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Herbicide Resistances and Epigenetic Mechanisms

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Ai miei genitori
Francesco e Maria Grazia,
ai miei nonni
Mario, Carla,
Mario e Giovanna,
alla mia compagna Chiara,
a chi ha creduto in me
e a tutte le persone a me care

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Preface

This thesis results from my PhD in Earth and Environmental Sciences - Cycle XXXV. The thematic PhD scholarship was funded on the EPIRESISTENZE research project, funded by Lombardy Region, D.G. Agriculture, food and green systems, call for funding for research projects in the agricultural and forestry sector.

The experiments were carried out mainly at the Laboratory of Plant Biodiversity and Biosystematics of the University of Pavia (Botanical Garden), partly at the Laboratory of Immunology and Genetic Analysis of the University of Pavia, partly at the Lombardy Region Plant Protection Service Laboratory in Vertemate con Minoprio (CO), partly at the Laboratory of Biology and Pneumology Unit - c/o Policlinico San Matteo in Pavia. Growth trials were conducted at Agricola 2000 S.c.p.A. in Tribiano (MI). Soil chemical and physical analysis were conducted at MAC – Minoprio Analisi e Cartificazioni Laboratory in Vertemate con Minoprio (CO).

All phases of the project were supervised by Prof. Maura Brusoni, Prof. Enrica Capelli and prof. Anna Maria Picco of the Department of Earth and Environmental Sciences of the University of Pavia. Some experimental activities were carried out in collaboration with Dr. Beniamino Cavagna, Dr. Francesca Gaffuri, and the team of the Lombardy Region Plant Protection Service Laboratory, others with Dr. Marta Guarise and the team of Agricola 2000 S.c.p.A.

This PhD research has deepened my knowledge about the complexity of biological mechanisms underlying plant adaptation to environmental stressors. Specifically, it allowed me to learn a lot about herbicide resistances, widening and improving my skills on analytical techniques to study genetics and epigenetics basis of this complex topic. Moreover, this PhD experience has also allowed me to focus on plants systematic, plants genetic variability, microbiology and statistics.

The collaborations with the Laboratory of Immunology and Genetic Analysis, the Laboratory of Micology and Lombardy Region Plant Protection Service Laboratory have

undoubtedly been fundamental experiences for my professional growth as a researcher, allowing me to experience at first hand the work in laboratory, interacting with professionals.

Abstract

Herbicide resistance (HeR) is a major threat to worldwide agricultural systems and is an example of the adaptive evolution of weeds in response to human selective pressures. This has resulted in the evolution of global resistance to a wide range of herbicides in many weed species. To date, chemical control has represented the most efficient tool for managing weeds.

Regulation (EC) No. 1107/2009 *concerning the placing of plant protection products on the market*, which established rules for the authorization for sale, use and control of plant protection products in the European Union (Union), recognizing the precautionary principle, has limited the number of plant protection products available on the market. This has led to the repeated use of a narrower range of plant protection products with the same mechanism of action, a fact that has favored the development of herbicide-resistant populations, leading to severe yield losses worldwide, estimated at around 35 % in rice crops.

Generally, weed resistance to herbicides is linked to two main mechanisms: Target Site Resistance (TSR), which involves a DNA mutation on genes expressing herbicide target proteins, and Non-Target Site Resistance (NTSR), involving metabolic processes of detoxification. Besides, weeds will often exhibit resistance to more than one herbicide, which may or may not be in related chemical families. We recognize *cross-resistance* as the ability to withstand herbicides from different chemical families due to a genetic mutation and/or metabolic mechanism. We also recognize *multiple-resistance*, as the ability to withstand herbicides from different chemical families due to one or more mechanisms. Recent studies highlight how herbicide resistance occurrence might also be influenced by epigenetic processes (i.e. DNA methylation, miRNA expression). In fact, epigenetic adaptation is induced by environmental stressors, which act as trigger of organisms response. If we consider that epigenetic mechanisms might be triggered by herbicides as stressors, we could hypothesize that plants response towards chemical control is the result of a complex physiological pathway. In addition, biotic and abiotic factors could play an

important role in the development of herbicide resistance since they stimulate epigenetic mechanisms that in turn influence the expression of genes involved in herbicide resistance occurrence.

Chapter 1 presents information about the productive reality of rice cropping in Italy, provides a description of the paddy field's environment of the Lombardy region and reports a detailed depiction of current European and Italian legislation related to the sustainable use of plant protection products (PPP). Further, herbicide classification methods and a detailed description of currently known genetic and physiological mechanisms leading to herbicide resistance occurrence are reported. *Echinochloa* P. Beauv. species are characterized, focusing on the hazard they represent for Italian rice cropping. Finally, the aims of the project are explained.

Chapter 2 is dedicated to field work and describes the preliminary survey conducted through the questionnaire and the sampling phase, with the collection of resistant *Echinochloa* spp. specimens and of paddy soils. Results of resistant *Echinochloa* specimens geo-localization within the rice farming territory in the Lombardy region, have been presented to the XI International Agriculture Symposium "AGROSYM 2022" (online conference) held in October 2020. Herbicide resistance appeared to be spread throughout the whole rice-cropping area of the Lombardy region.

Chapter 3 is devoted to the floristic and vegetation analysis of weeds phytocoenoses present in all the visited paddies. Plants species surveyed belong to the alliance *Oryzo sativae-Echinochloion oryzoidis* O. Bolòs & Masclans 1955, typical of paddies. The abundance of therophytes and ruderal species is indicator of a high disturbed environment.

In Chapter 4 are described the analysis of chemical/physical characters of paddy soils. Soils with a silt/clay texture, richer in macronutrients and soils with a coarse/sandy texture, poorer in macronutrients were identified.

Chapter 5 describes the paddy soils microbiota. The presence of fungal and bacterial strains was analyzed by two methodologies: the conventional plate culture and the

metagenomic analysis. Through conventional plate culture method, *Trichoderma* and *Penicillium* were recorded as the mainly present fungal strains in surveyed soils. These fungi are able to establish relationships with plants at the rhizoplane level. They are called *rhizo-competent* fungi, as they influence plant health and growth by limiting the actions of pathogens. Through metagenomic analysis, many bacterial and fungal orders were identified. Among them, *Methanosarcinales (Archaea), Actinomycetales (Bacteria)* and *Malasseziales (Fungi)* are the most abundant and peculiar of a highly disturbed environment.

Chapter 6 focuses on the identification of *Echinochloa* spp. specimens collected. Two different methods were employed: morphometric approach allowed to discriminate a large part of the whole collection of *Echinochloa* spp. specimens. Samples of doubt identification were analyzed by PCR and RFLP to be univocally classified. 84 specimens were classified as *E. oryzicola* and 72 as *E. crus-galli*.

Chapter 7 is devoted to the analysis of *E. crus-galli* and *E. oryzicola* intraspecific variability by means of microsatellites markers (SSR). An improvement of the whole analytical methodology was performed, in order to obtain better microsatellites fingerprints on agarose gel and to analyze these molecular markers as codominant data. Codominant data analysis in those allopolyploid species was carried out through R 3.6.3 (packages *pegas* and *StAMPP*). As concerns *E. crus-galli*, a higher genetic variability was surveyed in resistant population in comparison to susceptible ones. Results obtained from barnyardgrass were published on Diversity Journal (MDPI). As concern *E. oryzicola*, no difference in genetic variability was assessed between resistant and susceptible biotypes. In general, a high degree of intraspecific variability was assessed in each species.

Chapter 8 is divided in 4 sections.

In the first, the screening of TSR in the whole *Echinochloa* spp. specimens collection was described. The presence of mutated specimens was detected by means of the RFLP markers. About 13% of the samples possess mutations conferring resistance to ASL and ACCase inhibitors herbicides. Among these samples, some were detected as heterozygous (possess a wild type and a mutated allele variants).

The second and third sections are devoted to the analysis of epigenetic mechanisms acting in the regulation of herbicide resistance in *Echinochloa* spp.. Only wild type specimens were considered. In order to maintain controlled experimental conditions and to exclude biotic and abiotic factor of stress that could affect epigenetic adaptive responses in tested plants, with the exception of the herbicide stress only, controlled growth and herbicide application tests were performed.

In the second section, the expression of genes involved in herbicide detoxification and of miRNAs targeting these genes and regulating the translation of their mRNAs were analyzed. In general, miRNAs and genes showed two different trends: when herbicide does not stimulate miRNAs transcription, the target genes could be expressed, leading to herbicide detoxification, hence resistance; otherwise, when herbicide triggers miRNAs transcription, they down-regulate the expression of target genes reducing their detoxification ability. Results obtained from the analysis of the expression of miRNAs targeting genes involved in bispyribac-Na detoxification in resistant *E. crus-galli* specimens were published on Plants Journal (MDPI).

In the third section, the level of DNA methylation was analyzed. Findings revealed that DNA methylation is triggered by herbicide administration and that methylation level increases more in susceptible biotypes in comparison to resistant ones, inhibiting the expression of genes involved in herbicide detoxification.

In the fourth section, the incidence of *epiresistances* (resistances regulated by epigenetic mechanisms) is analyzed in relation to edaphic abiotic factors, soils microbial diversity and meteorological conditions. A greater incidence of *epiresistances* was surveyed in paddies characterized by lower microbial soil diversity and where higher air temperature, air humidity and rainfalls volume were recorded.

Chapter 9 reports final considerations and future perspectives of those analysis.

Riassunto

La resistenza agli erbicidi (HeR) rappresenta una grave minaccia per i sistemi agricoli di tutto il mondo ed è un esempio dell'evoluzione adattativa delle infestanti in risposta alle pressioni selettive dovute all'azione dell'uomo. Ciò ha portato all'evoluzione della resistenza verso una vasta gamma di erbicidi in molte specie di erbe infestanti. Ad oggi, il controllo chimico rappresenta lo strumento più efficiente per la gestione delle infestanti.

Il Regolamento (CE) n. 1107/2009 — *Immissione dei prodotti fitosanitari sul mercato dell'Unione europea*, che stabilisce le norme per l'autorizzazione alla vendita, all'uso e al controllo dei prodotti fitosanitari stessi, riconoscendo il principio di precauzione, ha limitato il numero di quelli disponibili sul mercato. Ciò ha portato ad un utilizzo reiterato di una minore gamma di prodotti fitosanitari con medesimo meccanismo di azione, fatto che ha favorito lo sviluppo di popolazioni resistenti agli erbicidi, causando a gravi perdite di rendimento a livello mondiale, stimate intorno al 35% nelle colture di riso.

Generalmente, si riconoscono due tipologie di resistenze agli erbicidi: la resistenza target site (TSR), causata da una mutazione *missense* del DNA in geni che esprimono proteine bersaglio degli erbicidi, e la resistenza non-target site (NTSR), che coinvolge i processi metabolici di disintossicazione dagli erbicidi. Spesso, le infestanti mostrano resistenza verso numerosi erbicidi. Riconosciamo sia casi di resistenza incrociata (*cross-resistance*), ossia resistenza a erbicidi che inducono il medesimo tipo di alterazione fisiologica nella pianta, sia casi di resistenza multipla (*multiple-resistance*), ossia resistenza a erbicidi che inducono differenti tipi di alterazione fisiologica nella pianta. Studi recenti evidenziano la resistenza agli erbicidi potrebbe anche essere influenzata da meccanismi epigenetici (ad esempio metilazione del DNA, espressione di miRNA). Di fatto, l'adattamento epigenetico è indotto da fattori di stress ambientali, che innescano risposte adattative degli organismi. Se consideriamo che i meccanismi epigenetici potrebbero essere innescati dallo stress rappresentato dagli erbicidi, si può ipotizzare che la risposta delle

piante al controllo chimico sia il risultato di un complesso pathway fisiologico. Inoltre, i fattori biotici e abiotici potrebbero rivestire un ruolo importante nello sviluppo della resistenza agli erbicidi dal momento che stimolano i meccanismi epigenetici che, a loro volta, influenzano l'espressione dei geni coinvolti nella resistenza agli erbicidi.

Il capitolo 1 riporta informazioni sulla realtà produttiva della coltivazione del riso in Italia, fornisce una descrizione dell'ambiente della risaia della regione Lombardia e una rappresentazione dettagliata della attuale legislazione europea e italiana relativa all'uso sostenibile dei prodotti fitosanitari. Inoltre, vengono qui riportati i metodi di classificazione degli erbicidi e una descrizione dettagliata dei meccanismi genetici e fisiologici attualmente noti legati alla manifestazione di resistenza agli erbicidi. Vengono descritte le specie del genere *Echinochloa* P. Beauv., concentrandosi sul pericolo che esse rappresentano per la coltivazione del riso in Italia. Infine, vengono spiegati gli obiettivi del progetto di dottorato.

Il capitolo 2 è dedicato al lavoro di campo. Descrive l'indagine preliminare condotta attraverso il questionario e la fase di campionamento, con la raccolta dei campioni resistenti *di Echinochloa* spp. e dei terreni di risaia. I risultati ottenuti della geo localizzazione degli esemplari resistenti di *Echinochloa* nel territorio a vocazione risicola della regione Lombardia sono stati presentati a *XI International Agriculture Symposium "AGROSYM 2022"* tenutosi nell'ottobre 2020. La resistenza agli erbicidi sembra essere diffusa in tutta l'area di coltivazione del riso della regione Lombardia.

Il capitolo 3 è dedicato all'analisi floristica e vegetazionale delle fitocenosi infestanti presenti in tutte le risaie visitate. Le specie vegetali esaminate appartengono all'alleanza *Oryzo sativae-Echinochloion oryzoidis* O. Bolòs & Masclans 1955, tipica delle risaie. L'abbondanza di terofite e specie ruderali è indicatrice di un ambiente altamente disturbato.

Nel capitolo 4 viene descritta l'analisi dei caratteri chimico/fisici dei terreni delle risaie. Sono stati identificati terreni con tessitura limosa/argillosa, più ricchi di macronutrienti e terreni a tessitura ghiaiosa/sabbiosa, più poveri di macronutrienti.

Il capitolo 5 descrive il microbiota dei terreni di risaia. La presenza di ceppi fungini e batterici è stata analizzata con due metodologie: quella colturale e quella

metagenomica. Attraverso il metodo colturale su piastra, *Trichoderma* e *Penicillium* sono stati registrati come i ceppi fungini principalmente presenti nei terreni esaminati. Questi funghi sono in grado di stabilire relazioni con le piante a livello del rizoplano. Sono chiamati funghi *rizo-competenti*, in quanto influenzano la salute e la crescita delle piante limitando le azioni dei patogeni. Attraverso l'approccio metagenomico, sono stati identificati molti ordini batterici e fungini. Tra questi, *Methanosarcinales (Archaea), Actinomycetales* (*Batteri*) e *Malasseziales (Funghi)* sono i più abbondanti e tipici di un ambiente altamente disturbato.

Il capitolo 6 si concentra sull'identificazione degli esemplari di *Echinochloa* spp. raccolti. Sono stati impiegati due diversi metodi: l'approccio morfometrico ha permesso di discriminare gran parte dell'intera collezione di campioni di *Echinochloa* spp. I campioni di dubbia identificazione sono stati analizzati mediante PCR e RFLP, per poter essere classificati in modo univoco e certo. 84 esemplari sono stati classificati come E. *oryzicola* e 72 come *E. crus-galli.*

Il capitolo 7 è dedicato all'analisi della variabilità intraspecifica in *E. crus-galli* e *E. oryzicola* mediante l'utilizzo di marcatori microsatelliti (SSR). È stato effettuata una ottimizzazione dell'intera metodologia analitica, al fine di ottenere migliori tracciati elettroforetici dei microsatelliti su gel di agarosio e di analizzare questi marcatori molecolari come dati codominanti. L'analisi dei dati codominanti in queste specie allopoliploidi è stata effettuata attraverso il software R 3.6.3 (packages *pegas* e *StAMPP*). Per quanto riguarda *E. crus-galli*, è stata rilevata una maggiore variabilità genetica nelle popolazioni resistenti rispetto a quelle sensibili. I risultati ottenuti da questo studio sono stati pubblicati sulla rivista Diversity (MDPI). Per quanto riguarda *E. oryzicola*, nessuna differenza nella variabilità genetica è stata valutata tra biotipi resistenti e sensibili. In generale, è stato valutato un alto grado di variabilità intraspecifica in ciascuna delle due specie.

Il capitolo 8 si articola in 4 sezioni.

Nella prima, viene descritto lo screening delle resistenze TSR sull'intera collezione di campioni di *Echinochloa* spp. La presenza di campioni mutanti è stata rilevata mediante i marcatori RFLP. Circa il 13% dei campioni possiede mutazioni che conferiscono

resistenza agli erbicidi inibitori dell'ASL e dell'ACCasi. Tra questi campioni, alcuni sono stati rilevati come eterozigoti (possiedono una variante allelica mutata e una wild type).

La seconda e la terza sezione sono dedicate all'analisi dei meccanismi epigenetici che agiscono nella regolazione della resistenza agli erbicidi in *Echinochloa* spp.. Sono stati considerati solo esemplari wild type. Al fine di mantenere condizioni sperimentali controllate ed escludere fattori ecologici biotici e abiotici di stress che potevano influenzare le risposte adattative epigenetiche nelle piante testate, ad eccezione del solo stress erbicida, sono stati eseguiti test di crescita controllata e di applicazione degli erbicidi.

Nella seconda sezione, è stata analizzata l'espressione dei geni coinvolti nella disintossicazione da erbicidi e dei miRNA che si appaiano e legano i mRNA di questi geni, regolandone l'espressione. In generale, i miRNA e i geni analizzati hanno mostrato due diversi comportamenti: quando l'erbicida non stimola la trascrizione dei miRNA, i geni target possono essere espressi, portando alla disintossicazione degli erbicidi, quindi alla resistenza; alternativamente, quando l'erbicida stimola la trascrizione dei miRNA, questi down-regolano l'espressione dei geni bersaglio, riducendo la loro capacità di disintossicazione. I risultati ottenuti dall'analisi in *E. crus-galli* dell'espressione di geni coinvolti nella disintossicazione da bispyribac-Na dei loro rispettivi miRNA regolatori sono stati pubblicati su Plants Journal (MDPI).

Nella terza sezione, è stato analizzato il livello di metilazione del DNA. I risultati hanno rivelato che la metilazione del DNA è innescata dalla somministrazione di erbicidi e che il livello di metilazione aumenta maggiormente nei biotipi sensibili rispetto a quelli resistenti, inibendo l'espressione dei geni coinvolti nella disintossicazione degli erbicidi.

Nella quarta sezione, viene analizzata l'incidenza delle *epiresistenze* (resistenze regolate da meccanismi epigenetici) in relazione a fattori abiotici edafici, diversità microbica dei suoli e condizioni meteorologiche. Una maggiore incidenza di *epiresistenze* è stata riscontrata nelle risaie caratterizzate da una minore diversità microbica del suolo e dove sono stati registrati temperatura dell'aria, umidità dell'aria e volume delle precipitazioni più elevati.

Il capitolo 9 riporta le considerazioni finali e le prospettive future di questo lavoro.

Chapter 1 – Introduction and aims of the project

This chapter presents information about the productive reality of rice cropping in Italy, mainly focusing on the regions in which is more extended (Piedmont and Lombardy). Furthermore, it provides a description of the paddy field's environment and the most critical and vulnerable ecosystems present in the rice-growing territory of the Lombardy region. Then, it reports a detailed depiction of current European and Italian legislation related to the use of phytosanitary products. Further, herbicide classification methods are reported, together with a detailed description of currently known genetic and physiological mechanisms leading to herbicide resistance occurrence. Weed species belonging to *Echinochloa* P. Beauv. genus, the case study in this PhD thesis, are characterized, focusing on the hazard they represent for Italian rice cropping. Finally, the aims of the project are explained.

1.1 Rice cultivation in Italy – production reality

In Europe, Italy is the main producer of rice (*Oryza sativa* L.). More than 50 % of the European area devoted to this crop is located in Italy. In the 2022 crop year, rice cultivation covered an area of about 205.605 ha, mainly located within the Lombardy and Piedmont provinces (ENR[1], 2022). Italian areas cultivated during 2022 are shown in the Table 1.1 below, listed by Regions and Provinces:

Table 1.1: rice cultivated areas in the provinces of Lombardy and Piedmont

Region	Province	ha	% on total
Lombardy	Mantova	957.86	1.03%
	Cremona	--	0.00%
	Lodi	2014.90	2.17%
	Milano	12144.61	13.07%
	Pavia	77782.57	83.72%
	Total	**92148.00**	**100 %**
Piedmont	Alessandria	7574.96	6.67%
	Biella	3882.93	3.42%
	Cuneo	131.96	0.11%
	Torino	341.42	0.30%
	Vercelli	70151.13	61.83%
	Novara	31373.69	27.65%
	Total	**113456.09**	**100%**

These data show the relevance of rice cultivation in northern Italy (Lombardy and Piedmont regions).

In particular, in Lombardy, the area cultivated with rice increased in 2015/16 compared to previous years, with a growth of 5.4%. Currently 92.148 ha are cultivated with

[1] ENR: Ente Nazionale Risi - https://www.enterisi.it/servizi/notizie/notizie_homepage.aspx

rice confirming the positive trend of the last period. Furthermore, there has been a gradual replacement of the areas invested in *Indica* varieties, more exposed to international price dynamics and growing international trade pressures, in favor of *Japonica* ones, more sensitive to global market dynamics (ISTAT, 2019).

The contribution of rice cultivation to the PPB (Production at Basic Prices) of cereals at the regional level stood at 22% in 2016. About 40% of Italian rice production satisfies a large part of domestic consumption, while the remainder is destined for the European market, helping to limit imports of the grain from third countries (ISTAT, 2019). In this framework, Italian rice farming is now facing important challenges, mainly related to the competition with rice systems from Southeast Asian countries, characterized by lower production costs. Furthermore, it has to deal with the increased demand of global market with regard to the quality and health characteristics of food production and the huge innovative products request.

Specifically, in 2016, in an area of about 230.000 hectares, 1.593.465 tons of rice were harvested with an agronomic yield of 6.81t/ha, recording a production increase of +5.8% compared to 2015. The amount of net milled production was about 1 million tons. To sum up, the two-year period 2015/16 showed an increase in exports of about 15% to third countries and an increase of 2% to the EU market. Of particular significance there are also the agreements that the European Commission is negotiating for free trade with India, Thailand, Japan and the Mercosur economic area. In essence, rice farming represents a sector of great importance for Italian agriculture, in particular for the Lombardy region (ENR, 2022).

1.2 Rice cultivation in Lombardy – territory and environmental characterization

Lombardy is the second Italian rice producer region (ENR 2022). Its cultivation is mainly distributed in the provinces of Pavia and Milano (Figure 1.1) (ERSAF 2019 – SIARL/SISCO 2019 – DUSAF 2019).

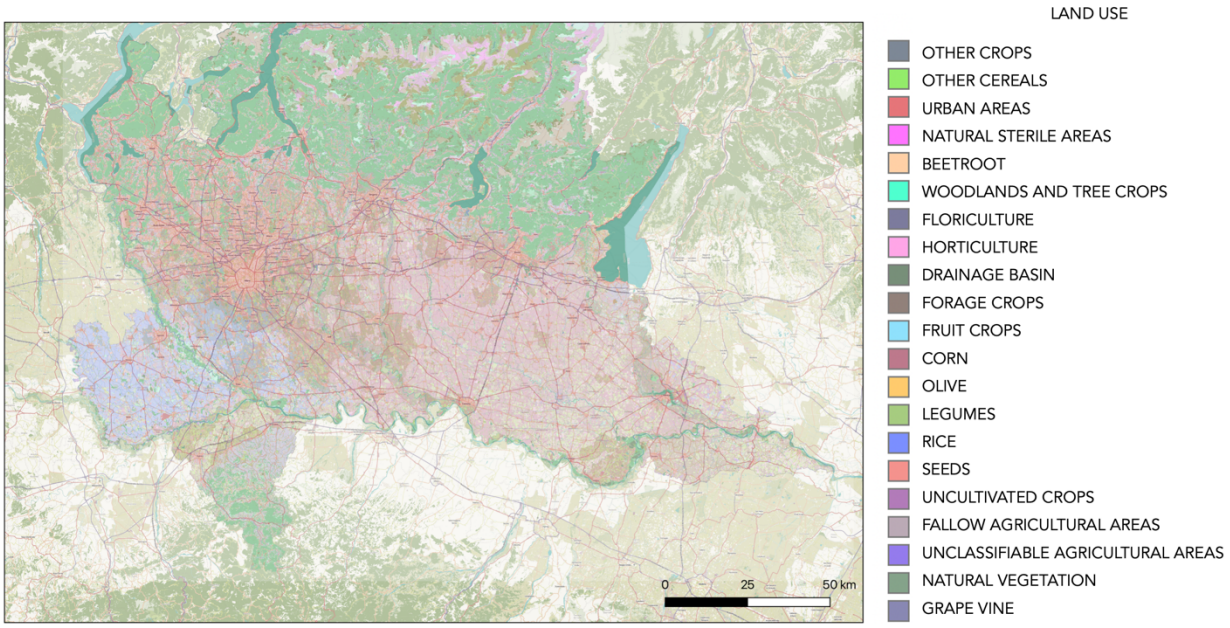

Figure 1.1: Lombardy Region soil data map (ERSAF 2019 – SIARL/SISCO 2019 – DUSAF 2019)

The submerged rice field represents a peculiar ecosystem in which weed phytocoenoses possess equally peculiar characteristics. The weed flora of rice paddies is characteristic of marshy or otherwise water-saturated habitats and can be attributed to the alliance *Oryzo sativae-Echinochloion oryzoidis* O. Bolòs & Masclans 1955. It is represented by weed coenoses, mostly annuals, that infest fields of rice (*O. sativa*). These are generally species of tropical origin, accidentally introduced by humans, that periodically appear and then spread in areas where rice is grown (Figure 1.2).

Figure 1.2: particular of submerged paddy in the municipality of Carbonara Al Ticino (PV).

Ecologically, they prefer water-soaked soils and high temperatures. For this reason, the life cycle of these species is rather short and confined to the summer months only. These are coenoses consisting mostly of therophytic species. Abundant and frequent species are: *Leersia oryzoides* (L.) Sw., *Echinochloa* P. Beauv. spp., *Cyperus difformis* L., *Panicum dichotomiflorum* Michx., *Heteranthera reniformis* Ruiz et Pav., *Schoenoplectus mucronatus* (L.) Palla, *Alisma plantago-aquatica* L., *Oryza sativa* L. var. *sylvatica* Chiappelli. Thus, numerous weed species develop in the rice field and differ according to the nature of the soil, quality of irrigation water, cultivation techniques, and crop rotation. They are all characterized by rapid development: in fact, they often overpower rice in height and have a strong growth capacity. Average presences of 6-7 plants/ m^2 are enough to reduce rice production by as much as 70 to 80 % (Smith, 1988).

Considering the highly vulnerable environment in which rice cultivation is performed, its production also assumes a very high relevance in the characterization of the landscape. As a matter of facts, the submersion cultivation system of rice deals an important relation with the ecosystem. In the Lombardy region the EU recognized the environmental

relevance of rice cropping and designated a series of Special Protected Areas (SPAs) to preserve vulnerable animal and plant species or wetlands of international importance for migratory waterfowl. The most extended Special Protection Areas (SPAs) within the rice farming territory are *Risaie Della Lomellina* (SPA IT2080501) and *Boschi del Ticino* (SPA IT2080301) (Figure 1.3).

