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Original paper

In vitro evaluation of fungicide and bio-fungicides against isolates of *Alternaria solani* Sorauer

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Abstract In vitro control of *Alternaria solani* included studies of different fungicides, essential oils basil and ethyl extract of *Allium cepa*. In Romania in the case of tomato culture it is very low due to several production constrains including diseases. One such disease is the early blight caused by fungus *Alternaria solani* (Ell.) Martin. The isolate used in this study was obtained from tomato and it was cultivated on three culture media: potato dextrose agar (PDA), malt extract agar and Czapek Dox medium. Using chlorothalonil, thiophanate methyl, prochloraz, tebuconazole and cooper sulphate, they were evaluated in order to control colony growth of fungi, at the concentrations recommended in agriculture by the specialized literature. The extract of Allium concentration with three levels (3%, 75% and 100%) significantly stronger effect on reducing mycelia growth, reducing spore germination and causing high inhibition percentage of *A. solani*. The fungal effect of the essential oil basil is very low being close to that of the control.

Keywords *Alternaria solani*, leaf spot, tomato, *in vitro*, fungicide, plants extract.

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Introduction

Genus *Alternaria* Nees ex Fr. (syn. *Macrosporium* Fr., *Rhopalidium* Mont.) is a genus of dematiaceous fungi. The colonies are effusive, usually gray, blackish-brown or black. The mycelium is immersed in the substrate, sometimes superficial, consisting of septate hyphae, brown-olive or brown. The stroma is very rare. Thirst and hypopodia are absent. Conidiophores are macronematous, mononematous or simple or irregular and weakly branched, brown-light or brown, septate, with conidial scars. Conidiogenic cells are polyretic, sympodial, sometimes monotretic, healing. Conidia are solitary or catenated (blastoconidia) ovoid or oblique often rostrate, olive brown or brown, smooth or warty, with transverse septa and often longitudinal and oblique.

It parasitizes a very large number of plant species, being a polyphagous pathogen. Host plants attacked: *Allium, Carthamus, Cheiranthus, Chrysanthemum, Cichorium, Citrus, Cruciferae, Cucurbitaceae, Datura, Daucus, Dianthus, Gossypium, Nicotiana, Oryza, Passiflora, Raphanus, Ricinus, Sesamum, Solanum, Sonchus.*

The *Alternaria solani* Sorauer causes damping off of tomato seedlings. Common names used for the disease at various stages of plant and fruit development include seedling blight and damping off on seedlings, foot rot and collar rot on young plant stem, stem blight and canker on stem and brankes, early blight and leaf spot and leaves, blossom blight on the calyx, black rot and hard rot on fruit. Plant infection may result in a complete loss of yield through foliage destruction and direct fruit damage by the pathogen, as well as sun blotch on defoliated plants (ROTEM [14]).

Most currently grown tomato cultivars are susceptible to early blight to varying degrees, so that foliar fungicides are frequently used to manage the disease. The most effective early blight control measure consists of frequent applications of fungicides starting early in the growing season before the first symptoms appear (MAHADEVASWAMY [7]).

As an alternative to synthetic fungicides, and in congruence with improvements in integrated pest management, a search for natural products has become a very important task. Essential oils are plant volatiles containing monoterpenes, sesquiterpenes and phenyl propionoids. Many researchers have examined the influence of essential oils on fungi that are plant pathogens, and fungi important in food industry, and proved that such plant compounds could provide a solution (SITARA & al [15]; SOKOVIĆ & al [16]; TANOVIĆ & al [17]).

Materials and Methods

Alternaria solani was isolated from the infected fruit of tomato collected from Arges region. After performing their pathogen city test their culture was maintained on potato dextrose agar medium, Czapek Dox and malt agar, 27± 1°C (MARDARE & al [8]). The sterilized tissue was dried on sterile filter paper on a clean bench, plated on potato dextrose agar (PDA; potatoes 20 g, glucose 20 g, agar 20 g) and incubated at 25°C. Mycelium growth was observed and transferred to a new plate containing sterilized potato dextrose agar. Pure cultures obtained through hyphal tip method and single spore subculture techniques.

