Chapter 1
In Vitro and *In Vivo* Bioassays

Mercedes Verdeguer

1 Introduction

The plant's environment is determined by all the physical and chemical factors that characterize the habitats, and also by the effects that other co-occurring organisms induce on them. The functional study of plant's behaviour in their environment linked ecology and physiology in a new discipline, the ecophysiology (Pardos 2005). Ecophysiologists, or physiological ecologists deal with ecological questions like the mechanisms that regulate and control growth, reproduction, survival, abundance, and geographical distribution of plants, as these processes are affected by interactions of plants with their physical, chemical, and biotic environment. The knowledge of these ecophysiological patterns and mechanisms helps to understand the functional significance of specific plant traits and their evolutionary heritage. Ecophysiological techniques have greatly advanced understanding of photosynthesis, respiration, plant water relations, and plant responses to abiotic and biotic stress, from immediate to evolutionary timescales. Many important societal issues, as agriculture, climate change, or nature conservation, benefit from the application of an ecophysiological perspective (Lambers et al. 2008). Plants have adapted to an incredible range of environments, and the fields of ecological and environmental plant physiology have provided tools for understanding the survival, distribution, productivity, and abundance of plant species across the diverse climates of our planet (Ainsworth et al. 2016).

One of the branches of Ecophysiology focuses on the study of the physiological interactions of plants with other plants, animals and microorganisms. Some plants have the capacity of inhibiting the growth or development of surrounding plants by releasing chemical compounds known as allelopathic compounds or allelochemicals. Plants introduce allelochemicals into the environment through foliar leaching,

M. Verdeguer (✉)
Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Valencia, Spain
e-mail: merversa@doctor.upv.es

A. M. Sánchez-Moreiras, M. J. Reigosa (eds.), *Advances in Plant Ecophysiology Techniques*, https://doi.org/10.1007/978-3-319-93233-0_1

root exudation, residue decomposition, volatilization and debris incorporation into soil (Inderjit and Keating 1999). Most allelochemicals are secondary metabolites, which are obtained through branching of the main metabolic pathways of carbohydrates, fats and aminoacids (Lotina-Hennsen et al. 2006). Secondary metabolites act sometimes as allelochemicals; however, the terms 'allelochemical' and 'secondary metabolite' should not be used as synonyms (Whittaker and Feeny 1971).

In this chapter, *in vitro* and *in vivo* assays for the study of plant interactions, especially for the study of the effects of potential phytotoxic secondary metabolites (allelochemicals), are in detail described.

2 *In Vitro* Bioassays

The research of phytotoxic chemicals with potential utilization as herbicides is one of the areas in which allelopathic studies are more promising nowadays (Macías et al. 2001; Benvenuti et al. 2017). These studies need to have into account the following focus points: (1) the knowledge of the organism that release the chemicals ('donor plant'); (2) the knowledge of the chemicals involved in the interaction; (3) the observation of the plant that acts as the receiver ('target plant') to discover the effects of the chemicals and their mechanism/s of action; and (4) the way the chemicals go from the donor to the target plant, as well as the transformations, induced by biotic and/or abiotic factors, that the allelochemicals could undergo in between, as this could essentially modify their effects (Macías et al. 2008). Analytical techniques and bioassays are basic tools for the detection and understanding of the effects of these compounds.

The most widely used bioassays are seed germination assays, carried out on Petri dishes with filter paper as the most common support (Leather and Einhellig 1988), or agar as an alternative; and seedling growth tests (Lotina-Hennsen et al. 2006).

2.1 Seed Germination Bioassays

Although seed germination bioassays seem simple, some decisions are important in order to have representative and useful results.

First, depending on the objectives of the study, a mixture of compounds or a single compound could be tested. This is sometimes a point of controversy.

Allelopathic activity in field situations is thought to be often due to the joint action of mixtures of allelochemicals rather than to the action of a single allelochemical (Einhellig 1995). For example, Lydon et al. (1997) reported that soil amended with pure artemisinin was less inhibitory to the growth of redroot pigweed than soil amended with a chemically more complex annual wormwood leaf extract. There are many more examples supporting the synergistic action of mixture compounds (Barney et al. 2005; Koroch et al. 2007). Araniti et al. (2013) found that the

inhibitory effects caused by *Calamintha nepeta* methanolic extract on *Arabidopsis thaliana* depended on the combined action of different molecules. Inderjit et al. (2002) argued that the understanding of the joint action of phytotoxins in allelopathy research is mainly hindered due to the lack of a well-defined reference model and to methodological problems. Determining which compounds are involved in causing phytotoxicity will be important in developing predictive models for toxicity. Although plant physiologists have successfully determined the mode of action of several individual allelochemicals, more research is needed to evaluate the mode of action of compounds in a mixture belonging to different chemical classes (Inderjit and Duke 2003).