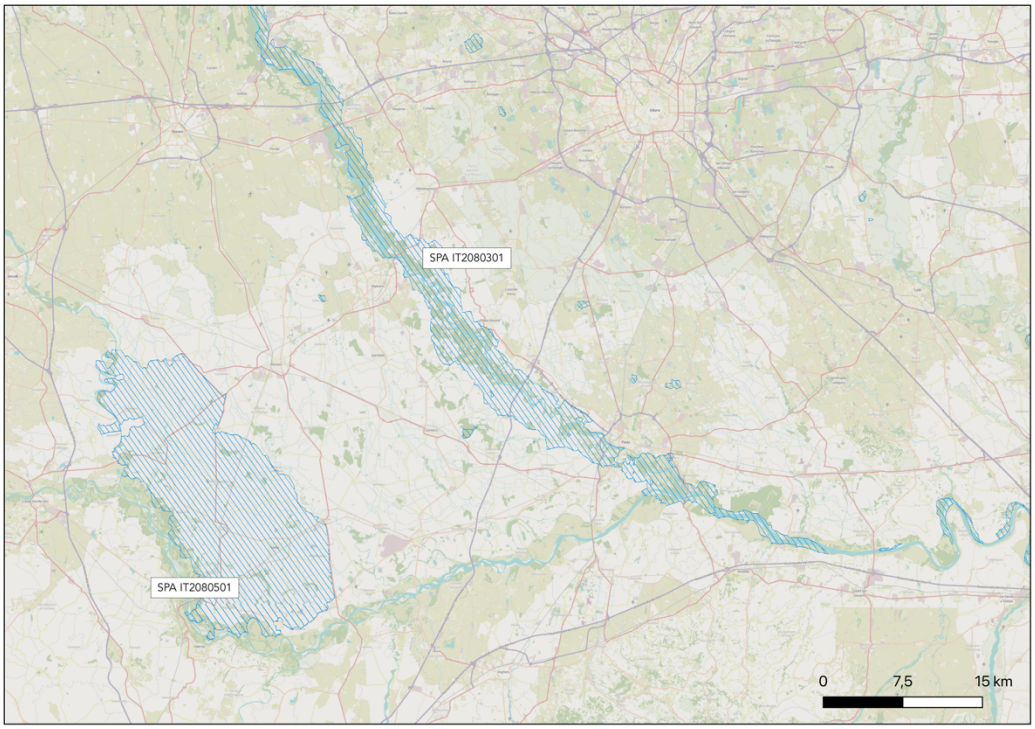

Figure 1.3: map of the Special Protected Areas (SPAs) Boschi del Ticino and of SPA Risaie della Lomellina. SPAs territory is marked in light blue texture (ERSAF 2019 – SIARL/SISCO 2019 – DUSAF 2019)

Water balance is also related with paddy field environmental characteristics, as the considerable volumes of water used for cultivation go largely to feed the aquifers and resurgences of the plain.

Furthermore, soil represents an important component of the agricultural ecosystem, characterized by microorganism communities biodiversity that play a key role in the degradation of organic matter and the recycling and decomposition of nitrogen and other nutrients. Any disturbance of the environment can result in modifications in the activity of microbiota and, consequently affect soil fertility. Therefore, in this context, cultivation methods assume great importance, and among them the use of plant protection

products (PPPs), which can increase environmental risks and affect the public's opinion of the entire sector.

1.3 European legislation on plant protection products

Usage of plant protection products is regulated both at international (European[2]) and national (Italian[3]) level.

On July 15th, 1991, the European Parliament and the Council enacted the **Directive 91/414/EEC** concerning the placing of plant protection products on the market. This Directive concerns the authorization, placing on the market, use and control within the European Community of plant protection products in commercial form and of active substances used to protect plants or plant products against harmful organisms. Member States shall prescribe that plant protection products may not be placed on the market and used in their territory unless they have authorized the product in accordance with this Directive and shall ensure that a plant protection product is not authorized unless its active substances are listed in Annex I and any conditions laid down therein are fulfilled.

On December 16th, 2008, the European Parliament and the Council enacted the **Regulation (EC) No 1272/2008** on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. This Regulation harmonizes the criteria for classification of substances and mixtures, and the rules on labelling and packaging for hazardous substances and mixtures. It also aims at establishing a classification and labelling inventory of substances.

On October 21th, 2009, the European Parliament and the Council enacted the **Regulation (EC) No 1107/2009,** repealing Council Directives 79/117/EEC and 91/414/EEC. This Regulation provided rules for the authorization of plant protection products in

[2] https://eur-lex.europa.eu/homepage.html

[3] https://www.salute.gov.it/portale/home.html

commercial form and for their placing on the market, use and control within the European Union. It also set out rules for the approval of active substances, adjuvants and co-formulants, safeners and synergists, which plant protection products contain or consist of. The purpose of these provisions is to ensure a high level of protection of both human and animal health and the environment and to improve the functioning of the internal market through the harmonization of the rules on the placing on the market of plant protection products, while improving agricultural production. The Directive recalls the precautionary principle in order to ensure that active substances or products placed on the market do not adversely affect human or animal health or the environment. In particular, Member States shall not be prevented from applying the precautionary principle where there is scientific uncertainty as to the risks with regard to human or animal health or the environment posed by the plant protection products to be authorized in their territory.

In Chapter I, subsection III, Article 18 - *Work programme* is highlighted the problem of herbicide resistance management of target pests in a global vision of environmental sustainability and human health. Chapter II lays down detailed requirements and conditions for approval of active substances, safeners, synergists and co-formulants. Chapter III is devoted to plant protection products (requirements and content, authorization for use), whereas Chapter IV concerns adjuvants. Plant protection products shall be used properly. Proper use shall include the application of the principles of good plant protection practice and compliance with prescribed conditions. It shall also comply with the provisions of Directive 2009/128/EC and, in particular, with general principles of integrated pest management. Chapter IX on emergency measures lays down the regulatory procedure to be taken immediately where it is clear that an approved active substance, safener, synergist or co-formulant or a plant protection product which has been authorized in accordance with this Regulation is likely to constitute a serious risk to human or animal health or the environment, and that such risk cannot be contained satisfactorily by means of measures taken by the Member State concerned.

On October 21th, 2009, the European Parliament and the Council also enacted the **Directive (EC) 128/2009**. This Directive establishes a framework to achieve a sustainable use

of pesticides by reducing the risks and impacts of pesticide use on human health and the environment and promoting the use of integrated pest management and of alternative approaches or techniques such as non-chemical alternatives to pesticides. The Directive applies to pesticides that are plant protection products as defined in Regulation (EC) No. 1107/2009.

1.4 Italian legislation on plant protection products

In Italy, plant protection products must be authorized by the Ministry of Health[4] in order to be placed on the market and used, for example, in the agricultural sector, in accordance with the provisions laid down in Regulation (EC) No. 1107 of 21/10/2009.

On August 14th, 2012, Directive 2009/128/EC was transposed by **Decreto Legislativo n. 150** - *Attuazione della Direttiva 2009/128/CE che istituisce un quadro per l'azione comunitaria ai fini dell'utilizzo sostenibile dei pesticidi (Decree No. 150 – Directive 2009/128EC implementation)* which established a framework for Community action to achieve the sustainable use of pesticides for the adoption of the National Action Plan (NAP) for the sustainable use of plant protection products.

On January 22th, 2014, Italy adopted the NAP through the **Decreto Interministeriale** - *Adozione del Piano d'Azione Nazionale per l'uso sostenibile dei prodotti fitosanitari, ai sensi dell'articolo 6 del decreto legislativo del 14 Agosto 2012, n° 150 (Interministerial Decree - Adoption of the National Action Plan for the Sustainable Use of Plant Protection Products, following article 6 of Legislative Decree n° 150, August 14th 2012),* periodically updated in accordance with Legislative Decree No. 150. On January 22th, 2014, the final text of the NAP was adopted by Decree of the MIPAAF (Ministero delle Politiche Agricole Alimentari e Forestali - Ministry of Agriculture Food and Forestry). The general objectives that the NAP aims to achieve are: 1) to reduce the risks and impacts of plant protection products on human health and the environment; 2) to promote the application of integrated pest

[4] https://www.salute.gov.it/portale/home.html

management, organic farming and other alternative approaches; 3) to protect users of plant protection products; 4) to protect consumers; 5) to safeguard the aquatic environment and drinking water; 6) to conserve biodiversity and protect ecosystems.

On March 6th, 2015, the Council of Lombardy Region approved the first edition of *Linee guida per l'applicazione in Lombardia del piano di azione nazionale (PAN) per l'uso sostenibile dei prodotti fitosanitari* - D.g.r. No. X/3233 (*Guidelines for the implementation in Lombardy of the National Action Plan for the Sustainable Use of Plant Protection Products*).

The Regional guidelines were subsequently implemented and updated with the issuance of the D.g.r. No. 1376/2019 and D.g.r. No. 5836/2021

1.5 Herbicide classification

Herbicides can be classified in several ways. One is based on when they are applied, that is, in pre-emergence or post-emergence of the crop. Herbicides applied in pre-emergence can be effective against grasses or broadleaves, while those applied in post-emergence may be selective (specific target) or non-selective (broad target). Another way to classify herbicides is based on their mode of action (MoA), that is defined as the type of physiological (biochemical or biophysical) alteration through which the herbicide causes its phytotoxic action on the plant to which it is applied (GIRE 2022). Modes of action are: 1) Lipid biosynthesis inhibitors; 2) Amino acid biosynthesis inhibitors; 3) Plant growth regulators; 4) Photosynthesis inhibitors; 5) Nitrogen-metabolism inhibitors; 6) Pigment inhibitors; 7) Cell-membrane disruptors; 8) Seedling-growth inhibitors. In 2022, HRAC[5] (Herbicide Resistance Action Committee), an international organization administered by CropLife International and operated by members of the agrochemical industry which supports global efforts in the fight against herbicide resistant weeds, published an updated version of a conceptual map for the classification of herbicides. Three main groups are

[5] HRAC website: https://www.hracglobal.com/

defined: herbicides acting on light activation of reactive oxygen species (ROS), cellular metabolism and cellular division and growth (Figure 1.4).

It is important to consider that herbicides belonging to a specific group have the same mode of action even though they may belong to a different chemical family. For example, pyrimidinyl benzoates, sulfonylurea and imidazolinone are different molecules of Acetolactate Synthase inhibitor herbicides. The MoA of herbicides is crucial to understand the management, classification, organization, and hierarchy of the herbicides. It also provides an insight into herbicide resistance, which continues to be a problem for a sustainable agricultural management. The excessive and inaccurate use of herbicides has led to increased development of resistance among weeds, causing injury and destruction of useful plants in agriculture and landscape. Managing the resistance onset will be a great challenge to deal with in order to obtain either a sustainable and an efficient cropping.

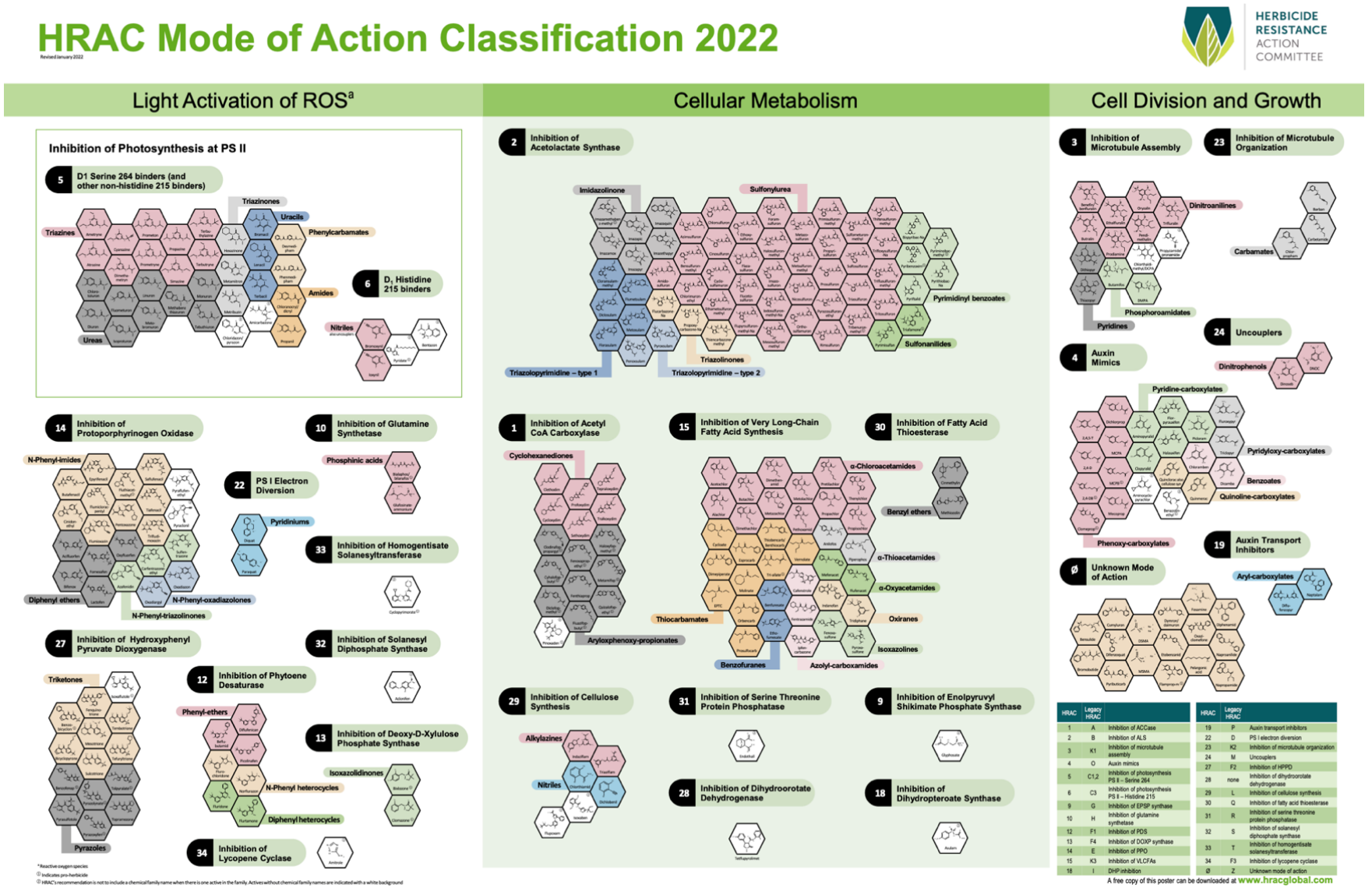

Figure 1.4: HRAC mode of action 2022 classification

1.6 Herbicide resistance

Herbicide resistance is defined as the natural and heritable ability of some individuals in a weed population to survive the dose of herbicide normally used to control them. In all weed populations there are a limited number of plants that can survive herbicide treatment. It's rare that a weed population is resistant only to the selecting herbicide. It is often also resistant to other herbicides having the same mechanism of action (MoA), with which, however, it has never been in contact. This phenomenon is called *cross-resistance*. In more severe and complex cases to manage, a resistant population is able of surviving simultaneously to herbicides having different mechanism of action, This latter case is defined as *multiple resistance* (GIRE 2022[6]).

Weed management is one of the most critical aspects in agriculture. Each year are estimated considerable worldwide yield losses due to the negative influence that weeds are able to exert on the crop's production, in terms of quantity and quality. Chemical control in the form of herbicides has so far represented the most effective tool for managing weeds. However, as a result of strict European regulations concerning the placing of plant protection products on the EU market (Reg EC/1107/2009), the repeated use of an increasingly narrow range of herbicides, that target the same metabolic pathway, has selected for herbicide resistant populations (Deyle et al., 2013; Mascanzoni et al., 2018; Cusaro et al., 2022a). Thus, resistance is an evolutionary phenomenon, forcing the usage of several herbicides or control methods that may be more expensive and/or less effective. Moreover resistance can persist for several years, even in the absence of the herbicide selector, due to the seed stock (or seed bank) in the soil (GIRE 2022). In addition, artificial selection of agronomic traits in rice (*Oryza sativa* L.) which are useful to humans has unintentionally promoted the evolution of crop-like weed biotypes. As a result, the weeds can evade chemical control and eradication from fields, allowing them to spread throughout the agroecosystem (*Vavilovian mimicry*) (McElroy et al., 2014; Ye et al., 2019). The annual worldwide cost of crop losses caused by weed infestation of crops is estimated around 32

[6] GIRE – Gruppo Italiano Resistenza Erbicidi website: http://gire.mlib.cnr.it/index.php

billions of USD (Kubiak et al., 2022). Thus, weeds pose a serious threat to food security (Deyle et al., 2013).

Currently, more than 480 cases (species × site of action) of herbicide resistance have been reported globally: this phenomena is distributed over 251 weed species and encompasses resistance to almost all herbicide mode of action (MoA) (Markus et al., 2017). Once resistance is significantly frequent within a population, it might spread rapidly to other populations by pollen or seed, and potentially can be transmitted to other species via hybridization (Powles et al., 2010; Markus et al., 2017). An increase in the application frequency of a particular herbicide will probably be accompanied by commensurate resistance to that herbicide. Hence, this is a very complex phenomenon resulting from the synergy of multiple factors, both genetic and non-genetic.

Herbicide resistance can be classified into two main categories: target site resistance (TSR) and non-target site resistance (NTSR).

TSR is mainly caused by DNA missense mutation[7], results in different amino acids being encoded at a particular position in the resulting protein. Missense mutations alter the function of the resulting protein. This nucleotide change leads to the expression of a protein with different amino acids than the *wild type* individual, thus causing an altered folding of the protein itself. The change in the structure of the enzyme prevents the binding of the herbicide at its site of action, nullifying its toxic effect (Figure 1.5). Since many herbicides are designed to target specific enzymes or proteins, most of the times target-site resistance involves a mutation only of a single gene (Yuan et al, 2007).

[7] In genetics, a missense mutation is a point mutation in which a single nucleotide change results in a codon that codes for a different amino acid. It is a type of nonsynonymous substitution.

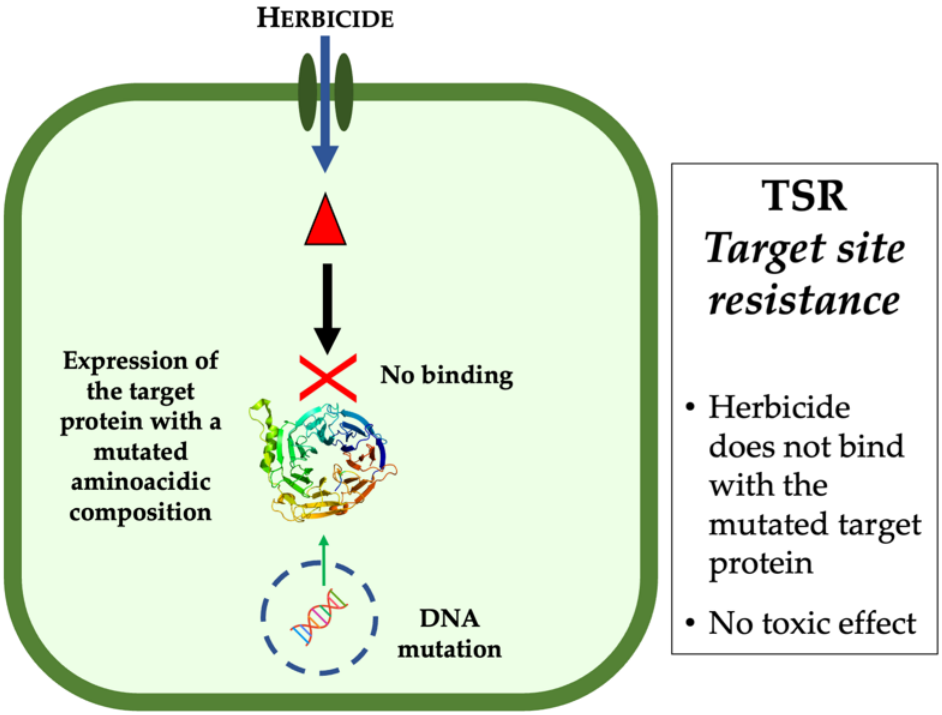

Figure 1.5: scheme of Target Site Resistance (TSR)

On the contrary, the process of NTSR involves many physiological processes, including regulatory mutations[8] in genes not targeted by herbicide, that may lead to decreased herbicide absorption, herbicide translocation and enhanced herbicide detoxification (Figure 1.6). Particularly, the occurrence of NTSR via enhanced detoxification is considered of particular importance because it can confer unpredictable resistance to multiple MoA, including chemical compounds never used before to control weeds.

[8] Regulatory mutations: mutations causing protein overproduction (Deyle et al., 2013)

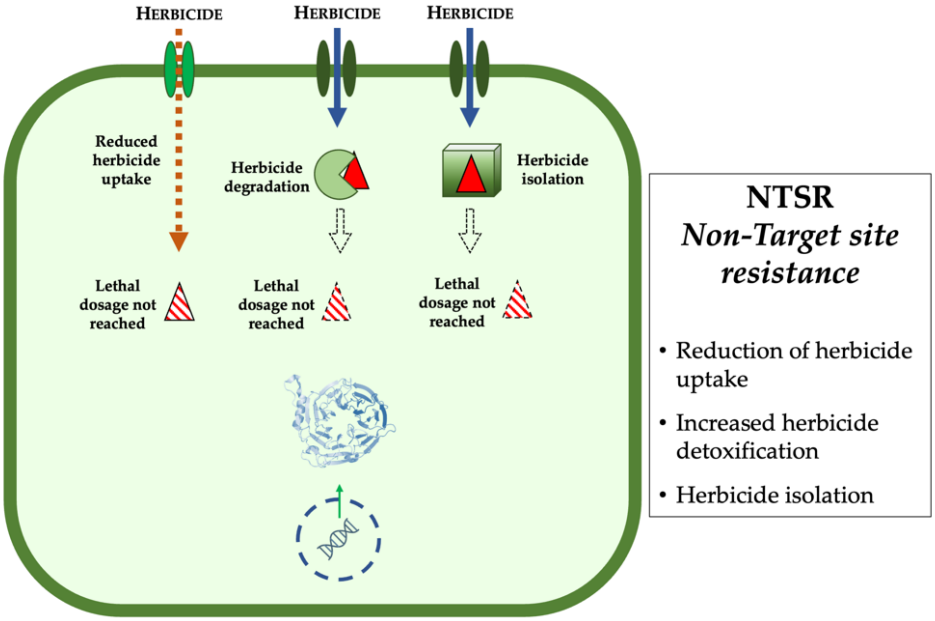

Figure 1.6: scheme of non-Target Site Resistance (NTSR)

NTSR caused by a plant detoxification process can be summarized by a four-phase schema. In phase I, herbicide molecules are activated so as to expose certain functional groups to phase II enzymes. Typical phase I detoxification reaction is oxidation, which mainly involves P450 superfamily monooxygenases. In phase II, the metabolic intermediate is conjugated with a hydrophilic molecule that allows the end product of phase II detoxification to be recognized by phase III transducers. In phase III, the conjugated molecule is transported into the vacuole or extracellular space. ABC transporters are the most common group of transporters involved in this phase of detoxification. Finally, phase IV involves further degradation of the conjugated molecule in the vacuole or extracellular spaces. In summary, there are many plant detoxifying proteins that could be involved in NTSR. However, to date, only four gene families are known to be involved in this process: cytochrome P450s, Glutathione-S-Transferases, ABC transporters and glycosyltransferases.

It has recently been suggested that, in addition to TSR or NTSR, herbicide resistance might also be influenced by epigenetic processes. Epigenetic regulation has been associated with numerous cellular processes, such as developmental programming, gene expression, embryonic development, transposon silencing, genome stability, and plant stress responses. Several studies have indicated how in *Homo sapiens* the evolution of

resistance to certain drugs was associated with epigenetic regulation as well as genetic variability (Ingelman-Sundberg et al., 2016). In light of this, it is likely to hypothesize that the expression of enzymes involved in the herbicide detoxification network may be influenced by epigenetic mechanisms.

Epigenetics refers to traits that are potentially heritable but not due to mutations in the DNA sequence. Specifically, epigenetic modulation can occur at the pre- or post-transcriptional level: in either case, DNA expression is affected. In the first case, it consists of chemical modification of histones[9] (i.e., methylation) that makes DNA more or less accessible to transcription factors. In the second case, various types of small-non-coding RNAs (i.e. siRNA, miRNAs) are involved. These molecules cleave messenger RNAs, counteracting their translation to proteins, hence regulating genes expression. Epigenetic changes can be induced either spontaneously, genetically, or environmentally. For the most part, chromatin modifications are induced by genetic changes, such as gene duplication or transposon insertion. The latter changes are developmentally controlled in each generation. Epigenetic changes induced by environmental stresses are presumably more random and usually revert soon after their onset. However, under specific conditions pre transcriptional epigenetic modifications might persist for a longer period after stress exposure, providing a "stress memory" in an organism that could be inherited.

In the light of this, considering herbicides as stressors, it could be likely to assume that these latter trigger epigenetic response that could affect the onset of herbicide resistance (Figure 1.7). Furthermore, since epigenetics is also environmentally influenced, deepen the knowledge about this topic represents an ambitious challenge in order to optimize precision weed management (PWM) technologies. More targeted and sustainable strategies of controlling herbicide resistance could help to reduce chemical inputs, improving environmental and food health.

[9] Histones are highly basic proteins abundant in lysine and arginine residues that are found in eukaryotic cell nuclei. They act as spools around which DNA winds to create structural units called nucleosomes (Cox, Nelson, Lehninger. 2005)

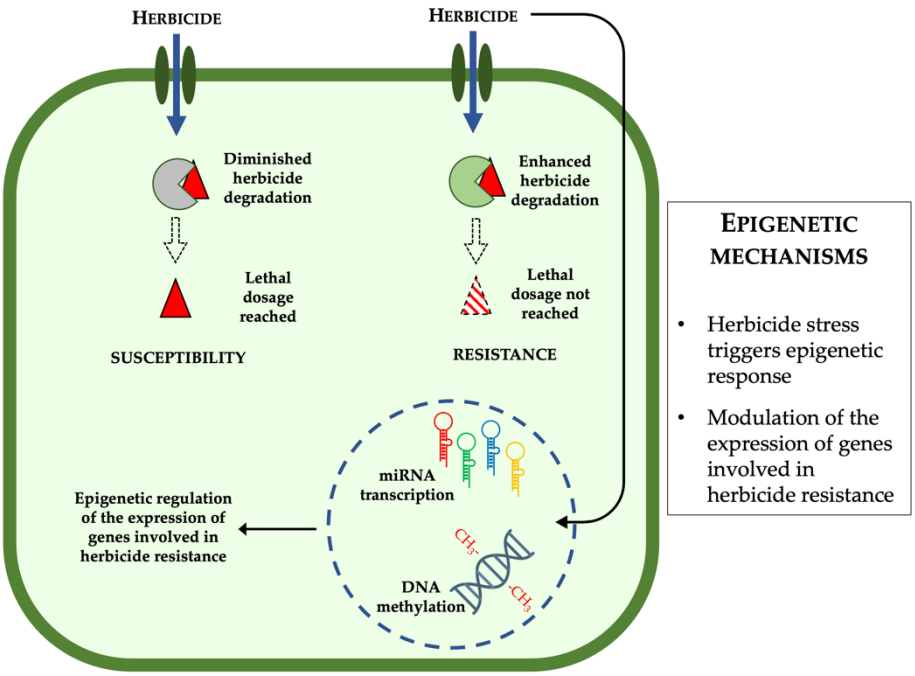

Figure 1.7: scheme of epigenetic mechanisms regulating resistance / susceptibility

1.7 *Echinochloa* P. Beauv species in Italian rice fields

Among the most widespread and noxious weeds infesting Italian rice fields of the northern Italy, the species of the genus *Echinochloa* P. Beauv. are counted to be the worst to which farmers have to face with. The genus *Echinochloa* belongs to the tribe *Paniceae* R. Br. subfamily *Panicoideae* A. Br., family *Gramineae* Juss (= *Poaceae* Barnh.). There is some disagreement about the species that constitute this genus: in fact, species classification may result a pretty difficult task due to the high morphological variability of these weeds (Ruiz-Santaella et al., 2006). From data collected to date, it is estimated that the genus *Echinochloa* may include 20 to 50 annual and perennial species. These plants are widely distributed in the tropical and warm temperate regions of the world. Many of these species are among the most important annual weeds of the summer season. They prefer marshy places such as rice fields, although they can also be found in dry soils. Thanks to their broad ecological tolerance, they are very successful competitors and may cause severe reduction in crops

yields (Vidotto et al., 2007). Each year, worldwide yield losses are estimated to be around 35 % due to the negative influence that *Echinochloa* weeds are able to exert on the crop's production, in terms of quantity and quality (Oerke et al., 2004; Pan et al., 2022).

