Biological Activity of Plant Extracts and Essential Oils

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Contributor: Regina Fragkouli , Maria Antonopoulou , Elias Asimakis , Alexandra Spyrou , Chariklia Kosma , Anastasios Zotos , George Tsiamis , Angelos Patakas , Vassilios Triantafyllidis

The development and implementation of safe natural alternatives to synthetic pesticides are urgent needs that will provide ecological solutions for the control of plant diseases, bacteria, viruses, nematodes, pests, and weeds to ensure the economic stability of farmers and food security, as well as protection of the environment and human health. Unambiguously, production of botanical pesticides will allow for the sustainable and efficient use of natural resources and finally decrease the use of chemical inputs and burden.

biopesticides plant extracts essential oils

1. Introduction

Climate change and environmental degradation are severe threats worldwide, and their consequences can cause serious impacts on our planet. Recognizing the importance of these threats to humanity, on 11 December 2019, the EU Commission presented the European Green Deal, which consists of a set of policy initiatives that aim to neutralize climate by 2030 and render Europe the first climate-neutral continent by 2050 [1]. One of these initiatives is the reduction of greenhouse gas emissions by at least 55% by 2030 compared to 1990 levels. To achieve 2030 climate targets, the EU Commission has also adopted a set of strategies in various sectors such as transportation, industry, energy, and agriculture [2].

Amongst them, the Farm to Fork strategy is characterized as the heart of the European Green Deal and aims to accelerate the transition to a sustainable food system. The objective of this strategy is to ensure food safety in an environmentally sustainable manner, simultaneously maximizing environmental, health, and social benefits. To accelerate the transition to sustainable and healthy food systems, this strategy aims to reduce pesticide use by 50% by 2030 by applying low-input sustainable agriculture or simply alternative agriculture, amongst others [2].

Pesticides are any substance or mixture of substances of chemical or biological ingredients intended for repelling, destroying, or controlling any pest or for regulating plant growth [3]. The term “pesticide” applies to insecticides, herbicides, fungicides, rodenticides, molluscicides, wood preservatives, and various other substances used to control pests. Pesticides also include plant growth regulators, defoliants, and desiccants. Their use has increased 50% since 1950, and it is estimated that 2.5 million tons of industrial pesticides are now used each year [4]. Moreover, global pesticide use is expected to show an increasing trend in the future, and it is expected to reach a value of 4.5 million tons by 2030 [5][6].

Although pesticides have a principal role in crop production, intensive and improper use of them can cause numerous detrimental effects on human health and the environment and reduce the safety of agricultural products, which has raised major public and scientific concern in the last few decades [7][8][9]. For humans, dermatological, gastrointestinal, neurological, carcinogenic, respiratory, reproductive, and endocrine effects are representative adverse health effects that have been associated with pesticide exposure [10].

The human and environmental health risks that are associated with the use of chemical pesticides, as well as the aims set by the Farm to Fork strategy, have led to an increasing demand for the development of alternative eco-friendly pesticide formulations. Biopesticides have long been recognized as attractive alternatives to synthetic chemical pesticides for pest control because they present important properties, with their non-toxic nature being the most significant [11][12][13].

Biopesticides aim to control plant-damaging pests, insects, and fungi and are generally categorized into three groups: (i) microbial biopesticides (containing microorganisms like bacteria, fungi, viruses, and protozoan or entomopathogenic nematodes as active ingredients that attack specific pest species), (ii) biochemical biopesticides (containing naturally occurring substances that control pests via non-toxic mechanisms), and (iii) plant-incorporated protectants (containing substances produced by plants from genetic material that has been added to the plant) [11][12]. The practice of using plant derivatives in agriculture has a long history of at least two and a half millennia, dating back to ancient Greece and Rome [14]. Botanical pesticides are characterized by bioactive mixtures/extracts/compounds from plant materials that serve as insecticides and repellents but also as bactericides, fungicides, herbicides, and nematicides [15]. In general, botanical pesticides contain numerous compounds that can be volatile and belong to different chemical groups such as aldehydes, ketones, alcohols, heterocycles, ethers or oxides, phenols, esters, amines, amides, flavonoids, and terpenes, amongst others. All of these compounds are produced as secondary metabolites and can present activities against pests, insects, and pathogenic fungi. Representative examples are the well-documented antimicrobial and antioxidant properties that present various terpenoids and phenolic compounds [13]. However, few biopesticide formulations have been commercialized up to now. The main limitations concern their reduced storage stability and sensitivity to environmental conditions, as well as the high production cost, which should be overcome in the near future. In this direction, the improvement of the formulation to increase and maintain the activity of biopesticides could be a solution [13]. Moreover, the use of widely available plants as raw materials can also contribute to overcoming the existing limitations.

