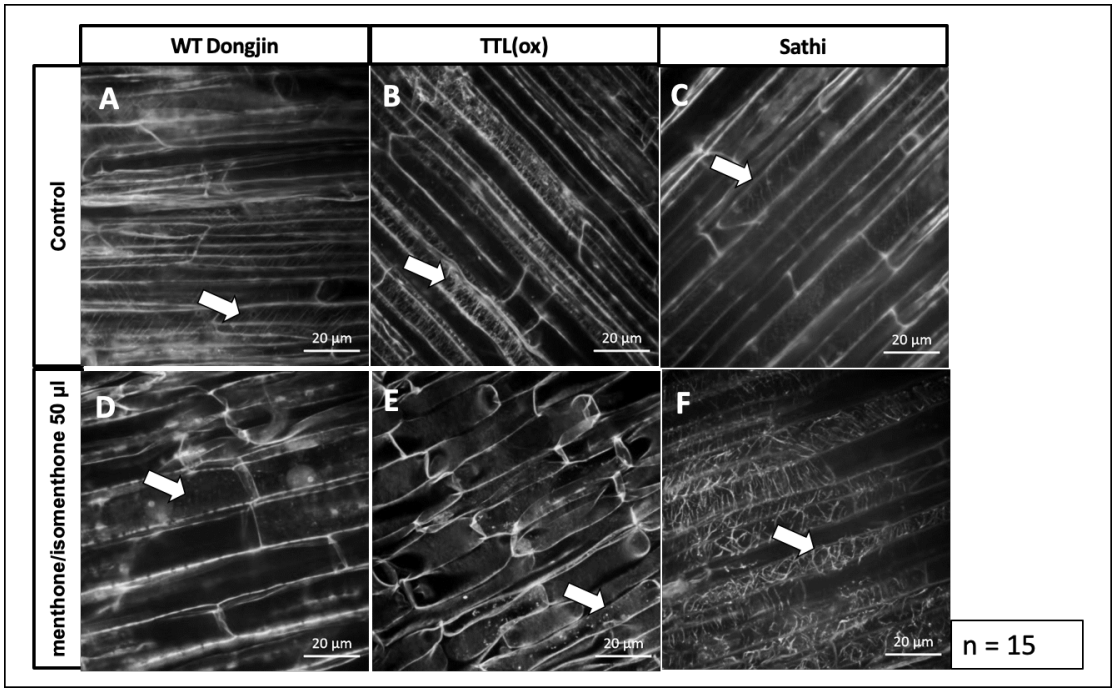



**Figure 3.22:** Effect of menthone/isomenthone on Rice microtubules. Roots of Dongjin (A) and TTL (ox) (B) show the microtubules which get disrupted after exposure to menthone/isomenthone (D and E). In case of Sathi roots (C)m the microtubules bundled after menthone/isomenthone treatment (F). Arrows show the microtubules.

The exposure of rice seedlings to menthone clearly has an effect on growth of rice (**Fig. 3.23 A, B**). The coleoptile length does not change significantly but the length of roots are inhibited after exposure to menthone.

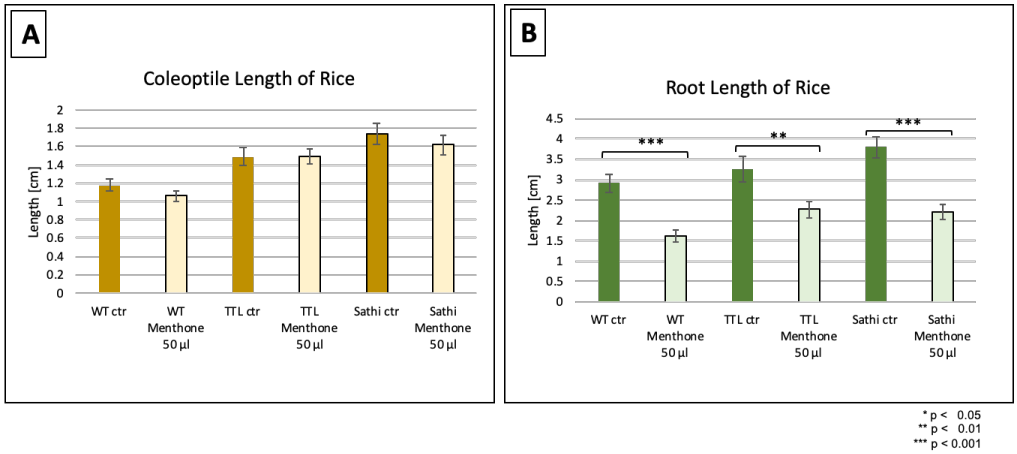



**Figure 3.23:** Effect of menthone/isomenthone on growth of rice seedlings. The length of coleoptile (A) and roots (B) was measure in the three genotypes of rice used in the study. The differences in the coleoptile were not significant between control and treated plants of each genotype. The root length was affected between the control and treated plants of each genotype.

## 3.5 Efficiency of compounds and plant oils as bioherbicides

### 3.5.1 Effects on germination

In order to check whether the extracted oils from the mentha species have an effect on growth, germination tests were made using cress, tomato, winter wheat and purple tansy (**Fig. 3.24**).

A volume of 1 and 10µl of different oils and compounds were used. The germination test of cress shows that limonene, α-pinene and β-pinene have no effect on the germination of Tomato seeds. *Mentha longifolia* extract, menthone/isomenthone and linalool at a concentration of 10µl do not let the seeds of cress grow. The effect of *M.longifolia* and menthone/isomenthone at 1µl reduces germination by 80%. Linalool at 1µl reduces germination by 25% (**Fig. 3.24 A**).

In case of purple tansy that limonene, α-pinene and β-pinene don't have significant effect on the germination of seeds. *M.longifolia* extract at a concentration of 10µl do not let the seeds grow while at 1µl the germination is reduced by 43%. menthone/isomenthone reduces germination by 60%. Linalool at 1µl reduces germination by 35% and at 10µl by 80% (**Fig. 3.24 B**).

Tomato seedlings are not affected significantly by limonene, α-pinene and β-pinene. However, linalool at 10µl stops germination while with 1µl the germination is reduced by 80%. Similarly, 10µl extract of *M.longifolia* inhibits germination by 95% while with 1µl germination is reduced by 50%. menthone/isomenthone at 10µl reduces germination by 70% and 1µl by 25 % (**Fig. 3.24 C**).

Winter wheat seedlings are not affected significantly by limonene, α-pinene and

β-pinene. However, linalool at 10µl inhibits complete germination while with 1µl the germination is reduced by 80%. Similarly, 10µl extract of *M.longifolia* inhibits germination by 95% while with 1µl germination is reduced by 40%. menthone/isomenthone at 10µl reduces germination by 70% and 1µl by 50% (**Fig3.24D**).

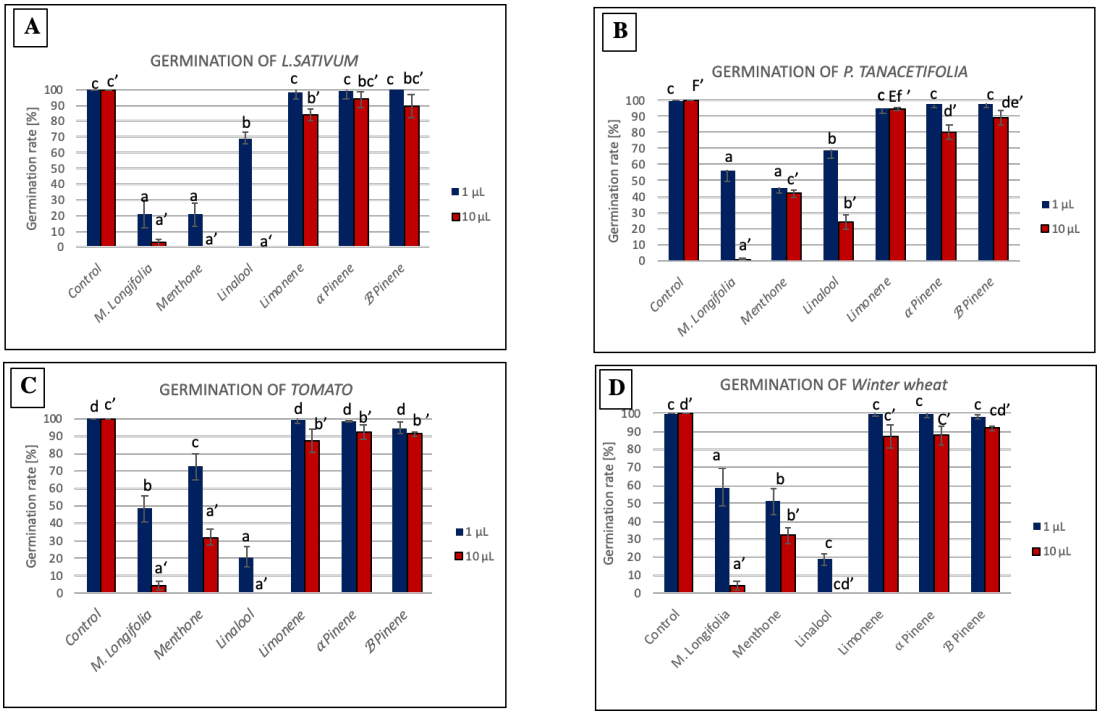



**Figure 3.24:** Effects of menthone/isomenthone, linalool limonene, α-pinene, β-pinene and *M.longifolia* oils were tested on *L.sativum* (A*), P.tanacetifolia* (B), Tomato (C) and winter wheat (D). Two volumes 1µl and 10µl of the oil and compounds were used to check the effects on the plants.

In case of *Rumex* seeds were grown and tested for germination rate on different extracts (**Fig. 3.25 A**). 10µl of menthone/isomenthone inhibits growth. On the contrary linalool and limonene seem to enhance germination. (**Fig. 3.25 B**).

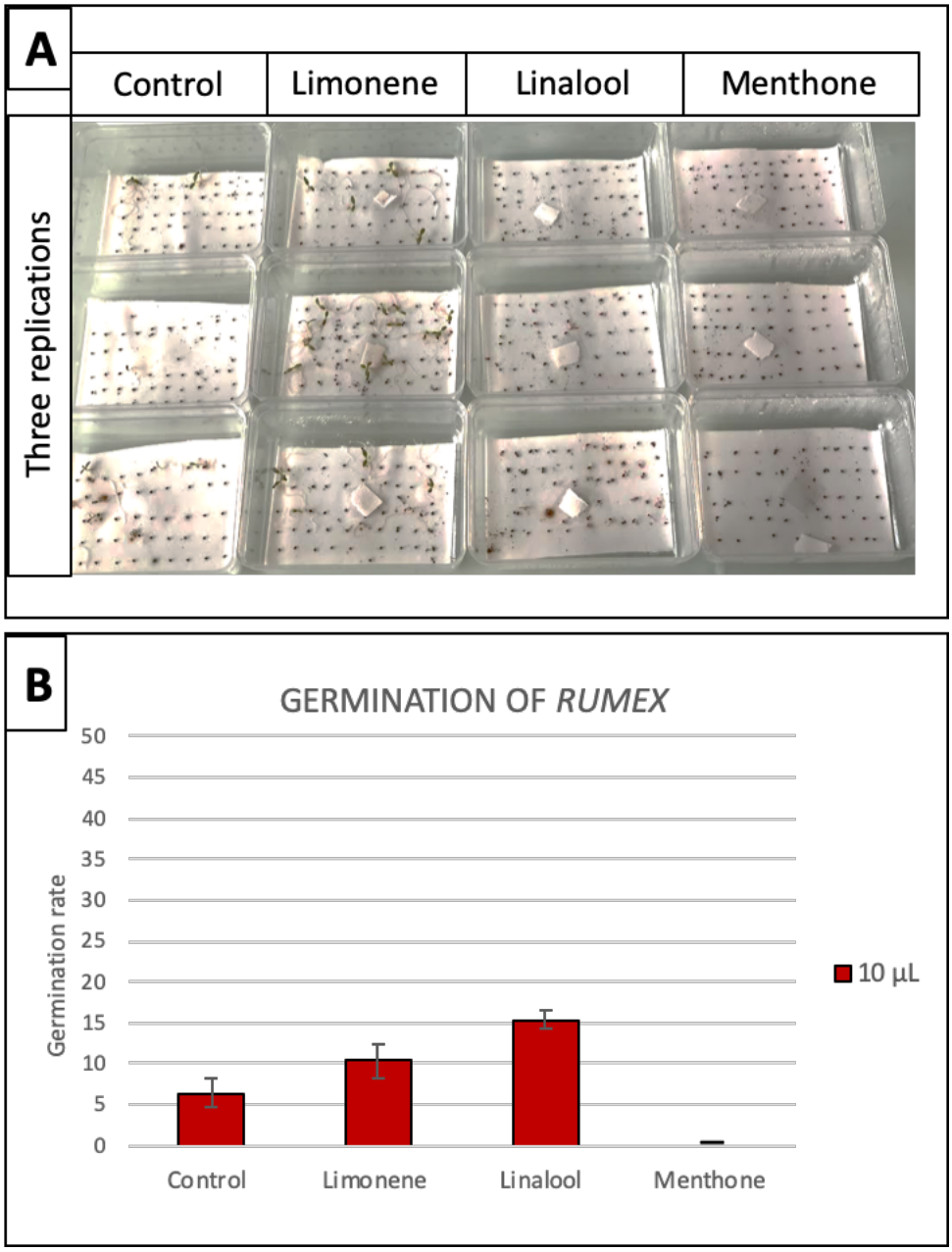



**Figure 3.25:** Effect of limonene, linalool and menthone/isomenthone on growth of *Rumex* seeds were grown with the compounds in a plastic box (Fig3.25A). The germination test was done to see effects on *Rumex*. 10µl of compounds was used to see the effects (Fig3.25B)

### *3.5.2* Effects on growth of *convolvulus*

The effect of 50µl in (2)g of extracted oils and compounds in / on Soil were tested on *convolvulus* plants by applying them along with the formulated substances on the surface of soil and by placing them with the convolvulus cuttings **(Fig. 3.26 A**). Fresh weight of plants grown with *A.rugose* oil and menthone/isomenthone show increase in

mass. *M.spicata crispa* oil, Limonene and Linalool have effects which are not so visible. Fresh weight decreases in plants grown with *M.longifolia* oil. Similar to the effect on fresh weight, shoot length and number of leaves (**Fig. 3.26 B, C**) are more in plants treated by *A rugosa* oil and menthone/isomenthone. *M.Spicata crispa* oil, limonene and linalool do not have effects while there is a decrease in number of leaves and shoot length (**Fig. 3.26 B, C**) in plants grown with *M.longifolia* oil. A higher concentration of limonene in both applied and rhizome treatments enhances shoot length, fresh weight and number of leaves. Plants treated with menthone/isomenthone show a reduction in shoot length, fresh weight and number of leaves. Application of linalool treated substance on top does not let the plants germinate while the ones where they were treated in rhizome show reduced shoot length, fresh weight and number of leaves.

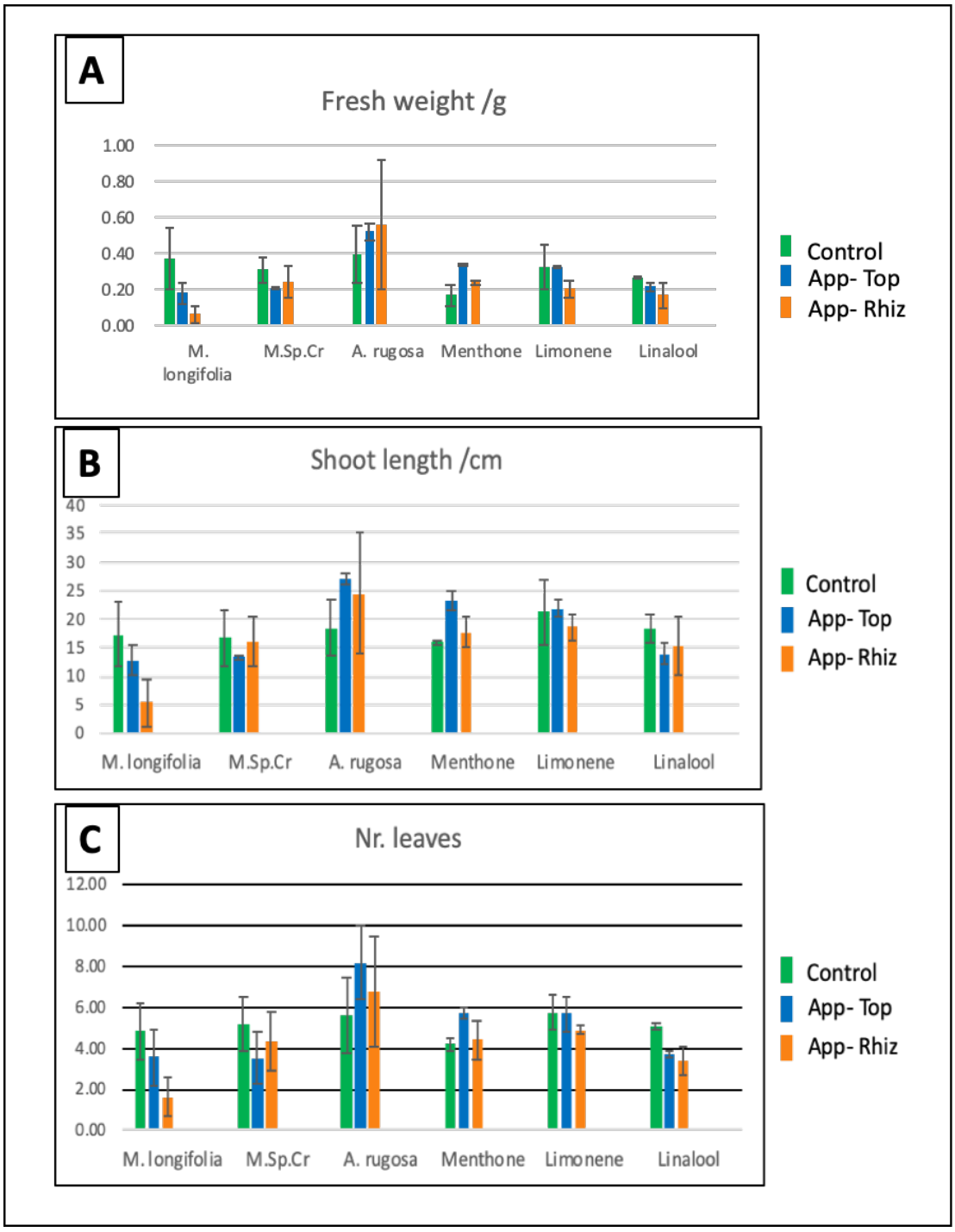



**Figure 3.26:** Effect of oils, menthone/isomenthone, limonene and linalool on c*onvolvulus* using formulated material. (A) 50µl of the respective oils and compounds were added into 2g of formulated substance. This was either placed on the surface of soil (App-Top) or was placed inside the soil (App-Rhiz). Fresh weight of the convolvulus plants was measured to see the effect of the oils and compounds on growth. (B) The shoot length was measured to compare the effects of the oils after two weeks of treatment. (C) The leaves of the *convolvulus* plants were also counted to see effects of the oils and compounds in the treatment plants compared with control plants.