Essential oils or aqueous or solvent extracts contain a mixture of compounds. Therefore, it is necessary to test them as a whole to know the effects that they could exert, but complementary tests with isolated compounds that are part of the mixture could also be done to better understand their activities and mechanism/s of action (Araniti et al. 2013). When more components are part of the mixture more difficult is to comprehend how they are acting and more complicated trials need to be performed.

The second step when planning bioassays is the selection of the target species. Lettuce seeds (*Lactuca sativa* L.) have been widely used because of their fast germination and high sensitivity. Some crop species have been also used for these purposes. There is concern that these species do not reflect what is occurring on natural ecosystems but they offer researchers a starting point from which to learn more about phytotoxicity and allelochemicals. There are some criteria that seeds must satisfy to be used in phytotoxic bioassays: being readily available; being affordable; germinating quickly, completely, and uniformly; and producing repeatable and reliable results. They must also be sensitive enough to respond to a variety of chemicals with different biochemical effects and to offer researchers a means by which to help identifying the mechanisms of action of active compounds. *Arabidopsis thaliana* (L.) has been also used as model species, as it is sensitive to a variety of potent allelochemicals and satisfies all of the selection criteria for target species (Pennacchio et al. 2005).

The most important consideration when choosing the species for developing a bioassay for an allelopathic study is to select the target species from both mono and dicotyledons (Lotina-Hennsen et al. 2006), because they have different metabolism and different responses to the phytotoxic compounds. It has been demonstrated that phytotoxic compounds cause different effects depending on the species they are acting against (Reigosa et al. 1999; Verdeguer et al. 2009a; Graña et al. 2013). The selected target species also depend on the objectives of the research. For studies of allelopathic compounds action in natural ecosystems it is necessary to select the species that are receiving the compounds in nature (Herranz et al. 2006). When searching new bioherbicides it is interesting to select different important weeds and crops as target species (Benvenuti et al. 2017) while when focusing in the mechanism of action is better selecting *A. thaliana* as target species (Reigosa and Malvido-Pazos 2007).

In vitro seed germination bioassays can be performed placing the seeds on filter paper (Dudai et al. 1999), agar (Pederson 1986) or other inert substrates, as sand or vermiculite (Dayan and Duke 2006). Filter paper is the most used substrate for many reasons, like its easy availability and management, low cost and the fact that usually limited quantities of natural compounds are available for testing. Depending on the thickness of the filter paper employed the requirements of water or water plus solvent solution of the compound(s) studied will be different. For example, Whatman n° 1 paper, which is one of the most used (Barnes and Putnam 1987; Reigosa and Malvido-Pazos 2007) is 87 g/m^2 and Whatman n° 3 paper, which has also been used for germination bioassays (Dudai et al. 1999), is 185 g/m^2. It is important that the filter paper is wet enough to allow seed germination but without excessive water to prevent fungal growth and to avoid anaerobic conditions that could difficult seed germination. Previous assays could be done with control seeds to determine the adequate water or solution supply.

Most allelochemicals or their mixtures (e.g. essential oils) are not soluble in water. In some studies, they are applied directly to the filter paper (Dudai et al. 1999; Verdeguer et al. 2009a), while in others, different solvents are used based on the compound's solubility to obtain aqueous solutions, like ethanol, dimethyl sulfoxide (Reigosa and Malvido-Pazos 2007) or Tween 20 (Angelini et al. 2003). When using solvents, it is necessary to include a control with the concentration employed to verify that they are not causing undesirable phytotoxic effects. It is important to have a good solution of the compound(s) studied, otherwise the results could be not as expected. For testing essential oils, Fitoil, a biological adjuvant containing 40% of soybean oil can be used to solubilize them in water (Verdeguer 2011).

Another aspect to consider when planning seed germination tests is the number of seeds and replications used. Normally, at least five repetitions are performed (Dudai et al. 1999; Angelini et al. 2003; Verdeguer et al. 2009a; Graña et al. 2013; Benvenuti et al. 2017). The number of seeds included can vary depending on the size of the seed and the purpose of the experiment, and also on the diameter of the Petri dish employed. The inhibitory potential of essential oils against different crops and weeds was tested in Petri dishes of 6 cm with 20 seeds each and 5 replications (Dudai et al. 1999). Benvenuti et al. (2017) used Petri dishes of 15 cm diameter and 50 seeds each with 3 repetitions, repeated twice (6 replications in total) to evaluate the effect of Compositae essential oils on *Amaranthus retroflexus* L. and *Setaria viridis* (L.) P. Beauv. Petri dishes of 9 cm diameter are commonly used for germination bioassays. Depending on the size of the seeds tested is recommendable to change the number of seeds placed in each Petri dish. Eight replicates with 24 seeds each were used for large-seeded species, whereas five replicates with 50 seeds each were used for small-seeded species for testing the phytotoxic potential of citral on weeds germination (Graña et al. 2013; Díaz-Tielas et al. 2014).