In vitro evaluation of fungicides against Alternaria solani

The efficacy of fungicides was tested against *A. solani* for radial growth inhibition on the Potato dextrose agar medium using poisoned food technique under in vitro condition. Twenty ml of poisoned medium was poured in each sterilized Petriplates. Suitable check was maintained without addition of fungicide. Mycelial disc of 5 mm taken from the periphery of 14 days old colony was placed in the centre of Petri incubated at 27 ± 1°C for 14 days and three replications were maintained for each treatment. The diameter of the colony was measured in two directions and an average value was recorded. Percent inhibition of mycelial growth of the fungus was calculated by using the formula by VINCENT [20].

$$I = (C-T)/C \times 100$$

where,

I = Per cent inhibition
C = Radial growth in control
T = Radial growth in treatment (fungicide)

Evaluation of various bio pesticides against *A. solani*

Development of the microwave assisted extraction procedure (MAE)

The plant material represented by red onion – *Allium cepa* was used to obtain the extract. For microwave assisted extraction (MAE) plant material was preliminarily processed by drying, grinding and sieving (BRAJESH & al [3]). The extract was obtained using a microwave equipment, model NEOS-GR (Microwave Extraction System from Milestone Inc), following the following experimental parameters: power, time, temperature. Following the establishment of the experimental protocol, a single experimental variant was performed using 12.5 g of plant dissolved in 300 ml of solvent (water: alcohol), at a power of 312 W, for 10 minutes (HORBOWICZ [5]). To highlight the antifungal efficacy of *Allium cepa* extract, it was incorporated into the medium in different concentrations using 4 experimental variants: V1 - 0.3%; V2 - 0.4%; V3 - 1%; V4 - 3%, V5 - 8%, V6 - 12%, V7 - 25%.

Elaboration of bioformulations

To obtain bioforms can be used for a basil oil (*Ocimum basilicum*) in pursuit of concentration: V1 – 600

ppm, V2 – 800 ppm, V3 – 1000 ppm, V4 – 2000 ppm, V5 – 3000 ppm. The emulsions were obtained at a temperature of 36°C and a stirring speed of 500 rpm, using an ISOLAB magnetic stirrer, for 5 minutes. Per cent inhibition of mycelial growth of the fungus was calculated by using the formula by VINCENT [20].

Results and Discussion

The pathogen Alternaria solani *in vitro* on culture medium

The pathogen *Alternaria solani* grown on PDA culture medium (potato glucose agar) forms whitish colonies with a radial development from the inoculation point. In the central area, the mycelial hyphae have a dark brown color on a radius of 1.5-1.7 cm. Mycelial hyphae are brown, septate, richly branched, often intertwined. On this culture medium, the growth of the fungus is very fast, in 4 days it reaches a diameter of 4 / 3.6 cm, after 10 days the surface of the Petri dish being completely covered (Figure 1).

In our *in vitro* observations we also observed a color change of the colonies from dark brown with white concentric zones to uniform olive throughout the mass after about two weeks. Being a hyphomycete, the fungus does not form conidiomes on the mycelial hyphae, differing directly from brown, sectarian, fasciculate conidiophores (Figure 2). On these conidiophores, brown, muriform conidia are formed (provided with transverse septa and with 1-2 longitudinal septa or incomplete obligations), short peduncled.

The fungus was also grown on malt-agar, after 4 days it forms whitish colonies with a fluffier appearance than on PDA, without the dark brown area in the center. After 10 days the color of the colonies turns gray. On this medium the dynamics of the pathogen is lower than in the case of PDA after 3 days the diameter being 2 / 2 cm, and after 10 days 6 / 6.8 cm.

Figure 1. *Alternaria solani in vitro*

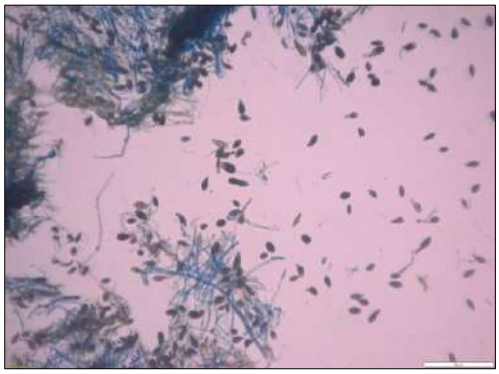


Figure 2. *A. solani* microscopic characters

On the medium Czapek Dox fungus *Alternaria solani* had a slow growth, the colonies having a gray color, with a muddy appearance, the diameter being 1 / 1 cm at 3 days and 6.5 / 6.6 cm at 21 days.