### 3.6 *Agastache rugosa* shoot as potential weed control

In order to test the potential of *Agastache rugosa* as a potent weed control, chopped *Agastache rugosa* shoot was put on the surface of the soil in pots with cress, tomato, winter wheat and *P.tanacetifolia*. In case of cress and tomato there is a slight increase in fresh wheat of plants grown with *A.rugosa* when compared to the control plants (**Fig. 3.27**). However winter wheat and *P.tanacetifolia* show a slight decrease in the fresh weight of the plants.

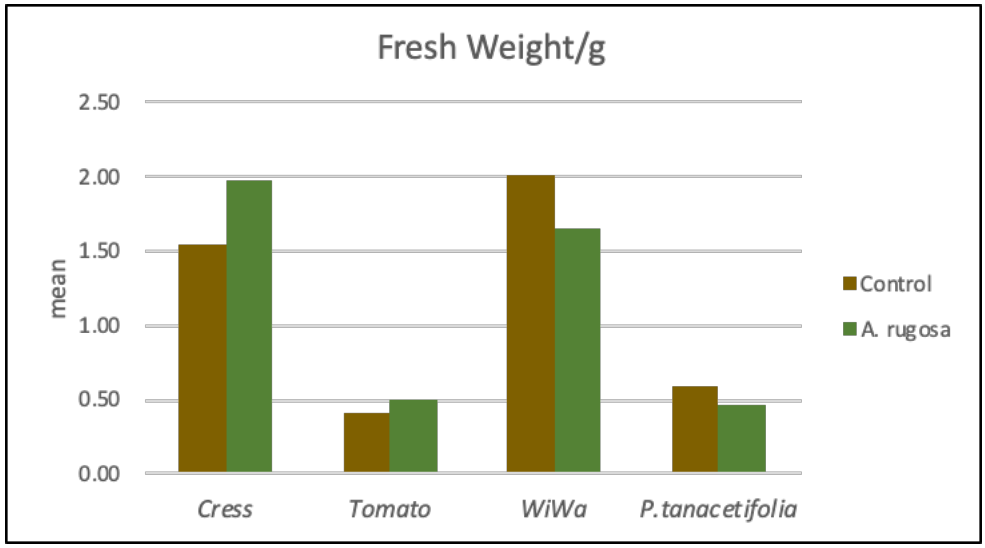



**Figure 3.27:** To test the efficiency of *Agastache rugosa*, the shoots of the *Agastache rugosa* plants were chopped into small pieces and placed on the surface of soil in the pots. Some soil was sprinkled on it.

### 3.7 Test of compounds as spray

In order to test the effect of the compounds on orchard plants like apple and grapes we tested concentrations of the compounds. When compared to the control plants, there was no difference between the 2 concentrations used of the compounds. Only linalool at 5% concentration is detrimental to the plants (**Fig. 3.28**).

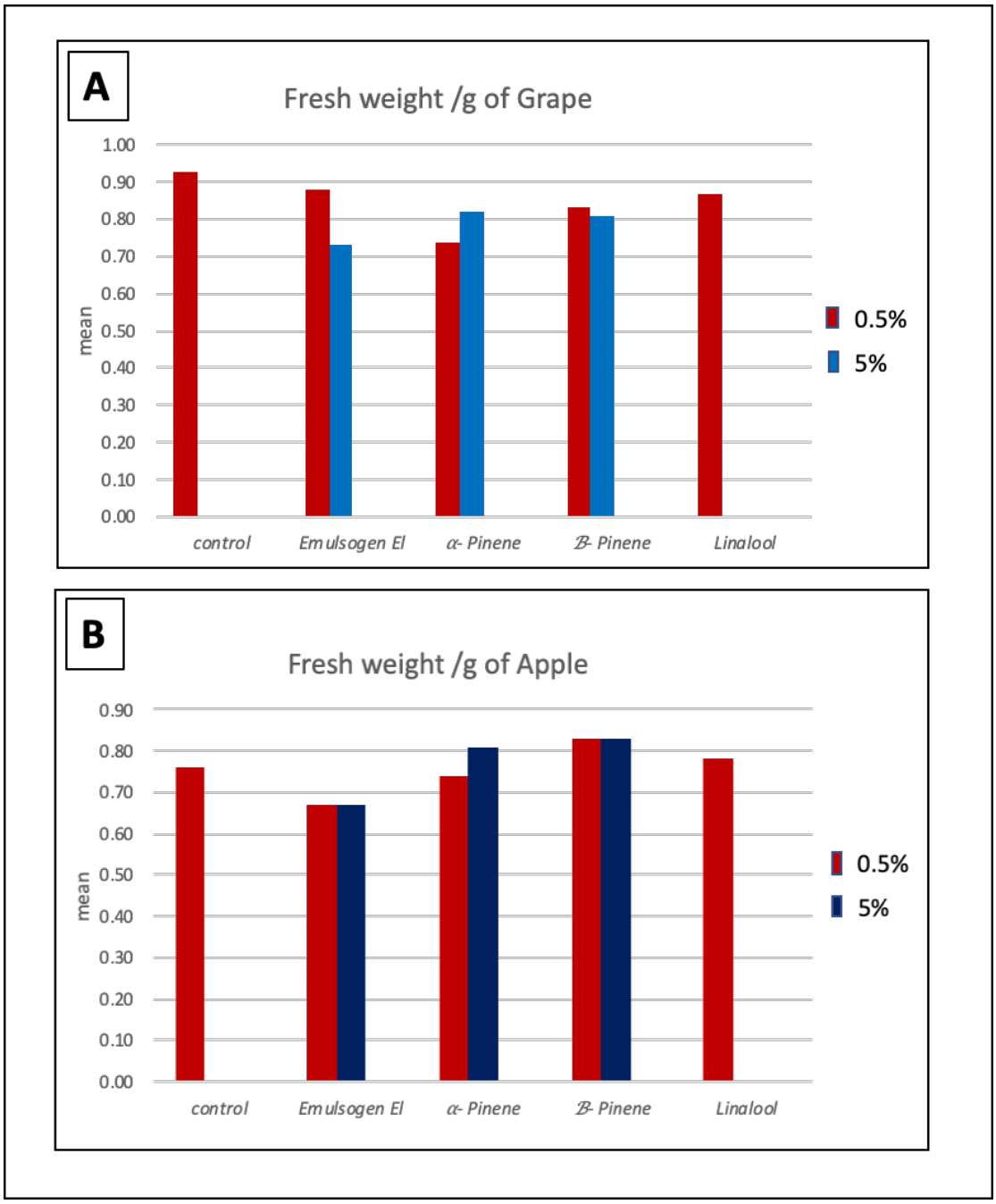



**Figure 3.28:** Effect of *mentha* related compounds on growth of Grapevine (A) and apple seedlings (B). To test the effect of α-pinene, β-pinene and linalool, plantlets of apple and grapevine were sprayed with the compounds in two concentrations (0.5 and 5 % v.v) and the fresh weight was measured after one week. Control plants were sprayed with only water. An additional control was done using only Emulsogen EL360.

### 3.8 Screening of compounds for effects on viability of HeLa Cells

Compounds from the mentha plants have clearly shown a mode of action specific to the cytoskeleton in case of plants. menthone, in particular, in case of BY2 cells, *Arabidopsis thaliana* and rice, shows a specificity to microtubules. It is well known that in case of cancer treatment, anticancer drugs target the cytoskeleton. To look at the potential of *mentha* related compounds as anticancer drugs, we tested the effect of α-pinene, β-pinene, menthone, limonene and linalool on HeLa cells. There was no significant effect of β-pinene on cell viability in any concentration of the compound. α-pinene on the other hand leads to cell death when used in higher concentration. When 0,25µl of *α*-pinene was used the viability was 100% and it reduced to 20% when 0,5µl was used and at 1,25µl and 2,5µl none of the cells were alive. In case of cells treated with menthone the effects are variable. 0.25µl, 1,25µl and 2,5µl of menthone have no effect on cell viability (**Fig. 3.29**). The cells treated with 0,5µl of menthone showed a reduction in cell viability. Limonene and linalool have very strong effects on cell viability even when the cells were treated with low amounts of the compounds.

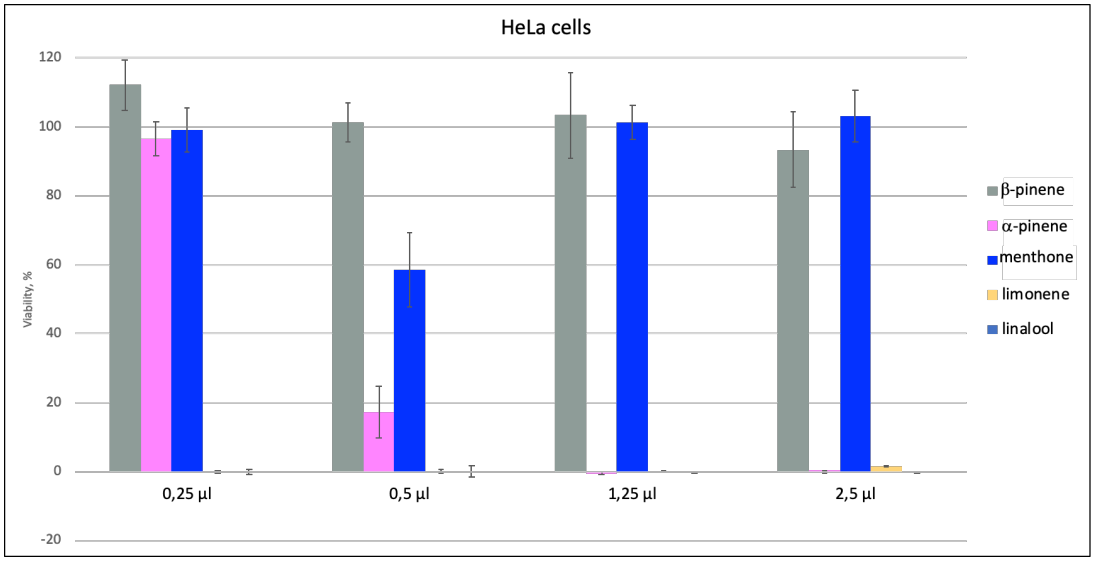


**Figure 3.29:** Effect of *Mentha* related compounds on HeLa cells. Different volumes of the compounds were used to see the effect on HeLa cell viability.

The effect of menthone on cells was tested and compared with control cells (**Fig. 3.30**). We can clearly see that at lower concentrations of 0.3 and 0.15 % v/v the effect is not high. However, when menthone was used in higher concentration (0.6 and 1.2% v/v) there was no cell alive. At cells which were treated with 0,15 % v/v menthone we could observed more multinuclear cells and apoptotic bodies, compared with the control. At a concentration of 0,3 % v/v menthone most cells were dead.

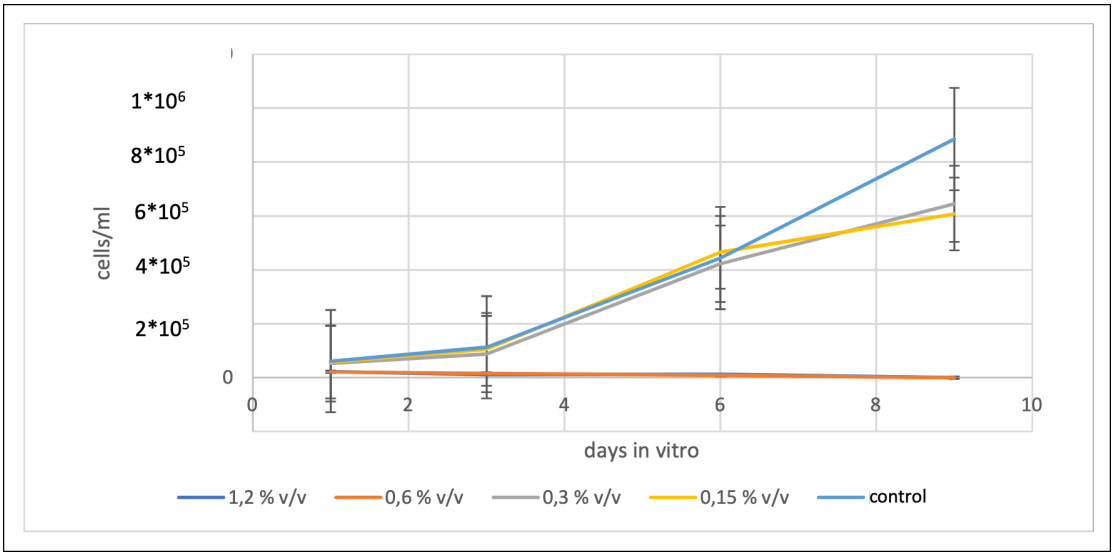



**Figure 3.30:** The proliferation of HeLa cells during 9 days after treatment with menthone. After treatment with 0,6 and 1,2% menthone the cells didn´t proliferate anymore. The proliferation of the cells treated with 0,3 and 0,15% was slightly impaired compared to untreated control cells.

# 4 Discussion

The world at present faces a global challenge of food security, because of the population growth and on the other hand loss of crops due to biotic and abiotic factors. One major reason for crop loss are weeds and the herbicides used to control the weeds are turning out to be unsustainable choices and also hazardous (Narwal 1999, Chamovitsh *et al*., 2017). With a growing concern about the hazardous effects of the synthetic herbicides (Gorrel *et al*.,1998; Kettles *et al*.,1997; Kogevinas *et al*.,1997, Kordali *et al*.,2009) and the problem of herbicide resistance (Dayan *et al.,* 2012; Soltys *et al.,* 2013) there is a demand for bioherbicides (Duke *et al*.,2000; Singh *et al.,* 2003; Chamovitsh *et al.,* 2017) which is a more sustainable solution. A very common screen for the search of bioherbicides are the naturally occurring allelochemicals which the natural world provides the plants with to cope with competition (Soltys *et al*., 2013; Duke *et al.,* 2000; Singh *et al*., 2003; Chamovitsh *et al.,* 2017; Macias *et al* 20017).Lamiaceae in general and *Mentha* group of plants are known to be allelopathic in nature, and so the present study focused on the *Mentha* related plants to get an insight in the mode of allelopathic action in these plants.

Using a comparative approach, the allelopathic effects of essential oils from different Mints (including the genus *Mentha*, but also Cat Mint (*Nepeta cataria*), and Corean Mint (*Agastache rugosa*), belonging to sisters clades within the *Mentheae* using the standard cress germination assay as readout was tested which clearly shows specific compounds to have an allelopathic effect. menthone/isomenthone, among other

compounds, on the basis of the correlation between bioactivity and chemical components, has been identified as a prime candidate for the allelopathic effect and our further results have confirmed this experimentally. To get a further insight in the mode of action, the effect of menthone/isomenthone in transgenic tobacco BY2 cell lines (expressing the microtubule marker AtTuB6 in fusion with GFP and actin labelled with GFP in GF11 lines) was followed and the results clearly show plant specific effects on microtubules and actin which on further experiments show specificity based on the compounds. The results showed specificity of extracted oils in context to cytoskeleton (microtubules and actin filaments). Using *Arabidopsis thaliana*, a dicotyledonous weed with GFP labelled tubulin and actin the results revealed a clear effect of menthone/isomenthone on microtubules. Similarly, microtubules in rice root were disrupted in general in case of treatment with menthone/isomenthone. The study also shows effects of the oils and compounds on weeds like *Rumex* and *convolvulus*, which are known to have problems in vineyards and meadows. The study paves way for the use of novel bioherbicides that would be environment friendly. Oil extracts of *Mentha* spp and the standard compounds which are present in the extracts show specific mode of action on the cytoskeleton. Since MTs are key to cell cycle and hence connected to cancer biology, we used HeLa cells, a model cell system to study cancer (Tan *et al.,* 2015), to see the effects of *Mentha* related compounds.

