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Phytotoxicity of coumarin on two weed species Trifolium and Medicago sativa and two plant pathogenic fungi Sclerotinia sclerotiorum and Aspergillus fumigatus

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Abstract

Coumarins are a group of secondary plant substances from the phenylpropanoid group. Numerous reports have been published on the biological activity of these compounds. These compounds occur in the legume family (*Fabaceae*), the citrus family (*Rutaceae*), and the umbelliferous family (*Apiaceae*). The phytotoxic effect of simple coumarin on clover and alfalfa seeds as well as the inhibitory effect on phytopathogenic fungi such as *Sclerotinia sclerotiorum* and *Aspergillus fumigatus* were tested. Physiological parameters such as root and shoot growth of these seeds as well as fungal growth were evaluated. First, the mentioned compounds were extracted at various concentrations including 0.001, 0.01, 0.1, and 1 mg/ml, and were prepared by diluting them in distilled water.

For fungi, different concentrations of coumarin in the culture medium were also prepared, including 0.25, 0.5, and 1 mg/mL, and the distilled water sample was considered as a control and performed in triplicate. Petri dishes with seeds and media with fungi were placed separately in an incubator at 25 degrees Celsius. The results showed that coumarin at all treatment concentrations, i.e. 0.001 mg/ml and more was harmful to the germination, root, and shoot growth of these seeds. Even in all concentrations of the fungi growing medium, e.g.0.25 mg/ml more damaged fungal growth. At a concentration of 1 mg/ml, it completely prevents the germination of trifolium and alfalfa seeds and the development of fungi. The ultimate goal of the research is to find natural herbicides to replace them with artificial herbicides that are harmful to the environment.

Keywords: Allelopathy, Apiaceae, Aspergillus fumigatus, Fabaceae, phenylpropanoid, Rutaceae.

1.Introduction

Allelopathy comes from the Greek words (allelon) for each other and (pathos) for illness or disease. The concept of allopathy does not include positive interactions. This is part of the knowledge of chemical ecology and refers to the inhibitory effect on the growth, development, or germination of another plant (Muller et al., 2000). The word interference is used to represent the effect of one plant on another plant as well as the effect of microorganisms on different plants. Disruptions include competition and injuries. The chemicals produced by allelopathy are called allelochemicals (Migani, 2003).

Chemicals that cause other damage can be released from living plants, secretions from dead plants and detached leaves, or through the chemical and microbial decomposition of plant residues. Terpenoids and alkaloids act as allelochemicals. Phenolic compounds, as secondary metabolites, can influence plant reproduction and growth (Indesit, 1996). Studies have shown that phenolic compounds influence biochemical and physiological processes (Leather and Einhellig, 1998). These are the age, concentration, and metabolic stage of the plant, climate, season, and environmental conditions. In addition, their production fluctuates qualitatively and quantitatively not only throughout the year but also depending on the variety, age, and type of organs. The results of allelopathy research are used in the areas of agriculture and forestry, biochemistry, microbiology, plant protection, soil science, natural sciences, plant pathology, and entomology. Coumarin is one of the phenolic compounds commonly found in dark plants (*Rutaceae*), legumes (*Fabaceae*), and *Apiaceae* (Razavi., 2011). In plants, coumarin is synthesized from phenolic propanoids and accumulates mainly in vacuoles. The main goal of allelopathy research is to find the cause of disruption of normal, natural conditions by chemicals and to introduce allelochemical compounds that prevent the growth of other plants and microorganisms in agricultural or natural ecosystems. Another goal of this science is to identify the allelochemical associations of plants and microorganisms with those of their environment, on which only limited research has been conducted (Migani, 2003). This research aims to study the biological responses (stem and root growth) of Trifolium and alfalfa seeds as well as the growth of the fungi Sclerotinia sclerotiorum and Aspergillus fumigatus to stress caused by the phenolic compound coumarin.

Clover

Trifolium alexandrinum is one of the plants belonging to the *Leguminosae* family, which has a relatively good potential to fix atmospheric nitrogen and increase soil nitrogen storage (Moawad et al., 1998; Unkovich and Pate., 2000). The quality of berseem forage is often similar to alfalfa, but in some conditions, the amount of its digestible elements is much higher and the amount of crude protein is lower than alfalfa (Shrestha et al., 1998). Compared to salinity, this plant is relatively sensitive to salinity, and hence improving its growth and development in salty soils has been considered (Francois et al., 1999). True clovers belong to the genus Trifolium and there are about 300 species of it in the world. Clover is the most important dicotyledonous fodder plant after alfalfa (Zamanian, 2006).

