S U M M A R Y

RESEARCH ON OBTAINING A PRODUCT BASED ON BIOLOGICALLY ACTIVE EXTRACTS EFFECTIVE IN ANTIFUNGAL PROTECTION OF PLANTS

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INTRODUCTION

Plants have the property of producing secondary metabolites with the role of inactivating pests. Depending on the method of obtaining, vegetal pesticides can be based on plant extracts or volatile oils (Vidyasagar and Tabassum, 2013). And they can be obtained from different parts of the plants, either fresh or dried, namely, leaves, flowers, roots, seeds or fruits. In general, dry plant parts are preferred, because through this process is reduced the amount of water, thus obtaining a higher yield of active substances (Abdulzahra et al., 2020; Chougule and Andoji, 2016). According to literature data (Gakuubi et al., 2016), plants with rich content of bioactive compounds and with activity against pests are part of the species families *Asteraceae, Fabaceae, Myrtaceae și and Solanaceae*. Phytochemical compounds of plant extracts, such as acids, alkaloids, flavonoids, saponins and terpenoids sunstance represent only a part of the biologically active substances used in formulation of plant protection products (Sanchez Pino et al., 2013).

CURRENT STATE OF KNOWLEDGE

The plant species *Tagetes erecta* L (Marigold) and *Trigonella foenum-graecum L* (Fenugreek), which are part of the *Asteraceae* and *Fabaceae* families, are spontaneously spread and cultivated globally in various geographical habitats, but also in the microsphere from the romanian territory, having a great significance for the agricultural, horticultural, pharmaceutical, food and nutraceutical industry. According to the specialized literature, a series of phytochemical studies were carried out on extracts obtained from the two types of plants, evidenced by rich composition in phytochemical compounds, especially flavonoids and polyphenols, alkaloid saponins, volatile oils, proteins and carbohydrates. All these compounds are scientifically proven for their antioxidant and antimicrobial properties. However, the analyzes on these two species of plants are limited to our countries, and the present research theses come to complete the picture of their use in the biopesticides industry based on plant biologically

active substances, with the aim of supporting and developing ecological agriculture and limiting the use of chemical conventional pesticides.

PERSONAL CONTRIBUTION

AIMS

The first objective of the present thesis was to obtain plant extracts rich in phytochemical compounds with antioxidant and antimicrobial properties, in an economical, efficient, environmentally friendly way, phenolic, antioxidant and germinal profile analysis.

A second objective was the association of the extract variant enriched with nutritious and antimicrobial phytochemical compounds with rhizobacteria with antifungal properties to recommend them in developing a potential ambivalent product (antifungal and phytostimulatory).

The doctoral thesis entitled "**RESEARCH ON OBTAINING A PRODUCT BASED ON BIOLOGICALLY ACTIVE EXTRACTS EFFECTIVE IN ANTIFUNGAL PROTECTION OF PLANTS**", is comprised of two parts and structured in six chapters.

PART I (Bibliographic Study) includes three chapters dedicated to the literature study (Chapters I, II and III), which comprises the current state of knowledge regarding biopesticides, the legislation regulating these types of products at the European level and are also presented plants such as *Tagetes erecta* and *Trigonella foenum-graecum* that are rich in phytochemical compounds with antioxidant and antimicrobial effect on plant pests.

PART II (Own Research) its focused on original research regarding various extraction methods, and complex characterization of phytobiological compounds extracted from the two types of plant species. This part, structured in three chapters, aims to address their potential use of obtained products in the plant protection industry.

Chapter VI includes extraction methods, characterization of phenolic compounds, antioxidant activity assessment and bio-germinative study of the six types of extracts on cucumber and radish seeds. The plant material studied was dried flowers of *Tagetes erecta* obtained from ecological culture an dried seeds of *Trigonella foenum-graecum*. The extraction methods used were maceration at room temperature and thermal assistance in the concentration phase, at temperatures of 35-40°C. The type of solvents selected belong to concentrated alcohols, propylene glycol (50%) and ethanol (70%). Thus, ethanolic extracts (40% and 70% ethanol) were obtained from each plant species at 1:10, 1:20 ratio and extracts in propylene glycol (50%), at 1:10, 1:20 ratio. The concentrations of phenolic compounds were determined, the antiradical activity was determined by the DPPH method, the content of dry matter was determined and thebehavior of the six variants of extracts was evaluated compared to the control (distilled water) and the solvents in which the extraction was carried out (ethanol 40 %, ethanol 70%, propylene glycol 50%) on cucumber and radish seeds, by applying the germination bioassay (at 0,10%, 0,50%, 1,00% and 1,50 % concentrations).