As plant-based natural pesticides have gained considerable attention in the few last years and development of them is still a growing trend, there is an urgent need to compile the current scientific knowledge about plants presenting biopesticidal effects, especially for the countries where the source plants are readily available and where conventional formulations comprising synthetic pesticides are both expensive and dangerous to humans and the environment. Being aware of the above, numerous researchers have focused on the evaluation of extracts and essential oils with biopesticidal properties from plants of Mediterranean countries.

2. Biological Activity of Plant Extracts and Essential Oils

Literature data indicate that plant extracts have promising antimicrobial, insecticidal, and herbicidal activity. Key findings of several recent studies focusing on the antimicrobial, insecticidal, and herbicidal activity of Mediterranean plant extracts and essential oils are presented. Their activity was also examined regarding plant bacteria, viruses, nematodes, and other pathogens. Although numerous studies have evaluated the biological activity of plant extracts and essential oils, in most cases the observed activity was not correlated with specific components. The biological activity was attributed to the synergistic effects of the different compounds [16]. Nevertheless, there were cases where the biological activity was correlated with specific compounds. Indicatively, γ-terpinene and myristicin were found to possess insecticidal activity and were effective on *Culex quinquefasciatus* larvae [17].

It is also worth mentioning that, in some cases, the observed activity significantly varies for different targets and even the same targets between essential oils/extracts of the same plant. For example, Pavela et al. [17] investigated the essential oils of *Crithmum maritimum* L. of different geographical origins and observed a significant differentiation in their insecticidal activity due to their phytochemical compositions. Furthermore, the activity of the essential oils of different parts of the plant was also found to vary. In a recent study, Zerkani et al. [18] observed significant differences in antimicrobial activity from the essential oils derived from different parts of *Pistacia atlantica*.

In addition, the same active compound has been reported to possess varied biological activity. Oil containing thymol as a major component was found by Ben Jabeur et al. [19] to present antimicrobial properties. Essential oils with thymol have also been suggested as potential plant-based insecticidal agents [16]. Essential oils with carvacrol and piperitenone oxide as major compounds have also been suggested [16][20] and reported to possess insecticidal activity. Up to now, a variety of assays have been used to evaluate the biological activity, such as antimicrobial, insecticidal, herbicidal, etc., of plant extracts and essential oils.

2.1. Commonly Used Assays for Evaluating Antimicrobial Activity

Various methods are used to evaluate antimicrobial activity in vitro. Among them, the most common are the agar dilution and disc diffusion methods. Agar dilution, otherwise referred to as the poisoned food method, is the method of choice when estimating antifungal activity [21]. The method is based on preparing solid media and adding a desired concentration of the extract to it. A certain volume of the extract can be mixed before the autoclaved medium is poured on Petri dishes or spread on their surface once it has solidified [22][23][24]. Subsequently, a small agar plug (4–7 mm in diameter) from an active fungal culture is inverted, with the mycelial surface facing down, and inoculated at the center of the agar plate. The inhibition is estimated by measuring mycelial growth in optimal conditions and comparing it with a control sample [22]. One or multiple concentrations of the extract can be used during the assay. Different concentrations can be used to determine the potency of the antifungal effect by measuring certain indices, such as half maximal effective concentration (EC_{50}) [23], the minimum inhibitory concentration (MIC), or the half inhibitory concentration (IC_{50}) of the extract/essential oil [19][25]. Variations of the agar dilution method have been successfully employed to test the antifungal capacity of various extracts against plant pathogenic fungi, such as *Verticillium dahliae* in olives [22]; *Zymoseptoria tritici* in wheat [19][25]; *Sclerotinia sclerotiorum* [23], *Fusarium oxysporum*, *Alternaria solani*, and *Pythium ultimum* in tomato [24][26]; and *Botrytis*