Alfalfa

Alfalfa belongs to the genus Medicago and family Fabaceae and its cultivar is M. sativa, a completely autotetraploid plant (Tan and Sperin, 2004). Medicago sativa is the most important fodder crop in the world, it contains many nutrients, including minerals and vitamins, especially vitamins A and C, is rich in calcium, has a low cellulose content, and a high yield, particularly higher than that of fodder systems. The following plant is a perennial plant and belongs to the sorghum family, which maintains its optimal density for several consecutive years in the absence of weeds. However, when weeds appear, especially on perennials of the wheat family, the optimal density of alfalfa gradually decreases (Karimi, 2007). As a perennial plant from the legume family, it is the most important forage plant for feeding livestock and represents the most important economic product after cereals (Barcaccia et al., 1999; Diwan et al. 1997).

Sclerotinia sclerotiorum

Sclerotinia sclerotiorum is one of the least specific omnivorous plant pathogens (Purdy, 1979). *Sclerotinia* affects more than 400 different plant species (Bland and Hall, 1994). Its hosts also include other crops such as peas, potatoes, sunflowers, clover, beans, lentils, etc. (Bailey 1996). Dispersal of *Sclerotina* occurs primarily via airborne ascospores, which are thought to be carried by wind over distances of several kilometers (Brown and Butler, 1936; Williams and Stelfox, 1979). To initiate infection, these ascospores require a food source (Purdy 1958), such as a flower petal (Abawi and Grogan 1975). Stem *sclerotinia* is the most serious disease affecting rapeseed products in China (Liu et al. 1990).

Aspergillus fumigatus

Aspergillus is one of the most widespread fungi in the environment and air, causing disease in animals and humans. Among the various species in this genus, fumigatus species play a more important role in pathogenicity and possess antigenic power, enzymes, and toxins. In this fungus, the conidia are smaller than here (Latge., 1999). In this experiment, the desired seeds were prepared in packets by Pakan Seed Company in Isfahan, and Sclerotinia and Aspergillus fungi were prepared and used in the Plant Anatomy Laboratory of Mohaghegh Ardabili University.

.2Materials and methods

2.1. Cultivation and maintenance stages

Clover and alfalfa seeds were disinfected separately. They were first washed with sodium hypochlorite solution for 3 minutes and then rinsed with distilled water. To produce the various coumarin gels, 20 mg of the substance was measured using a scale. The substance was then carefully weighed and poured into a microtube. To prepare the coumarin solutions, 2 drops of Tween 20 were added to solubilize the coumarin, which is insoluble in water. The contents of the microtube were then transferred to a balloon and the volume was increased to 20cc. The solution was shaken with a shaker for approximately 11 minutes. This resulted in a concentration of 1mg/ml. From the 1mg/ml solution, concentrations of 0.001, 0.01, and 0.1 mg/ml were prepared as stock solutions. To ensure accuracy in the test, 2 drops of Tween 20 were added to the control solution, which contained 2cc of distilled water. To examine each plate, 12 sterilized petri dishes were used. A piece of Whatman filter paper was placed at the bottom of each petri dish. Then, 11 desired seeds were placed in each petri dish. The same process was repeated for the control group, with 3 sterilized petri dishes prepared. The plates containing the seeds were then placed in an incubator set at a temperature of 21 degrees Celsius. The rate of germination was recorded daily for 6 days. Additionally, the length of the shoot and root was measured in different concentrations. As for the cultivation of fungi, please provide more specific information or refer to the appropriate documents for further details.

2.2. Preparation of culture medium and mushroom stock

To prepare the stock culture medium for the plates in the cultivation of fungi, a PDA (Potato Dextrose Agar) culture medium was used. The following steps were followed: 1. Weighed 3.8 grams of PDA powder. 2. Dissolved the PDA powder in a specified amount of distilled water to make a volume of 1000 ml. 3. Heated the solution until the powder was completely dissolved and the diluted solution became clear. 4. Pour 15 ml of the solution into each petri dish. 5. Using a corkscrew, remove a

a piece of *sclerotina* and place it in a part of a petri dish. 6. Place the petri dishes in an incubator for a week to allow the fungus to grow and fill the petri dish with its mycelium. Following these steps, the stock culture medium was prepared and the fungus was allowed to grow in the petri dishes.