If the germination tests are being maintained for more than 5–7 days, to study the effect of natural products in the development of the seedlings after germination, it also affects the number of seeds to be used, for example, *Portulaca oleracea* L., which is an important weed in Mediterranean summer crops has very small seeds and 20 or more seeds can be easily placed in a Petri dish of 9 cm diameter

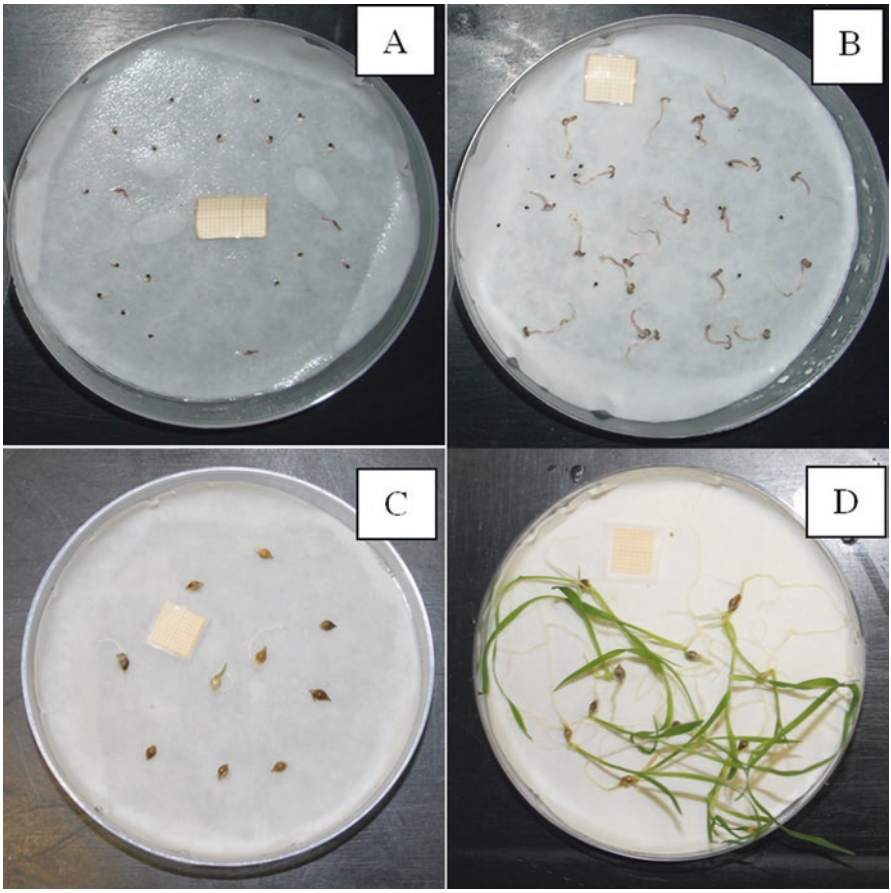

Fig. 1.1 *Portulaca oleracea* seeds after 3 days of incubation (**a**) (30 °C l2 h light/20 °C 8 h dark) and after 14 days (**b**). *Echinochloa crus-galli* seeds after 3 days of incubation (**c**) and after 14 days (**d**) in the same conditions

(Fig. 1.1a, b). *Echinochloa crus-galli* (L.) P. Beauv., which is an important weed in rice crops has greater seeds and is not recommendable to use more than 10 seeds in each Petri dish if the seedlings development has to be analysed, because when germination starts seedlings develop very quickly and take up a lot of space (Fig. 1.1a, b). In this case 5 repetitions of 20 seeds each could be used for *P. oleracea* and 10 repetitions of 10 seeds each could be used for *E. crus-galli*. It is important that the concentrations of compound(s) tested are the same in both cases. There is also a bit controversy with this issue because some authors stated that the doses received by seeds are different in both cases (when placing different seed number) but also the seeds requirements are different. As the doses of herbicides in field conditions are measured by surface area, we could use the same idea for the doses used in Petri dishes.

In a given soil volume containing a specific amount of phytotoxin, plants growing at low densities have a larger amount of toxin available per plant than at high densities, where the toxin is shared (and thus diluted) among many plants, receiving each plant a proportionately smaller dose. Lower phytotoxin concentrations could produce equivalent or greater inhibitory effects than higher concentrations when the amount available per plant is greater (Weidenhamer 1996). The degree of inhibition caused by an allelopathic compound will depend upon both phytotoxin concentration and the total amount of phytotoxin available (Weidenhamer et al. 1987).

Some species can present autotoxicity, like alfalfa, *Medicago sativa* L., (Chon and Nelson 2001) or tobacco, *Nicotiana tabacum* L. (Deng et al. 2017) or *Prosopis juliflora* (Sw.) DC. (Warrag 1995), among others. When testing these species, it is recommended to use well plate systems in order to separate the seeds and do not mask the effects of the allelochemicals tested with the autotoxins released by these seeds or seedlings. It is important to note that the International Seed Testing Association (ISTA) has developed the International Rules for Seed Testing, to uniform seed testing methods. The 2018 edition is available at the ISTA website. They are recommendations and testing methods for seeds designated for growing of crops or production of plants, but for spontaneous and medicinal plants or weed seeds had not been developed.