cinerea [27], *Penicillium allii* [28], *Stemphylium vesicarium* [29], and *Geotrichum candidum var. citri-aurantii* in decayed mandarin fruit [30]. Semerdjieva and colleagues used agar dilution to test the antifungal potential of essential oils against five fungal pathogens, including *Fusarium* sp. and *Rhizoctonia solani* strains isolated from stored potato, *Botrytis cinerea* from infected stored tomato, *Colletotrichum* sp. from anthracnose of bananas, and *Cylindrocarpon pauciseptatum* obtained from diseased grapevine [31]. Slight variations in the protocol involve inoculation of the agar containing the extract with a small volume from a liquid culture of the fungus [19][25] or with fungi-infected plant seeds [32] instead of an agar plug. Although the method is mostly used for fungal pathogens, Fu et al. [33] employed the agar dilution method to test the antibacterial potential of water extracts from aquatic weeds against 100 bacterial strains that were inoculated on agar plates by streaking.

On the other hand, the disc diffusion method is mostly preferred when screening extracts for antibacterial activity in vitro. However, it can be used for testing antifungal activity as well [18]. This method is based on spreading an amount of bacterial or fungal suspension (or an agar plug from an active fungal culture) on solid media, placing small paper discs (5–6 mm in diameter) soaked with a microvolume of the extract (e.g., 3–5 µL), incubating the plates in ideal growth conditions, and measuring the inhibition zones [21]. Disc diffusion was used to assess both antifungal and antibacterial activity of three subcritical carbon dioxide plant extracts from *Carum carvi*, *Thymus vulgaris*, and *Nigella sativa* [34]. The extracts were successful at inhibiting eight fungal pathogens, including the *Fusarium*, *Alternaria*, *Colletotrichum*, *Rhizoctonia*, and *Phoma* strains, as well as two bacterial phytopathogens belonging to the genera *Pectobacterium* and *Streptomyces* [34]. The study also employed another in vitro assay to test antimicrobial activity, the agar well diffusion method, which shares many similarities to the disc diffusion method. In its most common form, a volume (e.g., 50–250 µL) of the extract is applied in a central well (5–8 mm in diameter) on the agar plate, which is previously inoculated with the pathogen. Twenty-two water and water–glycol extracts were tested by this method for antimicrobial effect against the 10 previously mentioned plant pathogens [34]. The disc diffusion method was used to assess the antifungal capacity of essential oils from *Lavandula dentata* against strains of *Cercospora kikuchii*, *Cercospora sojina*, and *Septoria glycines* [35]; of pyroligneous acids identified in the bark of hybrid aspen trees against *Fusarium culmorum* [36]; and of extracts from seven plant species collected from the island of Lampedusa, in Italy, against *Penicillium italicum*, *Aspergillus carbonarius*, and *Drechslera gigantea* [37]. It was also used to test the antibacterial effect of nano-suspensions of *Chrysanthemum coronarium* and *Azadirachta indica* against *Escherichia coli* and *Staphylococcus aureus* strains [38] and of barnyard grass extracts against a tomato bacterial pathogen, *Pectobacterium carotovorum* [24]. Other applications of the method include screening against human pathogens. For instance, essential oils extracted from the aerial parts of *Origanum elongatum* were tested against nine pathogenic bacteria isolated from hospital patients [39], whereas essential oils from *Pistacia atlantica* were assayed against 12 human pathogens, 9 bacterial strains and 3 fungal strains [18].