2.3. Preparation of required concentrations of coumarin and their filtration

To prepare the culture media with different concentrations of coumarin for the cultivation of fungi, the following steps were followed: 1. Desired concentrations of 1, 0.5, and 0.25 mg/ml were chosen (with 3 repetitions for each concentration). 2. The solutions with coumarin concentration were filtered through a filter to remove any impurities. 3. After filtration, the solutions were added to the warm and liquid environment of the ship. 4. The resulting environments were distributed inside the Petri dishes5. All these operations were carried out under sterile conditions and within a UV hood to maintain cleanliness. 6. Once the culture media was prepared, the Petri dishes were placed in the refrigerator for 24 hours to cool and harden, making them ready for cultivation. To cultivate Sclerotinia and Aspergillus fungus in plates with coumarin material, the following steps were followed: 1. Circular pieces of fungi mycelium were removed using a corkscrew. 2. The mycelium discs were placed in a culture medium with coumarin concentrations. 3. The discs containing mycelium were placed in the middle of the plates.

2.4. Statistical analysis of data

The length of the root and stem of each seed in all tested concentrations was measured accurately using a millimeter ruler. The data was then analyzed using SPSS 21 software. To determine the significance of the differences between the test groups, Zanken's test was performed. The results showed that the differences between the test groups were statistically significant compared to the control group and among each other. In the case of measuring the radius of the mycelium of the fungi, the mycelium was measured and checked daily. The obtained data was analyzed using SPSS statistical software. The analysis showed that the differences in the radius of the mycelium among the different time points were statistically significant ($P \geq 0.05$).

3. Results

3.1. Effects of Coumarin on the Clovers and Alfalfa

The observed effects of increasing the concentration of Coumarin composition on seed germination and seedling growth of *Trifolium* and *Medicago* (alfalfa) were as follows: Figure 1 shows a decrease in stem growth of *Trifolium* and *Medicago* with increasing Coumarin concentration. As the concentration of Coumarin increased, the growth of stems in both Trifolium and Medicago (alfalfa) decreased. Figure 2 demonstrates a decrease in root growth of *Trifolium* and *Medicago* with increasing Coumarin concentration. As the concentration of Coumarin increased, the growth of roots in both *Trifolium* and *Medicago* decreased. Specifically, Coumarin in concentrations of 0.1 and 0.1 mg/ml gels had a strong negative effect on the germination of *Trifolium* and *Medicago*. In the concentration of 1 mg/ml Coumarin, none of the seeds were able to grow. These observations indicate that increasing the concentration of Coumarin composition negatively impacts seed germination and seedling growth of *Trifolium* and *Medicago*, with higher concentrations having a more pronounced effect.