To succeed in controlling these weeds, together with agronomic techniques of crop succession, chemical control is the most widely used method. The occurrence of herbicide resistant *Echinochloa* population has become over the years a serious problem that threats rice production sustainability and increases weed management costs. In Figure 1.8 is shown a geographical distribution of the resistances of *Echinochloa* spp. in the rice cropping area of the Lombardy region, listed by MoA (GIRE 2022).

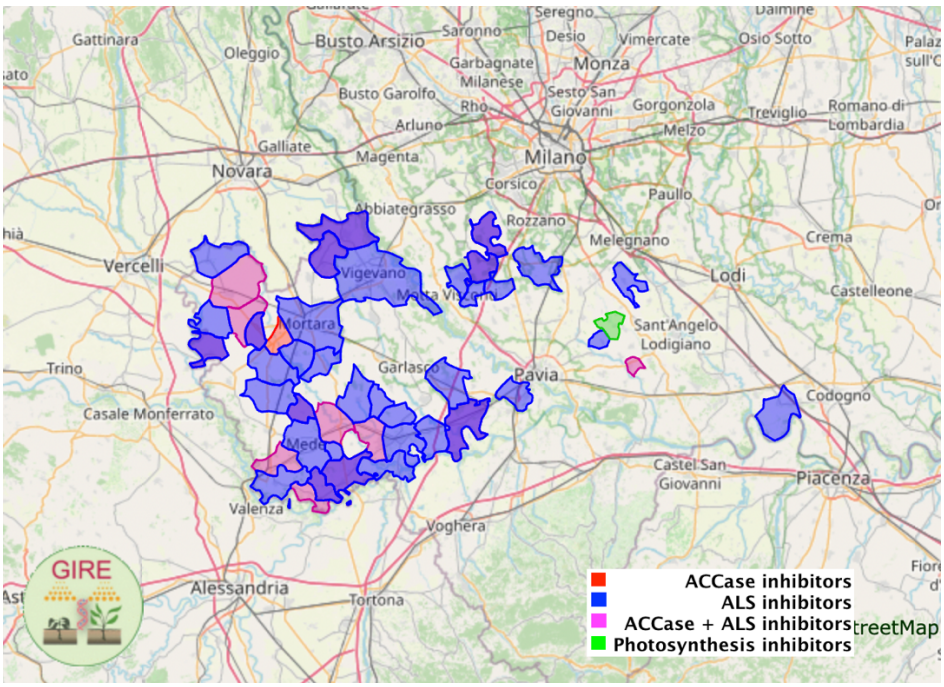

Figure 1.8: geographical distribution of *Echinochloa* spp. herbicide resistances in the Lombardy region (GIRE maps)

Resistances to acetolactate synthetase inhibitors (ALS) herbicides are the most frequent (blue areas), followed by cases of multiple resistance to Acetyl CoA Carboxylase (ACCase) and ALS inhibitors (purple areas), cases of ACCase (red areas) and photosynthesis inhibitors (green areas). As a matter of facts, ALS herbicides are by far the most used ones with more than 90% of rice fields treated at least once per year (Scarabel et al., 2012). In 2018, GIRE estimated that at least 40% of the Italian rice cultivated area is

affected by resistant populations. In 2022, there have been increased reports of weeds resistant to ACCase inhibitors, including species in the genus *Echinochloa* (ENR 2022). It is clear from these data that the problem of resistance in species of the genus *Echinochloa* poses a great threat to rice cultivation and its sustainability. It also shows how the phenomenon of resistance is not static, but is constantly evolving (Figure 1.9).

Figure 1.9: rice field infested by *Echinochloa* spp.

It's important to highlight that the difficulties in controlling *Echinochloa* species are not only related to their resistance status, but also to their biological characteristics. As a matter of facts, this weeds possess very high seed production and long seed dormancy, a C4 photosynthetic cycle which favors them in drought conditions, and high genetic variability, which plays a fundamental role in the adaptive response of organisms to varied environmental conditions, included herbicide administration (Mascanzoni et al., 2018, Cusaro et al., 2022a, Cusaro et al., 2022b).

Since *Echinochloa* species represent some of the most distributed and troublesome weeds in Italian rice cropping territory, this genus has been used as case study in this work.

Limited to the Lombardy region, the species of *Echinochloa* present are *E. colona* (L.) Link, *E. crus-galli* (L.) P.Beauv., *E. hispidula* (Retz.) Nees, *E. oryzicola* (Vasinger) Vasinger and *E. oryzoides* (Ard.) Fritsch. Moreover, *E. muricata* (P.Beauv.) Fernald and *E. crus-pavonis* (Kunth) Schult have been mistakenly recorded in the Lombardy region (Portale della Flora d'Italia[10], 2022). The most widespread resistant populations in the rice cropping area are of *E. crus-galli* (barnyardgrass / cockspurgrass, also known as "giavone rosso") and of *E. oryzicola* (late-watergrass, also known as "giavone bianco") (ENR / GIRE, 2022).

However, it's important to highlight that many of the *Echinochloa* species are hardly distinguishable, since they are characterized by a high polymorphism and by a huge variability of the characteristics usually considered in the identification process. The possibility to early recognize *Echinochloa* species in rice fields is crucial and represents a fundamental instrument for their management and to plan strategies of containment (Sparacino et al., 2007).

1.8 Aims of the project

Intense herbicide use but over time even sub-lethal herbicide doses and the repeated use of an increasingly narrow range of herbicides, cause a reduction in herbicide sensitivity in weed populations and lead to the evolution of herbicide-resistance (HeR) that represents one of the most critical aspects in weed management.

The PhD project aimed to investigate for the first time, with a multidisciplinary approach, the variability of herbicide resistances (HeR) in the genus *Echinochloa* P. Beauv. in the Lombardy region rice cropping area, assessing target-site based resistance (TSR), non-target-site based resistance (NTSR) and the role of the epigenetic mechanisms in the regulation of HeR occurrence.

[10] Portale della Flora d'Italia. http:/dryades.units.it/floritaly

The analysis of epigenetic factors affecting the evolution of herbicide resistance (HeR) required a multi-thematic research to achieve different sub-objectives. The research activities were developed in thematic sections each with its own methodology and results:

1. resistant *Echinochloa* populations mapping in the Lombardy rice-growing area
2. floristic and vegetation analysis of paddy's weeds
3. paddy soil physical and chemical analysis
4. paddy soil microbial and fungal community analysis
5. *Echinochloa* species identification by morpho-metric and molecular analysis
6. intraspecific biodiversity analysis in identified species
7. herbicide resistance analysis and epigenetic mechanisms involvement
8. assessing the incidence of resistances regulated by epigenetic mechanisms in relation to ecological factors

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WEBSITES

- https://www.opr.regione.lombardia.it/wps/portal/PROUE/OPR/sisco-siarl
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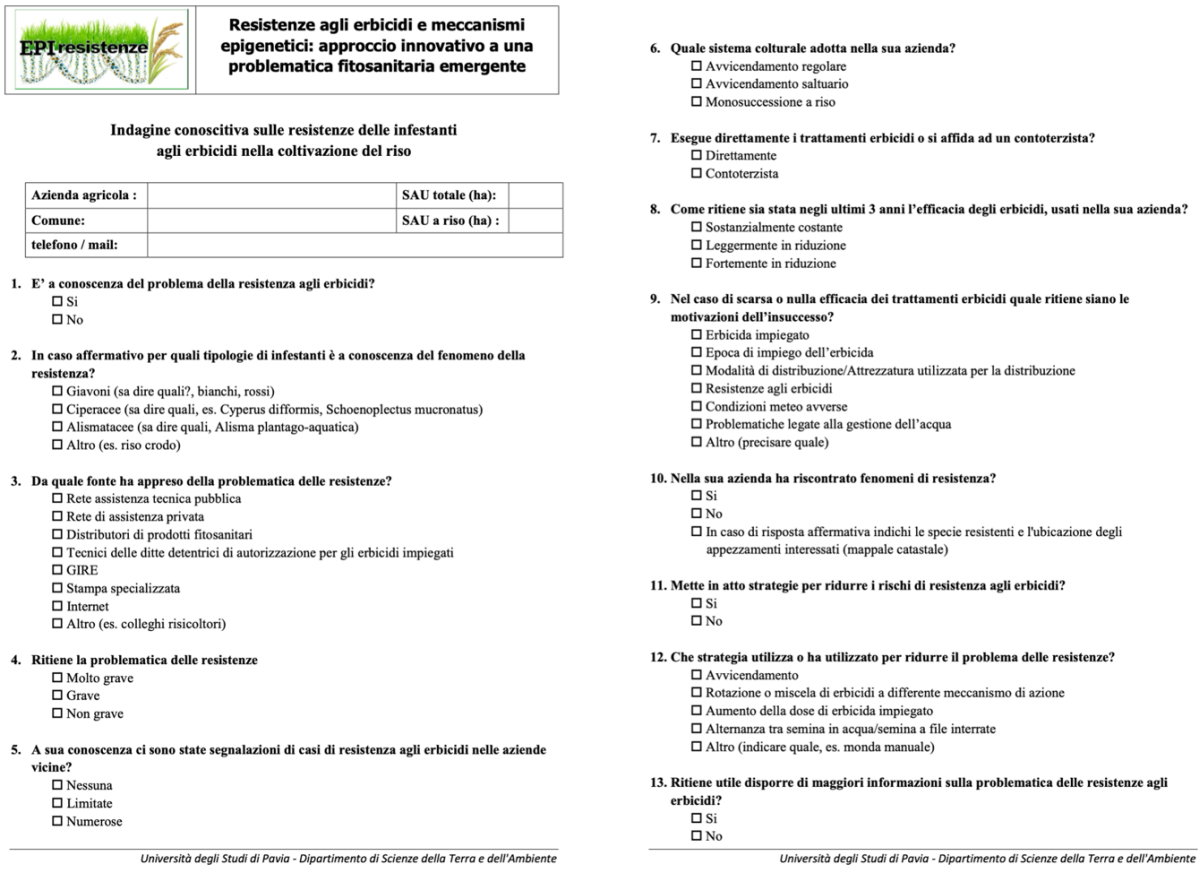
- https://www.regione.lombardia.it/wps/portal/istituzionale/HP/DettaglioServizio/servizi-e-informazioni/Enti-e-Operatori/Territorio/sistema-informativo-territoriale-sit/uso-suolo-dusaf/uso-suolo-dusaf
- http://wms.pcn.minambiente.it/

Chapter 2 – Field Work

Chapter 2 mainly reports information about the field work. In particular, in this chapter is described the preliminary survey conducted through the questionnaire submitted to rice farmers of the Lombardy region, aimed to obtain information about herbicide resistance occurrence on the territory. Further, is depicted the sampling phase, with the collection of resistant *Echinochloa* spp. specimens and of paddy soils. In this chapter the results of resistant *Echinochloa* specimens geo-localization within the Lombardy region rice farming territory are reported: obtained results have been presented to the XI International Agriculture Symposium "AGROSYM 2020" (online conference) held in October 2020.

2.1 Questionnaire submission – preliminary survey

A survey was conducted on the Lombardy rice-growing territory by means of a targeted questionnaire addressed to rice farmers in relation to the weed management practices applied in the last three years and the reporting of resistance phenomena. All records were useful for subsequent sampling purposes. Submitted questionnaire is reported in Figure 2.1.



Resistenze agli erbicidi e meccanismi epigenetici: approccio innovativo a una problematica fitosanitaria emergente

Indagine conoscitiva sulle resistenze delle infestanti agli erbicidi nella coltivazione del riso

Azienda agricola :		**SAU totale (ha):**	
Comune:		**SAU a riso (ha) :**	
telefono / mail:			

1. **E' a conoscenza del problema della resistenza agli erbicidi?**
   - ☐ Si
   - ☐ No
2. **In caso affermativo per quali tipologie di infestanti è a conoscenza del fenomeno della resistenza?**
   - ☐ Giavoni (sa dire quali?, bianchi, rossi)
   - ☐ Ciperacee (sa dire quali, es. Cyperus difformis, Schoenoplectus mucronatus)
   - ☐ Alismatacee (sa dire quali, Alisma plantago-aquatica)
   - ☐ Altro (es. riso crodo)
3. **Da quale fonte ha appreso della problematica delle resistenze?**
   - ☐ Rete assistenza tecnica pubblica
   - ☐ Rete di assistenza privata
   - ☐ Distributori di prodotti fitosanitari
   - ☐ Tecnici delle ditte detentrici di autorizzazione per gli erbicidi impiegati
   - ☐ GIRE
   - ☐ Stampa specializzata
   - ☐ Internet
   - ☐ Altro (es. colleghi risicoltori)
4. **Ritiene la problematica delle resistenze**
   - ☐ Molto grave
   - ☐ Grave
   - ☐ Non grave
5. **A sua conoscenza ci sono state segnalazioni di casi di resistenza agli erbicidi nelle aziende vicine?**
   - ☐ Nessuna
   - ☐ Limitate
   - ☐ Numerose

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6. **Quale sistema colturale adotta nella sua azienda?**
   - ☐ Avvicendamento regolare
   - ☐ Avvicendamento saltuario
   - ☐ Monosuccessione a riso
7. **Esegue direttamente i trattamenti erbicidi o si affida ad un contoterzista?**
   - ☐ Direttamente
   - ☐ Contoterzista
8. **Come ritiene sia stata negli ultimi 3 anni l'efficacia degli erbicidi, usati nella sua azienda?**
   - ☐ Sostanzialmente costante
   - ☐ Leggermente in riduzione
   - ☐ Fortemente in riduzione
9. **Nel caso di scarsa o nulla efficacia dei trattamenti erbicidi quale ritiene siano le motivazioni dell'insuccesso?**
   - ☐ Erbicida impiegato
   - ☐ Epoca di impiego dell'erbicida
   - ☐ Modalità di distribuzione/Attrezzatura utilizzata per la distribuzione
   - ☐ Resistenze agli erbicidi
   - ☐ Condizioni meteo avverse
   - ☐ Problematiche legate alla gestione dell'acqua
   - ☐ Altro (precisare quale)
10. **Nella sua azienda ha riscontrato fenomeni di resistenza?**
    - ☐ Si
    - ☐ No
    - ☐ In caso di risposta affermativa indichi le specie resistenti e l'ubicazione degli appezzamenti interessati (mappale catastale)
11. **Mette in atto strategie per ridurre i rischi di resistenza agli erbicidi?**
    - ☐ Si
    - ☐ No
12. **Che strategia utilizza o ha utilizzato per ridurre il problema delle resistenze?**
    - ☐ Avvicendamento
    - ☐ Rotazione o miscela di erbicidi a differente meccanismo di azione
    - ☐ Aumento della dose di erbicida impiegato
    - ☐ Alternanza tra semina in acqua/semina a file interrate
    - ☐ Altro (indicare quale, es. monda manuale)
13. **Ritiene utile disporre di maggiori informazioni sulla problematica delle resistenze agli erbicidi?**
    - ☐ Si
    - ☐ No

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Figure 2.1: submitted questionnaire

About 150 questionnaires were collected. In general, all farmers surveyed aware about the hazard that herbicide resistance represents. It was recorded that the efficacy of weed control programs is decrementing in the last three years and that *Echinochloa* species have been identified as the most troublesome weeds within the geographical area investigated. However, it should be noted that rice monoculture is still the most frequently

practiced cropping system. Finally, almost all farmers are in agreement with the usefulness to obtain information about herbicide resistance.

2.2 Sampling unit definition

A 5 x 5 km grid was superimposed on the land-use map of the Lombardy rice-growing area in order to create a sampling and monitoring scheme consisting of sampling units (SUs) under investigation. Each sampling unit (SU) corresponded to a paddy field where *Echinochloa* spp. resistant individuals were recognized.

On the basis of the TSR and NTSR resistance distribution maps reported by GIRE® (Italian Herbicide resistance working group) database and the questionnaires filled in by rice farmers, 30 farms (managed according to the principles of Directive 2009/128/EC, of which 1 following Operation 10.1.01 RDP 2014/2020) were selected in the province of Pavia and Milan, representing around 25% of the farms where herbicide resistance problems were found. In each farm, one or two paddy fields were considered for a total of 32 SUs.

Four specimens survived herbicide application were collected from each SU, for a total of 128 samples. These samples have been presumed "resistant" (R) for further analysis. In most cases, the plants that survived the herbicide treatment were underdeveloped, wilted and showed brown spots on leaves.

In addition, 12 *Echinochloa* spp. specimens have been collected from 3 SUs in 2 organic farms, while 16 specimens from untreated plots of InnovaTech srl. / Corteva Agriscience™ experimental trials[11]. Since these individuals have never been weeded, they have been considered as "susceptible control samples" (S) for further analysis.

A total of 39 SU have been visited and 156 *Echinochloa* spp. specimens have been collected. Sampling farms are reported in Figure 2.2 as yellow dots within rice cropping areas (light blue texture in Figure 2.2).

[11] Experimental field trials were carried out by InnovaTech Ltd./ Corteva Agriscience™ during the summers of 2019 and 2020.

Georeferencing was performed by means of QGIS 3.28 Firenze software (https://www.qgis.org/it/site/).

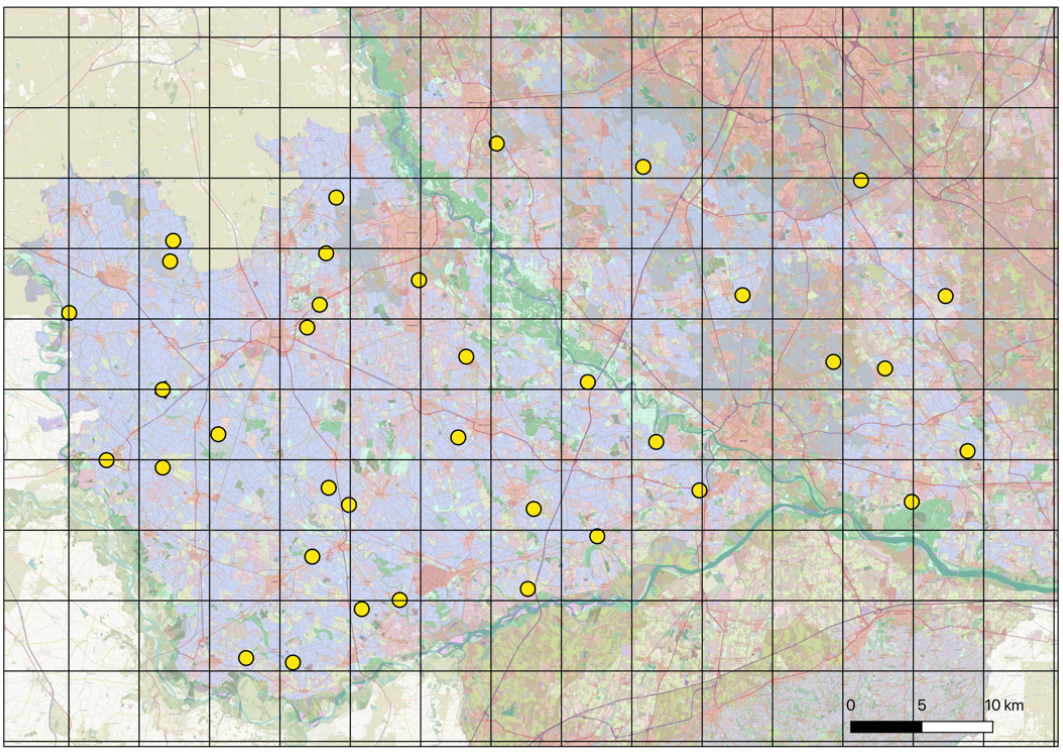

Figure 2.2: sampling scheme. Yellow dots correspond to farms in which resistant *Echinochloa* spp. specimens were surveyed. Light blue territories correspond to the rice cropping area (ERSAF 2019 – SIARL/SISCO 2019 – DUSAF 2019)

2.3 Sampling

Sampling was conducted from June to September in 2019 and 2020. In each SU has been performed:

1. Investigation about agronomic practices, seeding technique (wet or dry) and herbicides applied;
2. Phytosociological relevees (Braun-Blanquet, 1964) of the paddy's flora;
3. *Echinochloa* spp. specimens survived to herbicide application recognition and geo-localization;
4. Collection of a representative number of plants: 4 specimens in each SU, survived to herbicide (presumed resistant - R); 4 specimens in each SU from untreated plot or organic paddies (considered as susceptible - S). From each specimen, a complete stem, about 5 g of fresh tissues (leaves) and 100 seeds were collected. If seeds were not still developed when plant tissues were

collected, plants were labelled and seeds collected later. In SU where polymorphic *Echinochloa* spp. specimens were recognized, sampling of individuals was performed from each morphotype (Figure 2.3);

Figure 2.3: particular of sampling. *Echinochloa* specimens were labelled in the early summer (A) to proceed with seed collection when their development is complete (B).

5. Sampling of soil by the *non-systematic X method* (~ 1 kg of soil obtained from 12 collections at 2-25 cm depth) further to be used to analyze soil microbiota and chemicals there contained (Figure 2.4 - Lambkin et al., 2004);

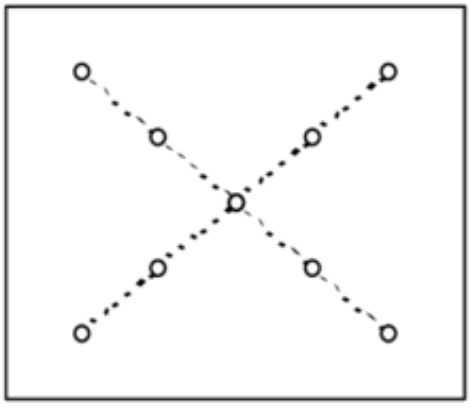

Figure 2.4: Schematic representation of the applied *non-systematic X soil sampling method* (Lambkin et al., 2004)

6. Recording of weather conditions during the summer within a 20-km radius of Corsico (MI), Lomello (PV), Castello d'Agogna (PV) and Vigevano (PV) municipalities – ARPA[12] stations.

[12] Agenzia Regionale per la Protezione dell'Ambiente - https://www.arpalombardia.it/Pages/ARPA_Home_Page.aspx

Collected samples were stored in the *Laboratory of Plant Biodiversity and Biosystematics* for subsequent analysis. In Table 2.1 are listed all collection sites.

Table 2.2: SU list. SU-ID: identification code; MUNICIPALITY: municipality of each farm; FARM: rice farms; FARM MANAGEMENT – SEEDING TECHNIQUE: rice cropping practiced / seeding technique.

SU - ID	MUNICIPALITY	FARM	FARM MANAGEMENT – SEEDING TECHNIQUE
PV 1	Ottobiano (PV)	Baino (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV 2	Sannazzaro de' Burgondi (PV)	Bellone (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV 3	Suardi (PV)	Magnani (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV4	Borgo San Siro (PV)	Dondoni (InnovaTech Exp.Tr.)	Untreated plot in 2019/2020
PV5	Cilavegna (St. Anna) (PV)	Groppelli (InnovaTech Exp.Tr.)	Untreated plot in 2019/2020
PV6	Zeme (PV)	Braggio (InnovaTech Exp.Tr.)	EC 128/2009 Wet seeding
PV7	Robbio (PV)	Greppi (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV8	Parona (PV)	Cassi (InnovaTech Exp.Tr.)	Untreated plot in 2019/2020
PV9	Vigevano (PV)	Marchesani (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV10	Rivoltella (PV)	Costanzo (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV11	Vigevano – Barbavara (PV)	Rabellotti (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
MI1	Mairano (MI)	Noè (InnovaTech Exp.Tr.)	Untreated plot in 2019/2020
MI2	Ozzero (MI)	Tacconi (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
DAGH	Robbio Daghetta (PV)	Daghetta Giovanni	EC 128/2009 – Wet seeding
PV12	Bascapè (PV)	Bazzini Pietro	EC 128/2009 – Dry seeding
PV13	Valle Lomellina (PV)	Gatti Massimo	EC 128/2009 – Dry seeding
PV14	Frascarolo-Sartirana (PV)	Rossignano Paolo	EC 128/2009 – Wet seeding
PV15.1	Zerbolò (PV)	Sala Pietro	EC 128/2009 – Dry seeding
PV15.2	Zerbolò (PV)	Sala Pietro	EC 128/2009 – Dry seeding
PV16	Belgioioso - St. Margherita (PV)	Foletti Angelo	EC 128/2009 – Dry seeding
PV17.1	Cozzo (PV)	Cascina Buscaiolo	EC 128/2009 - RDP Operation 10.0.01 Wet seeding
PV17.2	Cozzo (PV)	Cascina Buscaiolo	EC 128/2009 - RDP Operation 10.0.01 – Wet seeding
PV17.3	Cozzo (PV)	Cascina Buscaiolo	EC 128/2009 - RDP Operation 10.0.01 – Wet seeding
PV19	Pieve del Cairo (PV)	Maccagno	EC 128/2009 – Wet seeding
PV20	Genzone (PV)	Manzi	EC 128/2009 – Wet seeding
PV21	Roncaro (PV)	Gestioni Agricole	EC 128/2009 – Wet seeding
PV22	Zinasco Vecchia (PV)	Il Chicco	EC 128/2009 – Dry seeding
PV23	Pieve Albignola (PV)	Az. Ag. Genagricola	EC 128/2009 – Wet seeding
PV24	Santa Croce - S.Martino Siccomario (PV)	Carnevale Baraglia Francesco	EC 128/2009 – Dry seeding
PV25	Mortara (PV)	Ferraris	EC 128/2009 – Dry seeding
PV 26	Lomello (PV)	Pistone (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV 27	San Giorgio di Lomellina (PV)	Gilardi (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
PV 28	Dorno (PV)	Bellone	EC 128/2009 – Dry seeding
PV 29	Garlasco (PV)	Spina	EC 128/2009 – Wet seeding
PV 30	Giussago (PV)	Collivasone (InnovaTech Exp.Tr.)	EC 128/2009 – Wet seeding
MI 3	Carpiano (MI)	Cascina Pojago	EC 128/2009 – Wet seeding
PV 31	Carbonara al Ticino (PV)	Cascina Cavallera	EC 128/2009 – Wet seeding
PV 18	Lardirago (PV)	Tavazzani	Organic – Dry seeding
PV32.1	Candia di Lomellina (PV)	Tonelli	Organic – Wet seeding
PV32.2	Candia di Lomellina (PV)	Tonelli	Organic – Wet seeding

RDP: rural development program; EC 128/2009: Directive 128/2009 of European Community; Exp.Tr.: experimental trials

2.4 Resistant *Echinochloa* spp. specimens mapping

2.4.1 State of the art

In Italy, GIRE (Italian Working Group on Herbicide Resistance - www.resistenzaerbicidi.it) has been monitoring herbicide-resistant weed populations throughout the country since 1997 on the basis of reports from farmers. In addition, GIRE has produced and updated over the years herbicide resistance dynamics maps, in order to create a database to monitor the evolution of this phenomena (see Figure 1.8 in Chapter 1).