In vitro methods comprise the most common assays for antimicrobial screening since they are simple in terms of design and execution and provide useful and comprehensive results. On the other hand, in vivo and in situ assays are more challenging to set up and are thus less frequently used but generally provide more reliable data. Such an in situ antimicrobial assay was carried out by Steglińska and colleagues on potatoes [34]. In brief, water and subcritical carbon dioxide extracts (SCDE) from four plant species exhibited antifungal and antibacterial effects

when they were applied on potatoes. The in situ assay included immersion of potatoes in the plant extracts, application of 20 µL of bacterial or fungal suspension in three cuts (5 mm in diameter and 5 mm deep), and measuring the infestation rate after 2 weeks of incubation [34]. A similar test was conducted by Karim and colleagues, who created 2 mm-deep and 3 mm-wide wounds on mandarin fruit with sterile needles [30]. The cuts were inoculated with 30 mL of *Cistus* aqueous extract and 20 mL of a *Geotrichum candidum var. citri-aurantii* suspension. The incidence and severity of the fungal disease on the treated mandarin fruit was evaluated daily for 10 days [30]. Regarding antiviral activity, Hu et al. employed the half-leaf method to test the effect of nine compounds from the seeds of *Hyoscyamus niger* against a phytopathogenic virus, tobacco mosaic virus (TMV) [40]. The method is often used to test inactivation, protective, and curative effects of extracts against the selected pathogen and is based on smearing half of the surface of the leaf with the extract while leaving the other side with a control treatment. Depending on the type of effect that is being tested, the viral suspension is either mixed with the compounds and applied on the same side of the leaf or inoculated on the whole surface of the leaf [41].

2.2. Bioassays for Determining Pesticidal or Repellent Activity

Plant extracts can be submitted to a variety of assays to evaluate their insecticidal, acaricidal, nematocidal, or repellent potential, as well as their effect on oviposition. Standardized techniques include topical application, residual or surface contact, immersion in the extract or in a solution containing the extract, feeding bioassays, and fumigation [42][43]. Usually, the selected assay takes into consideration the unique biology of each pest or its developmental stage, since the egg and larval stages have different morphological and biological characteristics than the adult stage.

Among the previously mentioned techniques, topical application can be used for bioassays in most developmental stages. The technique is based on applying microvolumes of the extract directly on the body of the insect with a micropipette or a microsyringe [43]. It was used successfully for larvae of the lepidopteran *Spodoptera littoralis*. Different concentrations of *Origanum syriacum* subsp. s*yriacum* extract were mixed with 1 µL of acetone, and each solution was applied on the dorsal region of 80 larvae per dose [44]. Insecticidal bioassays using topical application of extracts with a microsyringe were similarly performed on the dorsal region of *Spodoptera frugiperda* larvae [45]. Topical application tests can also be performed on adult individuals. However, in this case, since adults of certain insects display high motility or flying ability, as a first step before the topical application of the extract, the insects are anaesthetized with CO_2 or on ice [44][46][47]. For instance, female *Musca domestica* flies were first anaesthetized and then treated with different doses of *Origanum syriacum* subsp. s*yriacum* extracts by applying a microvolume of the extract on the pronotum of the flies and measuring the effect after 24 h [44]. Topical application methods have been used to assay multiple insect species, such as *Pectinophora gossypiella*, *Thaumatotibia leucotreta*, *Helicoverpa armigera*, *Myzus persicae*, *Aphis craccivora*, *Aphis citricola*, *Aedes aegypti*, *Diaphorina citri*, *Tribolium castaneum*, *Trichoplusia ni*, and *Brassicogethes aeneus*. [46][47][48][49][50][51][52]. In the case of *Trichoplusia ni* larvae, an injection assay was also performed, with one microliter of test solution injected into the ventral hemocoel [48].

On the other hand, during residual contact techniques, individuals or groups of target organisms are exposed to residues of the bioactive compounds. The compounds are usually added uniformly on natural (e.g., leaves, fruit,