The best culture medium for the growth and development of the pathogen was PDA. This medium is rich in starch and glucose, two carbohydrates that are essential in fungi metabolism.

In vitro evaluation of fungicides

Among the different fungicides tested the maximum of 100 per cent inhibition was found in procloraz (97.84%), followed by tebuconazole (94.96%) and cooper sulphate (93.45), succeeded by chlorothalonil (37.88%) and thiophanate methyle (23.75%) of mycelial inhibition (Table 1). Thiophanate methyle was less inhibitory than either procloraz or tebuconazole at all investigated concentrations (Figure 3).

Chlorothalonil was less inhibitory than either prochloraz or tebuconazole at all investigated concentrations. Mycelial growth was reduced less than 10% by the lowest chlorothalonil concentration. The fungicidal effect of copper on the mycelium was exceptional at all concentrations tested, which shows the negative influence of copper on the respiratory processes in fungal cells (Figure 3).

Similar results were obtained by the author G. MAHADEVASWAMY & K.S. RAGHUWANSHIWHO [7] reported that the sistemic fungicide chlothalonil, tebuconazole and propiconazole at *Alternaria solani* causing early blight in tomato.

These results are in conformity with the earlier findings of those workers who reported systemic fungicides hexaconazole, propiconazole, penconazole, difenconazole, thiophanate methyl and carbendazim at various concentrations had significantly inhibited mycelial growth of *A. carthami* infecting safflower (MURUMKAR & al [10]; TAWARE & al [18]), *A. helianthi* infecting sunflower (AMARESH & NARGUND [1]), *A. alternata* infecting sesame (BAVAJI & al [2]).

Table 1. Statistic analysis of data obtained *in vitro* on PDA with fungicides after 10 days

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minim.	Max.
					Lower Bound	Upper Bound		
Chlorothalonil (0.2%) V1	6	37.8833	1.88340	.76889	35.9068	39.8598	35.71	41.26
Thiophanate V2 (0.1%) V2	6	23.7450	3.25770	1.32995	20.3263	27.1637	20.12	28.44
Prochloraz (0,1%) V3	6	99.1300	1.00735	.41125	98.0728	100.1872	97.91	99.99
Cooper sulphur (0,5 %) V4	6	93.3500	1.19204	.48665	92.0990	94.6010	91.30	94.28
Tebuconazole (0,1%) V5	6	94.9650	1.19375	.48734	93.7122	96.2178	93.18	96.78
Control	6	5.0000	.00000	.00000	5.0000	5.0000	5.00	5.00
Total	36	59.0122	38.62715	6.43786	45.9427	72.0818	5.00	99.99

