Investigation into the effects of essential oils on mycoflora and winter wheat seed germination

T. O. Rozhkova¹, I. A. Zhuravska², L. V. Nemerytska², S.V. Mozharovskyi², A.V. Matsyura³, S.V. Stankevych⁴, L.V. Popova⁵

Sumy National Agrarian University, 160 G. Kondrateiva St, 40021 Sumy, Ukraine
 ³ Zhytomyr Agrotechnical College, 96 Pokrovska St, 10031 Zhytomyr, Ukraine
 ³ Altai State University, Barnaul, Russian Federation
 ⁴ V.V. Dokuchaev Kharkiv National Agrarian University, v. Dokuchaevske, Kharkiv region, 62483, Ukraine

⁵ Odessa State Agrarian University, 13 Panteleimonovskaya St, 65000 Odessa, Ukraine

Corresponding author Email: rozhkova8@gmail.com

ORCID ID:

T. O. Rozhkova 0000-0002-0791-9736

I. A. Zhuravska 0000-0001-9332-1134

L. V. Nemerytska 0000-0001-7933-0587

S.V. Mozharovskyi 0000-0002-4613-0156

L.V. Popova 0000-0001-8624-2753

The mycoflora of winter wheat seed consists of various fungi which differ as to the parasitism rate. Their harmfulness is connected with their phytotoxic action and ability to produce mycotoxins. It has been proved that essential oils from different plants have an effective antifungal impact, that is why we studied the effectiveness of 13 essential oils which were produced by LLC "Aromatyka", Ukraine (cinnamon, orange, peppermint, rosemary, vervain, ylang-ylang, sandal, fennel, lavender, eucalypt, bergamot, frankincense, and geranium) on securing of a group of fungi from winter wheat seeds.

The seed mycoflora of Bogdana variety harvested in 2017-2020 which was grown in the North-Eastern Forest-Steppe zone of Ukraine (Sumy oblast) was analyzed. A fungi complex analysis was made on the media PGA. The impact of essential oils on seed germinating ability and the length of wheat sprouts on the 7th day has been determined. A longstanding studying of seed treatment by essential oils showed their effects on mycoflora formation. These plants affected not only the fungi amount but also changed their secure spectrum. The studies admit a negative impact of fungi on seed germination and plant development. The highest seed germination equaled 95% under its treatment with rosemary and orange oils. But under seed treatment with rosemary oil, there appeared Fusarium sp. and *Bipolaris sorokiniana* (Sacc.) Shoem., which have a negative impact on seed germination. Orange oil has increased the amount of fungi of Penicillium sp. The best results (on the basis of three indices) had sandal oil, under which the fungus colony has increased half and seed germination equaled 61%, the sprout length was maximal when applying other oils (58.8 mm).

The essential oils which fully inhibited seed and fungi development (cinnamon, fennel, vervain, geranium) were determined. The study of two of them as fungicides for treatments at the beginning of wheat flowering proved a similar effect to falcon on the change of the internal seed mycoflora.

Keywords: seed mycoflora, winter wheat, essential oils.

Introduction

Intensive chemicalization of agricultural production has a negative impact on the environment and on peoples' health. Pathogenic organisms cause significant losses of agricultural products every year. A search for alternative ways of protection with the purpose of reducing the pesticide load is an urgent problem today.

There are 3000 essential oils, from which only 300 are used by people (Burt & Reinders, 2003). The term "essential oil" was first suggested by Swiss scientist Paracelsus von Hohenheim in the 16th century. The earliest records as for techniques of essential oils production belong to Andalusian doctor Ibn Al-Baitar, though they were used in Shumery and in ancient Egypt. The International Organisation for Standardisation (ISO) gives a definition of essential oil as a product, which is received by distillation with water or vapor, by a mechanic treatment, or a dry distillation of natural materials. Essential oils are received from all parts of a plant, more often from grasses and spices, though new sources of these substances (like food and plant waste) are studied nowadays (Macwan et al., 2016; Ravindran & Jaiswal, 2016; Fengfeng et al., 2017; Nazzaro et al., 2017).