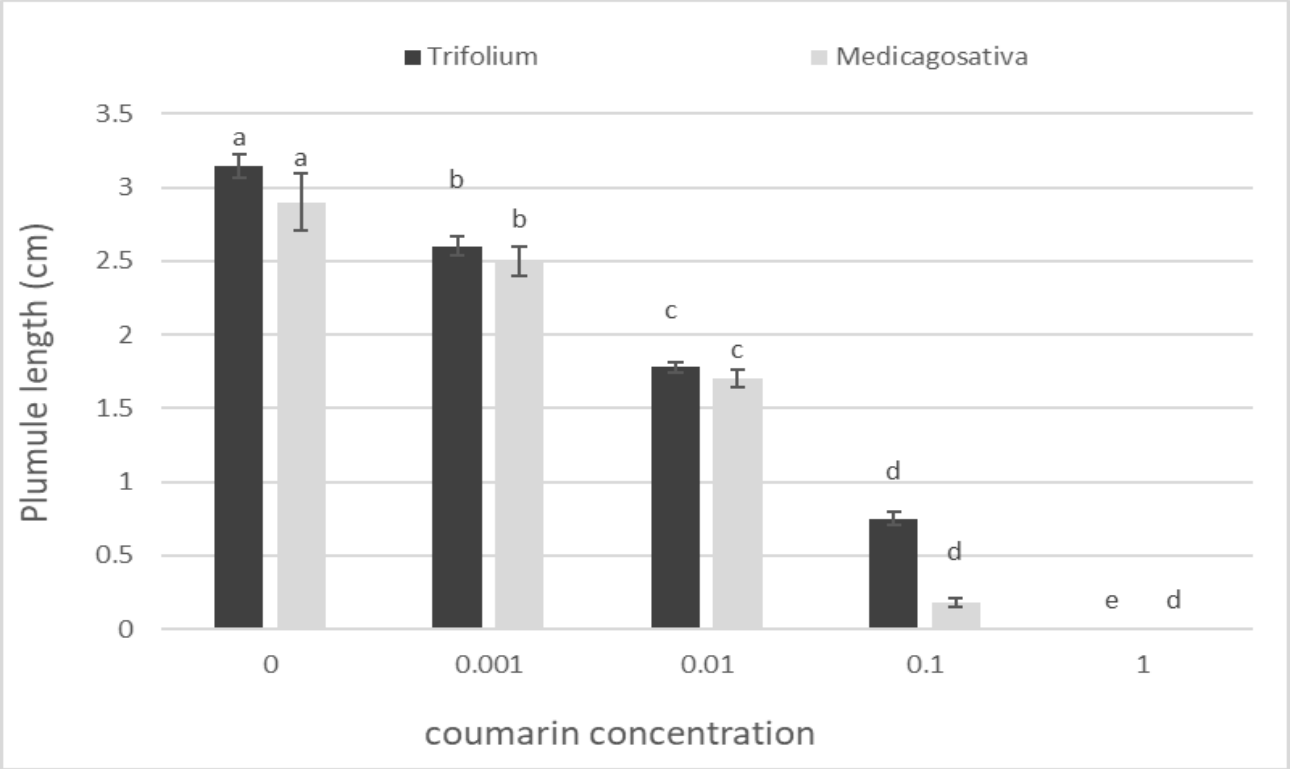

Figure1. The effect of coumarin in different concentrations on the germination of stems of *Trifolium* and *Medicago sativa*