The extracts obtained showed a high concentration of total polyphenols expressed in caffeic acid equivalents (CAE) mg/ml and flavonoid compounds expressed in rutin equivalents (RE) mg/ml. The variant of *Tagetes erecta* flower extract in 40%

ethanol presented the highest concentration of phytochemical compounds (CAE mg/ml 6,723±0,511 and RE mg/ml – 9,102±0,430), a more pronounced redox response (EC_{50} µl/ ml extract: 0,47±0.007 RSD=1,130) and a positive effect on the seeds stimulating germination and root growth about the solvent at all sample doses studied (0,10% - Gi% - 288,24%; 0,50% - Gi% - 122,88%; 1,00% - Gi% - 196,41% and 1,50% - Gi% - 17,41%), was selected for further antimicrobial (antifungal) study *Monilinia laxa, Fusarium graminareum* and *Aspergilius* niger strains and determining the synergistic antifungal activity associated with rhizobacteria belonging to the *Bacillus* species.

Chapter V includes the qualitative and quantitative determinations of the phenolic substances in the extract *Tagetes erecta* extract (ethanol 40%), (selected following the results recorded in the research in Chapter VI), the cocncentration of reducing sugar, soluble proteins, the determination of the antiradical activity by the DPPH, TEAC, ABTS, and CUPRAC methods, follow up of the bio-germinative study on wheat seeds and the determination of antifungal, prebiotic, antibiofilm and antipathogenic and synergism with *Bacillus* bacterial species.

The results of **Chapter V** consisted of the qualitative evaluation of total polyphenolic compounds expressed in gallic acid equivalents mg/ml- GAE and flavonoids, expressed in quercetin equivalents mg/ml - QE), but also quantitatively by the HPLC-HESI method, the soluble proteins content, the reducing sugars content, highlighting the specific bands of phenolic compounds in the vegetable residue by ATR-FTIR, the study of the phytotoxic/bio stimulatory activity on wheat seeds compared to the solvent (ethanol 40%), antimicrobial activity, as well as antiradical activity by DPPH, CUPRAC, FRAP and TEAC methods. To intensify the antifungal effect, synergy studies were carried out for the rhizobacteria stimulated with the *Tagetes erecta* flower extract. The results obtained indicated a higher composition of TPC – 15,12±0,27 GAE – mg/ml, compared to the concentration of total flavonoids, TFC – 2,01±0,03 QE mg/ml. As for the qualitative identification methods of phenolic compounds from the *Tagetes erecta* extract in 40% ethanol, it was carried out using HPLC-HESI and results obtained indicated 3.36% (27 compounds) of the total poliphenolic compounds quantified and 20,15% of the total flavonoids, of which the majority compounds were represented by quercetin – 383,74 mg/l, syringic acid – 30,71 mg/l, ellagic acid – 26,80 mg/l, 3,4-dihydroxybenzoic acid – 20,50 mg/l, myricetin – 13,64 mg/ml and chlorogenic acid – 11,85 mg/L. The extract had a density of 1,0526±0,0016 g/ml and a dry matter content of 3.6%. Regarding the dried flowers of *Tagetes erecta* vegetal material), the bands in the interval of 650-4000cm^{-1} highlights the presence of some primary biochemical components and macronutrients, including proteins, lipids, and also carbohydrates. According to literature reports, carbohydrates present an important role in plants as a nutrient.

Antiradical activity research by DPPH, CUPRAC, FRAP and TEAC spectrophotometric methods, shows that the extract recorded a high antioxidant activity throughout the 4 analysis methods, but however, the highest antioxidant activity was recorded through CUPRAC 65,81±1,51 Trolox mM/ml), compared to all other

studied methods. This was possible because both hydrophilic and lipophilic antioxidants can be analyzed with this method.

In the research of the germination bioassay regarding the evaluation of the behaviour of the *Tagetes erecta* flower extract in 40% ethanol, it was proven that it improved the growth of the roots of germinated wheat seeds, compared to the control but also the solvent at 0,50 % concentration (Gi RRG extract – 112,81%; RRG solvent – 73,41%).