Essential oils contain different secondary metabolites that have an allelopathic impact on plants, adversely affect insects and various microorganisms. The researchers consider these oils to be an alternative to pesticides. For this purpose, they are diligently studying them as insecticides (Titouhi et al., 2017), herbicides (Dutra et al., 2020), fungicides (Amini et al., 2016), and acaricides (Ribero et al., 2019). It has also been proved that essential oils affect plant growth and development, that is why in an agrarian sector they are used as inhibitors (oils inhibit the germination of potato tubers during storage) (Shukla et al., 2019) and stimulate plant growth (Dyadiuchenko et al., 2020). Seed treatment with thyme essential oil increases drought resistance of wheat (Ben-Jabeur et al., 2019). The wheat seeds contain various microorganisms: fungus, bacteria, viruses, etc. Some of them are phytopathogenic, which is why the seed is a source of infection for them. Fungi are the most studied among phytopathogenic microorganisms. It has been proved that fungus infection occurs in the period of plants growing, most often in the period of flowering. The hyphae appear in the seed which is being formed. The fungi inside the seed are dangerous not only for plants under their germination but also for animals and people because most of them produce mycotoxins. Field and seed infection is controlled in two ways: by spraying plants and treating the seeds with fungicides.

The essential oils from different plants are diligently studied as biofungicides against seed infection. The research on the effects of essential oils of different concentrations on fungi from wheat seeds (Acremonium sp., Alternaria sp., Arhrobotrys sp., Aspergillus sp., Cladosporium sp., Epicoccum sp., Fusarium sp., Penicillium sp., Rhizopus sp., Trichoderma sp. and Ulocladium sp.) in Turkey showed the highest anti-fungi activity of oils from clove and oregano. In Slovenia the researchers proved thyme oil (*Thymus vulgaris* L.) to have the highest anti-fungal action on mycelial growth of fungi (*Alternaria alternata* (Fr.) Keissl, *Alternaria infectoria* E.G. Simmons, *Aspergillus flavus* L., *Epicoccum nigrum* L. and *Fusarium poae* (Peck) Wollenw) of wheat seed mycoflora in vitro (Anžlovar et al., 2017). The effects of savory essential oil (Iran) on wheat infection by *Fusarium oxysporum*, grown in pots under artificial seeds infection with a pathogen, showed a lack of infection control as compared to fungicide treatment (Rahimian & Eisvand, 2016). Citral as a component of various essential oils (lemon myrtle, Litsea sp., lemongrass, lemon, eucalyptus, etc.) inhibits not only phytopathogens development but also reduces the amount of mycotoxins (patulin, aflatoxin, alternariol, and its derivative, alternariol monomethyl ether) (Liang et al., 2015; Wang et al., 2018; Wang et al., 2019).

The anti-fungal mechanism of essential oils has been already studied: distortion of the cell membrane, changes, and inhibition of fungi cell wall (Wu et al., 2008; Yutani et al., 2011), dysfunction of fungi mitochondria (Chen et al., 2013), the decrease in the amount of nitrogen oxide (Cotoras et al., 2013), etc.

The aim of the study was to establish the features of regulation of seed mycoflora by treatment with essential oils of various plants and to identify their impact on wheat germination.

Methods

The seeds of Bogdana variety were grown in the North-Eastern part of the Forest- Steppe region of Ukraine (Sumy oblast, Sumy National Agricultural University (SNAU)). The harvests of 2017-2020 were analyzed. The effects of 13 essential oils produced by LLC "Aromatyka", Ukraine (cinnamon, orange, peppermint, rosemary, vervain, ylang-ylang, sandal, fennel, lavender, eucalypt, bergamot, frankincense, and geranium) have been analyzed. The seeds were being rinsed with flow water for an hour and then doused with sterile water (100ml). Then 1ml of essential oil was added to each container. The seeds were kept in this solution for 2.5 hours. The seeds in the control were immersed in sterile water for the same period. The received material was dried on filter paper without rinsing in water and put into Petri dishes (20 in amount) on PGA. Dishes were put into a thermostat where the colonies and seed germination was going on under the temperature of 22–24 °C for 7 days.

The fungi were determined according to mycelia structure as well as to sporulation. As many as 100 seeds were analyzed in each variant. The germination capacity of seeds and the length of wheat sprouts were determined on the 7th day. The effectiveness of winter wheat spraying was tested in the experimental field of a Research and Manufacturing Complex of SNAU. The plants were sprayed with a hand-sprayer one time at the beginning of plant flowering (5.06.2020). It was a small-plot three-fold experiment. Falkon (Bayer) with a consumption rate of 2ml/11 was used as a standard. The same rate of consumption was used for fennel essential oil spraying; the amount of cinnamon essential oil was reduced (1.5ml/11) because in previous years, we have noted its phytotoxic effect.