inflorescences) or artificial (e.g., filter discs) surfaces, and the specimens are placed on them [43]. Such a residual contact assay was applied by Alkan and Gökçe [53] on egg masses of the Colorado potato beetle *Leptinotarsa decemlineata*. The eggs that were oviposited on potato leaflets were sprayed with 20 µL of six plant extracts to examine their ovicidal effect. The leaflets were then placed in petri dishes and egg mortality was recorded for 7 days [53]. Residual spraying was also used to apply plant essential oils on adult aphids (*Myzus persicae*) [54]. Other surface contact techniques that did not employ spraying were used to determine the acaricidal efficacy of different concentrations of an extract from *Onosma visianii* roots [55]. The mite that was subjected to the treatment belonged to the species *Tetranychus urticae*. A pipette was used to apply 20 µL of the various dilutions on one side of bean leaf discs (sized 2 cm^2), which were then placed on agar-containing plates. Various developmental stages of the mites were assayed. Adult females, nymphs, or eggs were transferred to the discs and incubated at fixed temperature and light conditions for 24 h or for up to five days after the treatment. Thus, this assay, with minor modifications for each case, was used to assess adult mortality, the number of oviposited eggs for live females, and the hatchability of eggs [55]. A similar study was carried out for *Saponaria officinalis*-synthesized silver nanocrystals against *Tetranychus urticae* [56]. Surface toxicity was also used to assess the larvicidal activity of *Tagetes minuta* essential oils to *Lucilia cuprina* flies. The applied protocol was based on transferring third-instar larvae of the fly in glass vials with filter papers impregnated with different dilutions of the essential oils [57]. Various residual or surface contact bioassays, with certain modifications in their protocols, were used to test the bioactivity of a variety of plant extracts and essential oils against eggs, larvae, and adult specimens of insects and mites [20][38][52][58][59][60][61][62][63][64][65][66][67][68][69][70][71][72][73][74][75]. For instance, Erdogan and Mustafa dipped tomato leaf discs into the test solutions instead of pipetting a volume onto their surface and then placed *Tuta absoluta* larvae on them [64]. Surface contact bioassays can be performed not only on a laboratory scale but also on a larger scale. For instance, extracts from leaves of *Agave americana* were used against the hemipteran *Brevicoryne brassicae* in field experiments performed at a cabbage farm. The application of the extracts was carried out by spraying parts of the leaves and the center of the adult plant [72].

Repellency, rather than acute toxicity or pest mortality, may also be assessed with modified surface contact methods. Typical repellency assays use filter papers that are treated with the extract on one half and the respective solvent on the other half and are subsequently placed in Petri dishes with the test samples [76]. Such repellency bioassays were carried out for larvae of the khapra beetle, *Trogoderma granarium* [61], and adults of *Tribolium castaneum* [76]. Ilyas and colleagues, on the other hand, treated guava fruits by immersing them in plant extract solutions. The treated fruits were subsequently offered to adult *Bactrocera zonata* flies that were kept in cages, and the number of individuals that settled on the fruits were recorded for 5 h per day for two days [70]. Mangang and colleagues also used a more sophisticated system, termed an “insect management unit,” to study the repellent properties of packaging material [76]. Pourya et al. also used an arena to perform repellency bioassays on adult *Callosobruchus maculatus* beetles [58]. The arena consisted of three plastic chambers that were connected by small tubes. The beetles were placed in the central chamber, the control cowpeas treated only with solvents were placed in the first test chamber, and the cowpeas that were treated with different concentrations of *Pistacia* essential oils were placed in the second test chamber [58].

Immersion techniques are especially suitable for developmental stages that take place within an aquatic environment, such as eggs or larvae of certain species. Therefore, immersion assays were performed on larvae of *Culex quinquefasciatus* mosquitoes [17][77][78][79]. The larvae were placed in 250 mL of solution containing 249 mL of distilled water and 1 mL of essential oils or a mixture (six different dosages were tested for each compound), and their mortality was recorded after 24 h of exposure to the treatment [78]. Similar approaches were used in other studies featuring larvae of other mosquito species, such as *Culex pipiens*, *Culex restuans*, *Aedes aegypti*, *Aedes albopictus,* and *Anopheles gambiae* [46][61][80][81][82]. Musso and colleagues used immersion techniques to study the larvae of the nematode *Panagrolaimus rigidus* [83]. Briefly, they placed 100 µL of suspension containing approximately 100 larvae in each well of a 96-well microplate. Then, they added 100 µL of essential oil solutions isolated from *Nepeta* plant species and incubated the microplates at 20 °C. Nematocidal activity was estimated by counting mobile and immobile roundworms using an optical microscope [83]. Immersion bioassays can be also performed to test the activity of extracts on insect eggs [84]. Eggs of the lepidopteran *Conopomorpha sinensis* were submerged in two different concentrations of various plant extracts for 10 s, and their hatching rate was measured for two days [84]. The use of solid formulations against the potato tuber moth *Phthorimaea operculella* can be considered a modified case of immersion methods [85]. The process was based on crude extracts that were mixed with talcum powder (magnesium silicate) as an inert carrier substrate. Moths were completely covered with the powdered extract, which was firmly attached to their cuticle. Mortality and other biological parameters of the moths were recorded after the application of the powder [85]. Immersion-based assays were carried out to study nematocidal activity against other species of nematodes, such as *Meloidogyne incognita* [86] and *Meloidogyne javanica* [87][88], as well as acaricidal activity against *Tetranychus cinnabarinus* mites using the slip-dip method [89].