From 2019 to 2020, GIRE recorded the presence of resistant *Echinochloa* spp. specimens in the provinces of Pavia and Milano (Figure 2.5 – red areas).

2.4.2 Results and discussion

Results obtained from the preliminary survey and the "in-field" recognition of resistant *Echinochloa* spp. specimens showed that herbicide resistance is currently spread throughout the whole rice-growing area of the Lombardy region, also in territories where GIRE had not yet signaled it. In Figure 2.5, green dots indicate the presence of resistant *Echinochloa* spp. specimens surveyed outside GIRE areas (red texture), while yellow dots specimens surveyed inside GIRE areas. What emerges from this geolocation is that the presence of resistant *Echinochloa* specimens is highly distributed throughout the territory of the Lombardy region and is expanding.

Mapping was performed by means of QGIS 3.28 *Firenze* software (https://www.qgis.org/it/site/).

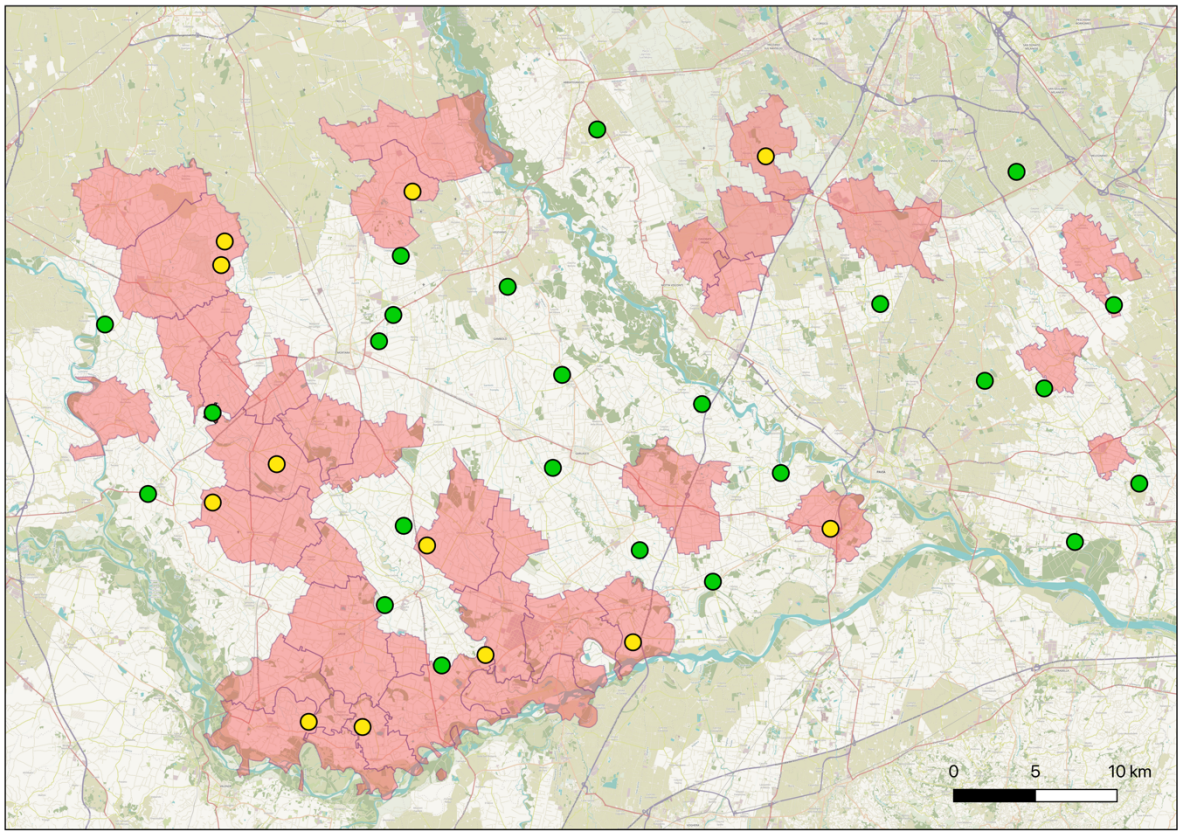

Figure 2.5: sampling sites mapping: green dots indicate the presence of resistant *Echinochloa* spp. specimens surveyed outside GIRE areas (red texture), while yellow dots specimens surveyed inside GIRE areas (ERSAF 2019 – SIARL/SISCO 2019 – DUSAF 2019 – GIRE 2019)

It is likely to assume that the presence of resistant *Echinochloa* spp. specimens even in areas not reported by GIRE is attributable to generally low levels of infestation probably related to the higher level of cropping systems diversification practiced in these areas, where crop rotation and dryland farming are more widely practiced (Mascanzoni et al., 2019).

The results obtained were the subject of an abstract and a poster titled *Mapping Of Herbicide Resistant Echinochloa Spp. Populations In Northern Italy (Lombardy Region)* presented at the international congress *XI International Scientific Agriculture Symposium "AGROSYM 2020"*, held as an online conference on October 8th and 9th, 2020.

The submitted abstract and poster are attached below.

2.2.3 ABSTRACT - XI International Scientific Agriculture Symposium "AGROSYM 2020", 8th to 9th October 2020, Bosnia and Herzegovina

MAPPING OF HERBICIDE RESISTANT *ECHINOCHLOA* SPP. POPULATIONS IN NORTHERN ITALY (LOMBARDY REGION)

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Abstract

This study is a part of the underway three-year (2019/2022) research project *EpiResistenze - Herbicide resistance and epigenetic mechanisms: innovative approach to an emerging phytosanitary problem,* funded by Lombardy Region, Directorate General for Agriculture. Italy is the first European rice producer nation, with about 230,000 ha dedicated to this cropping mainly located in northern regions. In Lombardy, the cultivation of rice is mostly carried out in aquatic environment and through single-crop cultivation. These agricultural practices and the repeated and constant use of a narrow range of herbicides favor the evolution of herbicide-resistant (He-R) weed populations that represent one of the most critical aspects in weed management. *Echinochloa* spp. are among the most difficult to control paddy weeds because of the development of resistance to a wide range of herbicide classes. One of the aims of *EpiResistenze* project is to map the evolution of *Echinochloa* spp. HeR in the Lombardy rice territory, analyzing HeR gene expression and focusing on the ecological (biotic and abiotic) factors related with HeR developement. The mapping of HeR populations was performed during the 2019 summer season. The resistant *Echinochloa* populations were georeferenced using Q-GIS3 software, and relative maps were produced. Preliminary results show that *Echinochloa* spp. HeR populations are widely spread throughout the rice-growing area of the provinces of Pavia, Milan, Lodi and Mantua, including areas where no resistance has been detected until now. The *Echinochloa* spp. has developed resistance especially against ALS, ACCase and photosynthesis inhibitors, also showing cases of "multiple resistance".

Keywords: *EpiResistenze, Echinochloa, Herbicide Resistance Mapping*

MAPPING OF HERBICIDE RESISTANT *ECHINOCHLOA* SPP. POPULATIONS IN NORTHERN ITALY (LOMBARDY)

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PROJECT FUNDED BY LOMBARDY REGION D.G. AGRICULTURE, FOOD AND GREEN SYSTEMS
CALL FOR FUNDING FOR RESEARCH PROJECTS IN THE AGRICULTURAL AND FORESTRY SECTOR

INTRODUCTION

ITALY IS THE FIRST EUROPEAN RICE PRODUCER NATION, WITH ABOUT 230,000 HA DEDICATED TO THIS CROPPING MAINLY LOCATED IN NORTHERN REGIONS. IN LOMBARDY, THE CULTIVATION OF RICE IS MOSTLY CARRIED OUT IN AQUATIC ENVIRONMENT AND THROUGH SINGLE-CROP CULTIVATION. THESE AGRICULTURAL PRACTICES AND THE REPEATED AND CONSTANT USE OF A NARROW RANGE OF HERBICIDES FAVOR THE EVOLUTION OF HERBICIDE-RESISTANT (HE-R) WEED POPULATIONS THAT REPRESENT ONE OF THE MOST CRITICAL ASPECTS IN WEED MANAGEMENT.

AMONG THE MOST DISTRIBUTED WEED SPECIES, THE GENUS *ECHINOCHLOA* (P.) BEAUV. (*POACEAE*) REPRESENTS ONE OF THE MOST PROBLEMATIC; IT INCLUDES ABOUT 50 SPECIES. SEVERAL STUDIES DEMONSTRATE THAT THESE BIOTYPES EXHIBIT DIFFERENTIAL HERBICIDE SUSCEPTIBILITY.

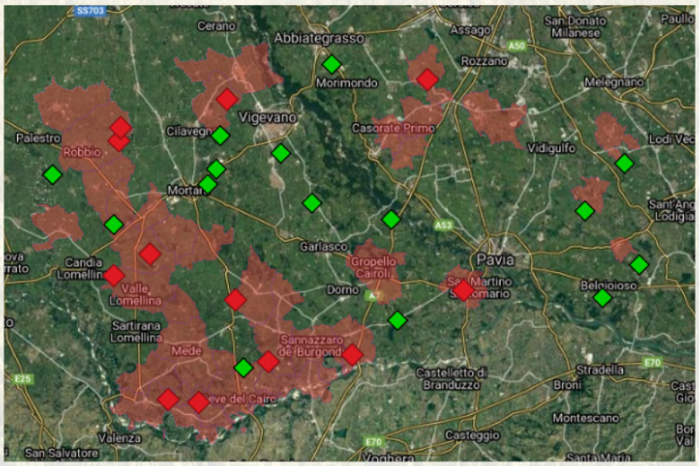
resistant *Echinochloa crus galli* P. Beauv

OBJECTIVES

ONE OF THE AIMS OF *EPIRESISTENZE PROJECT* IS TO MAP THE EVOLUTION OF *ECHINOCHLOA* SPP. HER IN THE LOMBARDY RICE TERRITORY, ANALYZING HER GENE EXPRESSION AND FOCUSING ON THE ECOLOGICAL (BIOTIC AND ABIOTIC) FACTORS RELATED WITH HER DEVELOPEMENT.

METHODOLOGY

RESISTANT *ECHINOCHLOA* POPULATIONS WERE SURVEYED DURING THE 2019 SUMMER SEASON AND GEOREFERENCED THROUGH Q-GIS3 SOFTWARE. A MAP OF THEIR DISTRIBUTION HAS BEEN PRODUCED BY OVERLAPPING THE DATA OF *ECHINOCHLOA* HER CASES REPORTED BY GIRE® (ITALIAN HERBICIDE RESISTANCE WORKING GROUP) WITH THE ONES DETECTED IN 2019 BY *EPIRESISTENZE PROJECT* TEAM.



RESULTS AND DISCUSSION

- THE RED AREAS REPRESENT THE MUNICIPALITIES WHERE GIRE® REPORTED CASES OF *ECHINOCHLOA* HER;
- THE RED SQUARES REPRESENT RESISTANT *ECHINOCHLOA* POPULATIONS WITHIN TERRITORIES WHERE GIRE® HAS REPORTED *ECHINOCHLOA* HER;
- THE GREEN SQUARES REPRESENT RESISTANT *ECHINOCHLOA* POPULATIONS WITHIN TERRITORIES WHERE GIRE® HAS NOT YET REPORTED *ECHINOCHLOA* HER;
- PRELIMINARY RESULTS SHOW THAT *ECHINOCHLOA* HER CASES ARE WIDELY SPREAD THROUGHOUT THE RICE-GROWING AREA OF LOMBARDY REGION: IN PARTICULAR, THE PROVINCES OF PAVIA, MILAN AND LODI ARE THE MOST AFFECTED.
- THE SURVEY OF *EPIRESISTENZE PROJECT* TEAM RECORDS *ECHINOCHLOA* HER CASES IN AREAS WHERE NO CASES HAVE BEEN DETECTED UNTIL NOW BY GIRE®.
- *ECHINOCHLOA* SPP. DEVELOP RESISTANCE ESPECIALLY AGAINST ALS, ACCASE AND PHOTOSYNTHESIS INHIBITORS, ALSO SHOWING CASES OF "MULTIPLE RESISTANCE".

CONCLUSION

A HIGH RISK OF RESISTANCE EVOLUTION IS ASSOCIATED WITH TRADITIONAL RICE CROPPING SYSTEMS CHARACTERIZED BY INTENSE RATES OF MONOCULTURE AND IN AREAS WHERE WATER-SEEDING IS WIDE-SPREAD, SUCH AS IN THE PROVINCE OF PAVIA AND MILAN. FOR A BETTER MANAGEMENT OF *ECHINOCHLOA* HER CASES, IT IS IMPORTANT TO IMPLEMENT THE KNOWLEDGE OF THE CAUSES THAT ARE AT THE BASIS OF THE ONSET OF THE PHENOMENA OF HER.

Figure 10.7: poster presented @ Agrosym 2020

2.5 References

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Chapter 3 – Floristic, vegetation and biodiversity analysis of paddy weeds phytocoenosis

Chapter 3 is devoted to the analysis of weeds phytocoenosis present in all the visited paddies.

3.1 Introduction

Weeds are considered the worse threat in rice farming. Severe weeds infestations may lead to rice yield losses, reducing the market value and quality of the rice grain. Weeds compete with crops for light, water and nutrients in the soil. They are by far the most difficult damaging organisms in agricultural production systems (Oerke et al., 2004). It has been estimated that without weed control, at a yield level of 7 to 8 t/ha, yield loss can be as high as about 90% (Ferrero, 2003). An average yield loss of 35% has been estimated for many crops worldwide (Oerke, 2004).

Chemical control has assumed over the years a crucial meaning in rice cropping to prevent these risks. For this reason, farmers have planned specific control treatments throughout the rice-growing season. More than 80% of the total consumption of pesticides utilized for crop protection are herbicides, with a total expense of about 110 million € /year (Ferrero et al., 2007). The use of synthetic chemicals for weed control has benefits for agricultural production, such as protecting crops and increasing farmers' profitability, but the use of these substances causes negative impacts on the environment and human health and affects the evolution of the organisms toward which the treatments are aimed (Sattin et al., 2006). In addition, chemical control is often associated with other agronomic techniques, such as crop rotation, which improves soil health, optimizes nutrients in the soil, and fights pest and weed pressure. Nowadays are also adopted organic or biodynamic agriculture, or agricultural management models integrated the Rural Development Programmes (RDP), which are more attentive to the resulting impacts on the environment. During the last 20 years in Italy, due to climate change and the lack of water availability in summer, rice dry seeding has been adopted more frequently, especially in the Piedmont and Lombardy region territories (Ferrero et al., 2021). New applications have characterized the agricultural management, in particular the development of mechanization, the diffusion of a broad range of herbicides, the turning from transplanting to direct seeding and the introduction of late, dwarf and less competitive rice varieties. All these changes determined an important modification of rice fields phytocoenoses, favoring the settlement of increasingly better

adapted weeds (Ferrero et al., 1999). In this scenario, the onset of herbicide resistance represent a critical issue to deal with, and the costs associated with this problem is significant. Moreover, in most of the cases, herbicide resistance is an heritable phenomena, passed on to subsequent generations (Delye et al., 2013).

Generally, the rice paddy weed phytocoenoses are characteristic of marshy or otherwise water-saturated habitats and belong to the alliance *Oryzo sativae-Echinochloion oryzoidis* O. Bolòs & Masclans 1955. It is represented by weed coenoses, mostly annuals, that infest fields of rice (*O. sativa*). The most widespread genera are *Echinochloa* (P.) Beauv., *Alisma* L., *Cyperus* L., *Schoenoplectus* (Rchb.) Palla and the exotic *Heteranthera* Ruiz & Pav. and *Leptochloa* (P.) Beauv. (Ferrero et al., 2007). As a matter of facts, weeds represent a dynamic component of the agricultural ecosystem, able to adapt rapidly to changes in management practices. It was recently discovered by Fang et al. (2015) how rice tends to compete with weeds by developing allelopathic responses. Allelopathy is a biological phenomenon that refers to the beneficial or harmful effects of one organism on another by influencing its growth, survival and reproduction through the release of chemicals into the environment (Stamp et al., 2003; Willis et al., 2007). In the study of Fang et al. (2015) was discovered how phenolic acids released from allelopathic rice triggers the expression of several miRNAs involved in plant hormone signal transduction, nucleotide excision repair and the peroxisome proliferator-activated receptor and p53 signalling pathways in *E. crus-galli*.

The purpose of this section of the research was to characterize and analyze weed phytocoenosis in rice fields where herbicide resistant populations of *Echinochloa* were identified.

3.2 Material & Methods

3.2.1 Field work

In each of the 39 visited SU, phytosociological relevés were carried out according to Braun-Blanquet (1964), considering plots of 10 m^2. Identified plant species were then listed with their percentage cover according to the Braun-Blanquet scale (Table 3.1).

Table 3.1: Braun-Blanquet cover abundance scale and midpoint of cover abundance values.

Braun-Blanquet scale	Cover-abundance values (%)	Midpoint of cover-abundance values (%)
5	75 – 100	87.5
4	50 – 75	62.5
3	25 – 50	37.5
2	10 – 25	17.5
1	1 – 10	5.0
+ or r	< 1	1.0

Specimens of doubtful identification were collected in the field and then catalogued and subsequently identified at the *Laboratory of Plant Biodiversity and Biosystematics* of the Department of Earth and Environmental Sciences, University of Pavia.

3.2.2 Collected samples identification

Recognition of vascular species samples was done through direct observation by stereo-microscope (Zeiss) at 10X and 40X magnification of the morphological traits, with reference to the *Flora d'Italia* by Pignatti (1982). The nomenclature referred to Pignatti (1982) and Conti et al. (2005).

3.2.3 Floristic Study

For every species detected biological form was assigned according to Raunkiaer's (1934) system, chorological type according to the indications found in Pignatti (1982) and

Ubaldi (2003) and by consulting the websites DAISIE (Delivering Alien Invasive Species Inventories Europe, www.europe-aliens.org) and ActaPlantarum (www.actaplantarum.org), the Primary and Secondary Grime Strategy according to Landolt et al. (2010), the status of native, exotic (naturalized or invasive) or cryptogenic according to Celesti- Grapow (2010), Banfi & Galasso (2010) and consulting the website ActaPlantarum.

Weighted spectra were produced for biological forms, chorological types, native/exotic status and Grime strategies.

1. ***Biological form*** - A classification system devised by Danish botanist Christen Raunkiaer, focused on the way plants overcome the adverse season. Specifically, by considering the positioning of dormant buds, Raunkiaer identified several biological forms (Phanerophytes, Chameophytes, Hemicryptophytes, Geophytes, Holophytes, Hydrophytes, Therophytes) (Raunkiaer, 1934).

2. ***Chorological type*** - A classification system that refers to a type of geographic distribution to which a set of species (or even genera or families) with similar range belongs. Specifically, according to Flora d'Italia (1982), the following are recognized: Eumediterranean, Boreal, Stenomediterranean, Orophytes, Eurasian, Atlantic, Cosmopolitan, Exotic and Cultivated. Depending on the source, these terminologies may change.

3. ***Exotic species*** - Exotic plant species, also referred to as "alien," are non-native organisms (i.e. not characteristic of the local flora), that spread outside their native range. They can be introduced by humans accidentally, through commercial activities, or intentionally for economic purposes (Szymura et al., 2016). Alien species can be occasional if they give rise to one or a few local populations that, being unable to reproduce, disappear after short periods; they may reappear only in the case of a new inoculation. If, on the other hand, alien species are able to reproduce in a new habitat, either vegetatively or sexually, generating numerous and fertile offspring capable of spreading rapidly over a wide area

and considerable distances from the parent plants and capable of competing with native species, they are termed *invasive* (Pyšek et al., 2004).The danger in these cases is that invasive species will replace native species, causing enormous damage to biodiversity. It is also possible that an alien species, although successfully inserted into the local flora, does not exhibit invasive behavior, as a case the increase in its population occurs mostly at the edge of older generations and over short distances. In such cases they are called naturalized.

4. ***Grime's Strategy*** - Grime's primary and secondary biological strategies represent behavioral categories to which different plant species belong according to their ability to survive in those habitats where conditions marginal to the ecological tolerance range, i.e., far from the optimum, are realized (Grime, 2001). Grime showed that the main limiting factors for plant growth and reproduction are: competition with other plants, environmental stress and disturbance to which the habitat is subjected. Among the primary ones we have: competitive (C), stress-tolerant (S) and ruderal (R). Competitive ones are able to grow very fast and rapidly in an undisturbed environment, but in the case of stress and disturbance they greatly decrease growth. The stress-tolerant and ruderals are both overwhelmed by the competitive in the environment without disturbance, but in the case of stress the former react by surviving while the latter often die. In the case of a highly disturbed environment only the ruderals succeed in producing seeds. Then there are the secondary categories, namely ruderals-competitive (C-R), ruderals stress-tolerant (R-S), ruderals stress-tolerant competitive (C-S) and ruderals stress-tolerant competitive (C-S-R).

3.2.4 Study of specific biodiversity

The following indexes were calculated:

1. ***Margalef's specific richness*** - is based on the species-number-of-individuals relationship (species-abundance) establishes a logarithmic relationship between the number of species detected and the number of individuals examined, calculated for different phytocoenoses, makes possible a comparison between them.

$$R = \frac{(S - 1)}{\ln N}$$

Where S is the number of species and surveyed and N is the number of analyzed individuals (Margalef, 1958).

2. ***Pielou's equitability*** - measures the degree of equidistribution of species abundances, varies between 0 and 1. Specifically, one corresponds to maximum equidistribution, and zero corresponds to minimum equitability, and means that there is strong dominance of one, two maximum three species in that plant community.

$$J = -\frac{\sum_i^S P_i \ln P_i}{\ln S}$$

Where S is the number of species and surveyed and P_i is the proportion of abundance of a single species (Pielou, 1977).

3. ***Simpson's dominance -*** This is an index of dominance; the value of this index increases as overall diversity decreases, that is, as the dominance of one or a few species over others increases. It measures the probability that two individuals randomly drawn from a sample belong to the same species. The higher this probability, the greater the dominance of one or a few species and therefore, the lower the diversity of the community examined.

$$\lambda = \sum_i P_i^2$$

Where P_i is the proportion of abundance of a single species (Simpson, 1949).

4. ***Shannon-Weaver's diversity*** - based on information theory. Information is a measure of the uncertainty of events; it is used as a measure of diversity because the greater the number of species and the more equal their distribution (equitability), the greater the uncertainty in assigning a randomly drawn individual from the sample to a given species. The greater the uncertainty, the greater the diversity. The diversity of a community can be compared to the uncertainty in predicting which species an individual randomly drawn from the sample belongs to; its value increases with increasing overall diversity i.e., richness and equitability indices of diversity.

$$H^1 = \sum_i P_i \ln P_i$$

Where P_i is the proportion of abundance of a single species (Shannon, 1948).

Being a logarithmic function, the index never takes high values (generally ranging from 1.5 to 3.5), varying from a minimum of H1 = 0 when only one species is present to a max of H max = ln S when all species are equally distributed.

3.2.5 Statistical Analysis

The entire analysis was carried out using R 3.6.3 software (R Core Team, 2019). A double heatmap – clustering analysis, based on "Jaccard" distance and "ward.D2" algorithm to maximize the variance between clusters (function "veg.dist" – package vegan (Oksanen, 2020)), was performed to obtain a graphical description of each weed species abundance in all the farms (function "heatmap.2" – packages gplots (Warnes et al, 2020), ggplot2 (Wickham, 2016), reshape (Wickham, 2007), dendextend (Galili, 2015). This analysis allowed to discriminate the most frequent weed species in each farm. In addition, farm with similar phytocoenoses were grouped together, while different separated.

3.3 Results and Discussion

3.3.1 Vegetation study

In Table 3.2 are reported the species surveyed during the sampling phase, listed with their Braun-Blanquet (1964) indexes. A total of 28 plant species have been identified.

Table 3.2: phytosociological relevees (Braun-Blaquet indexes). ID: SU identification code; MUNICIPALITY: municipality of each farm; FARM: rice farms; FARMING TECHNIQUE: seeding and rice cropping technique.