The last decision regarding seed germination tests is to define the duration. Depending on the objective, it can be established normally when all seeds have germinated or when there are not changes in germination rates after consecutive counts. Previous assays can be performed to determine how much time needs the majority of seeds to germinate.

Different parameters can be evaluated in germination tests. It is very important to use suitable indices of germination because the interpretation and the biological meaning of the assays depend of them (Chiapusio et al. 1997). For example, if it is considered only the total final seed germination rate (percentage of seeds germinated at the end of the experiment) delays on germination cannot be measured neither evaluated, while this is an important effect of allelochemicals on germination. The speed of germination (proportion of germinated seeds obtained in n days or hours) reflects better what happens during the germination process and is a good indicator for delays in germination. The speed of accumulated germination (cumulative number of seeds that germinate on time N since set up of the experiment) and the coefficient of the rate of germination (number of seeds germinated on time T) are less sensitive than the speed of germination to show delays on germination and their interpretation must be done carefully. Control and treated seeds must be compared at each exposure time for better conclusions. Other parameters that can be evaluated are the abnormal symptoms observed in seeds, and the radicle length, among others. If the tests are performed to investigate the phytotoxicity of some products on seed germination could be interesting to determine the concentration of product that causes 50% inhibition of germination rate (IC_{50}) and the concentration of product that causes 80% inhibition (IC_{80}). It is also important to verify if the

phytotoxic effects on seed germination are reversible or irreversible. For this purpose, the seeds that had been in contact with the phytotoxic product are transferred to water (Petri dishes are prepared as they were controls) after the germination tests, and their germination is evaluated again. If they germinate the phytotoxic effects are reversible. This is important for biodiversity conservation purposes.

A protocol for testing natural products phytotoxic effects on seed germination is here described. Seeds of different weeds, monocotyledons and dicotyledons are used in order to study different responses depending on the type of weed. For small-sized seed weeds, 20 seeds are placed in Petri dishes of 9 cm diameter between two layers of filter paper (50 g/m^2) wetted with 4 mL of the corresponding treatment, and 5 replicates are performed. For large-sized weed, 10 seeds and 10 replicates are used. The treatments applied are control (distilled water) and 4 different concentrations of the product assayed. The Petri dishes are incubated in a chamber with 60% HR, 30 °C 12 h light and 20 °C 8 h dark. Photos of Petri dishes are registered after 3, 5, 7, 10 and 14 incubation days and are processed with Digimizer and ImageJ to determine different parameters: number of germinated seeds per day, abnormal seeds, radicle length and seedling length, to calculate germination speed and rates. At the end of the assay also fresh and dry weight of treated plants can be registered.

2.2 Seedling Growth Bioassays

Seedling growth of the developed seedlings can be evaluated in order to determine the effects of one compound or a mixture of compounds on seedling growth after germination, if the seeds were able to germinate after the treatment, and this is performed as described in the previous part. Photos are registered from the Petri dishes after 3, 5, 7, 10 and 14 incubation days and processed with Digimizer and ImageJ to determine radicle and seedling length and other parameters of interest.

There are also specific assays performed to study the effects of natural products applied in post emergence, once the seeds have been germinated. In this assays seedlings of the target plants are produced following the protocols described in the previous section for the controls and then ten plants with uniform radicle length (5 mm) are selected and transferred to Petri dishes with the treatment to analyse the effects (Fig. 1.2). Five replicates are prepared for each treatment and the Petri dishes are incubated in the same conditions used for germination tests (Verdeguer 2011). Photos are registered after 3, 5, 7, 10 and 14 days since the plants are transferred and are then processed with Digimizer and ImageJ to measure radicle and seedling length. Other studies follow similar protocols using seedlings of 1–2 mm radicle length that are transferred to Petri dishes with the treatments and the growth is measured for 48 h (Dudai et al. 1999; Araniti et al. 2017).