## 4.1 Determination of taxonomic identity

Taxonomical identification is the key for biological studies and molecular markers are very good tools for identification of species in the present era. The use of molecular markers for identification of species was first developed for animals using mitochondrial markers (Hebert *et al.,* 2003). Molecular markers are a boon for identification of plant species especially through chloropastic markers (Thakur *et al*., 2019). In the present study, in order to authenticate the plants used in the study, the psbA-trnH intergenic spacer, a plastidic marker was used. The phylogenetic tree tree based on the sequences of the Psba-trnH region clearly shows that the sequences from the *mentha* species clustered separately from other tested genera of Lamiaceae family (*Agastache*, *Nepeta*, *Melissa* that all formed well supported separate clades). Previous studies have shown use of psbA-trnH markers for identification of genera within Lamiaceae family (Fabriyio de Mattia *et al.,* 2011) Previous studies have shown the use of markers to differentiate between species of the same genera (Jurges *et al*.,2018) and in the present study we could , based on the phylogenetic tree of the sequences obtained of psbA-trnH marker, show differences between different species of *Mentha* group of plants. Identification of species using DNA markers is more reliable than the traditional methods like chemical profiling or morphological charecterisation (Thakur *et al.,* 2019).

## 4.2 Effect of *Mentha* related oils and compounds

### 4.2.1 Effect on Germination

After the taxonomic identification of the plants of choice, the extracted oils were subjected to the cress bioassay for seed germination and based on the bioassay the GC profiles of the oils was analysed (**Supp Data1**). The rich chemical diversity of *Mentha* group of plants (Kokini, 1991) has been shown in previous studies (Cavalieri and Caporali 2010) to have inhibitory effect on seed germination. It was observed in the present study that the oils from most of the mentha plants chosen inhibited seed germination of cress except the oil from *M. suaveolens* (just confirmed as *M. spec.*), *M. x piperita*, and *Melissa officinalis* which inhibited cress germination only mildly. As a positive control citral rich Lemon Myrtle was used in the study and as per previous study (Chaimovitch *et al*., 2010), it showed strong inhibition. Based on the score of the bioactivity and the feasibility of the experiment, menthone/isomenthone was selected as a choice of compound. menthone/isomenthone is known to have inhibitory effects on seed germination (Martino*et al.,* 2010) apart from being antifungal and antimicrobial (Gulluce *et al.,* 2007; Mkaddem *et al*.,2009). As has been shown in previous study, *M.longifolia* (Mkaddem *et al.,* 2009) and A rugosa (Charles *et al*.,1990; Zielinska *et al.,* 2017) in our study clearly show abundance in menthone-isomenthone. A dose dependent study clearly shows the inhibitory effect of menthone on seed germination of cress which has been also previously reported (Martino *et al*.,2010; Ben-Ami *et al*.,2002; Synowiec *et al.,* 2017). With the identification of the compound (menthone/isomenthone) in the extracted oils of *M.longifolia* and *A.rugosa* and the

inhibitory effects of these two species on cress germination, the effect of *A.rugose* on growth of rice (as an example of monocotyledon) was studied. Monoterpenes are known to affect seed germination in monocots (Synowiecet *et al*., 2016; Chaimovitsh *et al*., 2012). Dongjin is not affected by *A.rugosa* speaks for an overall resistance to its allelopathic effects while Sathi seems to benefit from the cocultivation suggests a priminig effect. It is known that volatiles from plants help in priming of the host plants (Holopainen & Gershenzon, 2010; Reidlmeier *et al*., 2017; Arimura *et al.,* 2010). On the other hand the *A.rugosa* plants are also not affected by Dongjin like expected. But cocultivated with Sathi, a known allelopathic rice (Chung *et al.,* 2006; Kim and Shin 1998), *A.rugosa* has shorter height which suggests an allelopathic effect of Sathi represses the *A.rugosa* plant, while itself is getting an advantage. This supports previous studies showing competitive ability of rice above other plants (Olofsdotter *et al*., 2002).

### 4.2.2 Cell death estimation

Results of analyses of double stained BY2 cells indicated that all compounds in each used amount during 15 min, induced changes in plasma and nuclear membrane. The most active is pure menthone because it produced the I- and II-step-dying type of cells as well the dead ones. The second activity showed linalool because it produced the I- and I-step-dying type of cells. Both of these compounds in all concentrations eliminated the population of living cells. The limonene and oils from *A. rogusa* and *M. longifolia* had a similar to each other on the profile of the number of living, dying and dead cells.

They did not produce the important number of the II-step-dying and dead cell types. Moreover, the linalool also did not produce the dead cells. All of used compounds (but not menthone/isomenthone) acted as an inducer of changes as well as inhibitor, thus the changes did not go so far like in menthone/isomenthone. Is it possible that they can intercalate in cellular membranes and in the first step induced plasma membrane permeabilization, loss of protoplast integrity and its shrinkage and in the second step prevented changes in plasma membrane integrity by influence on plasma membrane composition and its dynamics (Sergiol. L *et al.,*2014). Similar changes are observed during plasmolysis, but plasmolysis is reversible and plant necrosis is irreversible (Douglas M. Templeton, Ying Liu, 2010). Shrunken protoplasts are often wrongly interpreted as an apoptosis-like process. In animals this process is mediated by cytoskeleton, but in plants (?). In animals this process needs plasma membrane integrity (Minina *et al.,* 2013). So, it is possible that plasma membrane of BY2 treated cells in all cases is only partially injured. It is important that during animal necrosis the swelling of cells and especially mitochondria are observed (Jason Karch *et al*., 2o15). In plants the swelling of mitochondria is possible, but cell wall prevents the swelling of cell protoplasts (Morimoto S1 at el., 2007) rather than cell components swelling. In our studies cells with shrinking cytoplasm and nuclei were observed (**Suppl. SF5**). Results of measurements as well as photos indicated that changes are similar to the necrotic typical for plants i.e. shrinkage of the protoplast and nucleus (van Doorn *et al.,* 2011). Moreover, sometimes the authors suggested that features of necrosis can be observed after 20 min (Reape *et al.,* 2008). In our experiments we conducted observations after

15 min of treatment and 5 min of staining. It is possible that the compounds act as antioxidant agent, we can suggested that application of compounds may act mechanically and not chemically. Slight mechanical stress also produces ROS, so after that they might be neutralized by oils. An increase in amount of ROS is a feature of necrosis (van Doorn *et al*., 2011). When the drop of oil was found we observed that all cells were dying and/or dead (**Fig. 4.1**).

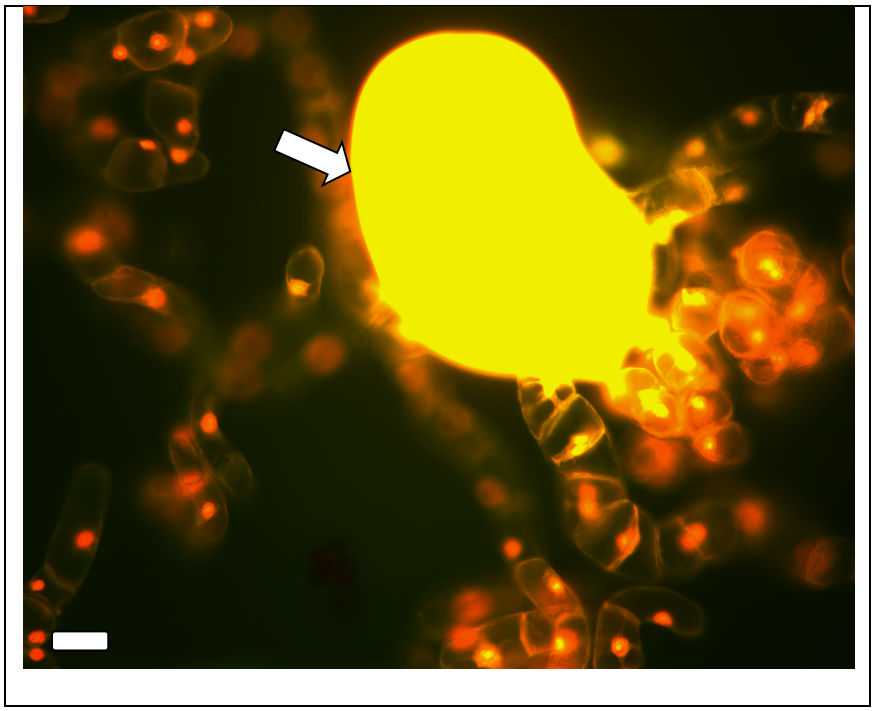


**Figure 4.1:** Drop of menthone/isomenthone (arrow) in a sample of BY2 cells stained with ethidium bromide and orange acridine. Scale bar = 30 µm.

### 4.2.3 Effect on the cytoskeleton

In order to check the specificity of mode of action from the different extracted oils and the compounds, BY-2 cells and *Arabidopsis thaliana* plants with fluorescently labelled actin and microtubule, and immunofluorescence of rice roots was used. Linalool which

in animals have been suggested to have anti microtubular effects (Di Sotto *et al.,* 2011), in the present study shows disruption of microtubules as well as actin filaments in case of BY-2 cell line. Similarly, limonene also led to disruption of microtubules and actin filaments. *A. rugosa* oil which is rich in menthone/isomenthone is also shown to have disrupted the microtubules which suggests the possible role of menthone/isomenthone as inhibitor of seed germination as discussed here, earlier. However, no effect of *A. rugose* oil was seen on actin filament. BY-2 cells have been previously shown to have microtubular disruption when treated with monoterpenes (Chaimovitsh *et al*., 2012).

*Arabidopsis* seedlings when treated with oil of *A. rugosa* clearly shows specificity in the mode of action by targeting the microtubules while the actin filaments remain intact. (Chaimovitsh *et al*., 2010) have shown the ability of citral to alter the microtubules while the actin filaments are intact. Immunofluorescence of rice roots treated with menthone/isomenthone showed disruption of microtubules in Dongjin and TTL (ox) lines while in case of Sathi the microtubules were bundled and more visible. This is similar to the results discussed earlier where Sathi seems to benefit on co cultivation with *A.rugose* plants. In order to get an insight into the reason for this phenotype of Sathi we did a gene expression study (**Suppl. Data 5.2.2**) which clearly shows differences in gene expression of allelochemical pathways suggesting different modes of action between Sathi and other rice genotypes used in the study. Our study using BY2 cells, *Arabidopsis thalian* and rice root clearly shows the target of menthone/isomenthone rich. *A. rugosa* is clearly the microtubules while

menthone/isomenthone on its own disrupts actin filaments too. Previous studies (Kriegs *et al.,* 2010) have also shown that menthol disrupts both microtubules and actin filaments in stomata. This suggests to the possible role of combinatorial effect of compounds from menthe plants. Although it has been suggested that there is a correlation between inhibitory action of monoterpenes and their chemical nature (Elakovich1988; Reynolds 1987; Vaughn and Spencer1993, Weidenhamer *et al.,* 1993), a new study has found no correlation between monoterpene structure and cytoskeletal disruption (Chaimovitsh *et al.,* 2017). We could clearly see in the present study a diverse mode of action in the oils of plants.

### 4.3 Trial as bioherbicide (Towards application)

To further check the potential of the extracted oils from the *mentha* species as bioherbicides and to see if they have an effect on growth, germination tests were made using cress, tomato, winter wheat and purple tansy. Herbicidal mode of action is based on the type of plant and therefore we included tomato as a dicot and winter wheat as a monocot among the cultivated plants. Clearly linalool has a strong effect on winter wheat while menthone/isomenthone and *M.longifolia* oil affects the germination of cress. The fact that some herbicides like trifluralin (Hess and Bayer, 1977), oryzalin (Morejohn *et al.*, 1987a,b;Hugdahl and Morejohn, 1993), propyzamide (Akashi *et al.*, 1988) and glyphosate (Yasuor *et al.*, 2006), use microtubules directly or indirectly as their targets in plant cells suggests for the potential use of menthone and menthone rich plants as possible weed controllers. The present study clearly shows specific mode of

action of oils and compounds which is essential to know for future use of these compounds as bioherbicides. (Chaimovitsh *et al*.,2012) have shown how citral sensitivity was more for mitotic MTs than cortical ones. Similarly compounds and oils in the present study had more or less sensitivity depending on whether the target was microtubule or actin. The specificity of target is a benefit for choice of target plants when the compound has to be used as a bioherbicide. The specificity in target also is a result of the additive or synergestic interactin of the compounds in the plants (Chotsaeng *et al.,* 2017)

*Rumex* which is a weed, very common in the alpine region is inhibited by treatment with menthone/isomenthone. The effect clearly seems to be similar to other monoterpenes which affect the microtubule and in turn cell cycle (Grano *et al*.,2013, Chaimovitsh et al 2012) A sustainable solution to the control of *Romex* would be beneficial for the farmers of the alpine region (Ammon *et al.,* 1999). Similar to the effect on Sathi rice, *A.rugose* oil and menthone/isomenthone seem to have a priming effect on *convolulus*. One reason why they don't have an inhibitory effect is that *convolvulus* cuttings were used where actin filaments are required for regeneration while the target for menthone/isomenthone and *A. rugose* oil is microtubule. The effect of the oils and compounds studied here need to be further studied on a field level to get a better insight in their development as bioherbicides.

## 4.4 Scope for cancer treatment (HeLa cells)

Plant based drugs are a major proportion of the pharmaceutical industry and in the present global health scenario the search for plant based drugs for diseases holds importance (Schmidt *et al*., 2007). Treatment of cancer has been a challenge for the medical fraternity and there is a continous search for drug for prevention and treatment of cancer. Studies have shown the potential of plant-based monoterpenes in cancer treatment (Faridi *et al.,* 2011; Bardon et al 2002; Weecharsangan *et al*.,2014). Essential oils from mint plants are known to have cytotoxic effects on the carcinogenic cells (Weecharsangan *et al*.,2014) which give a scope to *Mentha* oils as potential therapeutic and preventive drugs against cancer. *Mentha* species have been known to have therapeutic properties which come from their essential oils (Edris *et al.,* 2007; Mikaili *et al.,* 2013; Tazarani Najaran *et al.,* 2013; Hajlaoui *et al*., 2009) and also studies have shown their role as a possible cancer preventive and therapeutic compound (Nakamura *et al*., 2014; Crowell 1999). In the present study we tested the effects of the principle compounds constituting the essential oils of the plants used in the study. Effects of α pinene, limonene, linalool and menthone/isomenthone showed cytotoxicity to Hela cells. α pinene is a naturally occurring compound in pine needles and is known to have inhibitory effects on hepatoma cancer cells (Chen *et al*.,2014) by its effects on the cell cycle (Chen *et al* 2015). The effect of α pinene from mentha plants seems to have a similar effect as shown in the present study. Treatment of HeLa cells with α-pinene in a dose dependent manner clearly shows inhibitory effects on HeLa cells. It is known that limonene (Yu *et al*.,2018) has an anti-tumor effect in lung

cancer. Dietary uptake of limonene has shown decrease in mammary tumors (Elegbede *et al*., 1986) and the abundance of limonene in plants taken for this study shows the potential of these plants for anti-cancer drugs. Limonene inhibits the growth of Hela cells which is similar to previous studies done on other cancer cells (Yu *et al*.,2018; Elegbede *et al.*, 1986). Linalool which also showed cytotoxicity towards HeLa cells is known to arrest cell cycle and induces apoptosis in cancer cells (Chang *et al*., 2015; Chang and Shen 2014). menthone/isomenthone at higher doses lead to the death of cells but on a lower amount it led to apoptosis like event even with multinucleated cells and apoptotic bodies. Studies previously (Cheng *et al.,* 2008; Anwar *et al*.,2019) have shown that menthone/isomenthone inhibits cancer cell growth and could be used for treatment against cancer. Further studies need to be done to understand the mode of action of these compounds on HeLa cells. The effects of the extracted plant oils and the chemical constituents thereof need to be further studied, in depth, for potential use in cancer therapeutics.

## 4.5 Conclusion

In the present work, a hypothesis driven research moving towards application approach was taken where the plant oils were subjected to assays to find their allelopathic mode of action. Plants were the identity of plants used in the study was confirmed using psbA-trnH marker which has simultaneously provided a DNA barcode to differentiate between different plant species related to the *Mentha* group. Out of the oils extracted from different plants, specific mode of action could be observed in case of plants

containing menthone/isomenthone and β-pinene. menthone/isomenthone targets the microtubules as seen in

BY-2 cells, *Arabidopsis* seedlings and roots of rice. β-pinene targets the actin filaments in BY-2 cells, thereby both menthone/isomenthone and β-pinene showing specificity in their respective mode of action. The application based experiments provide evidence for future use of different plants from the study as potential sources of bioherbicides. *Rumex obtusifolia*, a very potent weed in the alpine region showed inhibition of germination after treatment with menthone and inhibition of growth after treatment with linalool. Similarly, oils extracted from *M. longifolia* and *M. spicata crispa* disrupt the growth of convolvulus. Furthermore, the effects of menthone were tested on cancer, HeLa, cells and it was found that menthone inhibits the proliferation of HeLa cells.