ID	MUNICIPALITY	FARM	FARMING TECHNIQUE	Abutilon theophrasti Medicus	Alisma plantago-acquatica L.	Amaranthus retroflexus L.	Ammania auriculata Willd.	Bidens frondosa L.	Bidens vulgata Greene	Butomus umbellatus L.	Chenopodium album L	Comellina communis L.	Cyperus difformis L.	Cyperus esculentus L.	Cyperus longus L.	Digitaria sanguinalis (L.) Scop.	Echinochloa spp.	Eclipta prostrata L.	Heterantera renifomis Ruiz & Pav.	Heterantera limosa (Sw.) Willd.	Lemna minor L.	Lindernia spp.	Murdannia keisak (Hassk.) Hand.-Mazz.	Oryza sativa L.	Oryza sativa L. varietà silvatica Chiappelli	Panicum dichotomiflorum Michx.	Polygonum persicaria L. (also called Persicaria maculosa Gray)	Portulaca oleracea L.	Schoenoplectus mucronatus (L.) Palla	Setaria glauca L.	Sorghum halepense (L.) Pers.
PV 1	Ottobiano	Baino	EC128_WS	--	1	--	--	1	--	--	--	--	--	--	--	r	3	--	--	--	3	--	--	5	--	--	r	--	r	--	--
PV 2	Sannazzaro de' Burgondi	Bellone	EC128_WS	--	--	--	1	--	r	--	--	--	--	--	--	r	2	2	1	--	4	--	--	5	--	r	r	1	--	--	r
PV 3	Suardi	Magnani	EC128_WS	--	--	--	1	1	--	--	--	--	--	--	--	1	3	2	--	--	2	--	--	5	--	--	1	--	1	--	--
PV4	Borgo San Siro	Dondoni	Untreated plot	--	--	--	--	--	--	--	--	--	--	--	--	r	3	--	2	--	1	--	--	5	--	--	--	--	--	1	1
PV5	Cilavegna	Groppelli	Untreated plot	--	--	--	--	--	--	--	--	--	r	--	--	r	3	--	--	--	1	--	--	5	--	--	--	--	--	--	--
PV6	Zeme	Braggio	EC128_WS	--	--	--	--	--	--	--	--	--	--	--	--	r	2	--	2	--	1	--	--	5	--	--	--	r	--	--	--
PV7	Robbio	Greppi	EC128_WS	--	--	--	--	--	--	--	--	--	--	r	--	--	3	--	--	--	3	--	--	5	r	r	--	--	--	--	--
PV8	Parona	Cassi	Untreated plot	--	1	--	r	--	--	--	--	--	--	--	1	--	2	--	--	--	1	--	--	5	--	--	--	--	--	--	--
PV9	Vigevano	Marchesani	EC128_WS	--	--	--	1	--	--	r	--	1	--	--	--	--	3	--	--	--	r	r	--	5	--	--	r	--	r	--	--
PV10	Rivoltella	Costanzo	EC128_WS	--	--	--	--	--	--	--	--	--	--	--	--	1	2	--	--	--	r	--	2	5	--	--	--	1	--	1	4
PV11	Barbavara	Rabellotti	EC128_WS	--	--	--	--	3	2	1	--	1	--	1	--	r	1	1	--	--	3	r	1	5	--	--	--	--	--	--	--
MI1	Mairano	Noè	Untreated plot	--	--	--	1	--	--	--	--	--	--	--	--	--	1	--	--	--	r	--	--	5	1	--	--	--	1	--	--
MI2	Ozzero	Tacconi	EC128_WS	--	--	--	r	--	--	--	--	--	--	--	--	1	1	--	1	--	r	--	--	5	--	--	1	--	--	1	1
DAGH	Robbio	Daghetta	EC128_WS	--	--	--	1	--	--	1	--	--	r	r	--	1	2	--	1	--	r	--	--	5	--	1	--	--	1	--	r
PV12	Bascapè	Bazzini Pietro	EC128_DS	--	--	--	--	--	--	--	--	--	--	--	r	r	1	--	r	--	--	r	--	5	1	--	--	--	--	--	--
PV13	Valle Lomellina	Gatti	EC128_DS	--	--	--	--	--	--	--	--	--	--	r	--	--	3	--	--	--	--	r	--	5	1	--	--	--	1	--	--
PV14	Frascarolo	Rossignano	EC128_WS	--	--	--	--	--	--	--	--	--	--	--	--	1	5	--	--	--	--	--	--	5	--	--	r	1	r	--	--
PV15.1	Zerbolò	Sala Pietro	EC128_DS	--	--	--	r	--	--	1	--	1	r	--	--	1	2	r	2	--	r	--	--	5	r	r	1	--	1	--	--
PV15.2	Zerbolò	Sala Pietro	EC128_DS	--	--	--	--	--	r	1	--	--	r	--	--	--	2	--	2	--	r	1	--	5	1	--	--	--	1	--	--
PV16	Belgioioso	Foletti	EC128_DS	--	--	--	--	--	--	--	--	--	--	--	--	1	1	--	1	--	--	--	--	5	--	--	--	--	r	--	r
PV17.1	Cozzo	Cascina Buscaiolo	RDP_WS	--	--	--	1	1	--	--	--	--	--	--	--	--	1	2	r	--	2	--	--	5	r	--	1	1	2	r	r
PV17.2	Cozzo	Cascina Buscaiolo	RDP_WS	--	r	--	r	--	2	2	--	1	--	--	--	r	4	2	2	--	3	r	1	5	--	--	1	r	1	--	--
PV19	Pieve del Cairo	Maccagno	EC128_WS	--	--	--	r	--	--	1	--	--	--	--	--	1	3	--	--	--	1	--	--	5	r	--	--	--	--	--	--
PV20	Genzone	Manzi	EC128_WS	--	--	--	1	1	--	--	--	--	1	--	--	--	4	--	1	--	2	--	--	5	--	--	--	--	1	--	--
PV21	Roncaro	Gestioni Agricole	EC128_WS	--	--	--	1	--	--	--	--	--	r	r	r	1	2	r	1	--	3	r	--	5	1	--	--	r	--	1	--
PV22	Zinasco Vecchia	Il Chicco	EC128_DS	--	--	--	r	--	--	--	--	--	--	r	--	3	1	--	1	--	r	r	--	5	1	1	--	--	r	--	1
PV23	Pieve Albignola	Az. Ag. Genagricola	EC128_WS	--	--	--	--	--	--	--	--	--	--	--	--	1	3	--	r	--	1	--	--	5	--	--	--	r	r	--	r
PV24	San Martino Siccomario	Baraglia	EC128_DS	--	--	--	--	--	--	--	--	--	--	--	--	--	1	--	--	--	--	--	--	5	--	--	r	--	r	1	--
PV25	Mortara	Ferraris	EC128_DS	--	--	--	--	--	--	r	--	--	--	--	--	r	1	--	--	--	r	--	--	5	1	--	r	--	--	1	1
PV 26	Lomello	Innovatech	EC128_WS	--	--	--	--	--	--	1	--	--	--	--	--	r	1	--	--	r	1	--	--	5	--	--	r	--	--	--	--
PV 27	San Giorgio di Lomellina	Innovatech	EC128_WS	--	--	--	--	--	--	--	--	--	--	--	--	--	2	--	1	r	1	--	--	5	--	--	--	--	r	1	--
PV 28	Dorno	Innovatech	EC128_DS	--	--	--	--	--	--	--	--	--	1	--	--	--	2	--	--	--	--	--	--	5	--	--	--	--	1	--	r
PV 29	Garlasco	Innovatech	EC128_WS	--	--	--	--	--	--	--	--	--	--	--	--	--	3	--	1	1	1	--	--	5	1	--	--	--	--	--	--
PV 30	Giussago	Innovatech	EC128_WS	--	--	--	--	--	--	--	--	--	1	1	--	--	2	--	r	--	1	--	--	5	--	--	--	r	--	--	1
MI 3	Carpiano	Cascina Pojago	EC128_WS	--	--	--	--	--	--	--	--	--	r	--	--	--	2	--	1	--	1	--	--	5	1	--	--	--	r	--	--

PV 31	Carbonara al Ticino	Cascina Cavallera	EC128_WS	--	--	--	--	--	--	--	--	--	--	--	--	--	3	--	1	--	--	5	--	5	--	--	--	--	--	--	--
PV 18	Lardirago	Tavazzani	ORG_DS	2	--	3	--	--	--	--	5	--	3	3	3	1	4	--	--	--	--	r	--	5	1	--	2	5	--	1	1
PV32.1	Candia di Lomellina	Tonelli	ORG_WS	--	--	--	--	r	--	--	--	--	r	--	--	1	3	--	1	--	1	--	--	5	--	--	--	r	1	--	1
PV32.2	Candia di Lomellina	Tonelli	ORG_WS	--	--	--	--	--	--	--	--	--	r	--	--	1	2	--	r	--	1	--	--	5	--	--	--	--	r	--	1

EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

The double heatmap - clustering analysis is shown in Figure 3.1. High abundances of species are highlighted with dark green color, while low ones with light green. The most spread weeds were *Echinochloa* spp., *Hetherantera* spp., *Lemna minor* L. and *Schoenoplectus mucronatus* (L.) Palla. Lardirago organic farm possessed the most diverse and richer phytocoenosis. *Oryza sativa* L. is a constant presence in each paddy.

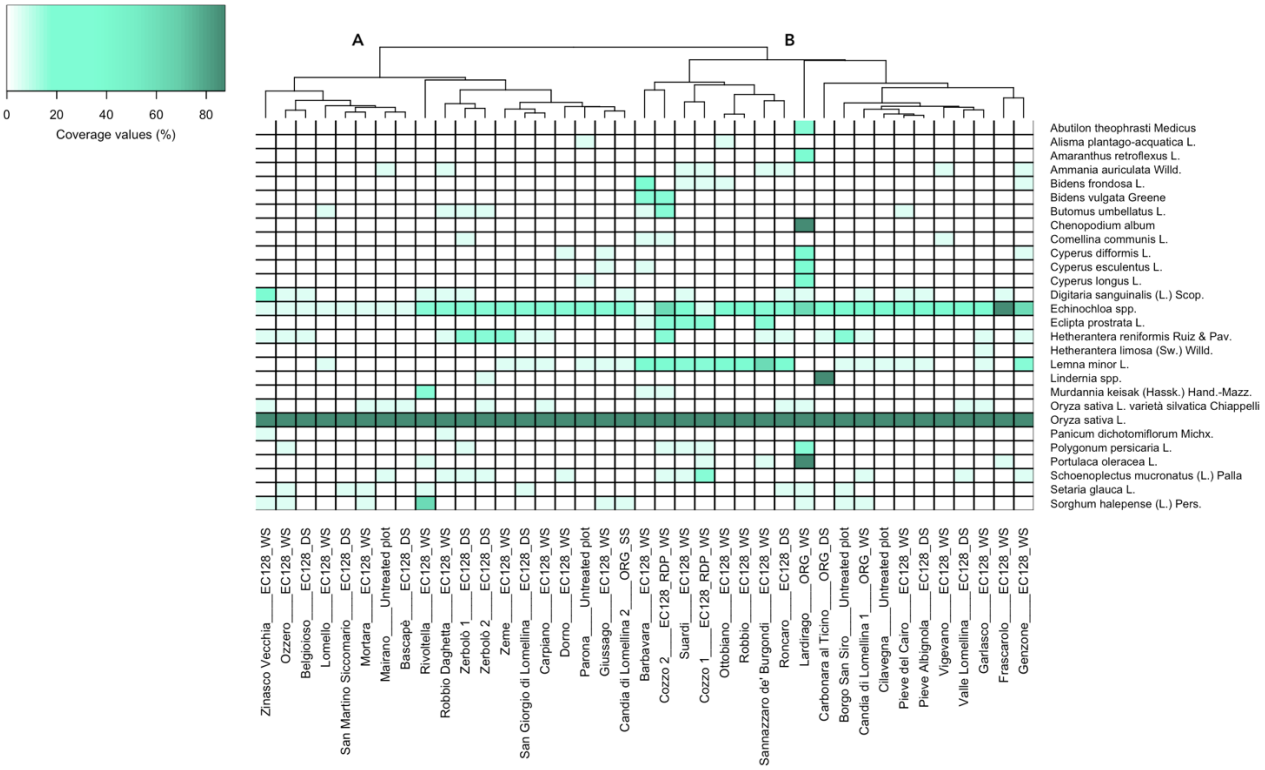

Figure 11.1: double heatmap – hierarchical clustering. High abundances of species were highlighted with dark green color, while low ones with light green. Abundance scale is based on midpoint of cover range values of Braun-Blanquet (1964). EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

Hierarchical clustering splits the SUs into two main clusters.

In cluster A are grouped 19 paddies in which lower levels of infestation and fewer plant species were observed. In general, it is noted that species of the genus *Echinochloa* are the dominant weeds, while other species are present sporadically (i.e. *Sorghum halepense* (L.) Pers., *Digitaria sanguinalis* (L.) Scop., *Murdannia keisak* (Hassk.) Hand.-Mazz., etc...). In cluster B are grouped 20 paddies in which generally higher levels of infestation and more diversified phytocoenoses were observed. Again, species of the genus *Echinochloa* are the dominant weeds. Here, other weeds are present in greater numbers and with greater abundance (i.e. *Bidens vulgata* Greene, *Butomus umbellatus* L., *Lemna minor* L., ecc…). *Echinochloa* species clearly represent the dominant weed infesting the paddies, with abundances higher than 40 / 50% particularly in paddies included in cluster B.

3.3.2 Floristic study

In Table 3.3 is reported the floristic study of the plant species surveyed in the visited SUs.

Table 3.3: floristic study

SPECIES	Raunkiær plant life-form	Chorology	Grime Strategy	Status
Abutilon theophrasti Medicus	Therophytes	Exotic	CR	Invasive Alien Species
Alisma plantago-acquatica L.	Hydrophytes	Cosmopolitan	CSR	Native
Amaranthus retroflexus L.	Therophytes	Cosmopolitan	R	Invasive Alien Species
Ammania auriculata Willd.	Therophytes	Cosmopolitan	R	Native
Bidens frondosa L.	Therophytes	Boreal	CR	Native
Bidens vulgata Greene	Therophytes	Boreal	CR	Native
Butomus umbellatus L.	Hydrophytes	Eurasian	CS	Native
Chenopodium album L.	Therophytes	Cosmopolitan	CR	Native
Comellina communis L.	Geophytes	Eurasian	CR	Invasive Alien Species
Cyperus difformis L.	Therophytes	Eurasian	SR	Alien (naturalized)
Cyperus esculentus L.	Therophytes	Eurasian	SR	Alien (naturalized)
Cyperus longus L.	Therophytes	Eurasian	SR	Alien (naturalized)
Digitaria sanguinalis (L.) Scop.	Therophytes	Cosmopolitan	R	Native
Echinochloa spp.	Therophytes	Cosmopolitan	CR	--
Eclipta prostrata L.	Therophytes	Neotropical	CSR	Alien (naturalized)
Hetherantera reniformis Ruiz & Pav.	Hydrophytes	Exotic	CS	Invasive Alien Species
Hetherantera limosa (Sw.) Willd.	Hydrophytes	Exotic	CS	Alien (naturalized)
Lemna minor L.	Hydrophytes	Cosmopolitan	CR	Native
Lindernia spp.	Therophytes	Eurasian	SR	--
Murdannia keisak (Hassk.) Hand.-Mazz.	Geophytes	Asian	CS	Invasive Alien Species
Oryza sativa L. varietà silvatica Chiappelli	Therophytes	Exotic	CR	Native
Oryza sativa L.	Therophytes	Exotic	CR	Native
Panicum dichotomiflorum Michx.	Therophytes	Exotic	CR	Invasive Alien Species
Polygonum persicaria L.	Hemicryptophytes	Cosmopolitan	R	Native
Portulaca oleracea L.	Therophytes	Cosmopolitan	R	Native
Schoenoplectus mucronatus (L.) Palla	Helophytes	Cosmopolitan	CS	Native
Setaria glauca L.	Therophytes	Cosmopolitan	CSR	Native
Sorghum halepense (L.) Pers.	Geophytes	Eurasian	C	Invasive Alien Species

Figure 3.2 represent an overall graphical analysis of the qualitative component of the specific biodiversity surveyed in the paddies of all the visited farms. Frequencies of qualitative components are based on the average coverage % of plants in all sampling sites.

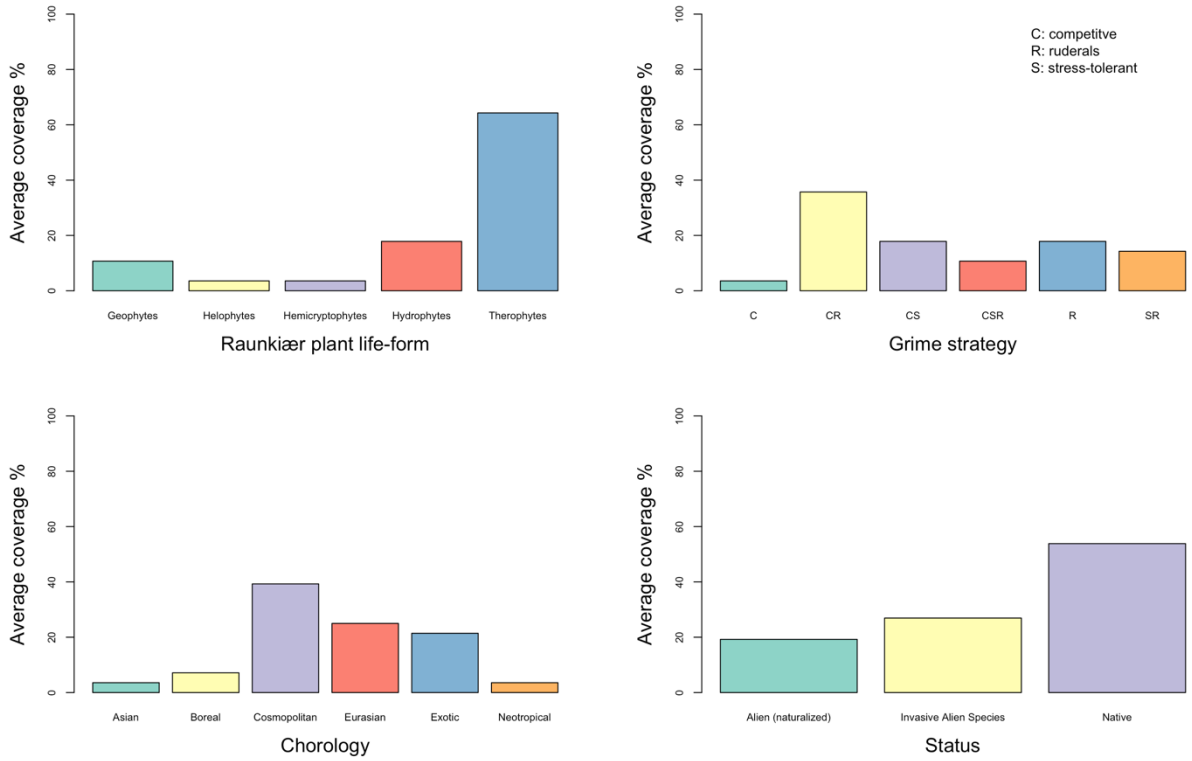

Figure 3.2: specific biodiversity qualitative component barplots

The most frequent Raunkiær plant life-form is *Therophytes* (> 60%). *Therophytes* are annual plants that complete their life cycle rapidly in favorable conditions and survive the unfavorable cold or dry season in the form of seeds. The most frequent Grime strategy is *Competitive / Ruderals* (~ 40%) and *Ruderals* (> 20%). The *Ruderal* strategy is common in severely disturbed, but potentially productive habitats. In this case, rapid colonization, rapid growth, and high reproductive rates are favored. The most frequent chorologic type is *Cosmopolitan* (> 40%). *Cosmopolitan* chorotype accounts plant species worldwide distributed. *Native* species are around 60% of the total, while *naturalized* and *invasive alien species* (IAS) ranged about 40% of the total.

Species surveyed in the visited rice fields are indicative of rapidly changing environments with high anthropogenic disturbance due to rice cropping.

3.3.3 Specific biodiversity study

In Table 3.4 are listed all the visited rice farms with the values of Margalef richness, Shannon Weaver diversity, Pielou evenness and Simpson dominance indexes of the surveyed phytocoenones.

Table 3.4: specific biodiversity quantitative component

Municipality	Management	Margalef richness	Shannon Weaver diversity	Pielou evenness	Simpson dominance
Ottobiano	EC 128/2009 – Wet seeding	2.1	1.23	0.59	0.35
Sannazzaro de' Burgondi	EC 128/2009 – Wet seeding	3.3	1.45	0.58	0.3
Suardi	EC 128/2009 – Wet seeding	2.4	1.61	0.73	0.29
Borgo San Siro	Untreated plot	1.8	1.25	0.64	0.38
Cilavegna	Untreated plot	1.2	0.76	0.47	0.54
Zeme	EC 128/2009 – Wet seeding	1.5	0.94	0.53	0.51
Robbio	EC 128/2009 – Wet seeding	1.5	1.02	0.57	0.39
Parona	Untreated plot	1.5	0.91	0.51	0.56
Vigevano	EC 128/2009 – Wet seeding	2.4	0.91	0.41	0.5
Rivoltella	EC 128/2009 – Wet seeding	2.1	1.43	0.69	0.31
Barbavara	EC 128/2009 – Wet seeding	3.3	1.73	0.7	0.25
Mairano	Untreated plot	1.5	0.75	0.42	0.67
Ozzero	EC 128/2009 – Wet seeding	2.4	1.04	0.47	0.56
Robbio Daghetta	EC 128/2009 – Wet seeding	3.3	1.3	0.52	0.44
Bascapè	EC 128/2009 – Dry seeding	1.8	0.43	0.22	0.8
Valle Lomellina	EC 128/2009 – Dry seeding	1.5	0.89	0.5	0.5
Frascarolo	EC 128/2009 – Wet seeding	1.5	0.91	0.51	0.45
Zerbolò 1	EC 128/2009 – Dry seeding	3.9	1.42	0.54	0.38
Zerbolò 2	EC 128/2009 – Dry seeding	2.7	1.3	0.56	0.41
Belgioioso	EC 128/2009 – Dry seeding	1.5	0.59	0.33	0.73
Cozzo 1	EC 128/2009 - RDP Operation 10.0.01 – Wet seeding	3.6	1.6	0.62	0.32
Cozzo 2	EC 128/2009 - RDP Operation 10.0.01 – Wet seeding	4.5	1.97	0.71	0.18
Pieve del Cairo	EC 128/2009 – Wet seeding	1.8	1.01	0.52	0.46
Genzone	EC 128/2009 – Wet seeding	2.1	1.42	0.68	0.32
Roncaro	EC 128/2009 – Wet seeding	3.9	1.46	0.55	0.34
Zinasco Vecchia	EC 128/2009 – Dry seeding	3.3	1.25	0.5	0.41
Pieve Albignola	EC 128/2009 – Wet seeding	2.1	0.9	0.43	0.5
San Martino Siccomario	EC 128/2009 – Dry seeding	1.2	0.42	0.26	0.81
Mortara	EC 128/2009 – Dry seeding	2.4	0.77	0.35	0.67
Lomello	EC 128/2009 – Wet seeding	1.8	0.6	0.31	0.73
San Giorgio di Lomellina	EC 128/2009 – Wet seeding	1.8	0.92	0.47	0.56
Dorno	EC 128/2009 – Dry seeding	1.2	0.77	0.48	0.6
Garlasco	EC 128/2009 – Wet seeding	1.5	1.12	0.62	0.44
Giussago	EC 128/2009 – Wet seeding	2.1	1.05	0.51	0.51
Carpiano	EC 128/2009 – Wet seeding	1.8	0.92	0.47	0.56
Carbonara al Ticino	EC 128/2009 – Wet seeding	0.9	1.12	0.81	0.35
Lardirago	Organic – Dry seeding	4.2	2.3	0.85	0.12
Candia di Lomellina 1	Organic – Wet seeding	2.7	1.24	0.54	0.41
Candia di Lomellina 2	Organic – Wet seeding	2.1	0.93	0.45	0.56

RDP: rural development program; EC 128/2009: Directive 128/2009 of European Community

Overall, the highest values of richness were recorded at the organic farms of Lardirago and Candia di Lomellina and at the farm of Cozzo in which integrated rice cropping is performed. High richness values are recorded also at Sannazzaro de'Burgondi, Barbavara, Robbio (Daghetta) and Zerbolò, where rice cropping follows Directive EC 128/2009. A similar trend was observed for the index of diversity, with high values recorded in the farm of Lardirago, Candia di Lomellina and Cozzo. The highest values of evenness

were found at Cozzo, Carbonara al Ticino and Lardirago. The highest dominance values were recorded at Bascapè, Belgioioso, San Martino Siccomario and Lomello. The values of these indices depict more diverse and balanced phytocoenoses in organic and integrated rice fields, less species-rich and diverse in those following Directive EC 128/2009.

3.4 Conclusions

This study provided a description of the weed phytocoenoses found in paddy fields managed by different agronomic techniques.

The results of the floristic analysis show that the weed flora is on the whole dominated by *Therophytes*, annual species that generally abound in ruderal environments and to which most of the crop weed species belong. Cosmopolitan are the most distributed species, followed by Eurasians. High intensity of disturbance is indicated by weeds belonging to Grime's category of Ruderal / Competitive, characteristic of habitats with high productivity and where competition is hindered by the action of disturbance, and of Ruderal, indicative of a habitat exposed to intense and repeated disturbance (Grime, 2001).

Quantitative analysis of biodiversity shows that the predominant weed species in all rice fields are those of the genus *Echinochloa*. Overall, the richness, diversity and equitability indices are rather low, while the dominance index is higher. In conclusion, paddy field communities are floristically rather poor, typical of situations where one or a few limiting factors influence their ecology, and characterized by low values of the indices of richness, diversity and equitability, and higher dominance index.

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Chapter 4 – Chemical-physical soil analysis

Chapter 4 is devoted to the chemical and physical analysis of paddy soils. Since the adaptive responses of an epigenetic nature of the weeds could be stimulated by particular edaphic chemical/physical conditions, a characterization of the soils of the SUs visited was performed.

4.1 Introduction

Rice-growing areas are characterized by soils that are specially managed for rice cultivation. During the growing season, management practices include field preparation, plowing, flooding, and maintaining a layer of standing water during cultivation, which is followed by drainage and field drying. For these reasons, rice soils are often found in landscapes where surface and internal soil drainage is poor, thus in deltas and adjacent floodplains, valleys and coastal plains.

The chemical composition of paddy soils is variable precisely because of continuous tillage processes that cause imbalance. For example, during the period of flooding, the oxygen supply to the soil is zero. Within a day or two after flooding, aerobic microbes consume the trapped oxygen and render the soil anoxic except for a thin surface layer. Drainage and drying reverse these changes, rebalancing the oxygen concentration in the soil (Ponnamperuma, 1981). As a matter of facts, paddy soils are characterized by anaerobic conditions that persist throughout the agricultural season. These soils occupy an important niche in the biosphere and their importance is widely recognized. Anaerobic soils are primary sources of nutrients for plants grown in rice fields or wetlands. The decomposition process of organic matter in the absence of molecular oxygen leads to the production of bioavailable nitrogen and phosphorus, which promote plant productivity (ENR[13], 2020). Furthermore, pesticides (herbicides, insecticides, fungicides, etc…) administration in paddy fields during the flooding season can easily cause soils pollution due to run-off or drainage of the chemicals and also due to their persistence. Therefore, it is crucial to know the chemical-physical and adsorption characteristics typical of each soil in order to predict the persistence of herbicides, and of pesticides in general, in rice fields and thus prevent their toxicity (Kibe et al., 2000).

As a matter of facts, crops and weeds, as well as microorganisms that inhabit this environment, are well adapted to live, survive and reproduce in presence of several stressors typical of agricultural lands (i.e. tillage, anaerobiosis, poor or rich micronutrients

[13] Ente Nazionale Risi - https://www.enterisi.it/servizi/notizie/notizie_homepage.aspx

availability, presence of pesticides in the substrate, etc…). If we consider that epigenetics (miRNAs, DNA methylation, etc…) acts in regulating plant adaptive responses to biotic and abiotic stresses and help restore cell homeostasis upon sudden environmental changes, it would be useful to analyze soil chemical and physical characteristics of each sampling unit (SU) in order to understand a possible relation between them, the adaptive responses of plants against such stressors and the onset of herbicide resistance.

The aim of this study was to obtain information about paddy soils chemistry and physics that will be useful to further analyze their relation with plants adaptation mechanisms leading to herbicide resistance occurrence.

4.2 Materials & Methods

4.2.1 Sampling

Sampling was conducted as described in Chapter 2, paragraph 2.3 "Sampling". Soil collection was conducted following *non-systematic X method* (Lambkin, 2004) in all the 39 visited SU. ~ 1 kg of soil was collected in each SU.

4.2.2 Chemical and Physical Analysis

The analysis of soil chemistry and physic were performed by MAC – Minoprio Analisi e Certificazioni s.r.l. in Fondazione Minoprio, Vertemate con Minoprio (CO), Italy. The following analyses were performed:

1. Soil particle size analysis (g/kg): *coarse soil, sand soil, silt soil, clay soil*
2. pH H_2O
3. pH $CaCl_2$
4. $CaCO_3$ concentration (g/kg): *CaCO3*
5. active $CaCO_3$ concentration (g/kg): *CaCO3_active*
6. organic matter (g/kg): *S_org*
7. organic carbon: *C_org*
8. total nitrogen (g/kg): *N_tot*
9. carbon/nitrogen ratio: *C/N*
10. cation exchange capacity (meq/100g): *CexC*
11. exchangeable calcium (meq/100g): *Ca*
12. exchangeable magnesium (meq/100g): *Mg*
13. exchangeable sodium (meq/100g): *Na*
14. exchangeable potassium (meq/100g): *K*
15. base saturation (%): *BS*
16. calcium to magnesium ratio: *Ca/Mg*
17. magnesium to potassium ratio: *Mg/K*
18. exchangeable sodium percentage (%): *exNa*
19. assimilable phosphorus (Olsen method) (mg/kg): *P_ass*

4.2.3 Statistical Analysis

Heatmap – hierarchical clustering analysis was performed, based on "Canberra" distance and "ward.D2" algorithm to maximize the variance between clusters (function "veg.dist" – package vegan (Oksanen, 2020), to obtain a graphical classification and characterization of soils based on their chemistry and physics and to analyze the contribution of all measured parameters to differentiate paddy soils (function "heatmap.2" - package gplots (Warnes et al., 2020)). Measured soil parameters were scaled in order to compare them among samples, since possess different units. Spearmann correlation was calculated in order to evaluate possible correlation among parameters analyzed (function "cor.test" – package stats (R Core Team, 2019)). Correlations were plotted as corrplot (function "corrplot" – packages GGally (Schloerke et al., 2021), Hmisc (Harrell, 2021), corrplot (Wei et al 2021)).