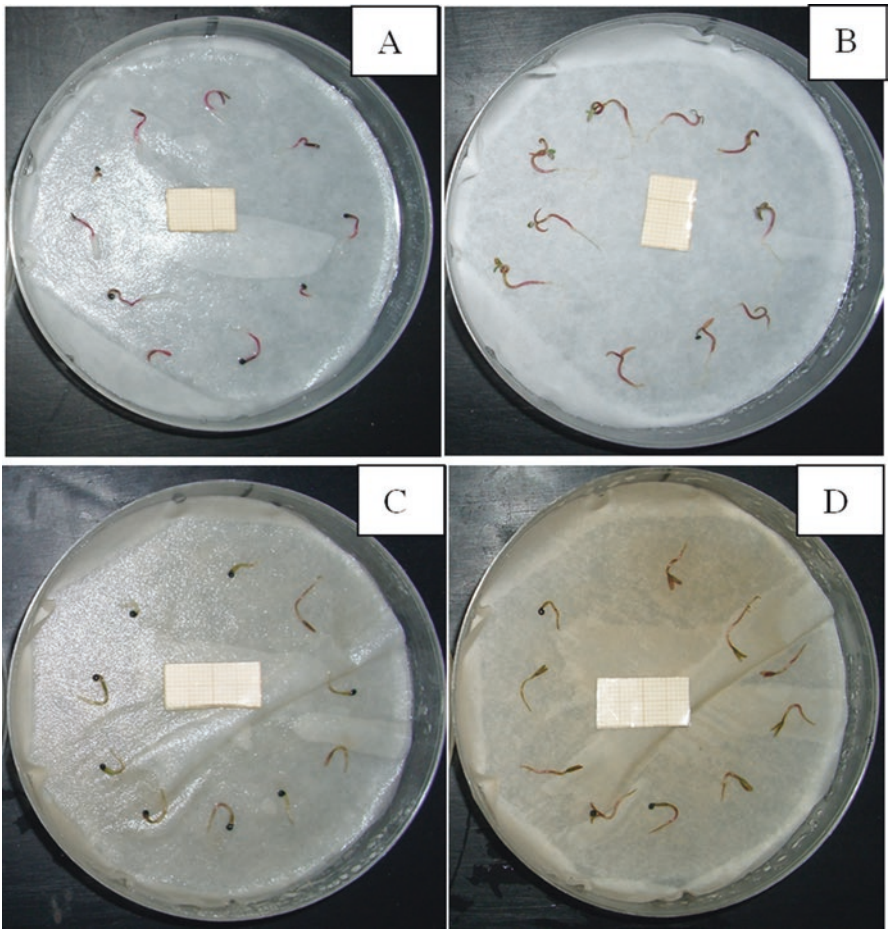

Fig. 1.2 *Chenopodium album* seedlings of control (**a**, **b**) and treatment of 100% concentration of *Eucalyptus camaldulensis* aqueous extract (**c**, **d**) after 3 and 14 days incubation (30 °C l2 h light/20 °C 8 h dark)

3 *In Vivo* Assays

There are few studies about the *in vivo* phytotoxic activity of natural products against weeds. One of the most important reasons is because of the lack of the natural products, which normally are laboratory obtained compounds without an adequate formulation for being applied in *in vivo* conditions, which is a limiting factor when planning assays. In the last years, groups working on this research area have made efforts to develop protocols for *in vivo* testing of natural products. In this section, some of these protocols will be described. Natural products can be tested in pre and post-emergence, and they can be applied by irrigation or spraying.

3.1 Pre-emergence In Vivo *Assays*

When testing natural products in pre-emergence against weeds there are two different possibilities: working with soil, and its seed bank, that has not been treated with herbicides, or working with peat substrate and sow specific weed seeds. Other important considerations are how, when and how often to supply the treatments. Assays with soil are better performed in trays than in pots, because soil in small pots can cause compaction problems, it is necessary a careful water management. It is recommendable to inventory the weeds that are growing in the field from which the soil is collected, and is imperative to correctly homogenize the soil collected before using it for the experiments.

In a greenhouse experiment, trays of 56.5 × 36.5 × 12 cm were filled with 5 cm perlite at the basis, and 5 cm soil from a citrus orchard where herbicides had not been applied for the last 5 years (Fig. 1.3). Three replications were prepared for each treatment. Treatments were applied in pre-emergence, and three aqueous extracts were tested: *Lantana camara*, *Eucalyptus camaldulensis* and *Eriocephalus africanus* at 100% dose. Controls were irrigated with water and the other trays were irrigated with the respective treatments (4 L per tray). Treatments were applied only once, and trays were irrigated once a week. Emerged plants were counted and