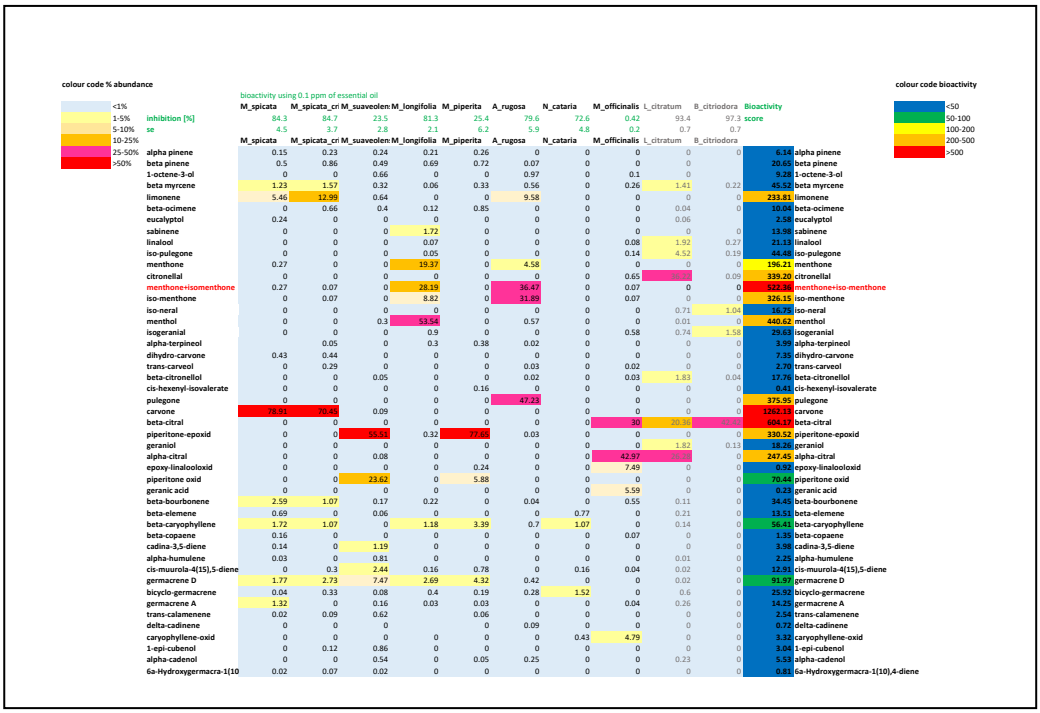
## 4.6 Outlook

The specificity of the plant oils and the compounds therein suggest to an intricate mode of action with reference to mode of actions based on factors other than the cytoskeleton (Latif *et al*., 2017). The present study paves way for further studies leading towards application as bioherbicides and treatment of cancer. As our studies show the specificity in the mode of action of plant oils and compounds, it can be further screened for potential use as weed control. *Rumex* as a weed in the alpine region and *convolvulus* in general are affected by the oils and compounds. Further field experiments are needed to validate the use of *Mentha* plants as weed controllers against *Rumex* and convolvulus.

The targets of most anti-cancer drugs are supposed to be the microtubules where the cell cycle is affected thereby controlling the cancer cells. Oils and compounds from *mentha* and *mentha* related plants show specificity to microtubules in case of mode of action. Also, it was observed that cell death in case of BY-2 cells is not random but programmed. The mode of action of the compounds and extracted oils suggests the possible potential of them as anticancer compounds. This needs to be further studied in detail with different cell types.

# 5 Appendix

## 5.1 Supplementary Figures




| bioactivity using 0.1 ppm of essential oil | M_spicata | M_spicata_cri | M_suaveolens | M_longifolia | M_piperita | A_rugosa | N_cataria | M_officinalis | L_citratum | B_citriodora | Bioactivity | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| inhibition [%] | 84.3 | 84.7 | 23.5 | 81.3 | 25.4 | 79.6 | 72.6 | 0.42 | 93.4 | 97.3 | score | |
| se | 4.5 | 3.7 | 2.8 | 2.1 | 6.2 | 5.9 | 4.8 | 0.2 | 0.7 | 0.7 | | |
| | M_spicata | M_spicata_cri | M_suaveolens | M_longifolia | M_piperita | A_rugosa | N_cataria | M_officinalis | L_citratum | B_citriodora | | |
| alpha pinene | 0.15 | 0.23 | 0.24 | 0.21 | 0.26 | 0 | 0 | 0 | 0 | 0 | 6.14 | alpha pinene |
| beta pinene | 0.5 | 0.86 | 0.49 | 0.69 | 0.72 | 0.07 | 0 | 0 | 0 | | 20.65 | beta pinene |
| 1-octene-3-ol | 0 | 0 | 0.66 | 0 | 0 | 0.97 | 0 | 0.1 | 0 | | 9.23 | 1-octene-3-ol |
| beta myrcene | 1.23 | 1.57 | 0.32 | 0.06 | 0.33 | 0.56 | 0 | 0.26 | 1.41 | 0.22 | 45.52 | beta myrcene |
| limonene | 5.46 | 12.99 | 0.64 | 0 | 0 | 9.58 | 0 | 0 | 0 | 0 | 233.81 | limonene |
| beta-ocimene | 0 | 0.66 | 0.4 | 0.12 | 0.85 | 0 | 0 | 0 | 0.04 | 0 | 10.04 | beta-ocimene |
| eucalyptol | 0.24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.06 | | 2.58 | eucalyptol |
| sabinene | 0 | 0 | 0 | 1.72 | 0 | 0 | 0 | 0 | 0 | 0 | 13.98 | sabinene |
| linalool | 0 | 0 | 0 | 0.07 | 0 | 0 | 0 | 0.08 | 1.92 | 0.27 | 21.18 | linalool |
| iso-pulegone | 0 | 0 | 0 | 0.05 | 0 | 0 | 0 | 0.14 | 4.52 | 0.19 | 44.44 | iso-pulegone |
| menthone | 0.27 | 0 | 0 | 19.37 | 0 | 4.58 | 0 | 0 | 0 | 0 | 196.21 | menthone |
| citronellal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.65 | 30.23 | 0.09 | 339.20 | citronellal |
| menthone+isomenthone | 0.27 | 0.07 | 0 | 28.19 | 0 | 36.47 | 0 | 0.07 | 0 | 0 | 522.36 | menthone+iso-menthone |
| iso-menthone | 0 | 0.07 | 0 | 8.82 | 0 | 31.89 | 0 | 0.07 | 0 | 0 | 326.15 | iso-menthone |
| iso-neral | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.71 | 1.04 | 16.76 | iso-neral |
| menthol | 0 | 0 | 0.3 | 53.54 | 0 | 0.57 | 0 | 0 | 0.01 | 0 | 440.62 | menthol |
| isogeranial | 0 | 0 | 0 | 0.9 | 0 | 0 | 0 | 0.58 | 0.74 | 1.58 | 29.65 | isogeranial |
| alpha-terpineol | | 0.05 | 0 | 0.3 | 0.38 | 0.02 | 0 | 0 | 0 | 0 | 3.99 | alpha-terpineol |
| dihydro-carvone | 0.43 | 0.44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7.38 | dihydro-carvone |
| trans-carveol | 0 | 0.29 | 0 | 0 | 0 | 0.03 | 0 | 0.02 | 0 | 0 | 2.70 | trans-carveol |
| beta-citronellol | 0 | 0 | 0.05 | 0 | 0 | 0.02 | 0 | 0.03 | 1.83 | 0.04 | 17.76 | beta-citronellol |
| cis-hexenyl-isovalerate | 0 | 0 | 0 | 0 | 0.16 | 0 | 0 | 0 | 0 | 0 | 0.41 | cis-hexenyl-isovalerate |
| pulegone | 0 | 0 | 0 | 0 | 0 | 47.23 | 0 | 0 | 0 | 0 | 375.95 | pulegone |
| carvone | 78.91 | 70.45 | 0.09 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1282.19 | carvone |
| beta-citral | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 20.38 | 30.63 | 604.17 | beta-citral |
| piperitone-epoxid | 0 | 0 | 55.53 | 0.32 | 77.65 | 0.03 | 0 | 0 | 0 | 0 | 330.52 | piperitone-epoxid |
| geraniol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.82 | 0.13 | 18.26 | geraniol |
| alpha-citral | 0 | 0 | 0.08 | 0 | 0 | 0 | 0 | 42.97 | 28.89 | 0 | 247.45 | alpha-citral |
| epoxy-linalooloxid | 0 | 0 | 0 | 0 | 0.24 | 0 | 0 | 7.49 | 0 | 0 | 0.59 | epoxy-linalooloxid |
| piperitone oxid | 0 | 0 | 23.62 | 0 | 5.88 | 0 | 0 | 0 | 0 | 0 | 70.44 | piperitone oxid |
| geranic acid | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.59 | 0 | 0 | 0.23 | geranic acid |
| beta-bourbonene | 2.59 | 1.07 | 0.17 | 0.22 | 0 | 0.04 | 0 | 0.55 | 0.11 | 0 | 34.45 | beta-bourbonene |
| beta-elemene | 0.69 | 0 | 0.06 | 0 | 0 | 0 | 0.77 | 0 | 0.21 | 0 | 13.51 | beta-elemene |
| beta-caryophyllene | 1.72 | 1.07 | 0 | 1.18 | 3.39 | 0.7 | 1.07 | 0 | 0.14 | 0 | 56.43 | beta-caryophyllene |
| beta-copaene | 0.16 | 0 | 0 | 0 | 0 | 0 | 0 | 0.07 | 0 | 0 | 1.35 | beta-copaene |
| cadina-3,5-diene | 0.14 | 0 | 1.19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.98 | cadina-3,5-diene |
| alpha-humulene | 0.03 | 0 | 0.81 | 0 | 0 | 0 | 0 | 0 | 0.01 | 0 | 2.25 | alpha-humulene |
| cis-muurola-4(15),5-diene | 0 | 0.3 | 2.44 | 0.16 | 0.78 | 0 | 0.16 | 0.04 | 0.02 | 0 | 12.91 | cis-muurola-4(15),5-diene |
| germacrene D | 1.77 | 2.73 | 7.47 | 2.69 | 4.32 | 0.42 | 0 | 0 | 0.02 | 0 | 91.97 | germacrene D |
| bicyclo-germacrene | 0.04 | 0.33 | 0.08 | 0.4 | 0.19 | 0.28 | 1.52 | 0 | 0.6 | 0 | 25.90 | bicyclo-germacrene |
| germacrene A | 1.32 | 0 | 0.16 | 0.03 | 0.03 | 0 | 0 | 0.04 | 0.26 | 0 | 14.23 | germacrene A |
| trans-calamenene | 0.02 | 0.09 | 0.62 | | 0.06 | 0 | 0 | 0 | 0 | 0 | 2.54 | trans-calamenene |
| delta-cadinene | 0 | 0 | 0 | | 0 | 0.09 | 0 | 0 | 0 | 0 | 0.72 | delta-cadinene |
| caryophyllene-oxid | 0 | 0 | 0 | 0 | 0 | 0 | 0.43 | 4.79 | 0 | 0 | 3.32 | caryophyllene-oxid |
| 1-epi-cubenol | 0 | 0.12 | 0.86 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.06 | 1-epi-cubenol |
| alpha-cadenol | 0 | 0 | 0.54 | 0 | 0.05 | 0.25 | 0 | 0 | 0.23 | 0 | 5.53 | alpha-cadenol |
| 6a-Hydroxygermacra-1(10 | 0.02 | 0.07 | 0.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.81 | 6a-Hydroxygermacra-1(10),4-diene |

**Figure SF 1:** Heat map for bioactivity and abundance of compounds in the oils extracted from the Mentha group of plants. The bioactivity and the abundance of compounds as shown in the heat map shows the high bioactivity of menthone/isomenthone and also their high presence in *A. rugosa* and *M. longifolia.*

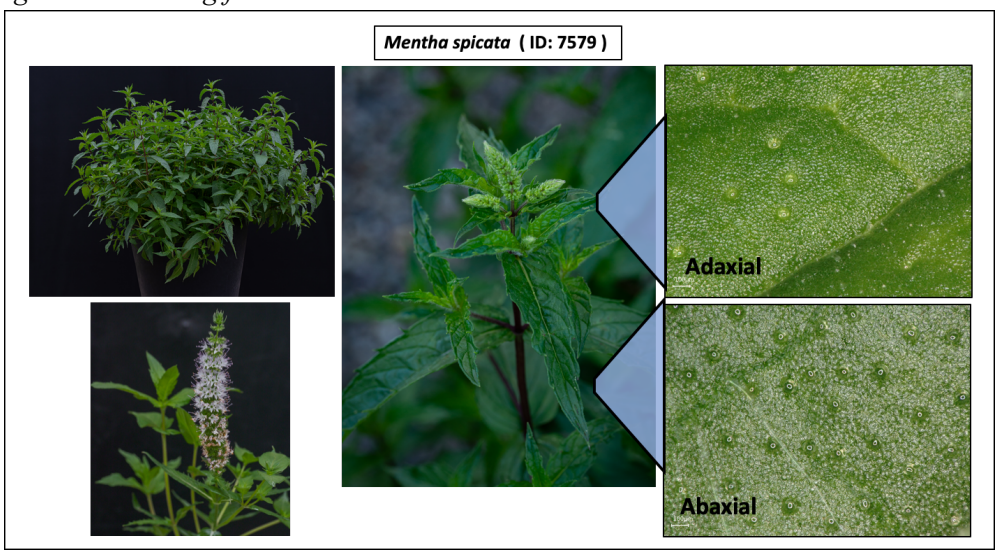



**Figure SF2:** Morphological details of *Mentha spicata* showing the overview of the plants with leaves and inflorescence. The adaxial and abaxial side of the leaves were observed to see differences in the glandular hairs and trichomes. Images for the magnified view of the leaves was taken using the Keycene microscope.

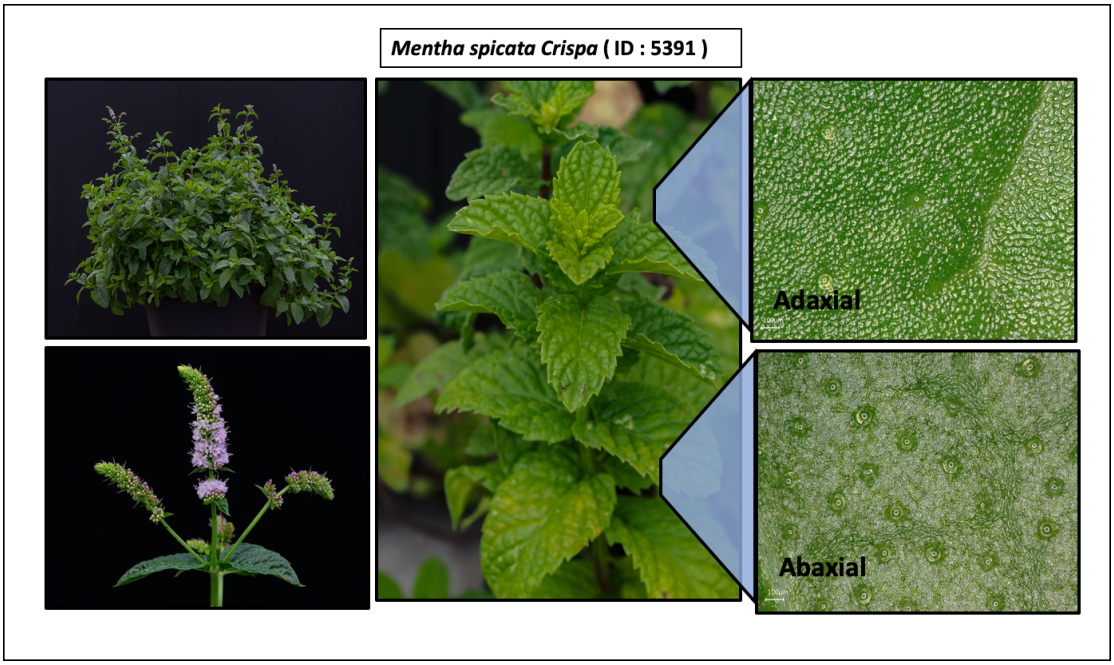



**Figure SF 3:** Morphological details of *Mentha spicata crispa* showing the overview of the plants with leaves and inflorescence. The adaxial and abaxial side of the leaves were observed to see differences in the glandular hairs and trichomes. Images for the magnified view of the leaves was taken using the Keycene microscope.

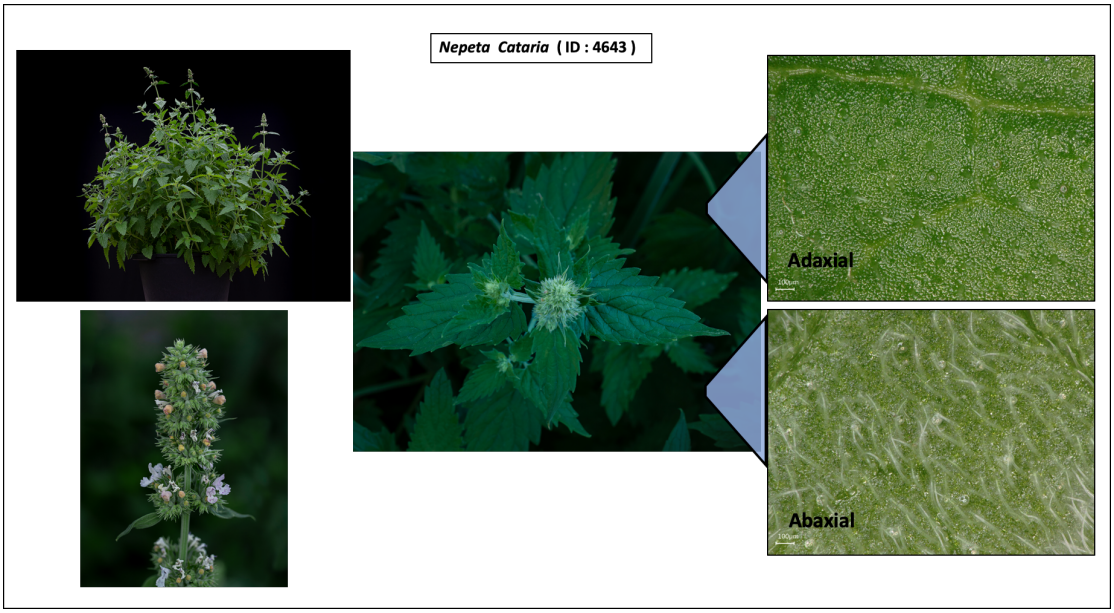



**Figure SF4:** Morphological details of *Nepeta cataria* showing the overview of the plants with leaves and inflorescence. The adaxial and abaxial side of the leaves were observed to see differences in the glandular hairs and trichomes. Images for the magnified view of the leaves was taken using the Keycene microscope.