The analysis of wheat seeds mycoflora was made after spraying on a media (PGA), after rinsing with water they were additionally kept in 1% solution of potassium permanganate during 1-2 min in order to study an internal composition of seed fungi (Naumova, 1951). The percentage of isolated fungi was calculated basing on the whole amount of secured colonies in a variant. The effect of spraying on the mass of 1000 grains was determined as well.

The statistical analysis of the results was made by the method of dispersion analysis, using standard programs «Microsoft Exel 2003».

Results

The analysis of mycoflora of winter wheat seed from the North-Eastern part of Ukraine started in 2007 and showed a significant presence of fungi of Alternaria sp. Seed germination observation during many years showed that there were no effects of these fungi on wheat seed germination. But a significant percentage of isolation of fungi (*Alternaria tenuissima* (Nees) Wiltshire, *A. alternata*, the complex of species *A. infectoria*, *A. avenicola*, and *A. arborescens*) in grain products anticipate the danger of a great amount of mycotoxins availability, which is not controlled in Ukraine. So, it was decided to find ways to regulate the number of fungi of Alternaria sp. in winter wheat seed. The analysis of essential oils' effects on the amount of these fungi started in 2017. The seeds were put into a 1% aqueous solution of cinnamon oil for 2.5 h. We showed satisfactory results as compared to other substances' effectiveness: the colonies amount reduced by half as compared to control (Table 1).

Effects of essential cinnamon oil (1% aqueous solution) on the mycoflora of wheat seed harvested in 2017.

Variant	Isolation of fungal colonies, number	Amount of colonies, number	Seed germination, %	Average sprouts length, mm
Control (water)	Alternaria sp. 75 Penicillium sp. 28 Cladosporium sp. 7 Monilia sp. 1	111	100	53.8
Cinnamon	Alternaria sp. 54 Penicillium sp. 3 Fusarium sporotrichioides Sherb. 3 Arthrinium caricicola Kunze: Fr 1	61	Have not germinated	-
LSD ₀₅	Alternaria sp. 3.9 Penicillium sp. 3.6	4	-	-

The amount of Alternaria sp. has reduced, herewith most of the isolation fungi were indeficated as one species *A. infectoria*, which contrary to other species produces only one mycotoxin. In 2017 fungi of Penicillium sp. were second as to determination among mycoflora representatives of wheat seed. The application of essential oil has fully reduced their amount. At the same time was

isolated F. sporotrichioides, which are the mycotoxins producers. The cinnamon essential oil has not given a chance to seeds to germinate.

In 2018 the spectrum of essential oils was broadened. Different results as to mycoflora changes of winter wheat seed were received (Table 2).

Table 2Effects of essential oils (1% aqueous solution) on the microflora of wheat seed, harvested in 2018

Variant	Isolation of fungal colonies, number	Amount of colonies, number	Seed germination, %	Average sprouts length, mm
Control	Alternaria sp. 54	115	100	60.4
	Penicillium sp. 12			
	Aureobasidium pullulans (de Bary &			
	Löwenthal) G.Arnaud 16			
	Nigrospora oryzae (Berkeley et			
	Broome) Petch 6			
	Monilia sp. 3			
	Other fungal colonies 24			
Orange	Alternaria sp. 10	74	95	16.3
	Penicillium sp. 63			
	Other fungal colonies 1			
Peppermint	Alternaria sp. 21	45	Have not	-
	Penicillium sp. 10		germinated	
	Trichoderma sp. 6			
	Monilia sp. 3			
	A. flavus 1			
	Aspergillus niger Tiegh. 1			
	Other fungal colonies 3			
Rosemary	Alternaria sp. 40	101	95	43.9
	Penicillium sp. 15			
	Trichoderma sp. 5			
	Chaetomium sp. 5			
	B. sorokiniana 4			
	Fusarium sp. 2			
	Other fungal colonies 30			
Cinnamon	Have not germinated	-	Have not germinated	-
LSD 05	Alternaria sp. 3.2	5.5	4,2	0.7
	Penicillium sp. 3.6			
	Other fungal colonies 2.8			

The amount of fungi colonies that had germinated from seed under its treatment with aqueous solutions of essential oils was admitted to decrease. In all variants was shown a decrease in the percentage of isolation of Alternaria fungi as compared to control. But a decrease in the amount of some species caused an increase in the amount or appearance of others. The essential oil from oranges reduced the percentage of isolation of Alternaria sp. up to 10%, but at the same time increased the number of colonies of Penicillium sp., due to which the length of wheat sprouts decreased.