In determining the antifungal activity of the extract, the following species of phytopathogenic strains were used*: Aspergillus niger, Fusarium graminearum* and *Monilinia laxa*. Qualitative antifungal screening was performed by embedding the extract into liquefied PDA medium cooled to 40-45°C. The *Tagetes erecta* extract was inoculated into the culture medium at 0,5%, 1,0% and 5,0% concentrations. The results of the reserch indicates that the extract reduced the mycelium diameter of *Fusarium graminearum* and *Monilinia laxa* strains. Highest percent inhibition was observed at the fungal strain *Fusarium graminearum* at the maximum concentration tested (5,0%), with a percentage of 54,17±5,89% (compared to the solvent, $p<0,05$), and in the case of the fungal *Monilinia laxa* strain, compared to the solvent ($p<0,05$)., the inhibition of the colony diameter was 52,29±2,60% at the 0,10% concentration. A statistically significant ($p<0,05$) increase was noticed in the *Aspergillus niger* strain in relation to the solvent.

The quantitative investigation of the antifungal activity was carried out in RPMI 1640 medium where the microdilution method was used in 24-well plates. The tested concentrations of the extract (0,1%, 0,5%, 1,0%, 1,5% and 3,0%) were made directly in the culture medium in a volume of 2 ml RPMI 1640 seeded with the inoculum fungal adjusted to the nephelometric standard 1 McFarland (10^6 CFU/ml). Thus, the minimum inhibitory concentration (MIC) was evaluated. For the strain *Monilinia laxa*, a statistically non-significant reduction in cell viability was observed relative to the solvent at all concentrations studied, and no significant reduction in viability was noticed by comparison to the positive control. For the *Fusarium graminareum* strain, the cell viability was decreased for the extract at the concentration of 3,0% (6,67±2,31%), the significant difference was made compared to the solvent activity. For *Aspergillus niger*, showed an increased cell proliferation compared to the control (solvent), at concentrations of 1,5% (93,24±1,91%) and 3% (95,95±1,91%).

For the antifungal evaluation effect, 10 µl of the sample were incorporated on the PDA medium, using concentrations of 0,1%, 0,5%, 1,0%, 1,5% and 3,0%. Thus, it was seen that the fungal strain *Monilinia laxa* was inhibited, and for the *Fusarium graminearum* strain, the inhibition was partially complete with significant differences in relation to the solvent at 0,1%, and 0,5% extract concentrations. In *Aspergillus niger* strain, the highest inhibition was seen at the lowest concentration (0,10%) in both extract and solvent.

During the evaluation of the microbial adhesion capacities to the inert substrate of the *Tagetes erecta* extract and the solvent (40% ethanol) on the investigated strains of pathogenic fungi, it was noticed that the microbial adhesion was statistically significant inhibited for the *Fusarium graminearum* strain (at 1.5 % and 3%

concentrations) and for the *Aspergilius niger* strain (1,0%, 1.5% and 3,0%). For *Monilinia laxa*, inhibition of adhesion was noticed for the positive control, but with a statistically significant by association with the extract. This phenomenon is probably due to the composition of the reduced content of sugar and polyphenols with antioxidant properties.

By studying the prebiotic and antibiofilm activity of the extract (concentrations 1,56-50,00%) on PGPR strains *(Bacillus licheniformis Bacillus amyloliquegaciens* and *Bacillus subtilis*), it was highlighted that the extract, but also the solvent express antibacterial activity. The MIC value was 12,50% at all three bacterial strains studied. In the bacterial strain, *Bacillus licheniformis*, a significant increase in cell viability was noticed regarding the solvent at the concentration of 1,56% (114,59±1,26%), but also for the positive control. Instead, for the *Bacillus amyloliquefaciens* strain it was observed that the extract sample statistically significant inhibited the microbial viability regardind to the solvent and the positive control.

At the same time, a synergistic effect regarding the stimulation of the adhesion of microbial cells to the abiotic substrate was highlighted between the *Tagetes erecta* flower extract and the *Bacillus licheniformis* strain. For the *Bacillus amyloliquefaciens* strain, no significant differences were noticed regardind to the positive control, but for the bacterial strain, *Bacillus licheniformis,* all variants tested significantly increased microbial adhesion. According to similar scientific results, the bacterial strain, *Bacillus subtilis*, through the presence of the component cyclic adenosine monophosphate (cyclic di-AMP) can act as an extracellular signal influencing the formation of the biofilm and the attachment of microbial cells to the biotic surface.