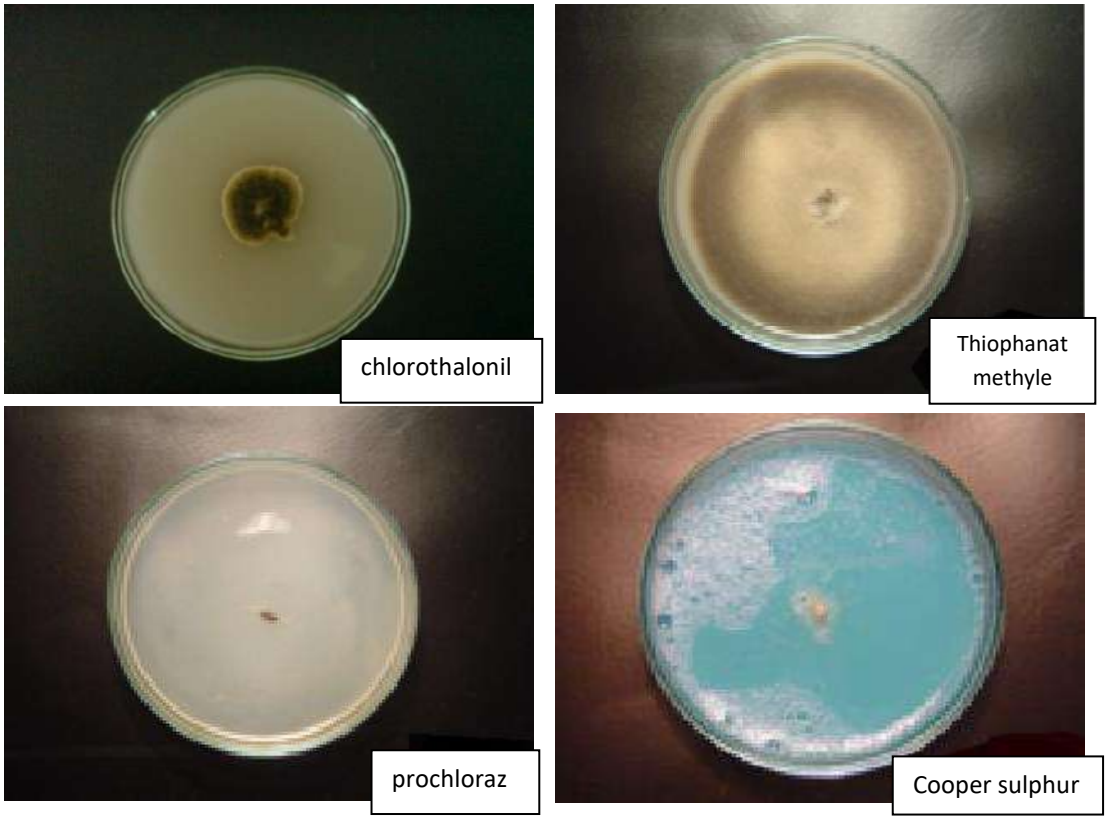


Figure 3. Growth of *A. solani* isolate on PDA amended with fungicides.

In vitro evaluation of bio-fungicides

There was an interaction between plant type and concentration level of the plant extracts on mycelia growth diameter of *A. solani*. The mycelia colony diameter decreased with an increase in concentration rate of the different plant extracts and essential oils.

The inhibitory effects of essential oils basil (*Ocimum basilicum*) against *Alternaria alternata* were tested at different concentrations (600-3000 ppm) *in vitro*. From the analysis of the figure it is observed that in all the tested concentrations, the fungal effect of the essential oil basil is very low being close to that of the control. Similar studies have been performed in Romania on the pathogen *Alternaria tenuis*, where the garlic extract does not have antifungal effect, for the dose of 20 µl/godery (MIRONAS & al [9]).

In current studies the most tested product was essential oils thyme where remarkable results were obtained in biological control, even at the lowest concentrations, 100-500 ppm (WUFENG & al [21]).

The fungicidal or fungistatic effect on the pathogen *Alternaria solani* was also tested in the case of the ethyl extract of *Allium cepa* in concentrations of 0.3%-12%. From the figure the fungicidal effect is observed at the