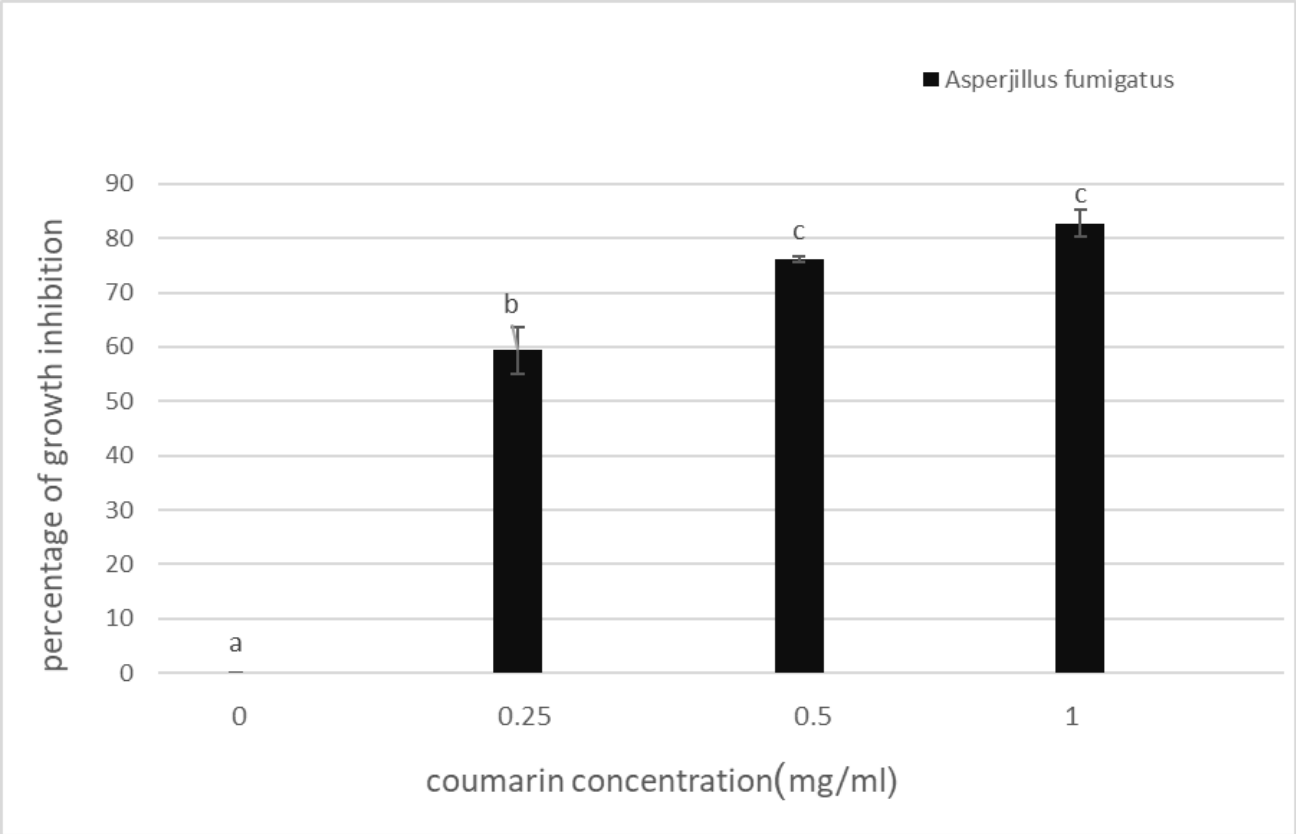

Figure 2. The effect of coumarin in different concentrations on the germination of the roots of *Trifolium* and *Medicago sativa*

3.2Effects of Coumarin on the Scrotina and Aspergillus fungi

3.2.1 Effects on Sclerotinia

Analysis of variance of the effect of coumarin on the vegetative growth of *Sclerotinia sclerotiorum* revealed that each container containing different concentrations of coumarin depending on the diameter of the fungal colony was significant with a probability of 0.05% because the culture medium contained 1 ml g/ ml showed no increase and media containing 0.5 and 0.25 showed radial growth, with the 0.25 concentration having the strongest radial growth. At a concentration of 1mg/ml coumarin, we observed an inhibitory effect of 100%, at lower concentrations this inhibition decreased. The inhibitory effect was 80.53% at a concentration of 0.5 mg/ml and the inhibitory effect was 47.7% at a concentration of 0.25 mg/ml (Figure 3). The percentage of inhibition was calculated and graphed using the following formula:

Percentage of inhibition of fungi mycelium growth = (dc/dt-dc)×100

In this formula, dc is the average diameter of the fungus colony in the control group and dt is the average diameter of the fungus colony in the treatment group.

3.2.2. Effect on Aspergillus fumigatus

The effect of coumarin on the vegetative growth of *A. fumigatus* showed that the medium with a concentration of 1mg/ml inhibited the radial growth of fungal mycelium by 73.82% compared to the control, and a concentration of 0.5 inhibited the radial growth of fungal mycelium by 76. 06%. The concentration is 0.25 inhibited the growth by 59.4%. As the concentration of coumarin increases, the percentage of inhibition also increases, so that at a concentration of 1mg/ml, coumarin snowed a sig-