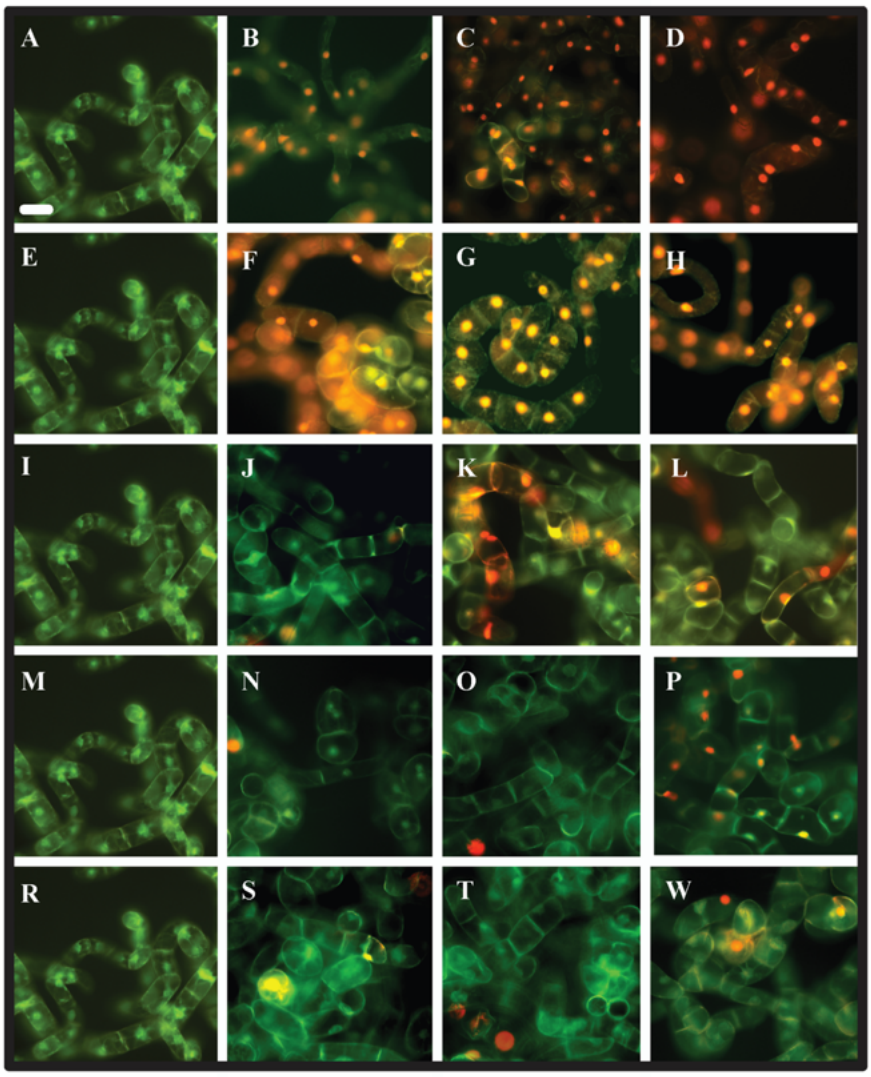



**Figure.SF5:** Representative photos of 3-d-old BY2 untreated cells (A,E,I,M and R) and these treated for 15 min with 0.1µl (B,F,J,N and S), 0.5µl (C,G,K,O and T) and 1µl of menthone (B,C and D), linalool (F,G and H), limonene (J,K and L) as well as oils isolated from *Agustache rogusa* (N,O and P) and from *Mentha longifolia* (S,T and W), than stained with ethidium bromide and orange acridine. Scale bar in A = 30µm and it is applied to all figures.

## 5.2 Competition between *Sathi* and *A. rugosa*

### 5.2.1 Morphological observations

For morphological observation different parameters were observed. In the first step the structure of trichomes on rice leaves were investigated. Trichomes are hairy like structures on the upper side of the rice leave and it has been postulated that trichomes

are part of the plants defense machinery against natural hazards (Xiao *et al*., 2017). For this reason, they might show differences in morphological appearance.

For observating this, leaves from Dongjin and Sathi were collected from control and cocultivated groups. They cut in small pieces and transferred in a solution of chloralhydrate overnight in order to bleach them. After bleaching plants were washed 3 times for 5 min with distilled water and stained with toloidin blue for 15 min. The stained leaves then were again washed with distilled water 3 times for 5 min, then transferred on a slide and observated with a binocular. The number and length of trichomes was measured within a defined area on each leaf section. Additionally, also the height of all plants were collected after 2 months of growth and for *A. rugosa* the status of its inflorescence (Number and length of buds) was measured.

As mentioned before, trichomes are postulated to be a part of the plants defense repertoire against natural hazards. For investigating if trichomes could also play a role related to allelopathy or resistance against allelopathic effects of other plants, leaves of Dongjin as non-allelopathic rice species, and Sathi as allelopathic rice species has been observed under a binocular.

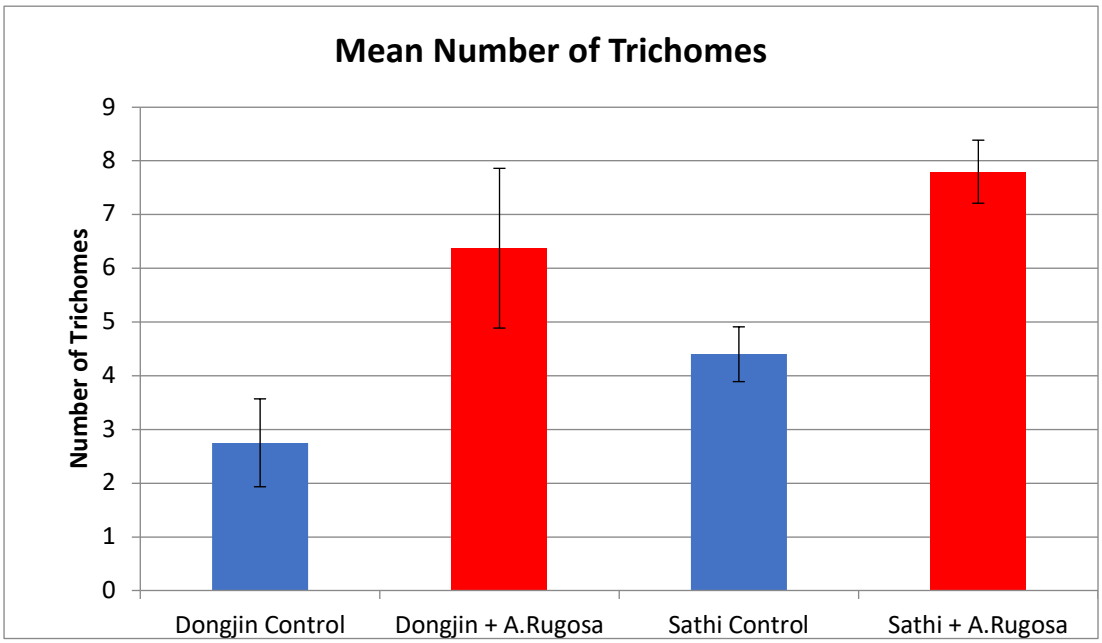



**Figure SF6:** Mean number of trichomes. The figure shows the difference of occurance of trichomes between control groups and cocultivated groups. Both rice species are reacting with a significant upscaling of their trichome number while cocultivated with *A.rugosa.*

The results are showing a significant raise of trichome number in both rice species after cocultivation with *A.rugosa*. If only the control groups are observed, Dongjin as non-allelopathic plant is producing less trichomes as Sathi does. The upscaling of trichomes (**Fig. SF6**), which is present in both species could be a response against *A.rugosa* which might be leading to upregulation of the defense mechanism.

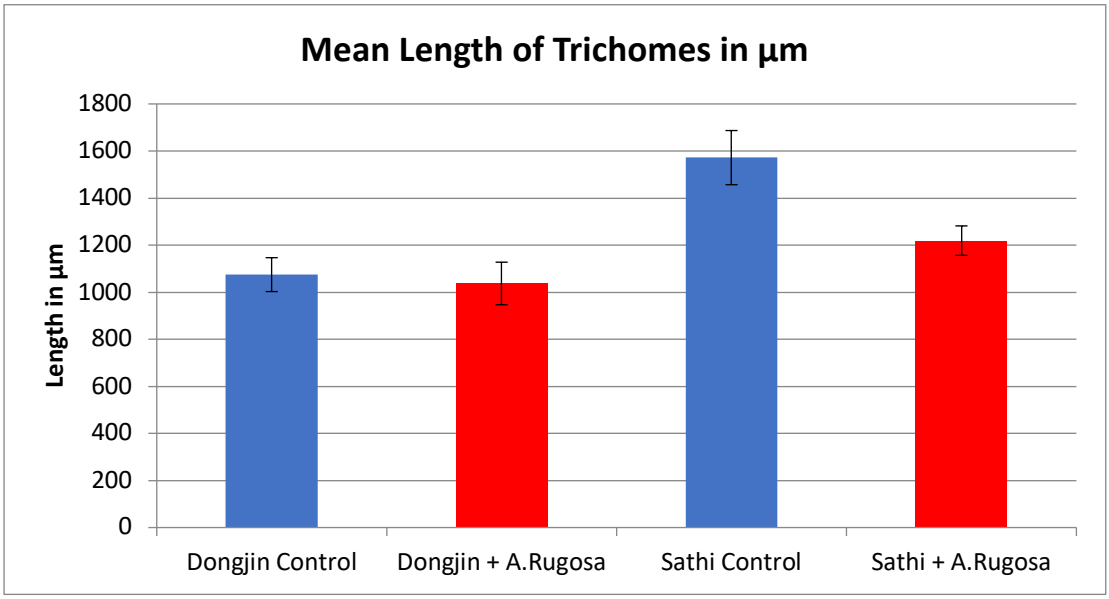



**Figure SF7:** Mean length of trichomes in µm. The figure shows not a significant change in the length of trichomes in the Dongjin species, but in Sathi the mean length of trichome reduces after cocultivation with *A.rugosa.*

The results for the length of the trichomes shows not a difference in between Dongjin control groups and Dongjin cocultivated with *A.rugosa*. Sathi is behaving different to Dongjin in this case. Sathi is reducing the mean length of its trichomes after cocultivation with *A.rugosa* plants. The reason for this behaviour is unknown and might be an reaction to *A.rugosa* volatile substances (**Fig. SF7**).

### 5.2.2 Gene expression studies: RNA extraction and cDNA synthesis

To get an insight to hypothetical differences in the expression pattern of phytoalexin related genes between Dongjin and Sathi, leaves of plants from control and cocultivated groups were collected from plants older than 2 months.

The leaves were frozen rapidly by liquid nitrogen, then crushed to a fine powder by mortar and pestle under RNAse free conditions. The powder was transferred into reaction tubes and freezed immedeately by liquid nitrogen for not allowing the samples

to thaw. RNA was extracted by Analytic Jena innuprep plant RNA kit according to the manufacturers advises.

The quantity of the obtained RNA was determined by photo spectroscopic investigation and the quality was verified by gel electrophoreses (1% Agarosegel).

After the RNA was extracted, cDNA was synthesized according to the following protocol:

In the first step the oligo(dT) primer as well as the dNTPs were added to the template dilution and incubated at 65°C for 5min. This step provides a annealing of the primers to the poly-A-tail of the mRNA without degrading the reverse transcriptase which is not tolerating such high temperatures.

The mastermix used for this step is described in the following:

**Table 1**: cDNA synthesis mastermix part 1

| Component | Volume [µl] |
|---|---|
| dNTP | 1,0 |
| Oligo(dT) Primer | 0,4 |
| RNA dilution | 14,6 |

In the second step the remaining components were added into the reaction tubes followed by further incubation at 42°C for 1h and a final cycle with 70°C for 10 to stop the reaction.

The complete Mastermix in detail is described in the following:

**Table 2**: cDNA synthesis mastermix part 2

| Component | Volume [µl] |
|---|---|
| Mastermix step 1 | 16 |
| RT-Buffer (10x) | 2,0 |
| Reverse transcriptase (M-MuLV) | 1,0 |
| RNase Inhibitor | 1,0 |

After the reaction is completed, the samples were cooled down to 12°C in the cycler for direct usage in the freezer at -22°C for long term storage.

To be able to compare the expression pattern of Dongin and Sathi in control and cocultivated conditions, a semi-quantitative PCR was performed.

Since the oryzalexins A-E and the momilactones A and B are together with sakuranetin are postulated to show the highest quantity in the mass of phytoalexins, primers were taken from puplicated papers (Toyomasu et al., 2008; Shimizu *et al*., 2012) to investigate the expression pattern especially for these substances (**Fig. SF8**). For normalising reasons, also a primer pair for actin as housekeeping gene was used.

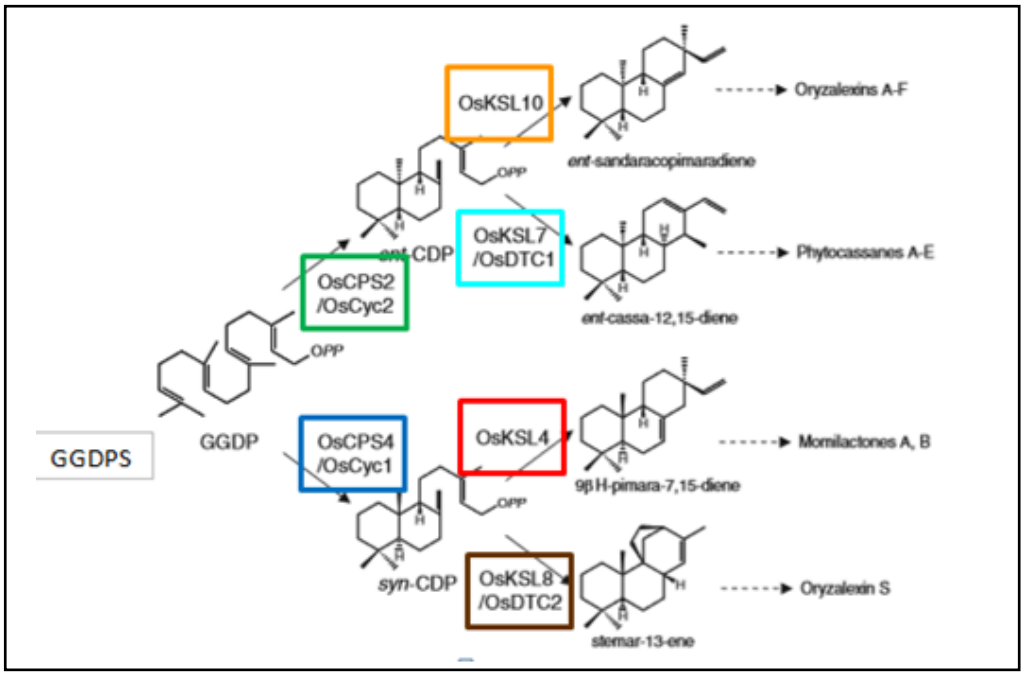



**Figure SF8:** Pathway of diterpene phytoalexine biosynthesis. The coloured squares are containing the enzymes responsible for the phytoalexin pathway in rice. Primers used in the following experiments were designed to perform a semi-quantitive PCR for observing the expression of the genes of these enzymes. Since sakuranetin is not related to this pathway, it is not shown in this figure.

The exact design of used primers is shown in the following:

**Table 3:** Primer details for semi-quantitative PCR.

| Name | Sequence 5‘ > 3‘ |
|---|---|
| Actin forward | CATGCTATCCCTCGTCTCGACCT |
| Actin reverse | CGCACTTCATGATGGAGTTGTAT |
| OsCPS2 forward | CGAGGAGCTTACTGTACGC |
| Os CPS2 reverse | TGAGCAGATCTCGATTGTG |
| OsCPS4 forward | CTGCAGCGCTATTAACAGAC |
| OsCPS4 reverse | AGTGTAGATGAGTCGGGGTAA |
| OsKSL4 forward | AAGAGGCTAAAGAGGCGA |
| OsKSL4 reverse | TTCAGTGGCTCAAAGATTACTC |
| OsKSL7 forward | ACCGAAGTGATGAAGCG |
| OsKSL7 reverse | CTGAGTTGAAGTGGCTCG |
| OsKSL8 forward | GAACATAGAGCGATTCAACGTA |
| OsKSL8 reverse | TCGTCCAGCCTAAATTCTG |
| OsKSL10 forward | CTGACAGCGGCAATACC |

| | |
|---|---|
| OsKSL10 reverse | CCTTAGGTGTGCGGTAGG |
| OsNOMT forward | CTAGCCGGATGCATGAAAGT |
| OsNOMT reverse | TGCACGTATAGGCACACACA |
| OsCOMT forward | AGGTGTTCGACCATCGTCTT |
| OsCOMT reverse | CACCGGAATTGAACATCAAA |

The Mastermix for the semi-quantitative PCR was created like described in the following:

The mastermix i used for preparing my samples is described in table below:

**Table4:** Mastermix for semi-quantitative PCR experiment.

| Component | Volume [µl]/Sample |
|---|---|
| Autoclaved, destilled water | 14,72 |
| 10X PCR Buffer | 2,0 |
| 10 mM dNTP mix | 0,4 |
| Forward Primer (20 µM) | 0,4 |
| Reverse Primer (20 µM) | 0,4 |
| Taq DNA Polymerase (5 U/ µl) | 0,08 |
| cDNA template (50ng/ µl) | 1 |

The PCR was performed under the following conditions:
**Table 5:** Used cycler setup for RT-PCR experiment.

| Step | Incubation time | Temperature |
|---|---|---|
| Activation | 3 min | 95°C |
| Denaturation | 30 sec | 95°C |
| Annealing | 45 sec | 57°C |
| Elongation | 45 sec | 68°C |
| Final elongation | 5 min | 68°C |

The quantitiy of the PCR products was determined by gelelectrophoreses (1% Agarosegel, 100V, 30min).