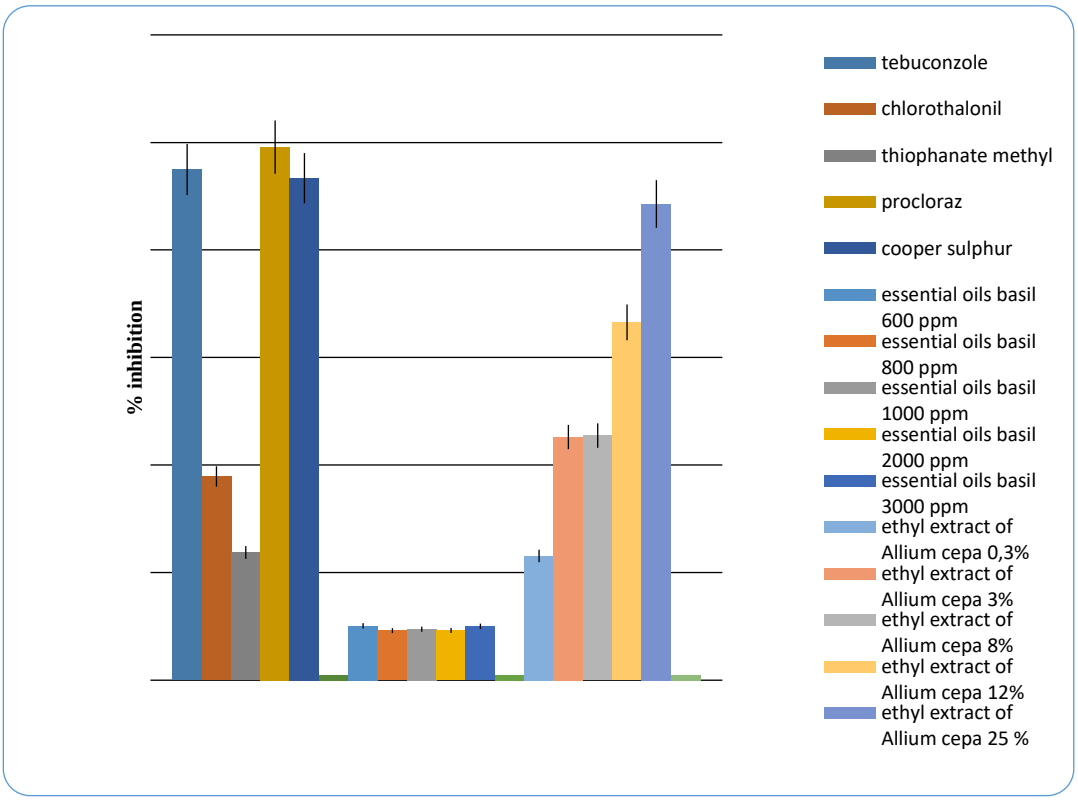
progressive increase of the extract concentration. The extract of *Allium* concentration with three levels (3%, 75% and 100%) significantly stronger effect on reducing mycelia growth, reducing spore germination and causing high inhibition percentage of *A. solani* (Table 2).

Table 2. Statistic analysis of data obtained *in vitro* on PDA ethyl extract of *Allium cepa* after 10 days

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	*Upper Bound*		
0,3% V1	6	13.5417	3.08044	1.25759	10.3089	16.7744	10.12	18.25
3 % V2	6	16.4017	.62863	.25664	15.7420	17.0614	15.44	17.10
8 % V3	6	49.7250	2.77178	1.13157	46.8162	52.6338	45.12	52.89
12 % V4	6	67.1683	2.59353	1.05880	64.4466	69.8901	64.12	71.25
25 % V5	6	75.5417	3.76960	1.53893	71.5857	79.4976	68.34	78.84
Control	6	5.0000	.00000	.00000	5.0000	5.0000	5.00	5.00
Total	36	37.8964	28.03069	4.67178	28.4122	47.3806	5.00	78.84

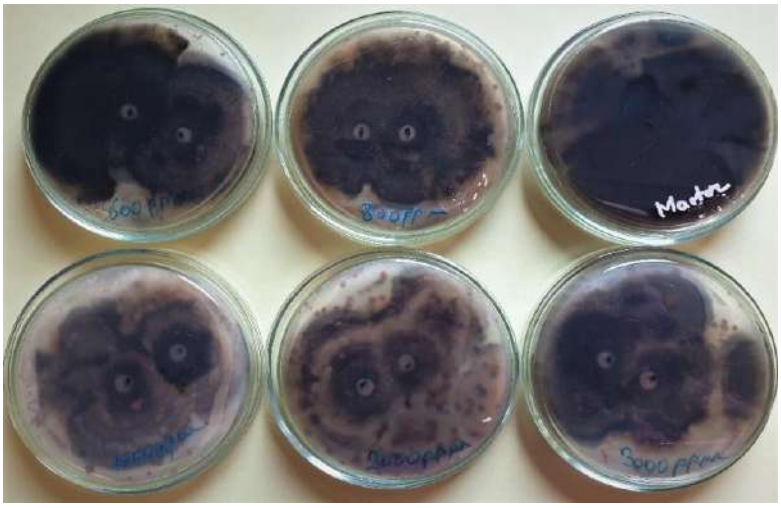
The existence of antifungal activity in the extracts may be attributed to the presence of allicin which is known for its broad spectrum antifungal activity. Allcin is the major compound, naturally present in garlic which is broken down into allicin, ammonium, and pyruvate under the action of alliinase enzyme (FOOKE [4]). Allicin is known to inhibit the germination of fungal spores as well as the hyphal growth (LEDEZMA [6]. Ajoene, another major bioactive compound of garlic might also be attributed to the antifungal property of the extracts (MUDYIMA & al [10], NAGANAWA & al [12]).