The oil from peppermint reduced the number of colonies by half, but the wheat seed was not able to germinate. Rosemary did not significantly reduce the percentage of fungi colonies but affected the spectrum of their species. The sprout length was decreased up to 43.9 mm. The availability of fungi of Alternaria sp. and Penicillium sp. was common for all variants. Under cinnamon oil application neither fungi colony nor wheat seed was germinated.

In 2019 A. pullulans and Alternaria fungi dominated in a control variant. Like in 2018, the effects of essential oils from plants on wheat seed mycoflora were different (Table 3).

Table 3Effects of essential oils (1% aqueous solution) on the mycoflora of wheat seed, harvested in 2019.

Variant	Isolation of fungal colonies, number	Amount of colonies, number	Seed germination, %	Average sprout length, mm
Control	A. pullulans 68 Alternaria sp. 41	135	99	62.2
	Trichoderma sp. 5			
	Penicillium sp. 3			
	N. oryzae 2			
	F. sporotrichioides 1			
	Other fungal colonies 15			
Ylang-ylang	A. pullulans 20	34	16	15.0
	Alternaria sp. 13			
	Other fungal colonies 1			

Sandal	Alternaria sp. 3	51	61	58.8
	A. pullulans 1			
	Other fungal colonies 47			
Eucalypt	Penicillium sp. 54	63	90	49.5
	Alternaria sp. 2			
	A. niger 1			
	Other fungal colonies 6			
Vervain	Other fungal colonies 1	1	Have not	-
Cinnamon	Have not germinated	-	germinated	-
Fennel	-		1	2.0
Lavender			Have not	-
			germinated	
LSD ₀₅	-	2.8	4	2.3

In a variant with seed steeping into a 1% aqueous solution of essential oil from vervain only one colony without sporulation germinated from one seed. None of the wheat seeds germinated. The application of ylang-ylang oil cut the number of colonies is almost 4 times, only 16% of seeds germinated and the length of their sprouts on the 7th day equaled 15mm on average. Sandal oil almost completely inhibited colonies *A. pullulans* and Alternaria sp., but the colonies of other fungi germinated without sporulation. Among the variants with the application of essential oils, 61% of seeds had a maximal sprout length. Eucalypt oil caused a significant percentage of isolation of Penicillium sp., which had fewer effects on sprouts length than in 2018 under the application of orange oil. The essential oils from cinnamon, fennel, and lavender prevented the colonies of fungi and wheat seed from germinating. In 2020 after essential oils treatment a greater amount of colonies germinated from seed as compared to the control variant, except

Effects of essential oils (1% aqueous solution) on the mycoflora of wheat seed, harvested in 2020.

for the variants with the application of cinnamon, geranium, and fennel (Table 4).

Variant	Isolation of fungal colonies, number	Amount of colonies, number	Seed germination, %	Average sprout length, mm
Control	Alternaria sp. 62 Trichothecium roseum (Pers.) Link 22 Trichoderma sp. 12 Acremoniella atra (Corda) Sacc. 10 Penicillium sp. 8 Mucor sp. 1	135	97	72.3
Bergamot	Other fungal colonies 20 Alternaria sp. 84 Tr. roseum 26 Trichoderma sp. 21 A. flavus 11 Penicillium sp. 5 Other fungal colonies 8	155	9	25.5
Frankincense	Alternaria sp. 77 Mucor sp. 10 Tr. roseum 24 A. atra 13 Trichoderma sp. 10 Penicillium sp. 8 Other fungal colonies 4	136	78	54.2
Lavender	Alternaria sp. 70 Tr. roseum 32 A. flavus 18 Trichoderma sp. 12 Penicillium sp. 6 Acremonium sp. 1 Other fungal colonies 15	154	Have not germinated	-
Eucalypt	Alternaria sp. 65 Mucor sp. 10 Tr. roseum 28 Penicillium sp. 21 Trichoderma sp. 12 A. niger 1	136	79	43.5
Cinnamon Geranium Fennel	Have not germinated	-	Have not germinated	-
LSD ₀₅	Alternaria sp. 7.4 Tr. roseum 4.3 Trichoderma sp. 3.6	3.1	3.3	0.9