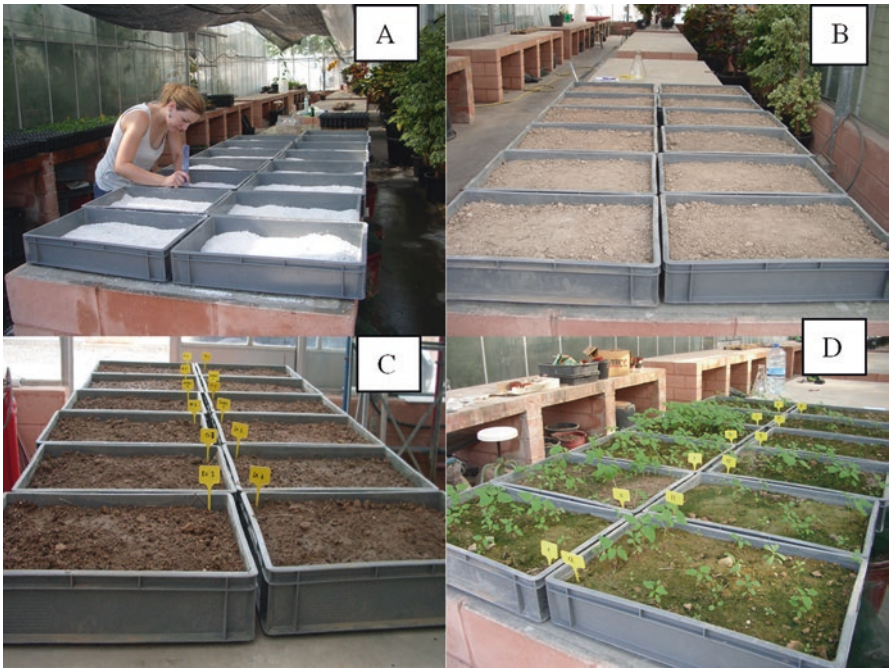

Fig. 1.3 Greenhouse *in vivo* experiments. Filling with perlite the basis of the trays (**a**), trays with soil before being treated (**b**), trays just after treatments application (**c**), trays 4 weeks after treatments application

classified, and the weed density was also calculated. Height, fresh and dry weight of the emerged plants was also registered. The experiment was finished when there were no significant differences in the number of emerged plants between control and treatments (Verdeguer et al. 2009b).

When using peat substrate, the experiments can be performed in seedbeds or pots. It is necessary take into account the same considerations about seeds size and number of repetitions that were exposed for *in vitro* bioassays. For example, in pots of 8 × 8 × 7 cm 20 small-sized weed seeds or 10 big-sized weed seeds can be placed.

3.2 Post-emergence In Vivo *Assays*

It is important to consider that treatments can be supplied by irrigation or spraying and their activity is influenced by the applying method (Fig. 1.4) (Graña et al. 2013; Díaz-Tielas et al. 2014; Castañeda 2017). As described in the previous section, assays can be performed with soil or with peat substrate. In assays with soil it is necessary to irrigate it to allow the germination of the seeds that are in the seed bank of the soil and the growth of the seedlings. In the assays with peat substrate, seeds are sown in pots or seedbeds, and when seedlings develop a thinning can be done if it is necessary. When the emerged plants reach the desired development stage, treatments can be applied by both methods, in order to determine which is the most effective.

The phenological stage in which the plant is treated is also an important factor to consider, because the activity of natural, and also synthetic products is very influenced by it. Previous assays could be performed with weeds in different stages to determine the best moment for application. For example, for *Arabidopsis thaliana*, treatments were applied when it had five fully expanded leaves (Graña et al. 2013; Araniti et al. 2017). For *E. crus-galli* it is recommended to apply herbicides for a best control when it has 2–3 true leaves and for *Conyza* species at the rosette stage. The treatments can be applied once (Verdeguer et al. 2009b; Castañeda 2017) or repeated times (Graña et al. 2013; Araniti et al. 2017). After the treatments are applied, phytotoxic effects can be evaluated. Mortality, observed damages (classified with a phytotoxicity scale), height, fresh and dry weight of the plants can be recorded to determine the herbicidal activity of the tested products.