In order to get an insight in the genetic mechanism of how Sathi is able to affect other plants like *A.rugosa* in this case, we did gene expression study based on the genes involved in production of phytoalexins (**Fig. SF8**). The hypothesis was, that phytoalexins are not only acting as agents against pathogens, but maybe also as allelochemicals against competing plants in the local area.

To get an insight to this, the genes of the most quantitative occurring phytoalexins in rice were investigated via semi-quantitative PCR to see possible differences in the pattern of pytoalexin related gene expression.Unfortunately, the primers OsKSL10; NOMT and COMT did not work properly, so they were excluded from the following results.

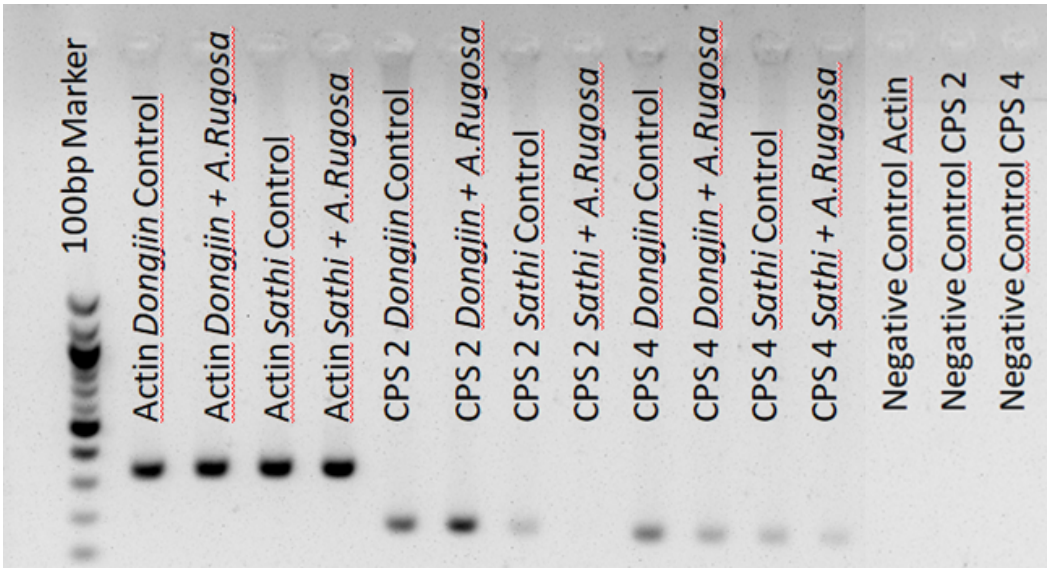



**Figure SF9:** Gel electrophoresis of CPS2 and CPS4. The pattern shows significant differences in the expression pattern of CPS2 and CPS4 in Dongjin and Sathi control plants. The expression of Dongin is in both genes higher as in Sathi in control groups. If the rices are cocultivated with *A.rugosa*, the expression activity in Dongjin is raising slightly while it is decreasing in Sathi in the case of OsCPS2. For OsCPS4 both species are decreasing their expression in cocultivation with *A.rugosa*. Actin was used as housekeeping gene and is showing a similar expression in all cDNA samples.

In this experiment the genes OsCPS2 and OsCPS4 (**Fig. SF9**) were investigated for their expression pattern in rice species. The results are showing differences in the rate of expression between Dongjin and Sathi under control conditions. Dongjin in this case is expressing both genes in a higher rate as *Sathi*. If the plants were cocultivated with *A.rugosa*, Dongjin is expressing OsCPS2 in a slightly higher rate while *Sathi* is reacting by a decrease of expression. For OsCPS4 both plants are reacting the same way by a decrease of expression activity. The expression of actin as a housekeeping gene keeps the same in all investigated groups.

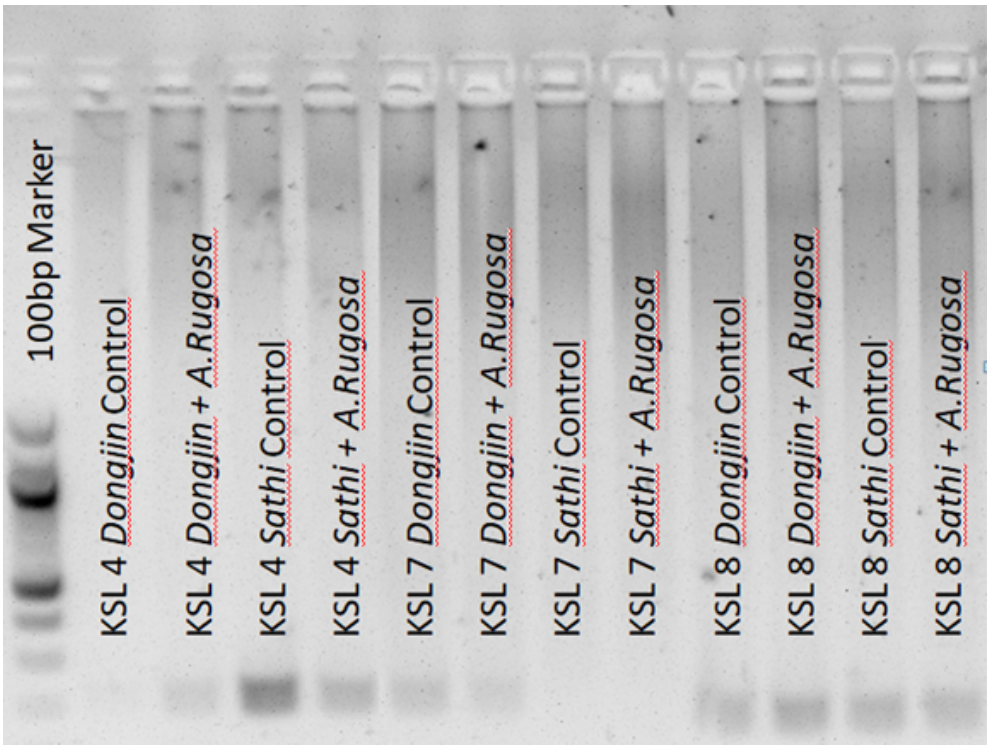



**Figure SF10:** Gelextrophoresis of KSL4; KSL 7 and KSL 8. The pattern shows significant differences in the expression pattern of KSL4 and KSL 7. While Dongjin under control conditions is expressing less KSL4, in cocultivation with *A.rugosa* its activity raises. *Sathi* is reacting the opposite way and expresses OsKSL4 in a higher rate under control conditions and decreases its activity in cocultivation. In the case of OsKSL7, the expression pattern of *Sathi* is that less that it is not measureable with this method. Dongjin is producing mRNA from OsKSL7 and is decreasing its expression slightly after cocultivation with *A.rugosa*. For OsKSL8 the expression pattern is the same for all plants and also do not change while in cocultivation.

In case of OsKSL4; OsKSL7 and OsKSL8 (**Fig. SF10**), also differences were observable between the investigated groups. While Dongjin is expressing less OsKSL4 than Sathi under control conditions, its activity raises in cocultivation with *A.rugosa* while Sathi is reacting vice versa by a decrease. If one is observing the pattern of OsKSL7, Dongjin is reacting to *A.rugosa* with a slightly decrease of expression activity, while in Sathi there is no mRNA detectable with this method in both cases, under control and cocultivated conditions. OsKSL8 is not showing significant differences for both rice species and is not changing in any way in cocultivation with *A.rugosa*.

### 5.2.3 Conclusions and Outlook

In the performed experiments *Sathi* is not only resistant to the allelopathic effect of *A.rugosa*, but also gaining an advantage by growing even higher in cocultivation. Also in performed experiments, Sathi is reducing its phytoalexin related gene expression in cocultivation. The mechanism below the resistance of Sathi to *A.rugosa* allelopathic substances, is still unknown and could not be answered by this work. But the decrease in phytoalexin gene expression, which are known to part as defence substances against pathogenes, is suggesting more than one possible hypothesis.

The decrease could be a direct influence and reaction of Sathi to *A.rugosa* allelopathic substances, which are weakening Sathi for pathogenic invasions and by that leading in the end to an long-term allelopathic effect. This could not observed in performed experiments since Sathi did not getting infected with anything under laboratory

conditions. Also, this hypothesis seems not to be very plausible, since Sathi is gaining benefits from the cocultivation.

Sathi is utalising *A.rugosa* as bodyguard against pathogenes and other plants, so *Sathi* might reduce its defences in presence of another allelopathic plant and takes advantages in form of using this free capacity for growth. This hypothesis would fit to the obtained decrease in phytoalexin related genes in cocultivation, but for a verification further research is needed.

Sathi is changing the pathway outgoing from GGDP. GGDP is also used in several other metabolic pathways from rice. If the usage of GGDP in the pytoalexin pathway is reduced, this could be a hint for a raised usage for other components. Also, this hypothesis needs further research for verification. Concluding the usage of phytoalexins an allelopathic agents in *Sathi* could not be verified by this work. The question how Sathi is repressing other plants in its local area keeps still a mystery.

# 6 References

**Aflatuni, A. (2003**). The use of plant origin substances against Galerucella sagitaria. In *Proceeding of the Nordic Association of Agricultural Scientists 22nd Congress "Nordic Agriculture in Global Perspective*.

**Akashi, T.,Izumi, K.,Nagano, E., Enomoto, M., Mizuno, K. and Shibaoka, H.(1988)**Effects of propyzamide on tobacco cell microtubules in vivo and in vitro. *Plant Cell Physiol.***29**,1053–1062.

**Ammon, H. U., & Müller-Schärer, H. (1999).** Prospects for combining biological weed control with integrated crop production systems, and with sensitive management of alpine pastures in Switzerland/Anwendungen biologischer Unkrautregulierung in integrierten Anbauverfahren im Ackerbau und in der Weidewirtschaft alpiner Regionen in der Schweiz. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz/Journal of Plant Diseases and Protection*, 213-220.

**Anthony Richard G, Teresa R. Waldin, John A. Ray, Simon W. J. Bright & Patrick J. HusseyArata Yoneda, Minori Akatsuka, Hidemasa Hoshino, Fumi Kumagai, Seiichiro Hasezawa**, **(2005)** Decision of Spindle Poles and Division Plane by Double Preprophase Bands in a BY-2 Cell Line Expressing GFP–Tubulin, Plant and Cell Physiology, Volume 46, Issue 3, Pages 531–538, https://doi.org/10.1093/pcp/pci055

**Anthony, R. G., Waldin, T. R., Ray, J. A., Bright, S. W., & Hussey, P. J. (1998)**. Herbicide resistance caused by spontaneous mutation of the cytoskeletal protein tubulin. *Nature*,*393*(6682), 260.

**Anwar, F., Abbas, A., Mehmood, T., Gilani, A.H. and Rehman, N.U. (2019)**. Mentha: A genus rich in vital nutra-pharmaceuticals—A review. *Phytotherapy Research*.

**Arimura, G. I., Shiojiri, K., & Karban, R. (2010).** Acquired immunity to herbivory and allelopathy caused by airborne plant emissions. *Phytochemistry*,*71*(14-15), 1642-1649.

**Babita Patni, Harish Chandra, Abhay Prakash Mishra, Sudhir Kumar Guru, Sara Vitalini, Marcello Iriti. (2018).** Rice allelopathy in weed management – An integrated approach, Cellular and Molecular Biology, E-ISSN: 1165-158X / P-ISSN: 0145-5680.

**Bajalan, I., Zand, M. & Rezaee, S., (2013).** The Study on Allelopathic Effects of *Mentha longifolia* on Seed Germination of Velvet Flower andTwo Cultivars of Wheat., 4(9), pp.2539–2543.

**Beckie, H., & Morrison, I. (1993)** Effect of Ethalfluralin and Other Herbicides on Trifluralin-Resistant Green Foxtail (Setaria viridis). Weed Technology,7(1), 6-14. doi:10.1017/S0890037X00036782

**Ben-Ami, M., Chaimovich, R., Chaimovitsh, D., & Dudai, N. (2002, August).** Essential oils as allelopathic agents: bioconversion of monoterpenes by germinating wheat seeds. In*XXVI International Horticultural Congress: The Future for Medicinal and Aromatic Plants 629*(pp. 505-508).

**Bettina Kriegs, Marcus Jansen, Ka Hahn, Helga Peisker, Olga Šamajová, Martina Beck, Silvia Braun, Andreas Ulbrich, František Baluška & Margot Schulz (2010)** Cyclic monoterpene mediated modulations of *Arabidopsis thaliana* phenotype,Plant Signaling & Behavior, 5:7, 832-838,

**Bogdanova, N., Feshchenko, S., Schürmann, P., Waltes, R., Wieland, B., Hillemanns, P., ... & Sohn, C. (2008)**. Nijmegen Breakage Syndrome mutations and risk of breast cancer.*International journal of cancer*,*122*(4), 802-806.

**Bremer, K., B. Bremer & M. Thuli (1998)** Classification of flowering plants. Internet http://www.systbot.uu.se/classification/overview.html

**Bruneton J (1995)** Pharmacognosy, Phytochemistry, Medicinal Plants. Lavoisier Publ. Londres, New York, Paris, 405–466.

**Budavari S, O'Neil MJ, Smith A, Heckelman PE (eds) (1989**) The Merck Index. An encyclopedia of chemicals, drug, and biologicals, 11th edn. Merck & Co., Rahway

**Burbott, A. J., & Loomis, W. D. (1967).** Effects of light and temperature on the monoterpenes of peppermint.*Plant physiology*,*42*(1), 20-28.

**Cavalieri, A., & Caporali, F. (2010)**. Effects of essential oils of cinnamon, lavender and peppermint on germination of Mediterranean weeds.*Allelopathy Journal*,*25*(2), 441-452.

**Chaimovitsh, D., Abu-Abied, M., Belausov, E., Rubin, B., Dudai, N., Sadot, E. (2010)**

Microtubules are an intracellular target of the plant terpene citral.The Plant Journal,61,399–408.

Chaimovitsh, D., Rogovoy, O., Altshuler, O., Belausov, E., Abu‐Abied, M., Rubin, B.& Dudai, N. (2012). The relative effect of citral on mitotic microtubules in wheat roots and BY2 cells. *Plant biology*,*14*(2), 354-364.

**Chaimovitsh, D., Rogovoy, O., Altshuler, O., Belausov, E., Abu-Abied, M., Rubin, B., ... & Dudai, N. (2012).** The relative effect of citral on mitotic microtubules in wheat roots and BY2 cells. *Plant biology*,*14*(2), 354-364.

**Chaimovitsh, D., Rogovoy, O., Atshuler, O., Belausov, E., Abu-Abied, M., Rubin, B., Ssdot, E., and Dudai, N. (2012)**. The relative effect of citral on mitotic microtubules in wheat roots and BY2 cells. Plant Biol. 14:354–364.

**Chaimovitsh, D., Shachter, A., Abu-Abied, M., Rubin, B., Sadot, E., & Dudai, N. (2017).** Herbicidal activity of monoterpenes is associated with disruption of microtubule functionality and membrane integrity.*Weed science*,*65*(1), 19-30.

**Chambers, H. L., & Hummer, K. E. (1994).** Chromosome counts in the Mentha collection at the USDA-ARS National Clonal Germplasm Repository.*Taxon*,*43*(3), 423-432.

**Chang M-Y, Shen Y-L (2014).** Linalool Exhibits Cytotoxic Effects by Activating Antitumor Immunity.*Molecules*. 19(5):6694-6706.

**Chang M-Y, Shieh D-E, Chen C-C, Yeh C-S, Dong H-P. (2015)**; Linalool Induces Cell Cycle Arrest and Apoptosis in Leukemia Cells and Cervical Cancer Cells through CDKIs. *International Journal of Molecular Sciences*. 16(12):28169-28179.

**Charles, D. J., Simon, J. E., Glowacki, C., & Widrlechner, M. P. (1990, September)**. Major essential oil constituents of Agastache spp. In *International Symposium on Medicinal and Aromatic Plants, XXIII IHC 306* (pp. 327-330).

Chemico-Biological Interactions, 188, Issue 2, 5, Pages 267-275 https://doi.org/10.1016/j.cbi.2010.03.0

**Cheng, F. & Cheng, Z., (2015).** Research Progress on the use of Plant Allelopathy in Agriculture and the Physiological and Ecological Mechanisms of Allelopathy. Frontiers in plant science, 6(November), p.1020.

