PhD School on Agriculture, Environment and Bioenergy

(http://sites.unimi.it/dottorato_aab/)

(XXXIX cycle, 2023-26)

Project draft

1. Field of interest

AGR/13

2. Project title

Exploiting plant extracts and plant-derived biologically active specialised metabolites as a source of new potential botanical herbicides

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4. Relevance of the topic and state of the art:

Synthetic herbicides, considered hazardous agents for the environment and human health, have had used to eliminate unwanted species in agricultural ecosystems for a century[1]. However, their massive and indiscriminate use in recent decades contributed in increasing soil, air and water pollution and induced the evolution of resistant weeds that have risen exponentially in the last thirty years[2,3]. Recently, the European Union has been activating several actions to restrict the use of numerous synthetic substances commonly used in pesticide formulations, characterised by harmful effects on the environment and human health[1]. European farmers already lost 60% of the substances available in 1991 (Directive 91/414 / EEC), which have not yet been replaced in the absence of alternatives. The European Conservation Agriculture Federation aims to promote the development of conservation agriculture in the European environment, which has as its fundamental objective "to conserve, improve and make more efficient use of natural resources, through integrated management of soil, water, biological agents and external inputs". Although this type of agriculture does not prohibit the use of herbicides, it does advocate their rational use and the incorporation of new products that have a lower residual effect and are more friendly to the environment, mainly prioritising the application of post-emergence herbicides [1]. Throughout the evolutionary process, plants have developed biosynthetic pathways to synthesise and accumulate a great variety of specialised metabolites, many of which are characterised by phytotoxic activity, playing a fundamental role in the interactions between living organisms in the natural environment[4]. These molecules tend to play more than one role in the metabolism of the producer plant, so there is a greater probability of finding compounds with multiple sites of action, valorising their bioherbicidal capacity[5]. The PhD project will focus on screening several extracts and/or pure specialised metabolites, selected for their low toxicological profile, on the most common European crops and their associated weeds to identify those characterised by the highest phytotoxic potential. The most interesting extracts will be chemically characterised to determine the biologically active compounds. Successively the pure molecules (isolated from extracts or selected through a literature survey) will then be deeply studied to elucidate their potential mode of action, on the model species *Arabidopsis thaliana*, through classical physiological/biochemical and new *-omics* (transcriptomic, proteomic and metabolomics) approaches.

5. Layout of the project (draft)

This agroecological project aims to investigate the phytotoxic potential of plant extracts, essential oils and/or plant-derived specialised metabolites to identify their mode of action and potential application for weed management in eco-friendly agriculture.

5.1. Materials & Methods:

The first step of the research will be achieved through a literature survey and selection of allelopathic species for extract/essential oils production or the most interesting plants' specialised metabolites, characterised by low environmental and human health toxicity. Successively, a rapid *in-vitro* and *in-vivo* screening will be carried out on germination/root growth of mono and dicot crops and the most widespread European noxious weeds (i.e. *Chenopodium album, Sinapis alba, Echinochloa crus-galli*, among others). The experiments, carried out in Petri dishes, will adopt a broad concentrations range of extracts/molecules. The dose responses curves will be fitted through nonlinear regression to determine several key parameters such as *i*) the inhibition threshold (lowest concentration required to start the inhibition), *ii*) the IC_{50} (concentration required to obtain 50% of inhibition), among others; parameters are essential for comparing crude extract/pure molecules phytotoxicity and for establishing a frame of milestones/references to which the following experiments can be compared.

The most promising phytotoxic extracts will be further analysed through bio-guided fractionation method, which integrates analytical procedures (i.e. liquid-liquid chromatography) with bioassays and represents a rapid and cost-effective methodology to discover biologically active specialised metabolites. Each fraction will be *in-vitro* assayed on seed germination and root growth (as previously described) to identify the most active fractions. The most biologically active fraction will be phytochemically characterised through spectrophotometric techniques.

The most interesting phytotoxic molecules will then be studied at a physiological, metabolic and molecular level.