Feeding bioassays were performed against adult aphids of the species *Myzus persicae*. In this case, different concentrations of *Origanum syriacum* subsp. s*yriacum* extracts were applied on cabbage, and 4 groups of 50 individuals were left to feed on it. Mortality was recorded 48 h after the application of the treatment [44]. Similar feeding assays were conducted for the leaf-cutting ants *Acromyrmex octospinosus* using extracts from *Mammea americana* seeds and *Nerium oleander* and *Nicotiana tabacum* leaves [90]. The insecticidal activity of *Brassica alba* mustard oil against the lepidopteran species *Cydia pomonella*, *Dendrolimus pini*, and *Spodoptera exigua* [91], as well as of *Eucalyptus* essential oils on *Sitophilus oryzae* and *Sitophilus granarius* [92], was also assessed by feeding bioassays. Feeding inhibition caused by *Satureja montana* essential oils was measured for *Spodoptera littoralis* larvae and *Myzus persicae* and *Leptinotarsa decemlineata* adults. The antifeedant activity was calculated by measuring the consumption of treated leaf discs and comparing it with the controls [88]. Different concentrations of extracts can be mixed and tested not only with a natural host but also with artificial larval diets. Such was the case of *Spodoptera frugiperda* (fall armyworm) larvae that were submitted to various concentrations of extracts from the aerial parts of *Senna crotalarioides* plants [93]. Similar feeding inhibition assays were conducted with other extracts isolated from various plant species, such as *Hyssopus officinalis*, *Lavandula intermedia*, and *Santolina chamaecyparissus* [87]; 14 plant species belonging to the families Asteraceae and Lamiaceae [16]; and with trans-anethole compounds from various Apiaceae species [73].

Fumigant bioassays can be conducted for volatile organic compounds. For instance, volatile essential oils isolated from bitter fennel (*Foeniculum vulgare*) and green anise (*Pimpinella anisum*) were tested for insecticidal activity

against *Macrosiphum euphorbiae* aphids, which infest tomatoes [42]. The tested essential oils were applied on filter papers, and the experiment was conducted on a small scale (only on tomato leaflets) and on a large scale both with whole plants and at the greenhouse level [42]. A different setup was used to test the insecticidal activity of lemongrass and rosemary essential oils against onion thrips, *Thrips tabaci*. Small *Allium schoenoprasum* seedlings with approximately 20 leaves were inserted separately into 50 mL test tubes. One milliliter glass tubes containing the essential oils were placed in each test tube along with 10 adult thrips for three days, and the mortality rate was calculated [94]. Other cases of fumigant bioassays with plant extracts and volatile essential oils have also been documented [58][60][69][92].