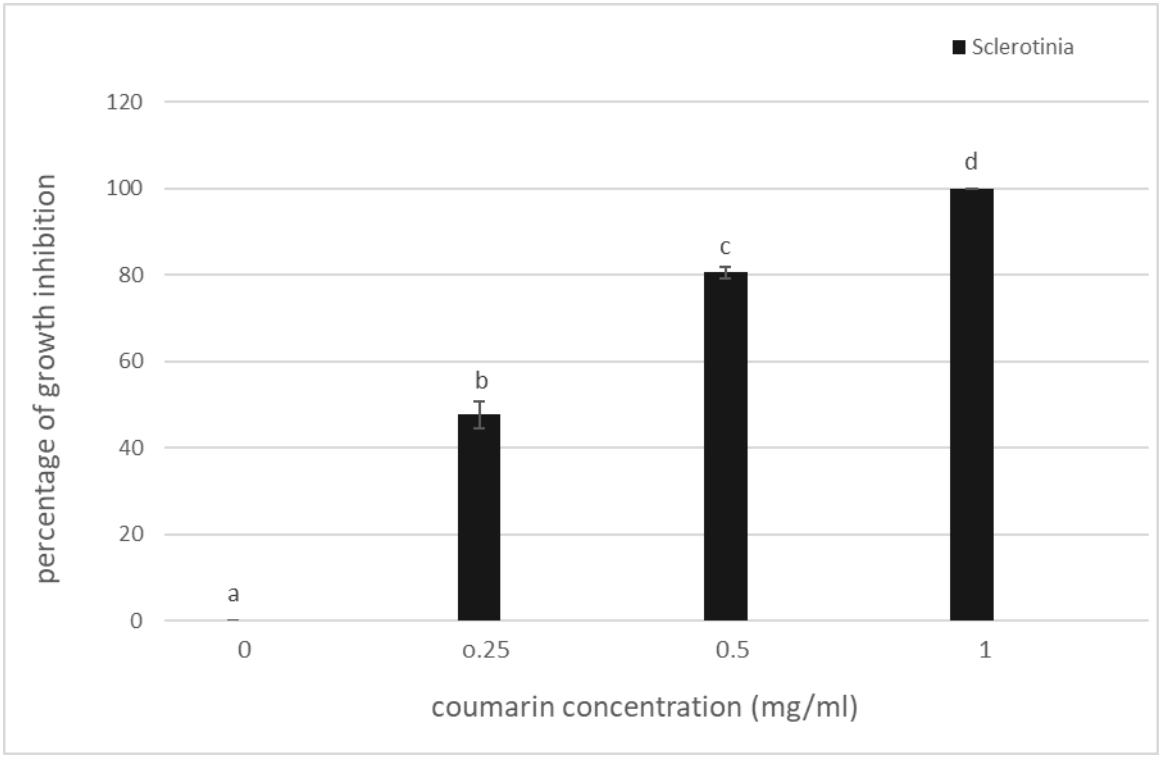

Figure 3. The amount of inhibitory effect of coumarin on the growth of*Sclerotinia* fungus

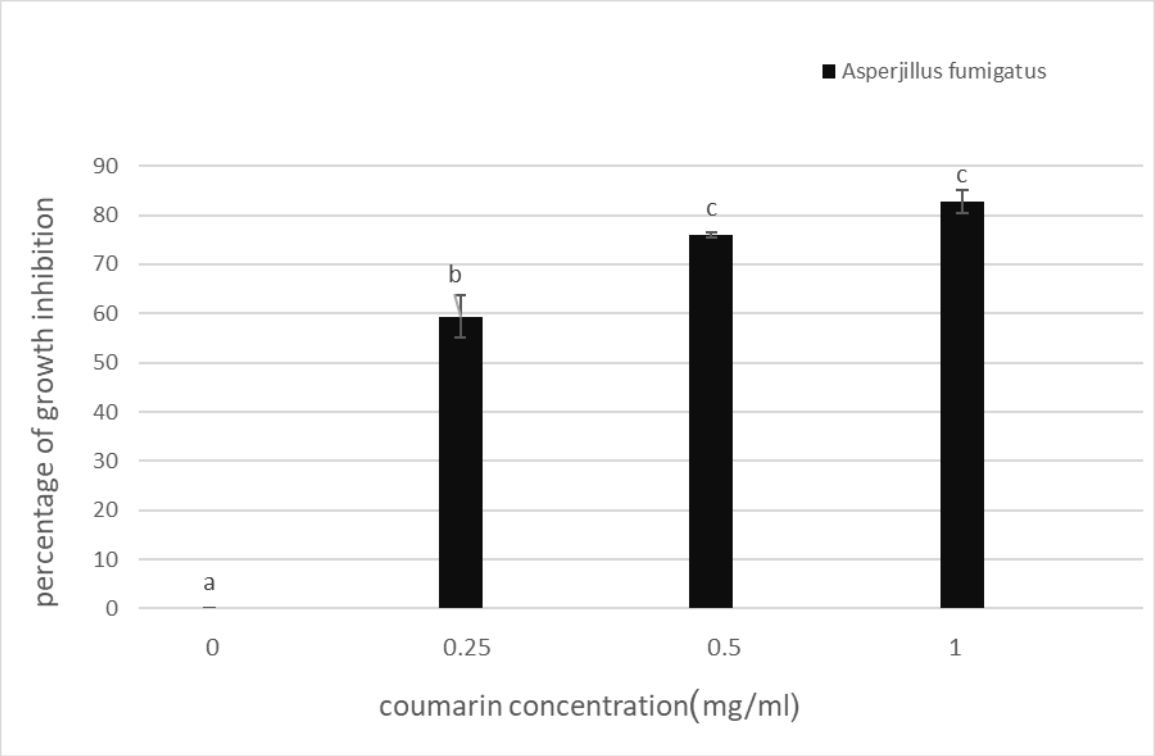

Figure 4. The amount of inhibitory effect of coumarin on the growth of Aspergillus fungus