The entire statistical analysis was carried out using R 3.6.3 software.

4.3 Results and Discussion

In Table 4.1 are listed the values of the analyzed parameters recorded in all the sample units.

Table 4.1: chemical and physical parameters analyzed

Sample	Farming technique	coarse_soil	sand_soil	silt_soil	clay_soil	pH_H2O	pH_CaCl2	CaCO3	CaCO3_active	S_org	C_org	N_tot	C/N	CexC	Ca	Mg	K	Na	BS	Ca/Mg	Mg/K	exNa	P_ass
Ottobiano	EC128_WS	6	471	425	104	5.7	5.3	0	0	18	10.6	1.2	8.8	10.9	2.94	0.72	0.1	0.06	35.05	4.1	7.2	0.55	52
Sannazzaro de' Burgondi	EC128_WS	60	531	375	94	7.1	6.7	0	0	21	12	1.4	8.6	11.8	7.73	1.07	0.25	0.13	77.8	7.2	4.3	1.1	100
Suardi	EC128_WS	10	281	595	124	7	6.6	0	0	28	16.4	1.7	9.6	15	8.66	1.66	0.16	0.09	70.47	5.2	10.4	0.6	30
Borgo San Siro	Untreated plot	6	651	275	74	5.8	5.3	0	0	12	7.1	0.9	7.9	7.5	1.99	0.47	0.22	0.08	36.8	4.2	2.1	1.07	74
Cilavegna	Untreated plot	4	481	435	84	5.6	5.1	0	0	20	11.4	1.4	8.1	10.6	2.63	0.71	0.19	0.03	33.58	3.7	3.7	0.28	44
Zeme	EC128_WS	0	591	345	64	5.7	5.3	0	0	25	14.6	1.6	9.1	11.4	3.38	0.7	0.21	0.06	38.16	4.8	3.3	0.53	53
Robbio	EC128_WS	0	411	465	124	5.5	5	0	0	26	15.2	1.7	8.9	14.5	4.39	1.08	0.25	0.1	40.14	4.1	4.3	0.69	30
Parona	Untreated plot	2	891	55	54	6	5.4	0	0	14	8.2	0.9	9.1	4.9	1.31	0.21	0.08	0.03	33.27	6.2	2.6	0.61	22
Vigevano	EC128_WS	4	531	395	74	5.6	5.2	0	0	13	7.8	1.1	7.1	7.2	1.69	0.2	0.08	0.02	27.64	8.5	2.5	0.28	47
Rivoltella	EC128_WS	4	491	405	104	6.2	5.6	0	0	17	9.9	1.2	8.3	10.9	3.42	0.94	0.14	0.03	41.56	3.6	6.7	0.28	43
Barbavara	EC128_WS	176	551	375	74	6	5.4	0	0	33	18.9	2.1	9	13.4	3.54	0.91	0.15	0.08	34.93	3.9	6.1	0.6	36
Mairano	Untreated plot	0	551	355	94	6.5	5.8	0	0	13	7.7	0.9	8.6	9.2	3.46	0.79	0.15	0.07	48.59	4.4	5.3	0.76	34
Ozzero	EC128_WS	78	471	445	84	5.1	5.7	0	0	18	10.4	1.2	8.7	10.6	4.23	0.57	0.19	0.05	47.55	7.4	3	0.47	38
Robbio Daghetta	EC128_WS	0	371	535	94	5.9	5.5	0	0	26	15.2	1.6	9.5	12.9	4.31	0.93	0.37	0.1	44.26	4.6	2.5	0.78	65
Bascapè	EC128_DS	6	411	535	54	6.7	6.1	0	0	27	15.6	1.7	9.2	11.4	6.34	0.83	0.13	0.3	66.67	7.6	6.4	2.63	56
Valle Lomellina	EC128_DS	6	361	545	94	5.4	5	0	0	14	8.3	1	8.3	9.9	2.92	0.72	0.21	0.05	39.39	4.1	3.4	0.51	62
Frascarolo	EC128_WS	158	451	425	124	6	5.5	0	0	14	8.2	1	8.2	11.3	4.94	1	0.14	0.07	54.42	4.9	7.1	0.62	24
Zerbolò 1	EC128_DS	128	711	235	54	6.1	5.6	0	0	26	15.3	1.7	9	10.3	3.51	0.49	0.06	0.06	40	7.2	8.2	0.58	9
Zerbolò 2	EC128_DS	88	631	315	54	6.6	6	0	0	31	17.9	1.9	9.4	11.8	5.09	0.83	0.21	0.1	52.8	6.1	4	0.85	46
Belgioioso	EC128_DS	36	380	518	102	6.6	5.8	0	0	27	15.5	1.4	11.1	14.4	8.06	1.46	0.16	0.1	67.92	5.5	9.1	0.69	29
Cozzo 1	EC128_RDP_WS	0	220	588	192	6.7	6.1	0	0	23	13.1	0.9	14.6	16.7	8.75	2	0.22	0.1	66.29	4.4	9.1	0.6	9
Cozzo 2	EC128_RDP_WS	4	650	268	82	6.5	5.7	0	0	20	11.7	0.9	13	8	2.8	0.68	0.12	0.03	45.38	4.1	5.7	0.38	8
Pieve del Cairo	EC128_WS	8	120	698	182	7.1	6.7	0	0	30	17.4	1.6	10.9	20.5	23.98	2.27	0.2	0.1	129.51	10.6	11.4	0.49	8
Genzone	EC128_WS	30	500	408	92	7.4	6.7	48	5	53	25.24	2.3	11	18.8	8.56	2.28	1.42	0.05	65.48	3.8	1.6	0.27	107
Roncaro	EC128_WS	0	420	438	142	6.4	5.9	0	0	19	10.9	0.9	12.1	12.7	4.89	1.02	0.19	0.25	50	4.8	5.4	1.97	20
Zinasco Vecchia	EC128_DS	0	620	288	92	5.6	4.9	0	0	11	6.4	0.6	10.7	8.9	4.84	1.03	0.19	0.26	71.01	4.7	5.4	2.92	78
Pieve Albignola	EC128_WS	4	300	588	112	8.2	7.3	43	6	18	10.54	0.9	11.7	11	7.01	0.89	0.22	0.04	74.18	7.9	4	0.36	60
San Martino Siccomario	EC128_DS	10	720	198	82	6.2	5.6	0	0	25	14.3	1.1	13	9.8	2.21	0.44	0.1	0.03	28.37	5	4.4	0.31	10
Mortara	EC128_DS	0	830	108	62	6.2	5.7	0	0	23	13.2	1	13.2	9.1	2.24	0.36	0.03	0.02	29.12	6.2	12	0.22	31
Lomello	EC128_WS	4	410	508	82	6.1	5.5	0	0	14	8.1	0.6	13.5	8.8	2.84	0.82	0.12	0.04	43.41	3.5	6.8	0.45	33
San Giorgio di Lomellina	EC128_WS	4	660	258	82	6.1	5.8	0	0	24	13.9	1.1	12.6	11.2	3.41	0.71	0.27	0.08	39.91	4.8	2.6	0.71	71
Dorno	EC128_DS	0	560	358	82	5.9	5.1	0	0	15	8.8	0.7	12.6	8.5	1.77	0.31	0.04	0.04	25.41	5.7	7.8	0.47	15
Garlasco	EC128_WS	14	520	378	102	5.8	5.4	0	0	16	9.2	0.8	11.5	9.8	3.02	0.54	0.23	0.07	39.39	5.6	2.3	0.71	32
Giussago	EC128_WS	12	580	328	92	6.4	5.7	0	0	16	9.5	0.8	11.9	9.6	3.95	0.71	0.34	0.03	52.4	5.6	2.1	0.31	45
Carpiano	EC128_WS	44	470	438	92	6.1	5.4	0	0	29	17.1	1.4	12.2	12.2	4.28	0.67	0.11	0.08	42.13	6.4	6.1	0.66	35
Carbonara al Ticino	EC128_WS	4	590	348	62	5.7	5.1	0	0	17	9.9	0.8	12.4	8.5	2.32	0.51	0.11	0.04	35.06	4.5	4.6	0.47	28
Lardirago	ORG_DS	82	620	318	62	6.3	5.7	0	0	21	12.2	1	12.2	9.2	3.46	0.88	0.35	0.01	51.09	3.9	2.5	0.11	63
Candia di Lomellina 1	ORG_WS	22	400	538	62	5.8	5.1	0	0	19	11.1	0.7	15.9	8.9	2.32	0.71	0.08	0.06	35.62	3.3	8.9	0.67	31
Candia di Lomellina 2	ORG_WS	4	340	598	62	5.3	4.8	0	0	35	20.3	1.8	11.3	13.8	2.48	0.79	0.12	0.06	25	3.1	6.6	0.43	18

EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

Heatmap – hierarchical clustering in Figure 4.1 shows a characterization of soils based on the analyzed chemical and physical parameters.

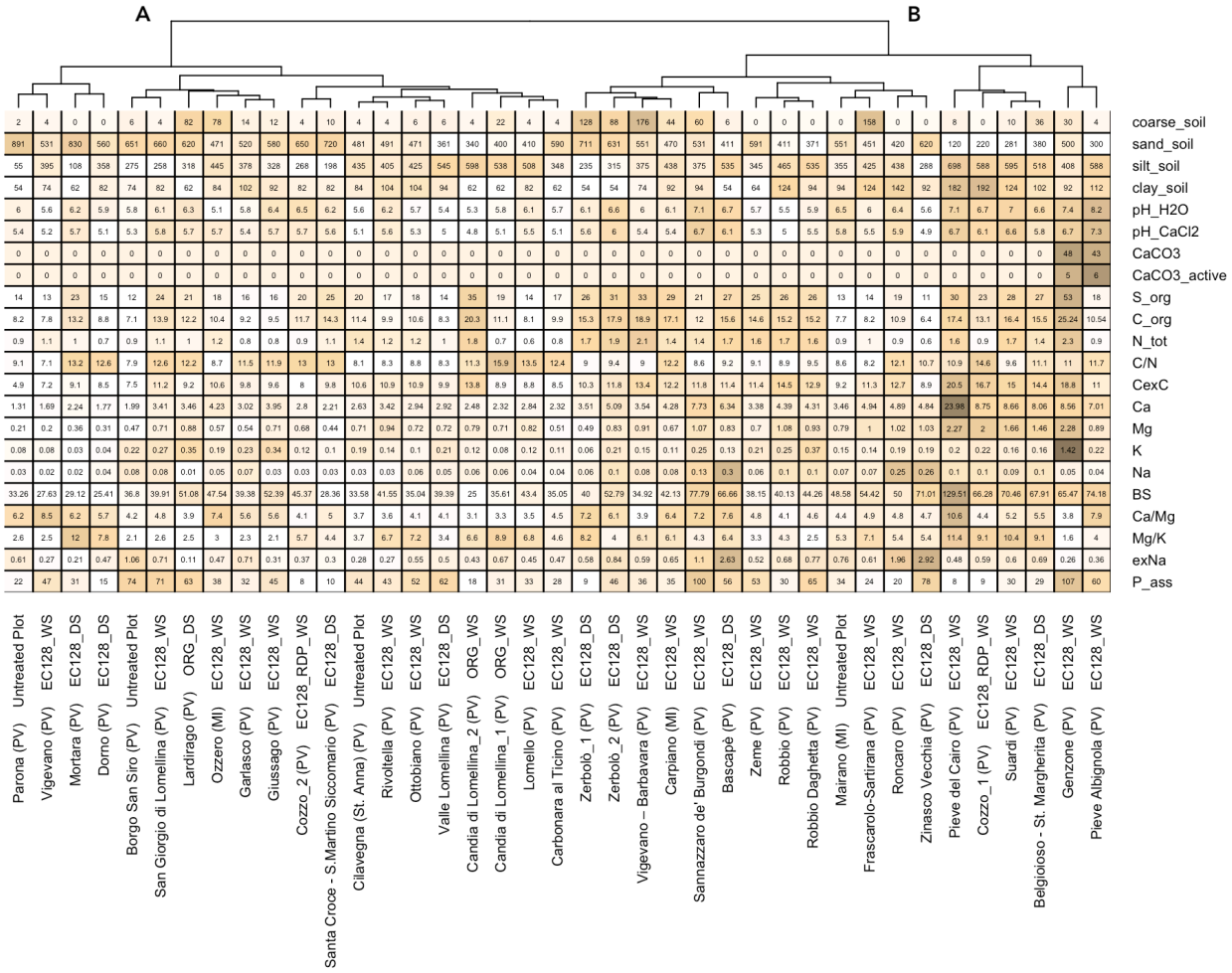

Figure 4.1: double heatmap - hierarchical clustering of soil chemical and physical features analyzed. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

Dark brown color indicates high values assumed by each variable, while low values were marked with white / ochre color. Hierarchical clustering groups soil samples into two clusters on the basis of the analyzed chemical and physical parameters.

Cluster A comprises 20 soil samples in which a low content of macronutrients was recorded, in comparison with soils comprised in cluster B. The only soil samples that possess a fine particle texture (silt) and high concentration of C, N and organic matter (S_org) belong to the organic paddies of Candia di Lomellina. Almost all samples of this

cluster possess a sandy texture and acidic pH. Cluster B includes 19 soil samples characterized by a high content of macronutrients (C, N, P, K, Mg, Na and Ca). In general, samples of cluster B are fine soils (silt/clay texture) richer in nutrients.

Correlogram in Figure 4.2 shows the correlations existing between the analyzed chemical / physical soil features. Red eclipses are positive correlations ($R \geq 0.8$), blue eclipses are negative correlations ($R \leq -0.8$). Stars indicate significant correlations ($P < 0.05$).

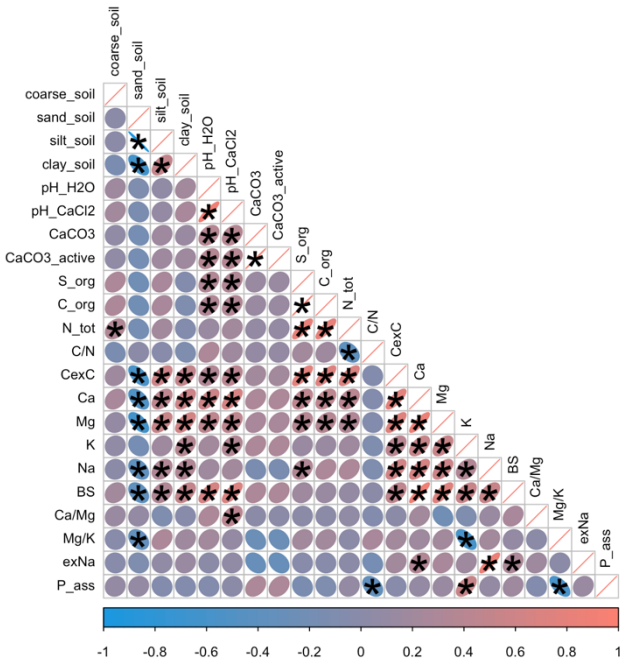

Figure 4.2: Correlogram of soils chemical and physical features

There are significant strong correlations (positive and negative) between particles size (sand, silt and clay) and Ca, Mg, K, Na and degree of saturation in bases (BS). In general, fine soils better retain micronutrients than coarse ones. The majority of farms characterized by fine soils is grouped in cluster B of the double heatmap – hierarchical clustering in Figure 5 (Pieve del Cairo, Cozzo1, Suardi, Belgioioso, Genzone and Pieve Albignola). Significant strong correlations resulted also between pH of H_2O and of $CaCO_3$ and C, N, organic matter, Ca, Mg and K.

4.4 Conclusions

Rice soils are specially managed soils for wet rice cultivation. Management practices include land leveling and construction of embankments to contain water, plowing and harrowing water-saturated soil, and maintaining a layer of standing water during the rice growing months. Soils are finally dried at harvest time.

All these practices lead to a continuous and sudden change in the ecological conditions of the paddy field, which is characterized as possessing very peculiar chemical/physical conditions (Ponnamperuma, 1981, Narteh and Sahrawat, 1999).

A broader understanding of the above mentioned chemical and physical characteristics would enable to obtain a better view of the ecology of paddy field ecosystem. This would allow to deepen the study of phenomena that regulate plant adaptive responses and lead to the manifestation of herbicide resistance, and to understand which are the most relevant biotic and abiotic edaphic factors in this context.

4.5 References

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Chapter 5 – Paddy soil microbiota analysis

Chapter 5 describes the microbiological analysis of paddy soils. The presence of fungal and bacterial strains was analyzed by two approaches: the conventional plate culture and the metagenomic approach.

In the former, soils collected were inoculated onto specific growth media. Subsequently, fungal and bacterial strains were isolated and identified.

In the second, eDNA (environmental DNA) was extracted from the soils collected and metagenomic libraries were subsequently produced through selective amplification of fungi (ITS barcoding) and bacteria (16S barcoding). Hence fungal and bacterial strains were identified.

5.1 Introduction

5.1.1 The Soil

Soil is defined as a complex structure consisting of physical, chemical and biological elements that, being in continuous interaction with each other, make it a living system. The soil is recognizable in the surface layer, more or less thin, of the Earth's crust and is affected by meteoric agents together with living organisms. It is organized in overlapping horizontal layers composed of minerals and organic matter which provide sustenance to plant growth and to the community of microorganisms present (United States Department of Agriculture - Natural Resources Conservation Service, 1999). Soil horizontal layers, that overlap in succession one after the other, are called horizons, each with its own physical, chemical and biological characteristics, which together contribute to defining the profile of the section of soil considered. Starting from the surface are recognized in order:

a. Horizon O, or organic layer: the layer of litter in turn divided into a surface layer (Oi) composed of unaltered leaves and twigs; central layer (Oe) consisting of partially degraded plant tissues; basal stratus (Oa) or humus layer, a homogeneous dark brown/black organic material resulting from the decomposition processes carried out by the microflora and microfauna present (Smith and Smith, 2017).
b. Horizon A, topsoil or surface soil: mineral soil derived from parental materials in which leached humus from the upper layer is also found. At this level it is sometimes observed the presence of an area characterized by maximum leaching or eluviation (horizon E) in which the minerals and the finest particles are lost together with the water that passes through the layer moving deeply (Smith and Smith, 2017).
c. Horizon B, subsoil: layer characterized by an accumulation of mineral particles and salts leached from topsoil (process of illuviation); the distinction between horizons B is based on the color, structure and type of accumulated material (Smith and Smith, 2017).
d. Horizon C: unconsolidated rocky parental material from which the soil originated; it is located below the zones of biological activity and has not been affected by pedogenetic processes (Smith and Smith, 2017).
e. Mother Rock (Smith and Smith, 2017).

Often in nature it is not possible to distinguish horizons A and B because they are mixed together in the so-called horizon Ap (p from plow, "plough") created by the anthropic action of tillage for agricultural purposes (Smith and Smith, 2017).

From a physical point of view, soil is defined by parameters such as color, depth, texture and porosity. The first is closely linked to the mineral composition of the soil and the abundance of organic matter present (a greater amount of humus is responsible for a darker soil) and is evaluated by means of the appropriate Munsen color system. The depth varies in different areas of the territory according to the slope, the degree of alteration of the parental material and vegetation. For classification purposes, the lower limit of soil has been conventionally set at a depth of 200 centimeters (Soil Taxonomy, 1999).

The particle size composition of the soil indicates the relative percentage distribution of sand, silt and clay particles within it. The texture affects the porosity of the soil, i.e. the volume of empty spaces in percentage ratio to the total volume of the soil considered. A soil characterized by coarse texture has wide pores that allow free movement of air, water and roots. On the contrary, soils in which the particles are reduced dimensionally are very compact and consequently poorly ventilated and difficult to penetrate by the root systems of plants. It is also responsible for some fundamental properties of the soil. Clay plays an important role in determining the water retention capacity and ion exchange between particles and soil solution. Water capacity or field capacity is the measure of the amount of water retained by the soil against gravity (i.e. how much water the pores of the soil can accommodate). The exchange of cations is the phenomenon of substitution of mineral cations (usually H^+, Ca^{2+}, K^+, Na^+, Mg^{2+}, Al^{3+}, NH^{4+}), weakly bound to the surface of soil particles, with other cations present in the soil. Mineral anions such as nitrate (NO_3^-), phosphate (PO_3^{4-}) and chloride (Cl^-) are instead repelled by negative charges placed on the surface of the particles, called colloids, and thus remain dissolved in the soil solution. In most agricultural soils present in the temperate zone, cation exchange is prevalent over anionic exchange and consistently affects soil fertility and quality, as the presence of negative charges prevents loss due to leaching of positively charged nutrients (Taiz and Zeiger, 2013).

Loams are soils containing sand, silt and clay in proportions ideal for agriculture: coarser particles promote drainage, while smaller ones have high nutrient retention capacity (Evert and Eichhorn, 2013).

5.1.2 The functions of soil

The soil performs fundamental functions for maintaining the proper functioning of the biosphere: a) it allows and supports the development of plants; b) governs the terrestrial biogeochemical cycles of elements such as carbon, nitrogen, sulfur and phosphorus; c) it is a source of mineral nutrients; d) its chemical composition and texture characterize the territory to which it belongs; e) it is home to the main decomposition processes carried out by the microorganisms present there; f) it assists and hosts the interaction phenomena of both positive beneficial and negative pathogenic nature between the root systems of plants and different organisms; g) fertility allows its use for agricultural purposes (Nakhro and Dkhar, 2010; Kirk et al., 2004; Garbeva et al., 2004; Nannipieri et al., 2003).

In paddy soils, methane (CH_4) is formed and emitted into the atmosphere. After irrigation, paddy soil becomes an increasing anaerobic environment and forms a suitable environment for methanogenic bacteria with decrease of soil redox potential. The dominant species are methanogen which influence directly formation of methane and the flux of methane emission into the atmosphere. These species are affected by various environmental factors such as the chemical and physical properties of soils, the application of inorganic and organic fertilizers, the pattern of irrigation and the phytocoenoses of these environments (Min et al., 1997).

5.1.3 Microbiota and soil microbiome

From a biological point of view, soil is a complex microhabitat populated by arthropods, nematodes, protozoa, fungi, bacteria, viruses and archaea, all connected to each other members of an intricate trophic network that makes use of the enormous amount of

nutrients released by plants in the soil. In particular, fungi and bacteria are responsible for more than 80% of the reactions that occur in the soil (Mendes et al., 2013). The coexistence in this environment of these organisms has been recently studied (De Menezes et al., 2017).

The difference in terminology between microbiota and microbiome should be clarified. The microbiota is the set of populations of microorganisms that colonize a given environment and maintain relationships with its components. When considering the totality of the genetic heritage, the term microbiome is used, that is the set of all the genes that the microbiota is able to express. In exclusive reference to fungal communities, the terms mycobiota and mycobiome have been adopted; considering the whole genome of plants and organisms closely related to them, we speak of phytobiome (Kim and Lee, 2020).

The region of the soil where microorganisms are present in greater abundance and exert their activity in a very intense way is undoubtedly the rhizosphere, or the area located immediately outside the root system of the plant. The maintenance of contact between roots and microbiota is facilitated by the low variation of humidity and the high concentration of nutrients due to the phenomenon of *rhizoposition*: the roots in fact release substances such as high and low molecular weight exudates (acids, vitamins, amino acids, polysaccharides, ectoenzymes), mucigels and lysates that can be used for metabolic purposes and make the soil an ideal place for the establishment of microbial communities (Dehò and Galli, 2014). In the rhizosphere the massive presence of bacteria and fungi has a beneficial influence on plants because, in addition to providing them with nutrients, they act as antagonists against plant pathogens by producing antibiotics and secondary metabolites or modulating their physiology (Tyc et al., 2017; Chapelle et al., 2016). Most of the bacterial groups characterized by the suppression of pathogens to prevent the onset of diseases belong to the phyla Proteobacteria, Firmicutes and Actinobacteria (Mendes et al., 2011). Another function to be attributed to the rhizospheric microbiota is the protection of the soil and plants from abiotic stresses such as drought and pollution. This contributes to increasing the resilience of the soil, i.e. its natural ability to react to perturbation events and return to equilibrium ecosystem conditions (Garbeva et al., 2004). In the rhizosphere Gram negative rod-shaped bacteria generally prevail while Gram positive are less represented.

We find in great abundance ammonifying, nitrifying (*Nitrosospira* and *Nitrosomonas* spp.) and denitrifying bacteria (*Pseudomonas* spp.) which need for their growth vitamin B and amino acids present in root exudates. This is also the seat of microorganisms that establish interactions with the plant itself: examples of great relevance are the symbiotic nitrogen-fixing bacteria (*Rhizobium* spp.) or free-living (*Azotobacter* spp.) that transform atmospheric nitrogen into forms assimilable by plants, mycorrhizal fungi such as endophytes belonging to the phylum *Glomeromycota*, which promote the intake of minerals by vascular plants, finally, rhizobacteria promoting plant growth (Plant Growth Promoting Rhizobacteria, PGPR) (Kim and Lee, 2020; Mendes et al., 2013). Analyzing the the soil in metabolic terms, it is possible to observe the so-called soil microbial loop, i.e. the process of supplying carbon to decomposer organisms in the rhizosphere by means of exudates released by the root system, which promotes the growth of the microbial population and the degradation of the organic matter of the soil. Predation by microbivores (protozoa and nematodes that feed on bacteria) results in the release of nutrients accumulated in microbial biomass, enriching the soil in mineral salts useful for the growth and development of the plants themselves (Smith and Smith, 2017; Coleman, 1994).

Together with the rhizosphere, the most studied soil compartment in terms of microbial composition is the bulk soil. It is the storage department of organic matter, water and minerals and hosts the biogeochemical reactions related to nutrient cycles. The microbial community plays a crucial role in ensuring the maintenance of these cycles and in increasing the availability of organic and inorganic substances by means of decomposition processes (Kim and Lee, 2020). The bulk soil microbiota is characterized by a high degree of heterogeneity given by variations in edaphic factors such as chemical properties related to elemental mineral composition, humidity and hydraulic conductivity, air, pH, ion exchange capacity, geographical position, depth of the soil and duration of the possible cultivation period (Taiz and Zeiger, 2013). It has been observed that the variability in microbial diversity is also partly due to the different size classes to which soil particles may belong: soils with finer texture are characterized by a more varied and composite bacterial and

fungal composition than sandy soils, consisting mainly of large mineral particles (Torsvik and Øvreås, 2002).