There was an interaction between plant type and concentration level on their effects on inhibition percentage. As the concentration of the plant extracts increased; the *A. solani* inhibition percentage also increased (Figure 4) Similar results were obtained by the author MUDYIWA & al [10] showed that the plant extracts had strong anti-*A. solani* activity and their effect increased with increase in concentration 50%, 75% and 100%.



Figures 4. Growth of *A. solani* isolate on PDA amended with essential oil basil.

The fungi toxic effects of all phyto extracts indicate the potential of selected plant species as a source of natural fungicidal material. Compared to the application of chemicals, in the case of biological products, the fungistatic effect on the pathogen is directly proportional to the concentration used. In our observations we did not observe a fungicidal effect of the bioformulations in all the tested variants. The quality of the bioformulations, the moment of their application, generally preventive, corroborated with the environmental conditions can ensure an optimal control of the morphological and physiological changes appeared during the pathogenesis process.



Figures 5. Growth of *A. solani* isolate on PDA amended with essential oil basil.

Figures 6. Growth of *A. solani* isolate on PDA amended with ethyl extract of *Allium cepa.*

Chemical control is the ultimate and easy solution of disease. But biological management is more acceptable to environment and human being. Lot of literature is available regarded to justify biological management of *Alternaria* leaf spot of *B. compestris*. Spray of soil isolates of *Trichoderma viride* at 45 and 75 days after sowing could manage *Alternaria* blight of Indian mustard (*Brassica juncea*) as effectively as mancozeb and other fungicides, which have been incorrigible later in multiplication trials (SOKOVIC & al [16]).

Interesting studies that may be an important side in biological control have been done on the pathogen *Alternaria alternata* which have shown that the treatment performed in combined form, consisting of both blue light irradiation emitted by LED-s, and growth in the presence of the Bengal Pink substance, induced the best inhibition and inactivation effects on colonies of fungi (POPA & al [13]).

Conclusion

The *in vitro* efficacy of classical fungicides has shown that tebuconazole and prochloraz are highly effective against this pathogen, and it is recommended to be used in the treatment of fruits before storage. In assessing the overall effectiveness of a plant protection product, other aspects, positive or negative, are taken into consideration: the duration of its biological activity (persistence of action), compatibility with different protection strategies

or cultural practices, application facilities, etc. The results of this study showed that the tested fungicides exhibited different levels of toxicity to *A. solani* isolates. Chlorothalonil and thiophanate methyl demonstrated the low toxicity to all tested *A. solani* isolates.

The direct biological efficacy of plant protection products depends on many factors, namely: the chemical nature and mode of action of the active substance, the concentration or dose of the active substance or commercially applied product, the form of pesticide conditioning, the duration of action of the active substance, the conditions environment (temperature, humidity, light, etc.), the development stage of the pathogen, the application equipment for the plant protection product etc.

The direct effectiveness is evaluated under conditions as close as possible to those of use in the agricultural practice of the product. This means, depending on the case, the evaluation in field experiments or in greenhouses.