**Cheng, H. M., Hsiang, C. Y., Chen, G. W., Wu, S. L., Chen, J. C., Huang, C. Y., ... & Ho, T. Y. (2008).** Inhibition of lipopolysaccharide-induced interleukin-1β and tumor necrosis factor-α production by menthone through nuclear factor-κB signaling pathway in HaCat cells. *Chinese Journal of Physiology*,*51*(3), 160-166.

**Choesin DN, Boerner REJ. (1991)**. Allyl isothiocyanate release and the allelopathic potential of Brassica napus (Brassicaceae). Am. J. Bot, 78, 1083-1090.

**Chotsaeng, N., Laosinwattana, C., & Charoenying, P. (2017).** Herbicidal Activities of Some Allelochemicals and Their Synergistic Behaviors toward Amaranthus tricolor L. *Molecules*,*22*(11), 1841.

**Chung, I. M., Kim, J. T., & Kim, S. H. (2006).** Evaluation of allelopathic potential and quantification of momilactone A, B from rice hull extracts and assessment of inhibitory bioactivity on paddy field weeds. *Journal of agricultural and food chemistry*,*54*(7), 2527-2536.

**Clark, G. S. (1998)** Menthol. Perfumer & Flavorist, v. 23, n.5, 3 – 46,

**Clark, R. J., & Menary, R. C. (1980).** Environmental effects on peppermint (*Mentha piperita* L.). I. Effect of daylength, photon flux density, night temperature and day temperature on the yield and composition of peppermint oil. *Functional Plant Biology*,*7*(6), 685-692.

**Coatti, G. C., Frangini, M., Valadares, M. C., Gomes, J. P., Lima, N. O., Cavaçana, N., ... & Singer, J. M. (2017).** Pericytes extend survival of ALS SOD1 mice and induce the expression of antioxidant enzymes in the murine model and in IPSCs derived neuronal cells from an ALS patient. *Stem Cell Reviews and Reports*,*13*(5), 686-698.

**David J. Newman and Gordon M. Cragg: (2007).** Natural Products as Sources of New Drugs over the Last 25 Years, Journal of Natural Products 70 (3), 461-477.

**Dayan, F. E., Owens, D. K., & Duke, S. O. (2012).** Rationale for a natural products approach to herbicide discovery. *Pest management science*,*68*(4), 519-528.

**Di Sotto, A., Mazzanti, G., Carbone, F., Hrelia, P., & Maffei, F. (2011).** Genotoxicity of lavender oil, linalyl acetate, and linalool on human lymphocytes in vitro. *Environmental and molecular mutagenesis*,*52*(1), 69-71.

**Dominguez, R., & Holmes, K. C. (2011).** Actin structure and function. *Annual review of biophysics*,*40*, 169-186.

**Dorman HJ, Kosar M, Kahlos K, Holm Y & Hiltunen R (2003)** Antioxidant prosperities and composition of aqueous extracts from Mentha species, hybrids, varieties and cultivars. Journal of Agricultural and Food Chemistry 51:4563–4569

**Douglas M.Templeton, YingLiu, (2010)** Multiple roles of cadmium in cell death and survival

**Dudai, N., Poljakoff-Mayber, A., Mayer, A.M. et al. J Chem Ecol (1999)** 25: 1079. https://doi.org/10.1023/A:1020881825669

**Edgar, Robert C. (2004):** MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Research 32(5), Pages 1792-1797

**Edris, A. E. (2007),** Pharmaceutical and therapeutic Potentials of essential oils and their individual volatile constituents: a review. Phytother. Res., 21: 308-323.

**Eiko Kawamura, Regina Himmelspach, Madeleine C. Rashbrooke, Angela T. Whittington, Kevin R. Gale, David A. Collings, Geoffrey O.** Wasteneys. MICROTUBULE ORGANIZATION 1 Regulates Structure and Function of Microtubule Arrays during Mitosis and Cytokinesis in the Arabidopsis Root

**Eisenreich W, Sagner S, Zenk MH, Bacher A (1997)** Monoterpene essential oils are not of mevalonoid origin. Tetrahedron Lett 38:3889–3892

**Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M. H., & Bacher, A. (1998)**. The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. *Chemistry & biology*,*5*(9), R221-R233.

**Elakovich, SD (1988)** Terpenoids as models for new agrochemicals. Pages 250–261 *in* Cutler HG, ed. *Biologically Active Natural Products—Potential Use in Agriculture*. Washington, DC:American Chemical Society.

**FabrizioDe Mattia (2011)** A comparative study of different DNA barcoding markers for the identification of some members of Lamiacaea, Food Research International, Volume 44, Issue 3, April 2011, Pages 693-702.

**Faridi, U., Sisodia, B. S., Shukla, A. K., Shukla, R. K., Darokar, M. P., Dwivedi, U. N. and Shasany, A. K. (2011),** Proteomics indicates modulation of tubulin polymerization by L-menthol inhibiting human epithelial colorectal adenocarcinoma cell proliferation. Proteomics, 11: 2115-2119. doi:10.1002/pmic.201000691.

**Felsenstein J. (1985):** Confidence limits on phylogenies: An approach using the bootstrap, Evolution, Volume 39, Issue 4, Pages 783-791.

**Franzios G Mirotsou M, Hatziapostolou E, Kral J, Scouras ZG & Mavragani-Tsipidou P (1997)** Insecticidal and genotoxic activities of mint essential oils. Journal of Agricultural Food and Chemistry 45: 2690–2694.

**Gaff, D. F., & Okong'o-Ogola, O. (1971)**. The use of non-permeating pigments for testing the survival of cells. *Journal of Experimental Botany*,*22*(3), 756-758.

**Galambosi, B. (1989**). Cultivation possibilities of essential oil plants in South-Finland. *Journal of Essential Oil Research*,*1*(4), 161-164.

**Gershenzon, J., & Croteau, R. (1990**) Regulation of monoterpene biosynthesis in higher plants. In: Biochemistry of the Mevalonic Acid Pathway to Terpenoids. (G.H.N. Towers, H.A. Stafford, eds.), Plenum Press, New York, pp. 99–160.

**Gershenzon, J., & Croteau, R. (1993**). *Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes, and diterpenes* (Vol. 333). CRC Press, Boca Raton, FL.

**Gershenzon, J., & Croteau, R. (1994**) Genetic control of monoterpene biosynthesis in mints *(Mentha*: Lamiaceae). In: Genetic Engineering of Plant Secondary Metabolism, Recent Advances in Phytochemistry, Vol. 28. (B.E. Ellis, G.W. Kuroki, H.A. Stafford, eds.), Plenum Press, New York, pp. 193–229.

**Gershenzon, J., Maffei, M., & Croteau, R. (1989)**. Biochemical and histochemical localization of monoterpene biosynthesis in the glandular trichomes of spearmint (*Mentha spicata*). *Plant physiology*, *89*(4), 1351-1357.

**Good, R. (1974)**. The geography of the flowering plants. 4 edition, London: Longman Group Limited.

**Gorell, JM, Johnson, CC, Rybicki, BA, Peterson, EL, Richardson, RJ (1998)** The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurology* 50:1346–1350

**Graña, E., Sotelo, T., Díaz-Tielas, C. et al. J Chem Ecol (2013)**

**Green, P. B. (1962)** Mechanism for plant cellular morphogenesis. Science 138: 1404-140

**Gul, P. (1994)**: Seasonal variation of oil and menthol content in Mentha arvensis Linn. Pakistan J. Forest. 44: 16-20.

**Gulluce, M., Sahin, F., Sokmen, M., Ozer, H., Daferera, D., Sokmen, A., ... & Ozkan, H. (2007).** Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. longifolia. *Food chemistry*,*103*(4), 1449-1456.

**Gupta R (1991)** Agrotechnology of medicinal plants. In: Wijesekera ROB (ed) The medicinal plant industry. CRC Press, Boca Raton, pp 43–57

**Hajlaoui, H., Trabelsi, N., Noumi, E. et al**., **(2009)** World J Microbiol Biotechnol 25: 2227.

**Harley R.M. et al. (2004)** Labiatae. In: Kadereit J.W. (eds) Flowering Plants · Dicotyledons. The Families and Genera of Vascular Plants, vol 7. Springer, Berlin, Heidelberg.

**Hebert PDN *et al*. (2003):** Biological identifications through DNA barcodes. Proc R Soc Biol Sci SerB 270:313–321

**Hedge C (1992)** A global survey of the biogeography of the Labiatae. In Harley RM & Reynolds T (eds) Advances in Labiatae Science. Royal Botanic Gardens, Kew 7–17.

**Hegnauer, R., (1989)**. Chemotaxonomie der Pflanzen. Band 8, Birkhäuser Verlag, Stuttgart, pp.689–695, 718
Herbicide resistance caused by spontaneous mutation of the cytoskeletal protein tubulin Nature volume 393, pages260–263 (**1998)**

**Hess, F.D. and Bayer, D.E. (1977)** Binding of the herbicide trifluralin to *Chlamydomonas* flagellar tubulin. *J Cell Sci.***24**, 351–360.

**Heywood, V. H. (1978)** Flowering Plants of the World. Oxford Press.
**Hisakazu Himane (2013).** Biosynthesis of Phytoalexins and Regulatory mechanisms of it in rice, Biosci. Biotechnol. Biochem., 77(6), 1141-1148.

**Hohenberger, P. et al., (2011).** Plant actin controls membrane permeability. Biochimica et Biophysica Acta - Biomembranes, 1808(9), pp.2304–2312.

**Holopainen, J. K., & Gershenzon, J. (2010)**. Multiple stress factors and the emission of plant VOCs. *Trends in plant science*,*15*(3), 176-184.

**Hugdahl, J.D. and Morejohn, L.C. (1993)** Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. *Plant Physiol.* **102**, 725– 740.

**Hui L, He L, Huan L, XiaoLan L, AiGuo Z.** (**2010)**. Chemical composition of lavender essential oil and its antioxidant activity and inhibition against rhinitis related bacteria. African Journal of Microbiology Research, 4(4), 309-313.Journal of Agricultural and Food Chemistry 43: 2384–2388.

**Ibrahim, M. A., Kainulainen, P., Aflatuni, A., Tiilikkala, K., & Holopainen, J. K. (2001)**. Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: with special reference to limonene and its suitability for control of insect pests.
**Inderjit, Cheng HH, Nishimura H (1999b)** Plant phenolics and terpenoids: transformation, degradation, and potential for allelopathic interactions. In: Inderjit, Dakshini KMM, Foy CL (eds) Principles and practices in plant ecology: allelochemical interactions. CRC, Boca Raton, pp 255–266.
**Inderjit, Dakshini KMM, Einhellig FA (1995**) Allelopathy: organisms, processes, and applications. American Chemical Society, Washington DC.
**Inderjit, Mallik AU (2002)** Chemical ecology of plants: allelopathy in aquatic and terrestrial ecosystems. Birkhäuser, Basal.
**Ishita Ahuja, Ralph Kissen and Atle M. Bones (2012).** Phytoalexins in defense against pathogens, Trends in Plant Science, Vol. 17, No. 2,

**Islam and Hisashi Kato-Noguchi. A.K.M. Mominul (2013)** *Mentha sylvestris*: A Potential Allelopathic Medicinal Plant, International Journal of Agriculture & Biology, ISSN Print: 1560–8530; ISSN Online: 1814–9596 13S–018/2013/15–6–1313–1318.

**Islam, A. K. M., & Kato-Noguchi, H. (2013).** Allelopathic potential of five Labiatae plant species on barnyard grass ('Echinochloa crus-galli'). *Australian Journal of Crop Science*,*7*(9), 1369.

**J. Abiodun Elegbede, Charles E. Elson, Martin A. Tanner, Asaf Qureshi, Michael N. Gould**, **(1986)** Regression of Rat Primary Mammary Tumors Following Dietary *d*-Limonene, *JNCI: Journal of the National Cancer Institute*, Volume 76, Issue 2, February, Pages 323–325.

**Järvi, A., Pessala, R., Hupila, I., Simojoki, P., Huhta, H., Virri, K., ... & Galambosi, B. (1994).** Yield potential of cold-tolerant culinary herbs (*Artemisia dracunculus*, *Levisticum officinale*, *Mentha x piperita*, *Agastache foeniculum*) grown in different latitudes in Finland. *Agro-Food'94: huomisen eväät: Tampere 15.-17.11. 1994, Tampere-talo*.

**Jason Karch, Jeffery D. Molkentin, (2015)** Regulated Necrotic Cell Death the Passive Aggressive Side of Bax and Bak, Circ Res. 116:1800-1809. DOI: 10.1161/CIRCRESAHA.116.305421

**Jürges J, Sahi V, Rios D, Reich E, Bhamra S, Howard C, Slater A, Nick P (2018).** Product Authenticity versus Globalisation - The Tulsi Case. PloS ONE 13, e0207763

**Kanatt, S.R., Chander, R. & Sharma, A., (2007)**. Antioxidant potential of mint (*Mentha spicata* L.) in radiation-processed lamb meat. Food Chemistry, 100(2), pp.451–458

**Kettles, MK, Browning, SR, Prince, TS, Horstman, SW (1997)** Triazine herbicide exposure and breast cancer incidence: an ecologic study of Kentucky counties. *Environ Health Perspect* 105:1222–1227.

**Kim, H.K. et al., (1999)**. HIV integrase inhibitory activity of *Agastache rugosa*. Archives of pharmacal research, 22(5), pp.520–523.

**Kim, K. U., & Shin, D. H. (1998).** Rice allelopathy research in Korea. *Allelopathy in Rice. IRRI, Los Banos, Philippines*, 39-44.

**Kogevinas, M, Becher, H, Benn, T, Bertazzi, PA, Boffetta, P, Bueno-de Mesquita, HB, Coggon, D, Colin, D, FleschJanys, D,Fingerhut, M, Green, L, Kauppinen, T, Littorin, M, Lynge, E, Mathews, JD, Neuberger, M, Pearce, N, Saracci, R (1997)** Cancer mortality in workers exposed to phenoxy herbicides, chlorophenols, and dioxins. An expanded and updated international cohort study. *Am J Epidemiol*.145:1061–1075.

**Kokkini S, Karousou R & Lanaras T (1995)** Essential oils of spearmint (carvone-rich) plants from the Island of Crete (Greece). Biochemical Systematics and Ecology 23: 287–297.

**Kokkini, S. (1991).** Chemical races within the genus *Mentha* L. In *Essential oils and waxes* (pp.63-78). Springer, Berlin, Heidelberg.

**Kordali, S.; Cakir, A.; Akcin, T.A.; Mete, E.; Akcin, A.; Aydin, T.; Kilic, H**. **(2009**) Antifungal and herbicidal properties of essential oils and *n*-hexane extracts of *Achillea gypsicola* Hub-Mor. And *Achillea biebersteinii* Afan (Asteraceae). *Ind. Crops Prod. 29*, 562–570.

**Kumar et al. (2016):** MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets, Molecular Biology and Evolution, Volume 33, Issue 7, Pages 1870–1874.

**Kunikowska A, Byczkowska A, Kaźmierczak A (2013)** Kinetin induces cell death in root cortex cells of *Vicia faba* ssp. *minor* seedlings. Protoplasma 250(4): 851–861

**Lambers H, Chapin FSIII, Pons TL. (1998).** Plantphysiological ecology. Springer-Verlag, Berlin, 540 pp.

**Lawrence, B. M. (1985).** A review of the world production of essential oils (1984). *Perfumer& flavorist*, *10*(5), 1-16.

**Lawrence, B.M. (1981)**. Progress in essential oils. Perfumer & Flavourist **6**: 73-75

**LawrenceBM. (1992)**.The spearmint and peppermint industry of North America. In: VerletN, editor.3rd International Conference on Aromatic and Medicinal Plants. Nyons, France, December 2–4; p. 59–90

**Ledbetter, M. C., and K. R. Porter**. **(1963)** "A" microtubule" in plant cell fine structure."The Journal of cell biology 19.1: 239-250.

**Li, X., Gruber, M. Y., Hegedus, D. D., Lydiate, D. J., & Gao, M. J. (2011).** Effects of a coumarin derivative, 4-methylumbelliferone, on seed germination and seedling establishment in Arabidopsis. *Journal of chemical ecology*,*37*(8), 880.

**Lichtenthaler, H. K., Rohmer, M. and Schwender, J. (1997)**, Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiologia Plantarum, 101: 643-652. doi:10.1111/j.1399-3054.1997.tb01049.x.

**Macías, F.A.; Molinillo, J.M.G.; Varela, R.M.; Galindo, J.C.G. (2007)** Allelopathy—A natural alternative for weed control. *Pest Manag. Sci.63*, 327–348.

**Maisch, J., & Nick, P. (2007).** Actin is involved in auxin-dependent patterning. *Plant Physiology*, *143*(4), 1695-1704.

**Martino LD, Mancini E, Almeida LFR, Feo VD. (2010)** The Antigerminative Activity of Twenty-Seven Monoterpenes. *Molecules*.; 15(9):6630-6637.

**McCaskill, D., & Croteau, R. (1999)**. Strategies for bioengineering the development and metabolism of glandular tissues in plants. *Nature biotechnology*,*17*(1), 31.

**McCaskill, D., Gershenzon, J. & Croteau, R. Planta (1992)** 187: 445. https://doi.org/10.1007/BF00199962

**Mikaili, Peyman et al. (2013)** "Pharmacological and therapeutic effects of *Mentha Longifolia* L. and its main constituent, menthol." *Ancient science of life* vol. 33,2 131-8. doi:10.4103/0257-7941.139059.