The fungal community hosted in the soil also contributes to the maintenance of the functions of the terrestrial ecosystem. In particular, saprophytic microfungi play a fundamental role in the processes of decomposition and recycling of nutrients. The fungal diversity reflects the different types of substrate that can occur within the organic matter; in fact, a succession of changes has been observed in the community of microfungi based on their ability to degrade the substrate. Higher concentration of fungi is found in the most superficial soil layers: horizon A or topsoil includes most of the active mycelium. In the soil of natural environments or influenced by anthropic activities such as agricultural practice, it is possible to distinguish the presence of keratinolytic microfungi (capable of digesting α-keratins, fibrous proteins very resistant due to the high content of cysteine) and keratinophils (which exploit for metabolic purposes materials naturally associated with keratins or deriving from their degradation) as the genera *Arthroderma* and *Chrysosporium* (Persiani et al. , 2011). In addition, importance should also be attributed to the numerous fungi (*Glomeromycota* spp. and many others) that establish mycorrhizal interactions with the root systems of vascular plants. The symbiotic association improves the absorption of nutrients (especially phosphate) and the detoxification capacity of the plant, stimulates the production of phytohormones, the primary immune response to any pathogens and increases the resistance of the plant to abiotic stresses (Kim and Lee, 2020).

Understanding the microbial composition of soils intended for agricultural exploitation is of fundamental importance to know the impact of the different cultivation techniques on the variety of microorganisms that contribute to the maintenance of cultivated plants in good health conditions and to evaluate the most efficient cultivation practices and attentive to the environmental balances characterizing the agroecosystem.

In 2010 the Earth Microbiome Project (EMP, https://earthmicrobiome.org/) was launched. It is an ambitious project involving scientists and independent research centers from all over the planet with the aim of creating the largest database of the terrestrial microbiome ever made. This will allow to evaluate in the future the impact of cultivation

techniques, economic activities and environmental pollutants and therefore the data that will derive from them will be fundamental to use strategies aimed at improving the environment and agri-food products. The EMP aims to outline a profile of each type of ecosystem by defining it based on the composition of the microbial communities present and the mutual interactions. The collected sequences are used as references for comparisons for identification purposes with those isolated from other environmental samples (Gilbert et al., 2014; Vogel et al., 2009).

Understanding the complexity of soil microbial composition and the impact of agricultural practices on soil microbiota is critical for more sustainable agriculture. The ability of soil to perform its functions is often referred to soil quality (Menta, 2011) which depends essentially on the high, but still partially unknown, biodiversity. It is well known that the set of microbial communities is only partly determined by environmental conditions (Schmidt et al., 2014) and it is known that individual taxa are influenced differently by the physicochemical characteristics of the soil, supporting the hypothesis that the structure of the microbiome can be manipulated (Leff et al. , 2015). Large-scale studies have shown that fungal communities show a clear pattern of geographic clustering (Peay et al., 2010) and the diversity of fungal communities is influenced by a variety of climatic and edaphic factors (Tedersoo et al., 2014; Maestre et al., 2015). Land use has long-term effects on the structure and diversity of soil microbiota (Goss-Souza et al., 2017). Both these parameters are consistently altered by high levels of nutrient intake linked to human activities (Leff et al., 2015).

The effects of agricultural management are complex and appear variable (Bunemann et al., 2006; Carbonetto et al., 2014). It is widely recognized that organic farming fosters greater abundance and diversity of microbiota and macrobiota, but data on microbial communities are still largely unknown (Postma-Blaauw et al., 2010). It is known that the adoption of limited processing systems, less harmful to soils, increases soil organic matter, water content and crop yields (Alvarez and Steinbach, 2009). However, the effects of different management on the microbiota are still poorly understood. The characterization of fungal and bacterial communities colonizing the same environment is important to

highlight the effects of their interactions due to a strong antagonism between bacteria and fungi (Barham et al., 2018; Shaw et al., 2019). A widely recognized indicator of soil quality is based on soil fauna (Cortet et al., 1999; Van Straalen, 2004; Parisi et al., 2005; Mint and Remelli, 2020). It may also be useful to monitor the effects of environmental changes on microorganisms within the soil (Jeffery and Gardi, 2010) to evaluate the relationship between microfauna and microbial components still little known.

Studies on the soil microbiome are extending to various places on the planet and it is increasingly evident that the resulting data will provide elements for precision cultivation techniques taking into account the conservation of the microbiota, an indispensable prerequisite for preserving the quality parameters of the soil itself and the quality of agri-food products. A recent study investigated the internal diversity of soil samples taken from tea plantations located around Lincang (Yunnan, China) by studying both α- and β-diversity: respectively the diversity within the sample consisting of the indices of richness in species and equitability (evenness of species) and the variation in species composition between different samples from different sites in the geographical area under examination (Kui et al., 2021). The analysis ended with the identification of the main bacterial taxa present in the investigated soils: the phyla *Proteobacteria* (30.90%), *Chloroflexi* (11.9%) and *Bacteroidetes* (6.4%), the families *Ktedonobacteraceae* (4.7%) and many other unidentified ones belonging to the class *Gammaproteobacteria* (3.5%). As far as the fungal component is concerned, the most represented phyla were *Ascomycota* (34.7%), *Basidiomycota* (16.6%) and *Glomeromycota* (7.7%), which showed an inversely proportional relationship between their relative abundance and the increase in soil pH values.

This study aims to analyze the soil microbioma of paddy field soils in the sampling units considered in the PhD project. Specifically, it aims to analyze bacterial and fungal communities by applying two different methodologies:

1. Conventional plate culture - pure isolation of the detected fungal colonies and analysis
2. Metagenomic approach - selection of communities of interest (Bacteria and Fungi) from the total extracted eDNA (environmental DNA)

5.2 Conventional plate culture - Materials & Methods

5.2.1 Sampling

Sampling was conducted, according to the methodology already described in chapter 2 "Field Work", paragraph "2.3 Sampling", in each paddy field where *Echinochloa* resistant specimens were surveyed. Soil samples were collected in each SU according to the *X non-systematic* sampling scheme (Lambkin et al., 2004) and were bagged, labeled and stored at – 20° C. Samples were thawed at the time of use.

5.2.2 Preparation of cultural substrate, inoculation and identification

Potato Destrose Agar (PDA)[14] was used as the culture medium: 42 g of PDA (Biolife Italiana srl – Milano - Italy) were dissolved in a total volume of 1000 ml with distilled water. In order to prevent the growth of bacteria and promote the development of fungal strains without contaminants, antibiotics were added to the substrate. Heat-resistant antibiotic Chloramphenicol, in the amount of 100 mg/l, was added before sterilization. Then, under hood and at room temperature, Penicillin G and Streptomycin in the amounts of 50 mg/l each. After autoclave sterilization (pressure of 1 atm, 120°C for 20 min) and after adding the antibiotics, the substrate was dispensed into Petri dishes. This process was performed totally in a vertical laminar flow hood to ensure maximum sterility.

Inoculation was performed dissolving 1 g of soil in sterilized distilled water, in a volume of 100 ml. To facilitate suspension, a magnetic stirrer (300 rpm for 20 min) was used. A solution of 1 ml was then taken and distributed in Petri dishes (operating under a fume hood). Five replicates (5 Petri dishes) were made for each sample. The plates were incubated at room temperature and checked weekly for up to two weeks (Caretta et al., 1987).

PDA was also used for the isolation of fungal colonies. Preparation of tubes useful for isolation of fungal colonies was carried out by suspending 42 g of PDA in 1000 ml of distilled water. Tubes were later sterilized by autoclave (pressure of 1 atm, 120°C for 60

[14] PDA is particularly suitable for the isolation and growth of molds and yeasts. Indeed, the concentration of glucose allows the development of yeasts and molds, while the low pH hinders the proliferation of bacteria.

min). PDA was allowed to solidify obtaining a slope of about 30° in the tubes, in order to facilitate the growth of the fungus in pure colony with air exposure. To identify fungal strains, any mycelium of doubtful identification was distributed from Petri dishes into the tubes and incubated until growth.

The study and recognition of fungal strains were carried out through a morphological, physiological approach (Samson et al., 2010). Through observations of macro-morphological features (growth mode, texture, color, and exudate production) and micro-morphometric features (morphology and size of the reproductive structures spores and conidia, with their respective conidiophores), the genus and, when possible, the fungal species were determined. Stereo and optical microscope was used for identification. Determination of the genus and/or species of the different fungal taxa was then carried out with the support of the main reference texts and appropriate monographs.

1. CROUS et al. (2007), BENSCH et al. (2010) for the genus *Cladosporium*
2. BISSET (1984; 1991 a,b,c) and RIFAI (1969) for the genus *Trichoderma*
3. DOMSCH, (1980) for the soil fungi
4. ELLIS (1971, 1976) for demiaceous *Hyphomycetes*
5. HANLIN (1990, 1998) for the division of the *Ascomycetes*
6. RAPER & FENNEL (1965) and KLICH (2002). for the genus *Aspergillus*
7. NELSON (1983) for the genus *Fusarium*
8. PITT (1979) for the genus *Penicillium*
9. SIVANESAN, 1987 for the genus *Cochliobolus*
10. SUTTON,1980 for the group of *Coelomycetes*
11. ZYCHA & Siepmann, 1969 for the order *Mucorales*

5.2.3 Fungal communities analysis

Presence of each fungal strains in each SU was recorded as average percentage abundance. A double heatmap – clustering analysis, based on "Canberra" distance and "ward.D2" algorithm to maximize the variance between clusters, was performed using the "vegdist" (package vegan - Oksanen et al., 2020) and the "heatmap.2" (package gplots – Warnes et al. 2020) functions, to obtain a graphical description of each fungal strain

abundance in each SU, allowing to discriminate the most frequent ones. In addition, paddies with similar fungal communities were grouped together, while different divided. The entire analysis was carried out using R 3.6.3 software (R Core Team, 2019).

5.3 Conventional plate culture - Results & Discussion

Table 5.1 records the average percentage abundance of each fungal strain surveyed in each SU. It could be noticed that morphometric identification may be a very difficult task and lead to a partial classification, since some characters present a high features variability.

Table 5.1: percentage abundance of fungal strains

Morphotype	Ottobiano	Sannazzaro de' Burgondi	Suardi	Borgo San Siro	Cilavegna	Zeme	Robbio	Parona	Vigevano	Rivoltella	Barbavara	Mairano	Ozzero	Robbio Daghetta	Bascapè	Valle Lomellina	Frascarolo	Zerbolò 1	Zerbolò 2	Belgioioso	Cozzo 1	Cozzo 2	Pieve del Cairo	Genzone	Roncaro	Zinasco Vecchia	Pieve Albignola	San Martino Siccomario	Mortara	Lomello	San Giorgio di Lomellina	Dorno	Gaidasco	Giussago	Carpiano	Carbonara al Ticino	Landriano	Candia di Lomellina 1	Candia di Lomellina 2
Bacteria and yeast	0.6	0.4	0.6	0.2	0	0	0.3	0	0.8	1	0.8	1	0.4	0.5	0.4	0.2	1	0.3	0.4	0.8	1	1	0.4	1	1	1	0.8	1	1	0	0	0.3	0	0.8	1	0.8	1	1	1
Alternaria spp.	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0.2	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0
Aspergillus flavus	1	0.2	0.2	0.2	0	0	0	0	0	0	0	0.4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.6	0	0	0	0	0	0	0	0.4	0	0
Aspergillus (yellow / orange)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Aspergillus niger	0	0.4	0.8	0.4	0.4	0.2	0	0	0	0	0	0.2	0.6	0.3	0	0	0.4	0	0	0.2	0	0	0	0.8	0	0	0	0	0	0	0.4	0.2	0	0	0	0	0.2	0	0
Aspergillus ochraceus	0	0.6	0.2	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aureobasidium spp.	0	0	0	0	0	0	0	0	1.4	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.4	0	0	0	0	0
White colony	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0
Cladosporium spp.	0	0	0.6	0	0	0	16.2	0	14.4	0	0	0	0	0.8	0	7.8	0.6	7.8	1.6	15.8	7.4	0.2	3	0	23.2	47.6	7.8	55.4	2.4	0	0	16.2	0	14.4	0	0	0	0.2	3.8
Clonostachys rosea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Cokeromyces spp.	0	0	0	0	0	0	0	0.8	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.8	0	0	0.2	0	0	0
Epicoccum spp.	0	0	0	0	0	0	0	0	0	0	0	3.2	0	0.3	0	0	0	0	0.2	0.4	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	3.2	0	0
Fusarium (white)	20	0	9.2	1.2	0.8	1.6	1.6	1.8	1	2.2	5.8	3.8	0	2	14	16	4.8	9	6.2	9.2	18.4	6.6	5.2	5.4	11	2.4	1.2	6.2	5	0.8	1.6	1.6	1.8	1	2.2	5.8	3.8	6.6	12.5
Fusarium (pink)	1.6	0.2	0.6	0.4	0	0	0.2	0	0.8	0	0	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0.8	0	0	0.6	0	0
Fusarium sporotrchioides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium (violet)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0
Geothricum (yellow)	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0
Geothricum (grey)	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0
Geothricum (lilac)	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	4
Brown colony	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sterile micelium	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0.2	0	0	0	0	1.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mortiriella alpina	0.2	0	0	0	0	0	0	0	0	0	0	0.2	0.4	0.2	0	0	0	0.2	0	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0
Mortiriella spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0.1

Mucor racemosus	2.4	0.8	2.2	9.2	3.8	13.2	0	0	0.4	0.2	17.8	0	0	0.2	0	0	1.2	0.6	0.4	0.2	1.2	0.6	8.2	1.8	20	20	18.8	0.8	6	3.8	13.2	0	0	0.4	0.2	17.8	0	0.6	0.9
Mucor spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0.2	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Myrotecium spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0	0	0
Peacilomyces lilacinus	0	0	0.2	0	0	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0	0	0	0	0	1	0
Penicillium (white)	1.6	0	0	0	0	0	0	0	0	0.4	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0	3	0	0
Penicillium (red)	0	2.2	1	0	0.6	0	0	0	0	0	0	0	0	0.3	0	0	0	4.6	0.4	0.8	0	0	0	0	0	0	0	0	0	0.6	0	0	0	0	0	0	0	0
Penicillium (yellow)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.6	0	0	0	0	0	0	0	0	0.2	0.4	0	0	0	0	0	0	0	20	0
Penicillium (grey)	0	0	0	0	0	0	0	0	0	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.6	0	0	0	2
Penicillium (brown)	0	0	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0	0	0	0
Penicillium sclerotiorum	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
Penicillium spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	2	0.2	0	0	0	0	0	0	0.2	0	0.4	0	0	2	0	0	0	0	0	0	0	0	0
Penicillium (green)	5	5.8	5	0.6	0	0.6	0	0	0	1.2	0.2	8.4	0	0.1	0	0	0	4.6	1.6	0.2	0	0	0	0	0	0	0	0	0	0	0.6	0	0	0	1.2	0.2	8.4	0	0
Talaromyces spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1
Phoma spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0	0	0	0	0	0	0	0	0	0
Rhizopus spp.	1.8	1	0.4	0.6	0.8	0	5.8	1.6	3.2	0.2	1.8	1.2	1	0.1	0	0	0	0	0	0	0	0	0	0	0	1.4	1.8	0	0	0.8	0	5.8	1.6	3.2	0.2	1.8	1.2	0	0
Trichoderma spp.	1.6	0	3	2	8	3.4	0	0	0	0	0.6	4.4	2.8	1	2.4	4.8	10.2	2.6	2.4	0.4	0	11	20	1.6	20	1	6.2	20	1.4	8	3.4	0	0	0	0	0.6	4.4	11	5.5
Violet colony	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

In Figure 5.1 are shown images and particulars of fungal colonies surveyed.

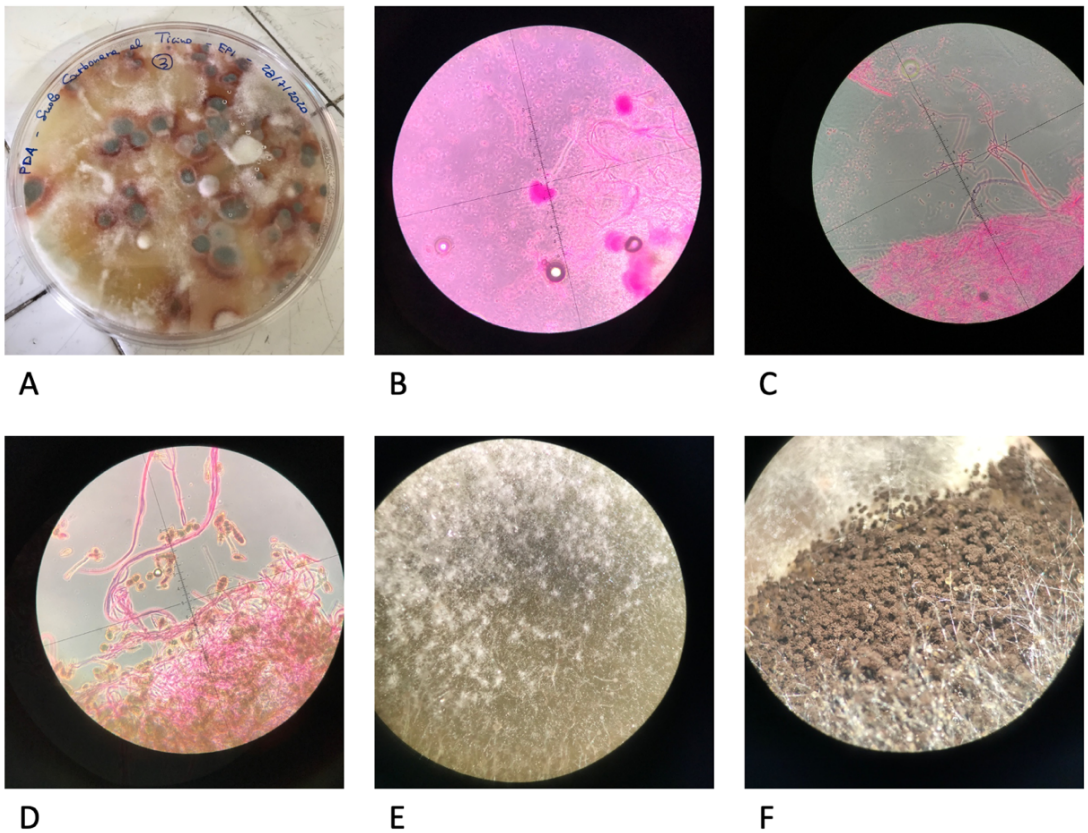

Figure 5.12: Images of fungal strains identified through cultural method. A: Plate with PDA and grown fungal colonies. B: Particular of *Aspergillus* spp. conidia. C: Particular of *Trichoderma* spp. conidia. D: Particular of *Alternaria* spp. conidia. E: Particular of *Trichoderma* spp. colony. F: Particular of *Mucor* spp. colony.

In Figure 5.2 a heatmap and cluster analysis were combined. The heatmap shows the abundance values of each fungal strain assumed in each soil sample analyzed by culture method. Specifically, higher abundance values are marked with dark blue color, lower values with lighter blue, absence with white color. Almost all SU possess comparable fungal communities, with a prevalence of *Cladosporium* spp., *Fusarium* spp., *Mucor racemosus* Bull., *Penicillium* spp. and *Thricoderma* spp.. Only *Cladosporium* spp. strain recorded a high abundance (~ 50%) in the soils of San Martino Siccomario and Zinasco Vecchia paddies.

Hierarchical clustering groups soil samples based on the similarity of their fungal communities. Two main clusters were identified: A and B. In cluster A are included 16 soil samples in which a fair abundance of *Fusarium* white colonies and *Trichoderma* spp. were recorded. In cluster B are grouped 23 soil samples: in most of them *Fusarium* white colonies were not recorded, while *Mucor racemosus* Bull., *Cladosporium* spp. and *Geothricum* (yellow) colonies are present in some samples.

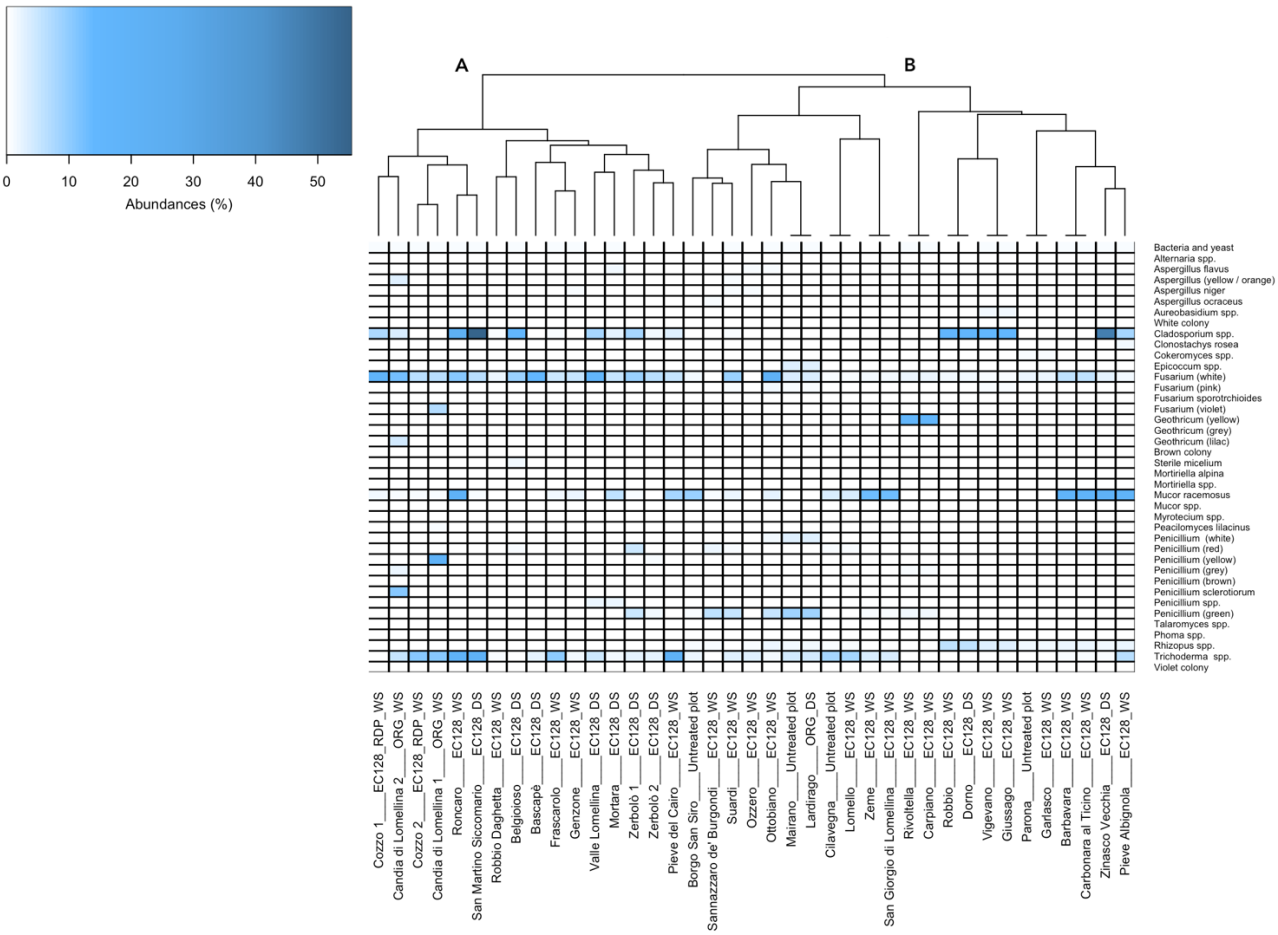

Figure 5.2: Double heatmap - cluster analysis based on the "Canberra" distance of collection sites and on the dendrogram produced by hierarchical clustering. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

In general, the biodiversity associated with paddy soils is not very high. Considering the fungal coenosis of soils the genus *Fusarium*, appears to be isolated with species, *Fusarium verticillioides* (white), *F. oxysporum* (white - pink) and *F. sporotricioides. Fusarium* is a widespread genus, especially in soil. Many species can cause severe diseases identified as necrosis, tracheomycosis, cancers or rots (Leslie and Summerell 2006). The genus *Fusarium* is of great importance for rice since it is capable of damaging different parts of the plant and producing complex alterations and symptoms (Summerell and Leslie, 2011).

The genus *Mucor* is a filamentous fungus found in soil, on plants, in decaying fruits and vegetables. *Penicillium* is a cosmopolitan genus with hundreds of species with a global distribution and, together with the genus *Aspergillus*, is among the major bio-degraders of organic matter (Pitt, 1979). With regard to rice cultivation, the genus is known

to produce some mycotoxins, particularly citrinin, which can contaminate caryopses (Abd-Allah & Ezzat, 2005). *Cladosporium* species are among the most common black (demiaceous) molds (Ellis, 1971) and are mainly isolated from soil and plant material, where they are frequently found as saprotrophs. In this context, *Cladosporium* and *Mucor* are the most represented *taxa*: the first is present in both dry and flooded paddy fields, while the second is mainly present in flooded paddy fields. The species of this genus are characterized by great physiological abilities, such as osmotolerance, halotolerance, thermotolerance, and psychrophilia (Zalar et al., 2007; Bensch et al, 2012; Li et al. 2012; Zhang et al., 2013; Godinho et al., 2015; Sandoval-Denis et al., 2015; Sandoval-Denis et al., 2016) and are commonly found associated with phylloplane and soil, as well as in a wide variety of environments, including marine environments (Liu et al., 2015).

Among the fungal strains described, *Trichoderma* and *Penicillium* are capable of colonizing soils and establishing relationships with plants at the rhizoplane level. They are called *rhizo-competent* fungi, as they are able to influence plant health and growth by limiting the actions of pathogens (Whipps, 1997).

5.4 Metagenomic approach - Materials & Methods

5.4.1 Sampling

Sampling was conducted in each paddy field where *Echinochloa* resistant specimens were surveyed. Specifically, soil samples were collected in each SU according to the *X non-systematic* sampling scheme (Lambkin et al., 2004) and were bagged, labeled and stored at – 20° C. Samples were thawed at the time of use.

5.4.2 DNA extraction and production of metagenomic amplicons for bacterial and fungi communities

Total eDNA was obtained from 250-500 mg of soil per sample using Macherey-Nagel™ NucleoSpin™ Soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. eDNA was then quantified on a Qbit fluorometer (ThermoFisher Scientific, Waltham, MA). Metagenomic amplicons of bacterial and fungi communities were obtained following PCR amplification, using primers linked to Illumina adapters. To produce bacterial amplicons, the V3-V4 hypervariable region of the prokariotic 16S rRNA gene was targeted, using the primers designed by Takahashi et al. (2014). To obtain fungi amplicons the ribosomal ITS1 region was targeted, by using primers BITS and B58S3 designed by Bokulich and Mills (2013).

The sequences of the primers used for bacteria and fungi are respectively:

- 16S bacterial primers (Mazzoli et al., 2020; Takahashi et al., 2014)
  Forward primer - pro341F: 5'- CCTACGGGNBGCASCAG -3'
  Reverse primer - pro805R: 5'- GACTACNVGGGTATCTAATCC -3'
- ITS1 fungal primers (Bokulich and Mills, 2013)
  Forward primer -BITS: 5'-ACCTGCGGARGGATCA (R=A or G) -3'
  Reverse primer -B58S3: 5'-GAGATCCRTTGYTRAAAGTT (Y=C or T)-3'

Adapters for sequencing on the Illumina® platform are bound to the primers; the sequences are listed below:

- ➢ adapter F: 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG - [locus specific sequence] -3'
- ➢ adapter R: 5'- GTCTCGTGCTCGAGATGTGTATAAGAGACAG - [locus specific sequence] -3'

DNA was amplified in 40l volumes using the following reagent concentration conditions in 40µl volumes: 1.0µl of extracted eDNA, 1.6µl of Primer Forward (0.4µM), 1.6µl of Primer Reverse (0.4µM), 8.4µl of GoTaq® Green Master Mix - Promega (0.4x), 27.4µl of H2O Nuclease Free.