It is crucial for novel biopesticides to show high specificity and activity only against their intended target pests. For that reason, similar bioassays can be executed to assess the safety of the compounds against non-target organisms, such as the ladybug *Harmonia axyridis*, *Eisenia fetida* earthworms, the green lacewing *Chrysoperla carnea*, honeybees, or *Trichogramma pretiosum* hymenoptera [44][45][63][67]. Non-target organisms may also include predatory mites, such as the species *Amblyseius swirskii*, which is widely used as a natural enemy for biological control of small pest species, including mites, thrips, and whiteflies [59]. Similarly, Pino-Otín and colleagues assessed the ecotoxicological impact of a biopesticide from *Artemisia absinthium* on the soil microbial communities of the earthworm *Eisenia fetida* and the plant *Allium cepa*. The changes in microbial communities were assessed with metagenomic amplicon sequencing of 16S rRNA, and toxicity tests on the onion plant were conducted on young bulbs. For the nematocidal assay, they estimated mortality by placing 10 adult earthworms on 500 gr of soil in 1 L plastic containers treated with different concentrations of the aqueous extract [95].

2.3. Bioassays for Determining Herbicidal Activity

Based on the average pesticide consumption of the EU-27 Member States during the period of 2010–2019, herbicides represent more than 30% of all pesticides used in the EU [96], whereas worldwide, herbicides account for 50% of all pesticides used, of which >75% are used in developed countries [13]. The reduction in herbicide use premises the adoption of suitable, alternative weed management strategies. However, farmers tend to focus on the short-term economic benefits, whereas the agroecological benefits of herbicide reduction are long-term oriented. In contrast to the use of synthetic herbicides, bioherbicides are an ecologically sustainable alternative that is a priority in the EU. These eco-friendly herbicides can be subdivided into microbial bioherbicides and bio-derived (biochemical) bioherbicides. Microbial bioherbicides are made of bacteria, fungi, or viruses, either in their active form (liquid formulation) or in their dormant form (dry formulation). Natural molecules extracted, in most cases, from plants are the active ingredients of bio-derived bioherbicides. However, botanical products can be heterogeneous as a concenquence of the bioactive component mixture’s presence either from the same or from purposefully mixed botanical sources. Physical analytical methods, such as chromatography, are inadequate for this purpose, as they are often not sensitive enough to the chemical complexities found in crude botanical extracts. Most often, a desired biological response is owed to a mixture of bioactive plant components, and the relative proportions of single bioactive compounds may vary from batch to batch, whereas the bioactivity remains within tolerable limits. Thus, physical or chemical analysis of a single component in such mixtures is not completely satisfactory [97]. The isolation of plant allelopathic substances and the evaluation of their phytotoxic effects can lead to the discovery of

new natural herbicides. For the above reasons, a decisive factor in the discovery of bioherbicides is the evaluation of the herbicidal activity of plant extracts by bioassays.

The herbicidal activity of plant extract evaluation can be estimated either at the laboratory scale using in vitro assays or in the field via pre- and postemergence assays. An in vitro assay evaluates the seed germination in Petri dishes. The inhibitory effects of the extract on weed seeds are determined by counting the germinated seeds (percent of germination), the root length of germinated seeds, the sprout length, etc. Firstly, it is crucial that the seed surface be sterilized to avoid possible inhibition of germination caused by fungal or bacterial toxins. The seeds are placed on a filter paper soaked by the extract [37] or covered by a soaked filter paper [98]. One concentration or multiple concentrations of the extract can be used during the assay [99]. The dishes are sealed with parafilm to avoid evaporation of the extract and incubated in certain temperature and photoperiod conditions. Variations of the method have been successfully employed to test extracts from various Mediterranean species against weeds such as *Melilotus officinalis* L., *Myosotis arvensis* (L.) Hill and Trigonella *besseriana* Ser. [98], and *Amaranthus retroflexus* L. and *Portulaca oleracea* L., *Stellaria media* (L.) Vill., and *Anagallis arvensis* [100]. The method can also be applied to germinating seedlings [36]. On the other hand, evaluation of the herbicidal activity can also be estimated in the field in pre- and postemergence assays. Morra et al. [101] evaluated the activity of *Sinapis alba* extract to the seeds of *Amaranthus powellii* and *Setaria viridis*. In preemergence assays, the solution of the extract is applied to the surface of the pot, whereas in postemergence assays, the extract either is sprayed or watered [24]. In preemergence assays, the emerged live seedlings, the plant height, and the dry weight are recorded, whereas in postemergence assays, the live plants per pot, the plant height, and the dry weight are determined [101].

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