In the study to determine the synergistic antifungal activity of the extract from the flowers of *Tagetes erecta* and rhizobacteria (*Bacillus* species), the strain variants of the *Bacillus* sp species and the extract concentrations were selected according to the results obtained for the minimal inhibitory concentration (MIC) assay, so that the extract leads to increased cell proliferation. To determine the synergistic antifungal activity on *Fusarium graminearum, Monilinia laxa* and *Aspergillus niger* strains, concentrations of 1.5% extract and solvent associated with the *Bacillus licheniformis* strain and 1.5% and 3% extract and solvent associated with the *Bacillus subtilis* strain were used. Thus, the results reveal that the selected variants showed good inhibition of mycelial growth by 28.33±2.08 mm and 26.67±1.53 mm in the case of the *Bacillus subtilis* strain enriched with concentrations of 1.5% and 3% *Tagetes erecta* on the *Fusarium graminearum* strain, while on *Monilinia laxa* was recorded a total inhibitory effect, both for the mixture with the microbial suspension of *Bacillus* sp, with extract and the mixture of *Bacillus* sp strains, with solvent. However, the synergistic effect between the extract and *Bacillus subtilis* strain was lower for the fungal strain of *Aspergillus niger*. An opposite effect was noticed in the case of the *Bacillus licheniformis* strain only in the *Fusarium graminearum* strain.

The two strains of *Bacillus* spp. from our study, showed variable antagonistic activity on the phytopathogenic strains, showing activity specifically on the *Fusarium graminearum* strain.

Chapter VI comprises general conclusions on the obtained results, and references.

Our research focused on obtaining a product from a natural source that could be a viable solution regarding plant protection, therefore six variants of extract from *Trigonella foenum-graecum* seeds and *Tagetes erecta* flowers were obtained. Thus, two extraction solvents were used, ethanol 70% and propylene glycol 50%, and the extraction process was made by maceration at ambient temperature and by slight thermal assistance during the concentration phase. The main reason for using these types of solvents and extraction methods was to extract compounds with potential phyto-stimulant and antimicrobial (fungicidal) effects in the highest possible quantity and, at the same time, not to damage potential extractable phytochemical compounds. According to the results obtained, on both species of plants, the most effective application for the extraction of phytochemical compounds was the thermally assisted one. This was evidenced by the increased concentrations in polyphenols and flavonoids, but also by a more pronounced redox activity, compared to the extract variants obtained in ethanol 70% and propylene glycol 50% (without thermal assistance). Following the bio-germinative study it was highlighted that the extract from *Tagetes erecta* flowers in 40% ethanol showed a strong phyto-stimulant effect on radish seeds, a slight phyto-stimulant effect was observed on cucumber seeds after treating them with *Trigonella foenum-graecum* extract (40% ethanol) only at the lowest concentration tested (0.10%).

Moreover, the research results obtained and presented in this thesis highlighted that the extract possesses a high antioxidant, antimicrobial and cytoprotective properties due to the compounds found in the composition. *Tagetes erecta* flower extract has shown a mild phyto-stimulatory effect on wheat seeds as well. Evaluation of the antifungal activity was carried out on solid and liquid media, observing that, the researched extract attenuated mycelial growth, especially for *Monilinia laxa* and *Fusarium graminearum* strains. The the property of causing disease of fungal strains is generally due to their character of forming bacterial biofilms on top of plant tissues and the application of *Tagetes erecta* flower extract could prevent the adherence of phytopathogenic agents, but also with the potency of rhizobacteria regarding antifungal activity, the property they possess. natural. As it appears from our study, the extract from the flowers of *Tagetes erecta* conditioned in 40% ethanol, in combination with strains of rhizobacteria (*Bacillus sp*), presented a viable biocontrol measure, showing their antagonistic activities against the phytopathogenic agent, especially against the *Fusarium graminearum* strain.

The research in this thesis confers novelty and originality by providing information that could be applied in the agricultural biotechnological industry, specifically for the development of an antifungal extract enriched with *Tagetes erecta* that could be the basis for a powerful biopesticide effective against phytopathogenic agents of plants and also, with a stimulating effect on the growth and development of vegetable seedlings and grain plants. This work also provides valuable and original information regarding obtaining in an efficient, safe, economical and environmentally

friendly way some plant extracts rich in phytochemical compounds with potential use in the plant protection and phytostimulation industry.

In the 150 pages of this thesis, there are 33 tables, of which 30 are original, 39 figures, of which 36 are original, and 233 references.

The results obtained during the doctoral studies were disseminated in 3 articles, of which 2 articles were published in ISI-rated journals (WOS) and 1 article was accepted for publication in an ISI-rated journal (WOS).