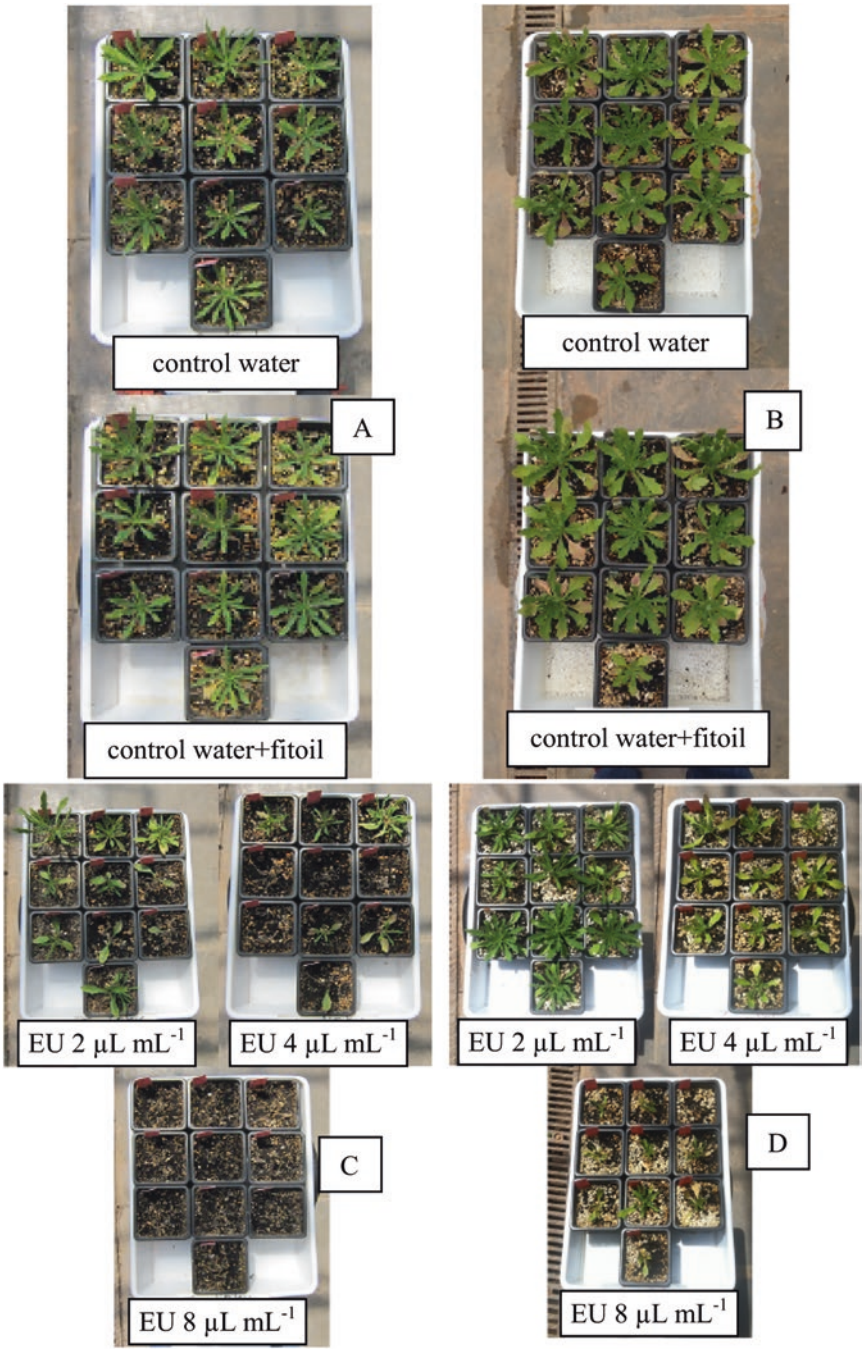

Fig. 1.4 Greenhouse *in vivo* experiment. Treatments with *Eucalyptus camaldulensis* essential oil in post emergence applied by irrigating (**a**, **c**) and spraying (**b**, **d**), *Conyza bonariensis* controls (**a**, **b**) and treated plants (**c**, **d**)

References

Ainsworth EA, Bernacchi CJ, Dohleman FG (2016) Focus on ecophysiology. Plant Physiol 172:619–621

Angelini LG, Carpanese G, Cioni PL, Morelli I, Macchia M, Flamini G (2003) Essential oils from Mediterranean lamiaceae as weed germination inhibitors. J Agric Food Chem 51:6158–6164

Araniti F, Graña E, Reigosa MJ, Sánchez-Moreiras AM, Abenavoli MR (2013) Individual and joint activity of terpenoids, isolated from *Calamintha nepeta* extract, on *Arabidopsis thaliana*. Nat Prod Res 27:2297–2303

Araniti F, Sánchez-Moreiras AM, Graña E, Reigosa MJ, Abenavoli MR (2017) Terpenoid trans-caryophyllene inhibits weed germination and induces plant water status alteration and oxidative damage in adult arabidopsis. Plant Biol 19:79–89

Barnes JP, Putnam AR (1987) Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). J Chem Ecol 13:889–906

Barney JN, Hay AG, Weston LA (2005) Isolation and characterization of allelopathic volatiles from mugwort *Artemisia vulgaris*. J Chem Ecol 31:247–265

Benvenuti S, Cioni PL, Flamini G, Pardossi A (2017) Weeds for weed control: Asteraceae essential oils as natural herbicides. Weed Res 57:342–353

Castañeda LG (2017) Control de *Conyza bonariensis* con aceites esenciales de plantas mediterráneas *Thymbra capitata, Mentha x piperita, Santolina chamaecyparissus* y *Eucalyptus camaldulensis*. Trabajo Final de Máster. Universitat Politècnica de València

Chiapusio G, Sánchez AM, Reigosa MJ, González L, Pellissier F (1997) Do germination indices adequately reflect allelochemical effects on the germination process? J Chem Ecol 23:2445–2453

Chon SU, Nelson CJ (2001) Effects of experimental procedures and conditions on bioassay sensitivity of alfalfa autotoxicity. Commun Soil Sci Plant Anal 32:1607–1619

Dayan F, Duke S (2006) Clues in the search for new herbicides. In: Reigosa M, Pedrol N, González L (eds) Allelopathy. Springer, Dordrecht, pp 63–83

Deng J, Zhang Y, Hu J, Jiao J, Hu F, Li H, Zhang S (2017) Autotoxicity of phthalate esters in tobacco root exudates: effects on seed germination and seedling growth. Pedosphere 27:1073–1082

Díaz-Tielas C, Sotelo T, Graña E, Reigosa MJ, Sánchez-Moreiras AM (2014) Phytotoxic potential of trans-chalcone on crop plants and model species. J Plant Growth Regul 33:181–194