In the previous years of investigation, a decrease in the percentage of Alternaria fungi was admitted, but in 2020 in the variants with the application of essential oils, more colonies germinated. It can be possibly explained by the fact that species *A. arborescens* dominated on control, and in other variants dominated *A. avenicola*. The essential oils reduced the amount of one Alternaria species which dominated the investigation of previous years. The application of bergamot oil caused a maximal amount of fungi colonies among all variants as well as had a negative impact on wheat seed germination. Seed treatment with frankincense and eucalypt oils resulted in a significant amount of secured Mucorales species. The eucalypt oil, like last year, caused an increase in the amount of Penicillium sp. These oils had a negative impact on seed germination. Seed treatment with frankincense oil caused the least impact on the number of fungal colonies. The application of lavender oil prevented the seed from germinating but stimulated the germination of a great amount of fungi colonies, which did not coincide with the results of the investigation conducted last year when no fungus was isolated. This fact indicates the need for many years of research into the effects of essential oils on the seed mycoflora. The fennel and cinnamon oils proved their effectiveness. The application of geranium oil demonstrated a similar reaction (Fig. 1).

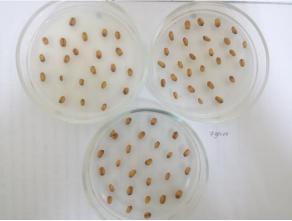


Fig. 1. Non-germinated wheat seeds without fungal colonies on the 7th day after treatment with cinnamon, fennel and geranium oils (2020).

The reason for choosing to further study the effectiveness of essential oils for spraying was their complete suppression of seed mycoflora. As an etalon, the plants were treated with Falcon at the beginning of flowering wheat. The analysis of the impact of plants spraying with essential oils on the inner mycoflora of wheat seed showed the results similar to the fungicide (Table 5).

Table 5 Effects of spraying on inner mycoflora formation of winter wheat seed as well as on 1000 seeds mass (SNAU, 2020).

Variant	Isolation of fungal colonies, %	Mass of 100 grains, g
Control	Alternaria sp. 62.7	38.16
	Tr. roseum 11.7	
	A. atra 7.4	
	Mucor sp. 3.7	
	F. poae 1.6	
	Rh. stolonifer 1.2	
	Other fungal colonies 11.7	
Falkon	Alternaria sp. 25.9	39.54
	A. pullulans 10.7	
	Tr. roseum 13.4	
	Cladosporium sp. 1.8	
	Penicillium sp. 0.9	
	Other fungal colonies 47.3	
Cinnamon	Alternaria sp. 35.6	32.8
	A. pullulans 17.8	
	Cladosporium sp. 4.2	
	Tr. roseum 0.9	
	Penicillium sp. 0.9	
	A. niger 0.9	
	Other fungal colonies 39.7	
Fennel	Alternaria sp. 34.8	36.66
	A. pullulans 20.7	
	Cladosporium sp. 5.4	
	Tr. roseum 3.3	
	A. niger 1.1	
	B. sorokiniana 1.1	
	Other fungal colonies 33.6	
LSD ₀₅	Alternaria sp. 2.6	0.4
	Tr. roseum 0.6	
	Other fungal colonies 0.7	

Spraying has significantly decreased the amount of Alternaria fungi, changed the spectrum of fungi which had an insignificant secure percentage, increased the number of fungi without sporulation, and caused the appearance of *A. pullulans*. Wheat treatment with essential oils solutions resulted in the decrease of the amount of *Tr. roseum*, as well as in the sporadical appearance of dangerous species: *A. niger* and *B. sorokiniana*. Spraying with preparations affected the mass of 1000 seeds. This index increased in a variant with Falkon spraying and decreased in a variant with essential oils treatment.

Discussion

Most investigations on studying the anti-fungal impact of essential oils were conducted under conditions in vitro, and their effectiveness as to a broad spectrum of fungi was achieved and proved. The essential oils of medium concentration *Zataria multiflora* Boiss., *Cuminum cyminum* L., *Foeniculum vulgare* Mill., Pinaceae, and *Heracleum persicum* Desf. ex Fisch. fully inhibited 10 nontoxic (*Fusarium solani* (Mart.) Sacc. and *Fusarium oxysporum* Schlecht.) and 11 toxicogenic (*Fusarium verticillioides* (Sacc.) Nirenberg, *F. poae* and *Fusarium equiseti* (Corda) Sacc.) fungi isolates (Naeini et al., 2010).