At the physiological level, the biological activity of isolated compounds will be assessed on plant metabolism to identify their potential target sites and modes of action on the model species *Arabidopsis thaliana*, using key concentrations calculated through dose-response curves built on each molecule. The most promising bioactive molecules will be deeply studied for morpho-physiology and molecular effects on plant systems. In this respect, the study will focus on *i*) seed germination, evaluating seed imbibition, germination frequency and rate, enzymes activity involved in the germination process and respiratory metabolism; *ii*) seedling growth parameters examining cell division, cell expansion rates, root morphology and root meristem anatomy and structure; *iii*) photosynthesis by measuring pigments content, PSII efficiency, stomatal resistance and internal CO_2 concentration, chlorophyll content, chlorophyll-*a* fluorescence using analytical techniques such as CIRAS, MultiSpeQ, among others; *iv*) water status by measuring water and osmotic potentials, relative water content and transpiration rate; *v*) membrane integrity, inorganic ions profile (ICP-MS system), ROS, ROS-scavengers activity and lipid peroxidation.

The most interesting molecules selected during the previous steps will be studied using key concentrations and exposure times through *-omics* techniques. In particular, transcriptomic, proteomic and metabolomic analyses will be carried out. In particular, the compounds will be applied at sub-lethal doses, thus enabling the detection of their primary effects on plants while excluding undesirable secondary effects. Transcriptome analysis will characterise all transcriptional changes determined by exposition to bioactive natural molecules. Indeed, integrating transcriptomic to other -omics profiles will support the development of metabolic models for studying bioactive compounds' modes of action. The experiments will be carried out on freeze-dried or lyophilised plant material and analysed through next-generation sequencing tools (NGS), GC-MS, and UHPLC-ESI-MS/MS approaches.

5.2. Schedule and major steps (3 years):

1st year: The activity of the first year will be divided into three different steps: *i*) literature survey and molecules selection focusing on those characterised by a low toxic effect on the environment and human health; *ii*) *in-vitro* screening, using a wide range of concentrations, on model species, crops and weeds to identify to confirm the phytotoxic activity of the selected molecules, extracts and/or essential oils; *iii)* bio-guided fractionation of the most phytotoxic extracts and identification of the most biologically active fractions.

2nd year: The activity of the first year will be divided into three different steps: *i*) chemical characterisation of the most bio-active fractions and essential oils through GC-MS and/or UHPLC-ESI-MS/MS approaches; *ii*) Screening of the pure molecules on the model species *A. thalina* (WT and mutants), identification of the main target organs, key concentrations and times of exposure; *iii*) transcriptomic analysis.

3rd year: The activity of the first year will be divided into three different steps: *i*) proteomic and metabolomic analysis; *ii*) data integration, validation, interpretation and management; *iii*) writing of the final project.

During the three years, the PhD student will statistically elaborate the data and divulge his/her research through original research article writing, congress and winter/summer school participation

6. Available funds

The PhD research activity will be useful in substaining the European project: HORIZON-CL6-2022-FARM2FORK-02-two-stage (Fair, healthy and environmentally-friendly food systems from primary production to consumption) (Project approved and in the financing phase). Action: HORIZON-RIA. Proposal number: 101084084-2. Acronym: AGROSUS. Title: Agroecological strategies for sustainable weed management in key European crops. Coordinat: Adela Sanchez Moreira (University of Vigo, Spain). UNIMI Scientific responible: Fabrizio Araniti. Total founding: 4.999.863,75 €.

7. Literature:

[1] Triantafyllidis, V., Mavroeidis, A., Kosma, C., Karabagias, I. K., Zotos, A., Kehayias, G., ... & Kakabouki, I. (2023). Herbicide Use in the Era of Farm to Fork: Strengths, Weaknesses, and Future Implications. Water, Air, & Soil Pollution, 234(2), 94.

[2] Qu, R. Y., He, B., Yang, J. F., Lin, H. Y., Yang, W. C., Wu, Q. Y., ... & Yang, G. F. (2021). Where are the new herbicides?. Pest Management Science, 77(6), 2620-2625.

[3] Duke, S. O., & Dayan, F. E. (2022). The search for new herbicide mechanisms of action: Is there a ‘holy grail’?. Pest Management Science, 78(4), 1303-1313.

[4] Motmainna, M., Juraimi, A. S., Uddin, M. K., Asib, N. B., Islam, A. K. M. M., & Hasan, M. (2021). Assessment of allelopathic compounds to develop new natural herbicides: A review. Allelopath. J, 52, 21-40.

[5] Gniazdowska, A., & Bogatek, R. (2005). Allelopathic interactions between plants. Multi site action of allelochemicals. Acta Physiologiae Plantarum, 27, 395-407.