Dudai N, Poljakoff-Mayber A, Mayer AM, Putievsky E, Lerner HR (1999) Essential oils as allelochemicals and their potential use as bioherbicides. J Chem Ecol 25:1079–1089

Einhellig FA (1995) Allelopathy: current status and future goals. In: Inderjit, Dakshini KMM, Einhellig FA (eds) Allelopathy: organisms, processes and applications. American Chemical Society, Washington, DC, pp 1–24

Graña E, Sotelo T, Díaz-Tielas C, Reigosa M, Sánchez-Moreiras AM (2013) The phytotoxic potential of the terpenoid citral on seedlings and adult plants. Weed Sci 61:469–481

Herranz JM, Ferrandis P, Copete MA, Duro EM, Zalacain A (2006) Effect of allelopathic compounds produced by *Cistus ladanifer* on germination of 20 Mediterranean taxa. Plant Ecol 184:259–272

Inderjit, Duke SO (2003) Ecophysiological aspects of allelopathy. Planta 217:529–539

Inderjit, Keating KI (1999) Allelopathy: principles, procedures, processes, and promises for biological control. Adv Agron 67:141–231

Inderjit, Streibig J, Olofsdotter M (2002) Joint action of phenolic acid mixtures and its significance in allelopathy research. Physiol Plant 114:422–428

Koroch AR, Rodolfo Juliani H, Zygadlo JA (2007) Bioactivity of essential oils and their components. In: Berger RG (ed) Flavours and fragrances: chemistry, bioprocessing and sustainability. Springer, Berlin/Heidelberg, pp 87–115

Lambers H, Pons TL, Chapin FS (2008) Plant physiological ecology. Springer, Berlin/Heidelberg

Leather GR, Einhellig FA (1988) Bioassay of naturally occurring allelochemicals for toxicity. J Chem Ecol 14:1821–1828

Lotina-Hensen B, King-Díaz B, Aguilar MI, Hernández Terrones MG (2006) Plant secondary metabolites. Targets and mechanisms of allelopathy. In: Reigosa MJ, Pedrol N, González L (eds) Allelopathy: a physiological process with ecological implications. Springer, Dordrecht, pp 229–265

Lydon J, Teasdale JR, Chen PK (1997) Allelopathic activity of annual wormwood (*Artemisia annua*) and the role of artemisinin. Weed Sci 45:807–811

Macías FA, Molinillo JMG, Galindo JCG, Varela RM, Simonet AM, Castellano D (2001) The use of allelopathic studies in the search for natural herbicides. J Crop Prod 4:237–255

Macías FA, Oliveros-Bastidas A, Marin D, Carrera C, Chinchilla N, Molinillo JMG (2008) Plant biocommunicators: their phytotoxicity, degradation studies and potential use as herbicide models. Phytochem Rev 7:179–194

Pardos JA (2005) Ecophysiology, a meeting point between function and management of forest ecosystems. Investigación Agraria: Sistemas y Recursos Forestales 14:277–291

Pederson GA (1986) White clover seed germination in agar containing tall fescue leaf extracts. Crop Sci 26:1248–1249

Pennacchio M, Jefferson L, Havens K (2005) *Arabidopsis thaliana*: a new test species for phytotoxic bioassays. J Chem Ecol 31:1877–1885

Reigosa MJ, Malvido-Pazos E (2007) Phytotoxic effects of 21 plant secondary metabolites on *Arabidopsis thaliana* germination and root growth. J Chem Ecol 33:1456–1466

Reigosa MJ, Souto XC, González L (1999) Effect of phenolic compounds on the germination of six weeds species. Plant Growth Regul 28:83–89

Verdeguer M (2011) Fitotoxicidad de aceites esenciales y extractos acuosos de plantas. mediterráneas para el control de arvenses. Tesis doctoral, Universitat Politècnica de València

Verdeguer M, Blazquez MA, Boira H (2009a) Phytotoxic effects of *Lantana camara*, *Eucalyptus camaldulensis* and *Eriocephalus africanus* essential oils in weeds of Mediterranean summer crops. Biochem Syst Ecol 37:362–369

Verdeguer M, Garcia D, Blázquez MA, Boira H (2009b) Potencial alelopático de extractos acuosos de *Lantana camara, Eucalyptus camaldulensis* y *Eriocephalus africanus* y posible uso como herbicidas naturales. In: Herbologia e Biodiversidade numa Agricultura Sustentável ,vol 1, pp 403–406

Warrag MOA (1995) Autotoxic potential of foliage on seed germination and early growth of mesquite (*Prosopis juliflora*). J Arid Environ 31:415–421

Weidenhamer JD (1996) Distinguishing resource competition and chemical interference: overcoming the methodological impasse. Agron J 88:866–875

Weidenhamer JD, Morton TC, Romeo JT (1987) Solution volume and seed number: often overlooked factors in allelopathic bioassay. J Chem Ecol 13:1481–1491

Whittaker RH, Feeny PP (1971) Allelochemics: chemical interactions between species. Science 171:757–770