Discussion and conclusion

In this project, it has been shown that with the increase of coumarin, the germination rate of Trifolium and medicaco seeds has decreased. , this decrease was in the length of roots and stems, The reduction of seed germination can be due to factors such as the reduction of mitotic divisions in the root meristem, the reduction of the activity of catalyzing enzymes and the disruption of the absorption of mineral ions in the presence of allelochemicals. On the other hand, the growth of plant pathogenic fungi, Sclerotina and Aspergillus fumigatus, decreased with the increase of coumarin. The negative effects of phenolic compounds on rapeseed plant growth have also been reported by (Baleroni, et al 2000). Razavi investigated the effects of coumarin compounds on the germination of lettuce seeds, and in this study, coumarin compounds had an inhibitory effect on the germination of lettuce plants. investigated the effect of six phenolic compounds on the germination of some weeds, the phenolic compounds in high concentrations had an inhibitory effect on the germination of the test bed seeds. Regarding the effect of coumarin on some plants such as corn, canola, Arabidopsis thaliana and … studies have been carried out.

Also, in Arabidopsis thaliana, the effect of coumarin on the terrestriality of this plant is that the results of this compound indicate the significant effect of this compound on the responsiveness of Arabidopsis thaliana. In another study, the effect of coumarin on the anatomy and growth indicators of roots, as well as the absorption of some ions and their transfer, has been done. The result of the story is about the negative effect of coumarin on the growth indicators and also the effect on root anatomy. Crop diversity also requires different outcomes for different plants.

It minimizes the chemical pesticide in the environment (Sotoodeh, 2017).In other research, they tested the effect of six phenolic compounds including (ferulic acid, gallic acid, coumaric acid, parahydroxybenzoic acid and vanillic acid) on the germination of some weeds including Chenopodium album،Plantago lanceolata L.،Amaranthus retroflexus،Solanum nigrum L. and Cirsium sp, the studies showed that high concentrations of phenolic compounds inhibited the germination of the seeds of the plants tested, while low concentrations of these compounds had no effect on the germination of the seeds, and even stimulated the germination of the seeds, they found that a combination of alchemical compounds mentioned can increase the inhibitory effects. (Reigosa, et al, 1999).

The use of secondary compounds of plants, extracts and essential oils as antimicrobial agents in food, herbal medicine and pharmaceutical industries is increasing (Isman, 2000). The effects of inhibiting the mycelium growth of Botrytis cinerea by thyme, cloves and mint were also investigated in 1997 and it was shown that thyme essential oil prevented the mycelium growth of the fungus (Antonov et al, 1997).

In another study, with the use of plant essential oil in the growth of the fungus that causes white rot of rapeseed (Sclerotinia sclerotiorum), they showed that the essential oil of Mentha piperita vulgari had a sufficient effect on the growth of the mushroom mycelium in a way that there was a significant difference with the control at the level of 1%. It has not shown any effect, and Foeniculum vulgaris fennel essential oil had the most effective inhibitory effect on the growth of Sclerotina fungus, and with increasing concentration of Thymus vulgaris thyme, the average diameter of the mushroom stem decreased. The results of this research showed that fennel can be used as a natural fungicide to control S. sclerotiourm. (Teimouri, S; Rahnama,k 2014).the mushroom stem decreased. The results of this research showed that fennel can be used as a natural fungicide to control S. sclerotiourm. (Teimouri, S; Rahnama,k 2014).

In another research progect with the effect of thymol on Sclerotina and Aspergillus fumigatus fungi, it was shown that the amount of antifungal activity increased with the increase in the concentration of thymol. (Nasrollahi, 2016).The effect of coumarin on Sclerotina and Aspergillus fumigatus fungi was significant and it is likely to have inhibitory effects on mycelial growth and spore production of plant and animal pathogenic fungi. Considering the large number of different groups of chemical compounds present in essential oils and extracts, it is highly probable that their antimicrobial activity cannot be related to a special mechanism, but different targets in the cell due to this compound are affected (Carson et al., 2002. Skandamis et al., 2001) through ultrastructural changes and mycelium analysis of the fungus, such as reducing the diameter and thickness of the mycelium walls (Zambonelli et al., 1996., De Biller beck et al., 2001, Nogueira et al., 2010) cell wall and plasma membrane (Knobloch et al., 1989) and changes occur in the mitochondrial membrane (Rasooli et al., 2006) .

Conclusion

The results of these tests confirmed the allopathic and antifungal effects of coumarin. According to these results, coumarin can be used to produce natural herbicides, and coumarin can also be used in the production of natural pesticides to deal with plant pests (including fungi).

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