**Minina EA, Filonova LH, Daniel G, Bozhkov PV (2013)** Detection and measurement of necrosis in plants. (in) Necrosis. Methods and protocols. Kimberly McCALL Charles Klein (eds). Springer Protocols. pp 229–248.

**Mkaddem, M., Bouajila, J., Ennajar, M., Lebrihi, A., Mathieu, F., & Romdhane, M. (2009).** Chemical composition and antimicrobial and antioxidant activities of *Mentha* (*longifolia L*. and viridis) essential oils. *Journal of food science*, *74*(7), M358-M363.

**Morejohn, L.C., Bureau, T.E., Molebajer, J., Bajer, A.S. and Fosket, D.E. (1987a)** Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. *Planta*, **172**, 252–264.

**Morejohn, L.C., Bureau, T.E., Tocchi, L.P. and Fosket, D.E. (1987b)** Resistance of Rosa microtubule polymerization to colchicine results from a low-affinity interaction of colchicine and tubulin. *Planta*, **170**, 230–241.

**Morimoto S1, Tanaka Y, Sasaki K, Tanaka H, Fukamizu T, Shoyama Y, Shoyama Y, Taura F. (2007**) Identification and characterization of cannabinoids that induce cell death through mitochondrial permeability transition in Cannabis leaf cells. J Biol Chem. 13;282(28):20739-51.

**Mostowy, S., & Cossart, P. (2012).** Septins: the fourth component of the cytoskeleton. *Nature reviews Molecular cell biology*, *13*(3), 183.

**Muller, Walter H., and Cornelius H. Mulle**r (**1964**). "Volatile Growth Inhibitors Produced by Salvia Species."Bulletin of the Torrey Botanical Club, vol. 91, no. 4, pp. 327–330. JSTOR, www.jstor.org/stable/2483297. Plant Physiology Jan 2006, 140 (1) 102-114; DOI: 10.1104/pp.105.069989

**Nagata, T., Nemoto, Y. & Hasezawa, S., (1992).** Tobacco BY-2 Cell Line as the "HeLa" Cell in the Cell Biology of Higher Plants. International Review of Cytology, Vol 132, 132(May), pp.1–30.

**Narwal, SS (1999)** Allelopathy in weed management. Pages 203–254 *in* Narwal SS, ed. *Allelopathy Update: Basic and Applied Aspects*. Volume 2. Enfield, NH: Science Publisher.

**Nick P, Heuing A, Ehmann B (2000)** Plant chaperonins: a role in microtubule-dependent wall-formation? Protoplasma 211, 234-244.

**Nick P, Schäfer E (1988)** Interaction of gravi- and phototropic stimulation in the response of maize (*Zea mays* L.) coleoptiles. Planta 173, 213-220.

**Nick P, Yatou O, Furuya M, Lambert AM (1994)** Auxin-dependent microtubule responses and seedling development are affected in a rice mutant resistant to EPC. Plant J 6, 651-663.

**Nick, P. (2008).** Microtubules as sensors for abiotic stimuli. In *Plant microtubules* (pp. 175-203). Springer, Berlin, Heidelberg.

**Nishida, N., Tamotsu, S., Nagata, N. et al. J Chem Ecol (2005)** 31: 1187. https://doi.org/10.1007/s10886-005-4256-y

**Olofsdotter, M., Jensen, L. B., & Courtois, B. (2002).** Improving crop competitive ability using allelopathy—an example from rice. *Plant Breeding*, *121*(1), 1-9.

**Oudhia P (2003)** Traditional and medicinal knowledge about pudina (*Mentha* sp. family: Labiatae) in Chhattisgarh, India. Botanical. Online, http://botanical.com.

**Pamela L. (1999**) Crowell, Prevention and Therapy of Cancer by Dietary Monoterpenes, *The Journal of Nutrition*, Volume 129, Issue 3, Pages 775S–778S

**Patra, D. D., Anwar, M. and Chand, S. (2000).** Integrated nutrient management and waste recycling for restoring soil fertility and productivity in Japanese mint and mustard sequence in Uttar Pradesh, India. *Agriculture, Ecosystem and Environment*, **80,**267–75.

**Petroski RJ and Stanley DW** (**2009)** Natural compounds for pest and weed control. J Agric Food Chem 57: 8171–8179.

**Pietrowska E, Różalska S, Kaźmierczak A, Nawrocka J, Małolepsza U. (2015)**. Reactive oxygen and nitrogen (ROS and RNS) species generation and cell death in tomato suspension cultures – *Botrytis cinerea* interaction. Protoplasma 252(1):307-19. doi: 10.1007/s00709-014-0680-6.

**Putham & DeFrank**. **(1983)** Use of phytotoxic plant residues for selective weed control, crop protection 2 (2), 173-181.

**Quader, H., Deichgräber, G., & Schnepf, E. (1986).** The cytoskeleton of Cobaea seed hairs. *Planta*, *168*(1), 1-10.

**Reape TJ, Molony EM, McCabe PF (2008)** Programmed cell death in plants: distinguishing between different modes. J Exp Bot 59(3) 435–444.

**Reynolds, T (1987)** Comparative effect of alicyclic compounds and quinones on inhibition of lettuce fruit germination. *Ann Bot*60:215–223.

**Riedlmeier, M., Ghirardo, A., Wenig, M., Knappe, C., Koch, K., Georgii, E., ... & Vlot, A. C. (2017).** Monoterpenes support systemic acquired resistance within and between plants. *The Plant Cell*, *29*(6), 1440-1459.

**S. Latif, G. Chiapusio, L.A. Weston. (2017)** Allelopathy and the Role of Allelochemicals in Plant Defence, Editor(s): Guillaume Becard, Advances in BotanicalResearch, Academic Press,Volume 82, Pages 19-54.

**Sagner, Silvia, Christoph Latzel, Wolfgang Eisenreich, Adelbert Bacher, and Meinhart H. Zenk. (1998)** "Differential incorporation of 1-deoxy-D-xylulose into monoterpenes and carotenoids in higher plants."*Chemical Communications* 2: 221-222.

**Saitou and Nei (1987)** The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees, Molecular Biology and Evolution, Volume 4, Issue 4, Pages 406–425.

**Sang, T. et al**. **(1997)** Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). American journal of botany, 84(9), Page 1120.

**Sano T, Higaki T, Oda Y, Hayashi T, Hasezawa S (2005)** Appearance of actin microfilament 'twin peaks' in mitosis and their function in cell plate formation, as visualized in tobacco BY-2 cells expressing GFP–fimbrin. Plant Journal 44, 595–605

**Schmidt BM, Ribnicky DM, Lipsky PE, Raskin I (2007)** Revisiting the ancient concept of botanical therapeutics. Nat Chem Biol 3:360–366

**Seigler, D. S. (1998).** Iridoid monoterpenes. In *Plant Secondary Metabolism* (pp. 353-366). Springer, Boston, MA.

**SergioLopez[a]BeatrizBermudez[a]SergioMontserrat-de la Paz[a]SaraJaramillo[b]Lourdes M.Varela[a]AlmudenaOrtega-Gomez[a]RocioAbia[a]Francisco J.G.Muriana[a](2014**) Membrane composition and dynamics: A target of bioactive virgin olive oil constituents. Biochimica et Biophysica Acta (BBA) - Biomembranes 1838, 6, Pages 1638-1656

**Sharma S & Tyagi BR (1991)** Character correlation, path coefficient and heritability analyses of essential oil and quality components in corn mint. Journal of Genetics 45: 257–262.

**Shasany AK, Khanuja SPS, Dhawan S & Kumar S (2000)** Positive correlation between menthol content and in and in vitro menthol tolerance in Mentha arvensis L. cultivars. The Journal of Biosciences 25: 263–2000

**Shimizu T, Lin F, Hasegawa M, Okada K, Nojiri H, Yamane H.** (**2012**). Purification and identification of naringenin 7-O-methyltransferase, a key enzyme in biosynthesis of flavonoid phytoalexin sakuranetin in rice. J Biol Chem.;287(23):19315-25,

**Singh, H.P., Kaur, S., Mittal, S. et al. J Chem Ecol (2009)** 35: 154. https://doi.org/10.1007/s10886-009-9595-7

**Singh, H.P.; Batish, D.R.; Kohli, R.K. (2003)**, Allelopathic interactions and allelochemicals: New possibilities for sustainable weed management. *Crit. Rev. Plant Sci. 22*, 239–311.

**Sivropoulou A, Kokkiki S, Lanaras T & Arsenakis M (1995)** Antimicrobial activity of mint essential oils. Journal of Agricultural and Food Chemistry 43: 2384–2388.

**Skrzypek, E. et al., (2015)**. Allelopathic effect of aqueous extracts from the leaves of peppermint (*Mentha* × *piperita* L.) on selected physiological processes of common sunflower (*Helianthus annuus* L.). Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 43(2), pp.335–342.

**Small E (1997)** *Mentha*-mint family (Lamiacae). In: Culinary Herbs. Ottawa, Ontario, Canada. NRC Research Press, 351–372.

**Soltys, D.; Krasuska, U.; Bogatek, R.; Gniazdowska, A. (2013)** Allelochemicals as bioherbicides—Present and perspectives. In *Herbicides-Current Research and Case Studies in Use*; Intech: Rijeka, Croatia, p. 662.

**Sylvie Bardon, (2002**) Valérie Foussard, Sophie Fournel, Agnès Loubat, Monoterpenes inhibit proliferation of human colon cancer cells by modulating cell cycle-related protein expression, Cancer Letters,Volume 181, Issue 2, Pages 187-194.

**Synowiec, A., Kalemba, D., Drozdek, E., & Bocianowski, J. (2017).** Phytotoxic potential of essential oils from temperate climate plants against the germination of selected weeds and crops. *Journal of pest science*, *90*(1), 407-419.

**Synowiec, A., Smęda, A., Adamiec, J., & Kalemba, D. (2016).** The effect of microencapsulated essential oils on the initial growth of maize (Zea mays) and common weeds (Echinochloa crus-galli and Chenopodium album). *Progress in Plant Protection*, *56*(3).

**Tan S, Grün C, Guan X, Zhou Z, Schepers U, Nick P (2015)** Gallic acid induces mitotic catastrophe and inhibits centrosomal clustering in HeLa cells. J Toxicol in vitro 30, 506-513
**Tate, J.A. and Simpson, B.B. (2003):** Paraphyly of Tarasa (Malvaceae) and diverse origins of the polyploid species. Systematic Botany, 28(4), Pages 723–737.

**Tayarani-Najaran, Z et al. (2013)**: "Antiemetic activity of volatile oil from *Mentha spicata* and *Mentha* × *piperita* in chemotherapy-induced nausea and vomiting." *Ecancermedicalscience* vol. 7 290. doi:10.3332/ecancer.2013.290.

**Thakur, V.V., Tiwari, S., Tripathi, N. et al. 3 Biotech (2019)** 9: 188. https://doi.org/10.1007/s13205-019-1724-6

The abnormal cell plates formed after microtubule disrupter herbicide treatment are enriched in callose **(2006)** Pesticide Biochemistry and Physiology, 84 (2), pp. 63-71

**Thompson DP (1989)** Fungitoxic activity of essential oils components on food storage fungi. Mycologia 81: 151–153.

**Tisserant, E. et al., (2014)**. Plant Cell Lines in Cell Morphogenesis Research. New Phytologist, 9 (1), p.135.

**Tomonobu TOYOMASU, Takuma KAGAHARA, Kazunori OKADA, Jinichiro KOGA, Morifumi HASEGAWA, Wataru MITSUHASHI, Takeshi SASSA & Hisakazu YAMANE (2008).** Diterpene Phytoalexins Are Biosynthesized in and Exuded from the Roots of Rice Seedlings, Bioscience, Biotechnology, and Biochemistry, 72:2, 562-567, DOI: 10.1271/bbb.70677,
**Tucker, A.O**. (**1992)**. The truth about mints. Herb Companion. 4:51–52.

**Tullihallitus (1984-1996)**. Ulkomaankauppa. Suomen viralliset tilastot 1984-1996. Helsinki: Tullihallitus.

**Turner, G.W., Gershenzon, J. & Croteau, R.B., (2000)** Development of Peltate Glandular Trichomes of Peppermint. Plant Physiology, 124(2), pp.665–679.

**Valdeyron, G., Dommée, B., & Vernet, P. (1977).** Self-fertilisation in male-fertile plants of a gynodioecious species: Thymus vulgaris L. *Heredity*, *39*(2), 243.

**van Doorn WG, Beers EP, Dangl JL, Franklin-Tong VE, Gallois P, Hara-Nishimura I, Jones AM, Kawai-Yamada M, Lam E, Mundy J, Mur LA, Petersen M, Smertenko A, Taliansky M, Van Breusegem F, Wolpert T, Woltering E, Zhivotovsky B, Bozhkov PV. (2011).** Morphological classification of plant cell deaths. Cell Death Differ.;18(8):1241-6. doi: 10.1038/cdd.2011.36.

**Vaughn, K. C. (2006).** The abnormal cell plates formed after microtubule disrupter herbicide treatment are enriched in callose. *Pesticide biochemistry and physiology*, *84*(2), 63-71.

**Vaughn, SF and Spencer, GF (1993**) Volatile monoterpenes as potential parent structures for new herbicides. *Weed Sci* 41:114–119.

**W.Q. Chen, B. Xu, J.W. Mao, F.X. Wei, M. Li, T. Liu, *et al.* (2014)** Inhibitory effects of α-pinene on hepatoma carcinoma cell proliferation, Asian Pac J Cancer Prev, 7), pp. 3293-3297.

**Weecharangsan, W., Sithithaworn, W., & Siripong, P. (1).** Cytotoxic Activity of Essential Oils of *Mentha* Spp. on Human Carcinoma Cells. *Journal of Health Research*, *28*(1), 9-12.

**Weidenhamer, JD, Macias, FA, Fischer, NH, Williamson, GB (1993)** Just how insoluble are monoterpenes? *J Chem Ecol* 19:1799–1803.

**Weiqiang Chen, Ying Liu, Ming Li, Jianwen Mao, Lirong Zhang, Rongbo Huang, Xiaobao Jin, Lianbao Ye**, **(2015**) Anti-tumor effect of α-pinene on human hepatoma cell lines through inducing G2/M cell cycle arrest, Journal of Pharmacological Sciences, Volume 127, Issue 3, Pages 332-338.

**Weir, T.L., Park, S.W., and Vivanco, J.M. (2004)** Biochemical and physiological mechanisms mediated by allelochemicals. Curr Opin Plant Biol 7:472– 479.

**Wink M** (**2003)** Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64: 3–19.

**Xiao K, Mao X, Lin Y, Xu H, Zhu Y, et al.,** (**2017**) Trichome, a Functional Diversity Phenotype in Plant. Mol Biol 6:183. doi:10.4172/2168-9547. 1000183.

**Yang RZ, Tang CS. (1988).** Plants used for pest control in China: a literature review. Econ Bot 42: 376–406.

**Yasuor, H., Abu-Abied, M., Belausov, E., Madmony, A., Sadot, E., Riov, J. and Rubin, B. (2006)** Glyphosate-induced anther indehiscence in cotton is partially temperature dependent and involves cytoskeleton and secondary wall modifications and auxin accumulation. *Plant Physiol.* **141**, 1306– 1315.

**Yasushi Nakamura, Yuki Hasegawa, Koji Shirota, Noboru Suetome, Takako Nakamura, Mullika Traidej Chomnawang, Krit Thirapanmethee, Piyatip Khuntayaporn, Panida Boonyaritthongchai, Chalermchai Wongs-Aree, Shigehisa Okamoto, Tomoaki Shigeta, Tomoaki Matsuo, Eun Young Park, Kenji Sato. (2014)**, Differentiation-inducing effect of piperitenone oxide, a fragrant ingredient of spearmint (*Mentha spicata*), but not carvone and menthol, against human colon cancer cells, Journal of Functional Foods, Volume 8, Pages 62-67,

**Yongquan Li, Ziling Sun, Xiaofeng Zhuang, Ling Xu, Shifei Chen, Mingzhi Li (2003)** Research progress on microbial herbicides, Crop Protection, Volume 22, Issue 2, Pages 247-252, ISSN 0261-2194.

**Yu, X., Lin, H., Wang, Y., Lv, W., Zhang, S., Qian, Y., Qian, B. (2018).** d-limonene exhibits antitumor activity by inducing autophagy and apoptosis in lung cancer. *OncoTargets and therapy*, *11*, 1833–1847. doi:10.2147/OTT.S155716

**Zielińska, S., Dąbrowska, M., Kozłowska, W., Kalemba, D., Abel, R., Dryś, A., ... & Matkowski, A. (2017).** Ontogenetic and trans-generational variation of essential oil composition in Agastache rugosa. *Industrial crops and products*, *97*, 612-619.

**Zimmermann W (1965)** Die Telomtheorie. Fischer, Stuttgart.

## Chemical List

| | |
|---|---|
| Evans Blue (2.5 %) | |
| n-Hexane | |
| α-Pinene | Sigma-Aldrich |
| β-Pinene | Carl Roth GmbH |
| β-Citonellol | Sigma-Aldrich |
| DMEM (Dubecco's Modified Eagle Medium) | Gibco Life Technologies |
| Geraniol | Sigma-Aldrich |
| Hoechst 33342 | |
| Limonene | Sigma-Aldrich |
| Menthone | Sigma-Aldrich |
| MTT Dye Solution | Promega |
| Penicillin/Streptomycin | Gibco Life Technologies |