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References

1. AMARESH Y.S., NARGUND V.B. *In vitro* evaluation of fungicides against *Alternaria heliathi* causing leaf blight of sunflower. *Indian J. Pl. Pathol*.; 22: 79-82 (2004).
2. BAVAJI M., KHAMAR M.D., NATH M.M. *In vitro* evaluation of fungicides and plant extracts on the incidence of leaf blight on sesame caused by *Alternaria alternata* (FR) Keissler. *Intern. J. Food Agric. Vet. Sci.* 2; 2 (3): 105-107 (2012).
3. BRAJESH KUMAR, KUMARI SMITA, BRAJENDRA KUMAR, LUIS CUMBAL, GUSTAVO ROSERO. Microwave-Assisted Extraction and Solid-Phase Separation of Quercetin from Solid Onion (*Allium cepa L.*) (2014).
4. FOCKE M., FELD A., LICHTENTHALER K. Allicin, a naturally occurring antibiotic from garlic, specifically inhibits acetyl-CoA synthetase. *FEBS Lett* 261:106-8 (1990).
5. HORBOWICZ M. Method of quercetin extraction from dry scales of onion. *Veget. Crops Res. Bull*. 57: 119-124 (2002).
6. LEDEZMA E., APITZ-CASTRO R. Ajoene the main active compound of garlic (*Allium sativum*): A new antifungal agent. *Rev Iberoam Micol*; 23(2):75-80 (2006).
7. MAHADEVASWAMY G., RAGHUWANSHI K.S. *In vitro* Evaluation of Fungicide and BioPesticides against Isolates of *Alternaria alternata* (Fr.) Black Spot. *Int. J. Curr. Microbiol. App. Sci.* 8(08): 2227-2234 (2019).
8. MARDARE E.S., CRISTEA S., GÂDEA M., TAMBA-BEREHOIU R. The influence of some abiotic factors on the development of *Alternaria* spp. pathogen ("in vitro") *Roumanian Biotechnological Letters*., 20(5): 10880-10884 (2015).
9. MIRONAS O., POHRIB C., MATEESCU R., BOCA E., JURCOANE S. The inhibitor effect of some vegetal extracts on the fungal strains of the *Alternaria* sp. and *Fusarium* sp., *Roumanian Biotechnological Letters*., 9 (1): 1523-1532 (2004).
10. MUDYIWA R.M., STEPHEN C., MARJORY T. Anti-*Alternaria solani* activity of onion (*Allium cepa*), Ginger (*Zingiber officinale*) and garlic (*Allium sativum) in vitro*, *International Journal of Plant and Soil Science* (2016).
11. MURUMKAR D.R., INDI D.V., GUD M.A., SHINDE S.K. and DESHPANDE A.N. Fungicidal management of leaf spot of safflower caused by *Alternaria carthami. J. Maha.Agric. Univ*. 34 (1): 54-56 (2009).
12. NAGANAWA R., IWATA N., ISHIKAWA K., FUKUDA H., FUJINO T, SUZUKI A. Inhibition of microbial growth by ajoene, a sulfur-containing compound derived from garlic. *Appl Environ Microbiol*; 62(11):4238-42 (1996).
13. POPA M., LIVADARIU O., DANAILA-GUIDEA S., NICULITA P., RISTICI J., RISTICI M. *In vitro* study regarding the testing of treatments with inhibiting effect on the pathogenic fungi of *Alternaria alternate*, *Roumanian Biotechnological Letters*., 13 (6): 4014-4021 (2008).
14. ROTEM J. The genus *Alternaria*: biology, epidemiology and pathogenicity. APS Press, St Paul, Minnesota (1994).
15. SITARA U., NIAZ I., NASEEM J., SULTANA N. Antifungal effect of essential oils on In Vitro growth of pathogenic fungi. *Pak. J. Bot*., 40(1): 409-414 (2008).
16. SOKOVIC M., MARIN P.D., BRKIC D., LEO J.L., GRIENSVEN D.V. Chemical composition and antibacterial activity of essential oils of ten aromatic plants against human pathogenic bacteria. *Food*, 1(2): 220-226 (2007).
17. TANOVIĆ B. *In vitro* effect of essential oils from aromatic and medicinal plants on mushroom pathogens: *Verticillium fungi cola* var *.fungicola*, *Mycogone perniciosa*, and *Cladobotryum* sp. *Archives of Biological Sciences, Belgrade*, 61(2): 231-237 (2009).
18. TAWARE M.R., GHOLVE V.M., UTPAL DEY. Bio-efficacy of fungicides, bioagents and plant extracts / botanicals against *Alternaria carthami*, the causal agent of *Alternaria* blight of safflower (*Carthamus tinctorius* L.). *Aftrican J. Microbio. Res*. 8 (13): 1400-1412 (2014).
19. VASUDHA A., KADAM D.N., DHUTRAJ D.V., PAWAR. *In vitro* evaluation of Different Fungicides against *Alternaria alternata* causing Leaf and Fruit Spot in Pomegranate. *Int. J. Curr. Microbiol. App. Sci.*, 7(10): 2292-2298 (2018).
20. VINCENT J.M. The esters of 4-hydroxybenzoic acid and related compounds. Part I. Methods for the study of their fungistatic properties. *J. Soc. Chem. Ind*., 66: 149-155 (1947).
21. WU FENG, XIAODONG ZHENG. Essential oils to control *Alternaria alternata in vitro* and *in vivo*. *Food control* 8(9):1126-1130 (2007).