But essential oils from different plants had different impacts on fungi. As follows from the data by Ayala-Zavala and others (2009) the essential oil from peppermint has to inhibit the development of *A. flavus* and *A. niger*. In our investigation, on the contrary, it provoked the germination of sporadical colonies of these fungi. The effectiveness of orange peel oil against *A. alternata* and *Alternaria dauci* (JG Kühn) JW Groves & Skolko, which germinated from carrot seed (in vivo) was proved while studying the effectiveness of extractions and essential oils from different plants (Lima et al., 2016). The anti-fungal effects of orange peel oil are attributed to the availability of monoterpene citrine, which has manifested the ability to inhibit the growth of mycelium *A. alternata* in vitro (Marostica et al., 2007). In our investigation orange essential oil has also reduced the amount of Alternaria colonies but has provoked a high percentage of isolation fungi of Penicillium sp. Cinnamon essential oil under conditions in vitro, by 100% has inhibited the growth of phytopathogenic fungi *F. oxysporum*, *Cylindrocarpon destructans* (Penz.) Penz. & Sacc., *Botrytis cinerea* Fr., *Colletotrichum gloeosporioides*, *Rhizoctonia solani* Kühn), except *F. solani*, its inhibition percentage equaled 66.59% (Ma et al., 2019). Our results have also shown a 100% fungi inhibition, but under conditions in vivo. The essential oils from fennel seed and leaves inhibited the mycelia growth of all tested phytopathogenic fungi: Alternaria sp., *Pencillium expansum* L., *R. stolonifer*, *Fusarium oxysporum* f. sp. *albedinis* and, *Aspergillus brasiliensis* (Sellam et al., 2015). The results of our investigation proved a 2-year long inhibition of germination of fungi colonies from wheat seed after its treatment with fennel oil.

It is more difficult to study the effects of the essential oils not on separate species but on a complex and especially, on seed mycoflora. The analysis on the impact of 6 essential oils (*Melissa officinalis* L., *Salvia officinalis* L., *Coriandrum sativum* L., *Thymus vulgaris* L., *Mentha piperita* L., *Cinnamomum zeylanicum* L.) on the mycoflora of wheat seed showed a decrease in the amount of fungi colonies on the 5th day, but on the 22nd day, the inhibiting effects decreased. A great amount of Alternaria fungi was admitted in all variants under oils application. Fungi of Cladosporium sp. have germinated in a variant with sage and coriander, Aspergillus sp. – with coriander and peppermint. The highest fungicide effects were admitted in balm mint and thyme oils. The cinnamon essential oil has effectively reduced the amount of fumonisin (Sumalan et al., 2013).

Our investigation showed the negative impact of essential oils on seed germination and wheat sprouting peculiarities. Some negative effects on seed germination and sprouts length were established when analyzing the effects of rosemary and oregano oils (in different concentrations) on the germination abilities of 5 wheat varieties. This fact was explained by a great amount of monoterpenes which, according to the previous data (Dudai et al., 2004), are powerful inhibitors of wheat seed germination. When the concentration of essential oils increased, the inhibition increased as well. The rosemary oil had less negative impact than that of oregano oil (Atak et al., 2016). The analysis of essential oils in the function of insecticides was combined with the analysis of their effects on seed germination *Triticum durum* Desf. The bergamot, fennel, and lavender oils decreased seed germination by 70% (Rossi et al., 2012). In our investigation, these oils have completely prevented seed germination *T. aestivum* L.

Conclusions

A long-standing studying of wheat seed treatment with essential oils has shown their impact on seed mycoflora formation. The essential oils affected not only the fungi amount but also changed the spectrum of their isolation. A decrease in the amount of fungi colonies was admitted in 2017-2019, but in 2020 their amount increased. The amount of Alternaria fungi decreased under the application of essential oils during the first 3 years, but in the last year of investigation, their amount increased due to specie change. The species *A. arborescens* dominated in control, and *A. avenicola* dominated in variants with the application of oils. The investigation failed to determine the effective essential oil for seed mycoflora regulation. The more the colonies amount decreased, the worse the seed germinated. In all variants, these substances inhibited plant germination and development. The highest seed germination equaled 95% under its treatment with rosemary and orange oils. Sandal oil had the best results (according to the combination of three indices), when the colonies amount decreased by half, seed germination equaled 61%, and the sprout length was maximal among all essential oils (58.8mm).

The essential oils which completely inhibited the seed and fungi development (cinnamon, fennel, vervain, geranium) have been determined. The analysis of two of them in the function of fungicides, under the treatment at the beginning of wheat flowering, proved to have a similar to Falkon impact on the changes of an internal seed mycoflora. But fennel and cinnamon essential oils decreased the mass of 1000 grains as compared to control.

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