Amplification was performed in a Biorad MJ Mini Personal Thermal Cycler the following amplification program: initial denaturation at 95°C for 3:00 minutes; denaturation at 95°C for 30 s. 31 (16S) and 29 (ITS) cycles of annealing at 55°C (16S) and 58°C (ITS) for 30s; extension at 72°C for 30s. Final extension at 72°C for 5:00 minutes.

Amplicon production was verified by electrophoretic analysis (100V x 60 min) of PCR products on 2% agarose gels and 1 X TAE buffer stained with ethidium bromide. The expected molecular weight (220bp for fungal amplicons and 450bp for bacterial amplicons) was verified in reference to 100 bp DNA ladder (Promega, Madison, Wisconsin, USA). Amplicons were visualized with Molecular Imager® Gel DocTM XR + (BIO-RAD, Hercules, CA, USA).

5.4.3 Sequencing

For sequence analysis, amplicons were sent to the sequencing center BMR Genomics SRL (Padua), where they were processed with the NexteraTM DNA Library Sequencing Preparation Kit (2020). The protocol applied is that for the 2x250pb pair-end sequencing method using Illumina®'s MiSeq platform.

5.4.4 Statistical analysis

The obtained sequences (reads) were subjected to an initial bioinformatic analysis to prepare them for analysis. The cleaned data were subjected to a final analysis with special softwares that allow the obtained reads to be compared with the sequences of fungi and bacteria in the reference databases. In our case, Greengenes for bacteria and UNITE for fungi. The amounts of reads obtained relative to each identified taxon were transformed into percentage frequencies data (abundance %).

The entire statistical analysis was performed by R 3.6.3 (R Core Team, 2019). Bacterial and fungal communities α-diversity was assessed by computing Chao 1, Abundance Coverage Estimator (ACE) and Shannon indexes at the taxonomic level of orders (function "estimateR" - package vegan - Oksanen et al., 2020). Diversity in composition among bacterial and fungal communities (β-diversity) was assessed, considering abundances at the taxonomic level of orders, by principal coordinates analysis (PCoA) (functions "ordinate" and "plot_ordination" - package phyloseq – McMurdie and Holmes, 2013) based on a distance matrix computed through "Mountford" distance and "multidimensional scaling" (MDS) method. PCoA was graphed using the "autoplot" function (package ggplot2 - Wickham, 2016). Soil bacterial and fungal relative abundances in each SU at the taxonomic level of Orders were graphed as heatmap (function "ggplot" - package ggplot2 – Wickham, 2016).

Double heatmap and hierarchical clustering based on "Canberra" distance and "ward.D2" algorithm to maximize the variance between clusters, was performed using the functions "vegdist" (package vegan - Oksanen et al., 2020) and the "heatmap.2" (package gplots – Warnes et al. 2020). The number of identified bacterial and fungal orders and soil chemical/physical parameters analyzed in Chapter 4 were considered, in order to provide a better description of paddies soils and to investigate a possible relationship between the presence of bacterial and fungal and edaphic chemical and physical characteristics.

A double heatmap – clustering analysis, based on "Canberra" distance and "ward.D2" algorithm to maximize the variance between clusters, was performed using the

functions “vegdist” (package vegan - Oksanen et al., 2020) and the “heatmap.2” (package gplots – Warnes et al. 2020)

Spearmann correlation was calculated in order to evaluate possible correlation among parameters analyzed (function “cor.test” - package stats – R Core Team, 2019) .

5.5 Metagenomic approach - Results & Discussion

5.5.1 Bacteria

α-diversity and richness indexes, investigated at the taxonomic level of orders, depicted a quite homogeneous situation among the different paddy soils, especially with respect to the Shannon diversity estimator (Table 5.2). The soil of Parona, managed following Directive EC 128/2009, is the only with lower richness and diversity values.

Table 5.2: Bacterial biodiversity and richness estimators calculated at the taxonomy rank of orders. Number of observed strains, Chao 1 richness estimator (Chao1) and associated standard error (SE.Chao1), Abundance Coverage Estimator (ACE) and corresponding standard error (SE.ACE), Shannon's index of biodiversity (Shannon).

Municipality	Observed strains	Chao 1	Chao 1 SE	ACE	ACE SE	Shannon
PieveAlbignola	91	91	0	91	4.4	3.57
Mortara	103	103	0	103	4.41	3.67
Robbio_Daghetta	100	100	0	100	3.76	3.53
Zinasco	90	90	0	90	3.94	3.47
SanMartinoSiccomario	87	87	0	87	3.92	3.42
ValleLomellina	98	98	0	98	4.32	3.67
PieveDelCairo	71	71	0	71	1.39	3.11
Frascarolo_Sartirana	66	66	0	66	3.99	3.44
Roncaro	116	116	0	116	4.94	3.71
Belgioioso	111	111	0	111	4.4	3.75
Zerbolo_2	103	103	0	103	4.35	2.92
Zerbolo_1	84	84	0	84	4.19	3.15
Bascape	88	88	0	88	3.86	3.48
Genzone	74	74	0	74	3.46	3.43
Cozzo_2	114	114	0	114	4.6	3.64
Ozzero	60	60	0	60	3.35	3.3
Cilavegna_StAnna	106	106	0	106	4.31	3.77
Cozzo_1	20	20	0	20	2.22	2.52
Suardi	137	137	0	137	5.36	3.75
Parona	25	25	0	25	0.98	1.78
Ottobiano	99	99	0	99	3.92	3.64
BorgoSanSiro	101	101	0	101	4.28	3.52
Rivoltella	91	91	0	91	3.63	3.58
Vigevano	94	94	0	94	3.73	3.59
Vigevano_Barbavara	113	113	0	113	3.89	3.67
SannazzaroDeBurgondi	94	94	0	94	3.73	3.56
Zeme	100	100	0	100	4.21	3.72
Mairano	94	94	0	94	3.97	3.69
Robbio	92	92	0	92	3.34	3.65
Dorno	125	125	0	125	4.47	3.64
Carpiano	84	84	0	84	2.83	3.37

Giussago	100	100	0	100	4.39	3.39
Garlasco	106	106	0	106	3.87	3.75
CarbonaraAlTicino	95	95	0	95	4.53	3.58
Lardirago	72	72	0	72	3.86	3.45
Lomello	94	94	0	94	3.45	3.59
SanGiorgioLomellina	106	106	0	106	4.03	3.5
CandiaDiLomellina_1	110	110	0	110	4.51	3.69
CandiaDiLomellina_2	93	93	0	93	3.96	3.59

In the PCoA (Figure 5.3), the first axis expressed 8% of variability and the second axis 3.3%. In the second and third quadrant are grouped 32 of the 39 soil samples, which are characterized by similar bacterial communities. In these farms soil all identified bacterial orders assume similar abundance values and there is no dominance of any of them. A smaller group located in the first quadrant includes soil samples of Dorno, Vigevano (Barbavara), Suardi, Mairano and Sannanzzaro De Burgondi. In these farms, a dominance of bacterial strains belonging to the orders *Actinomycetales, Bacillales, Streptophyta* and *Rikketsiales* was recorded. The soil of Zerbolò 2, which records the greatest presence of *Actinomycetales*, and that of Cozzo 1, where bacteria of the orders *Methanosarcinales* and *Desulfurococcales* are abundant, are separated from the other groups.

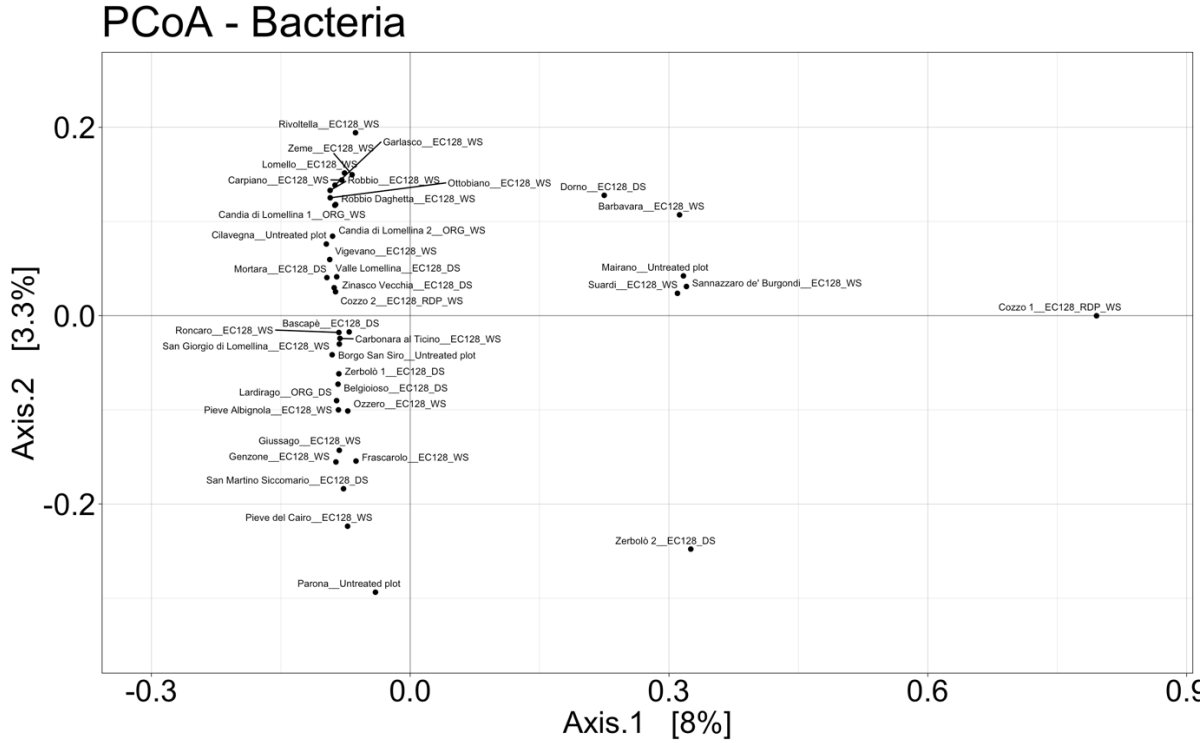

Figure 5.3: Principal Coordinates Analysis of soil bacterial communities. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

The heatmap graph in Figure 5.4 depicts all bacterial orders identified by metagenomic analysis in the analyzed soil samples based on their abundance. Specifically, dark blue color indicates high abundance values, while light blue/white low abundance values. Since as many as 214 bacterial orders were identified, the graph has been divided into 2 parts to facilitate its reading (Figure 5.4A and Figure 5.4B). For both heatmaps, the y-axis (Soil) repeats and shows the list of farms at which soil samples were collected, while the x-axis (Bacteria) lists bacterial orders surveyed.

Actinomycetales, *Bacillales*, *Clostridiales*, *Methanosarcinales* and *Rhizobiales* represent the most spread bacterial orders within almost all the paddies. In particular, Zerbolò 2 recorded a high abundance of *Actinomycetales* while Parona a high abundance of *Rickettsiales*. Cozzo 1 is characterized by high abundance of *Enterobacteriales* and *Nitrospirales*, nitrifying organisms.

Actinomycetales are an order of Gram-positive and anaerobic bacteria and can be found mostly in soil and decaying organic matter. They form symbiotic nitrogen fixing associations with over 200 species of plants, causing diseases in some species. *Bacillales* are an order of ubiquitous Gram-positive bacteria. *Clostridiales* are an order of Gram-positive bacteria. This group includes saprophytic organisms that ferment plant polysaccharides and that could be found in many places in the environment, most notably the soil. *Methanosarcinales* are an order of strictly anaerobic *Archaea* that survive by producing methane. Are typical of anoxic environment, such as paddies. *Rhizobiales* are an order of Gram-negative bacteria which fix nitrogen and are symbiotic with plant roots. *Rickettsiales* are an order of obligate intracellular parasites. *Enterobacterales* is an order of Gram-negative bacteria including diverse group of species living in distinct ecological niches and possessing a variety of biochemical characteristics. *Nitrospirales* order contains nitrifying bacteria which oxidize nitrite to nitrate (Brenner et al., 2005).

To sum up, these findings highlight that soil bacterial communities are mainly characterized by extremophilic anaerobic strains specialized in organic matter decomposition.

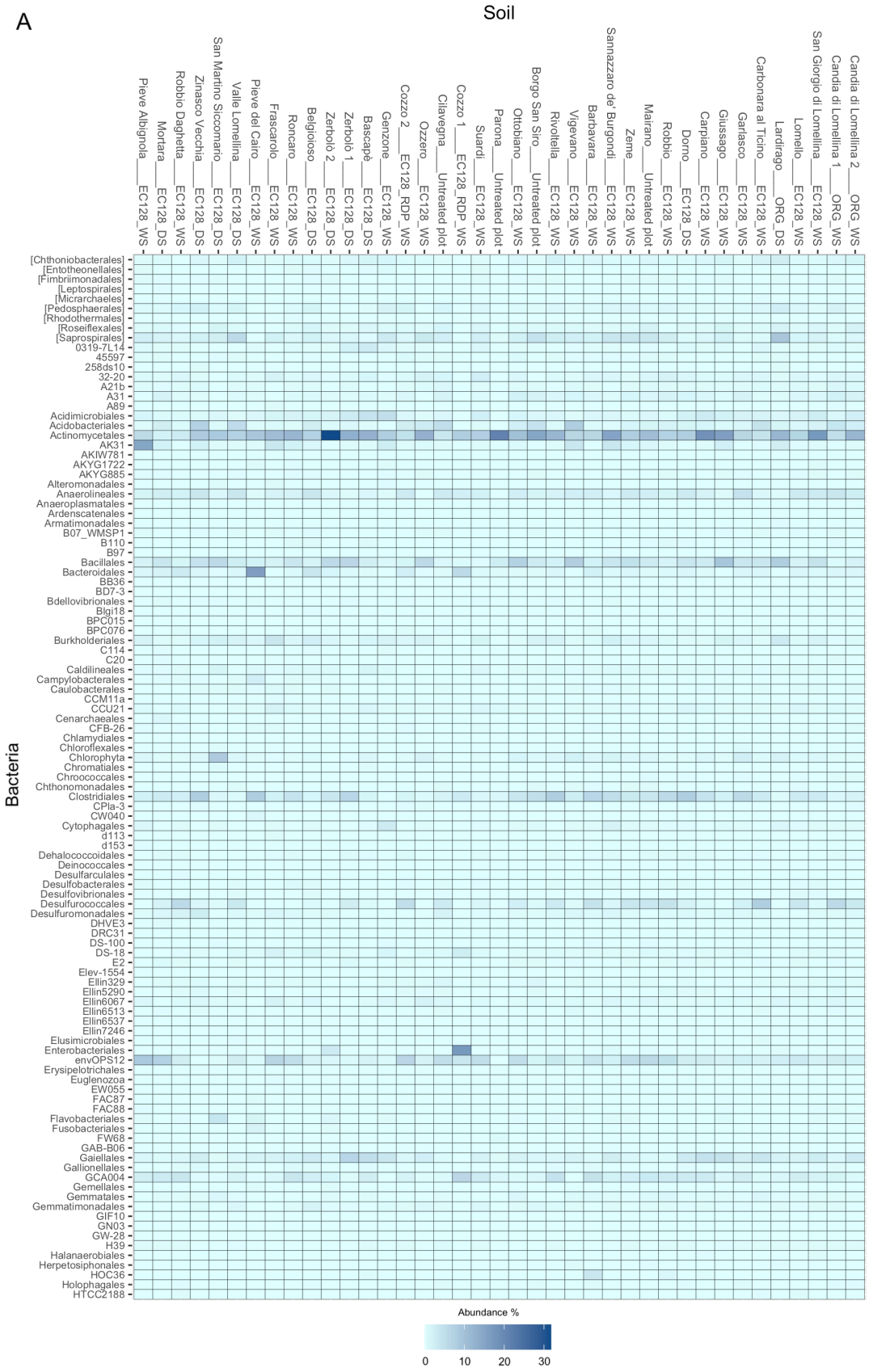

Figure 5.4 A: heatmap of bacterial orders in soils. High abundance is marked by dark blue color. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

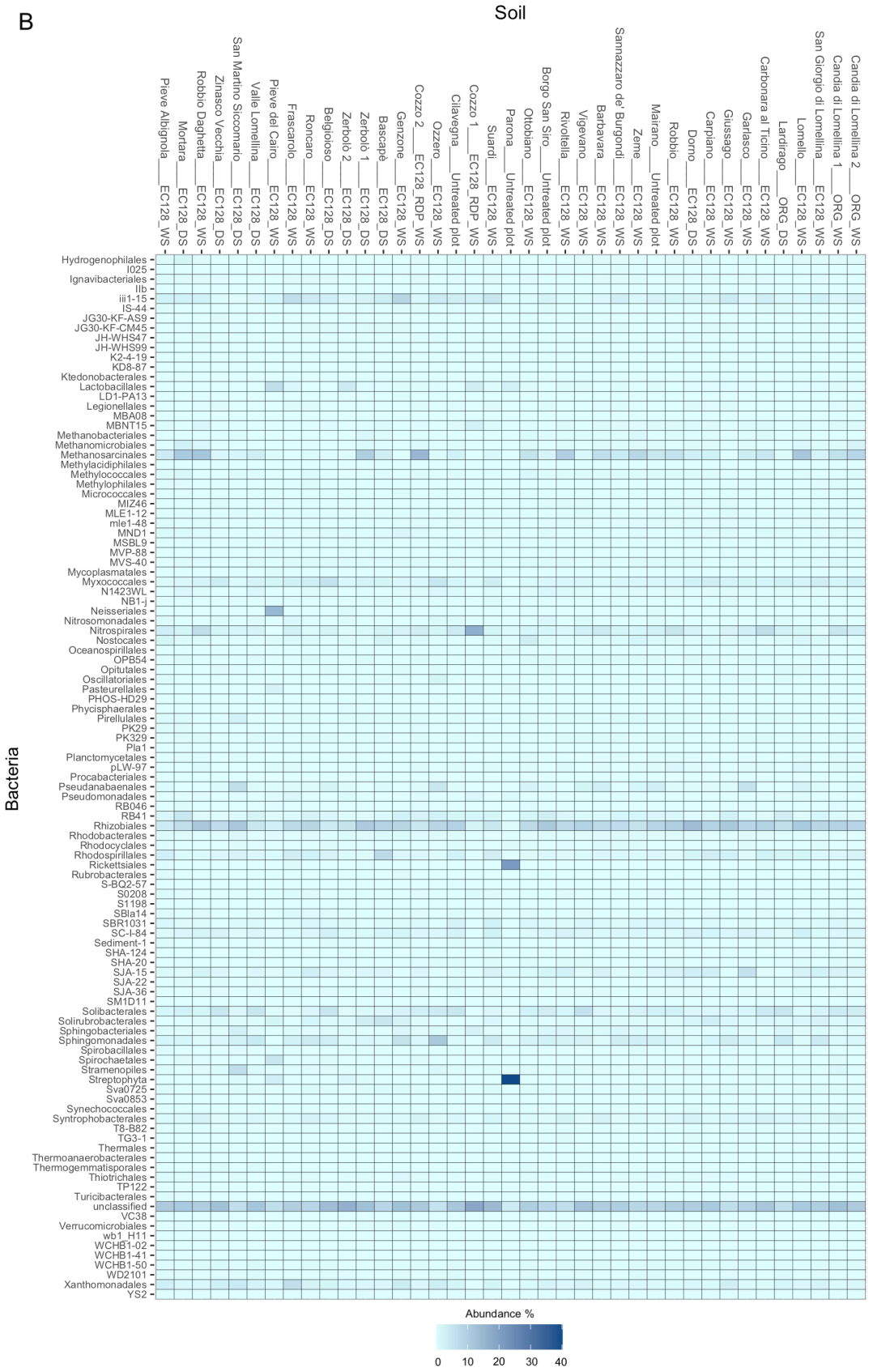

Figure 5.4 B: heatmap of bacterial orders in soils. High abundance is marked by dark blue color. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

5.5.2 Fungi

α-diversity and richness indexes, investigated at the taxonomic level of orders, depicted a quite homogeneous situation among the different paddy soils (Table 5.3). However, the soils of Piave del Cairo, Frascarolo (Sartirana), Roncaro, Bascapè, Suardi and Ottobiano managed following Directive EC 128/2009, and the soil of Cozzo 1, managed in reference to RDP Operation 10.0.01, present low richness and diversity values.

Table 5.3: Fungal biodiversity and richness estimators calculated at the taxonomy rank of orders. Number of observed strains, Chao 1 richness estimator (Chao1) and associated standard error (SE.Chao1), Abundance Coverage Estimator (ACE) and corresponding standard error (SE.ACE), Shannon's index of biodiversity (Shannon).

Municipality	Observed strains	Chao 1	Chao 1 SE	ACE	ACE SE	Shannon
PieveAlbignola	13	13	0	13	0.96	0.87
Mortara	16	16	0	NA	NA	0.89
Robbio_Daghetta	12	12	0	NA	NA	1.18
Zinasco	15	15	0	15	1.32	1.26
SanMartinoSiccomario	10	10	0	10	1.26	0.94
ValleLomellina	16	16	0	NA	NA	0.83
PieveDelCairo	4	4	0	NA	NA	0.57
Frascarolo_Sartirana	5	5	0	NA	NA	0.36
Roncaro	7	7	0	NA	NA	0.97
Belgioioso	10	10	0	10	0.95	1.6
Zerbolo_2	11	11	0	11	0.95	1.71
Zerbolo_1	14	14	0	14	0.96	1.72
Bascape	8	8	0	8	0.94	0.67
Genzone	11	11	0	NA	NA	1.87
Cozzo_2	13	13	0	NA	NA	1.79
Ozzero	13	13	0	NA	NA	1.11
Cilavegna_StAnna	16	16	0	16	0.97	1.72
Cozzo_1	5	5	0	NA	NA	0.63
Suardi	7	7	0	NA	NA	0.72
Parona	14	14	0	14	1.31	2.01
Ottobiano	5	5	0	NA	NA	0.63
BorgoSanSiro	16	16	0	16	0.97	1.6
Rivoltella	17	17	0	17	0.97	0.86
Vigevano	7	7	0	7	1.2	1.55
Vigevano_Barbavara	15	15	0	NA	NA	1.21
SannazzaroDeBurgondi	16	16	0	NA	NA	1.73
Zeme	14	14	0	NA	NA	1.27
Mairano	15	15	0	NA	NA	1.79
Robbio	15	15	0	15	0.97	1.6
Dorno	13	13	0	NA	NA	1.46
Carpiano	12	12	0	12	0.96	1.3
Giussago	16	16	0	16	1.32	1.7
Garlasco	12	12	0	12	0.96	1.01
CarbonaraAlTicino	16	16	0	16	0.97	1.9
Lardirago	17	17	0	17	1.33	1.76
Lomello	16	16	0	16	0.97	1.38
SanGiorgioLomellina	11	11	0	NA	NA	1.77
CandiaDiLomellina_1	14	14	0	NA	NA	1.3
CandiaDiLomellina_2	12	12	0	12	0.96	1.4

In the PCoA (Figure 5.5), the first axis expressed 34.6% of variability and the second axis 20.4%. This analysis showed two main groups of samples. A first group includes 12 paddy soils and lays between the second and third quadrant. In these soil samples, many

fungi orders were not identified by means of metagenomic analysis and a fair abundance of *Sordariales*, *Agaricales* and *Pezizales* was recorded. A second group includes all the other samples, which are spread essentially between the first and fourth quadrant. In these soils, a good presence of fungal strains of the order *Malasseziales*, species that are used to colonize unstable and disturbed environments, was found in these soils.

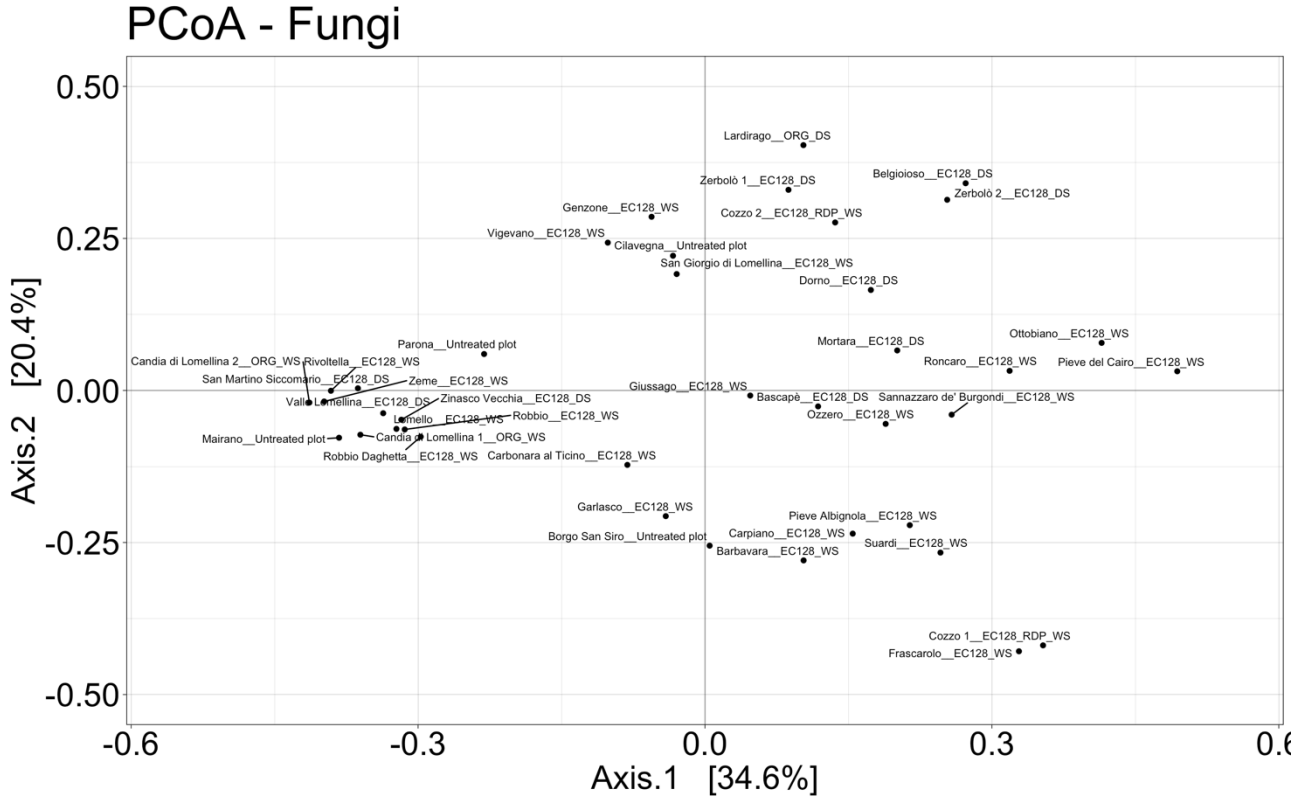

Figure 5.5: Principal Coordinates Analysis of soil fungal communities. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

In the heatmap in Figure 5.6 are graphed all the orders of Fungi identified by metagenomic analysis in the analyzed soil samples based on their abundance. Specifically, dark blue color indicates high abundance values, while light blue/white low abundance values. *Agaricales*, *Malasseziales*, *Mortirellales*, *Pezizales* and *Sordariales* represent the most spread fungal orders within almost all the paddies. Moreover, in almost all soils sample, many sequences of eDNA resulted unidentified.

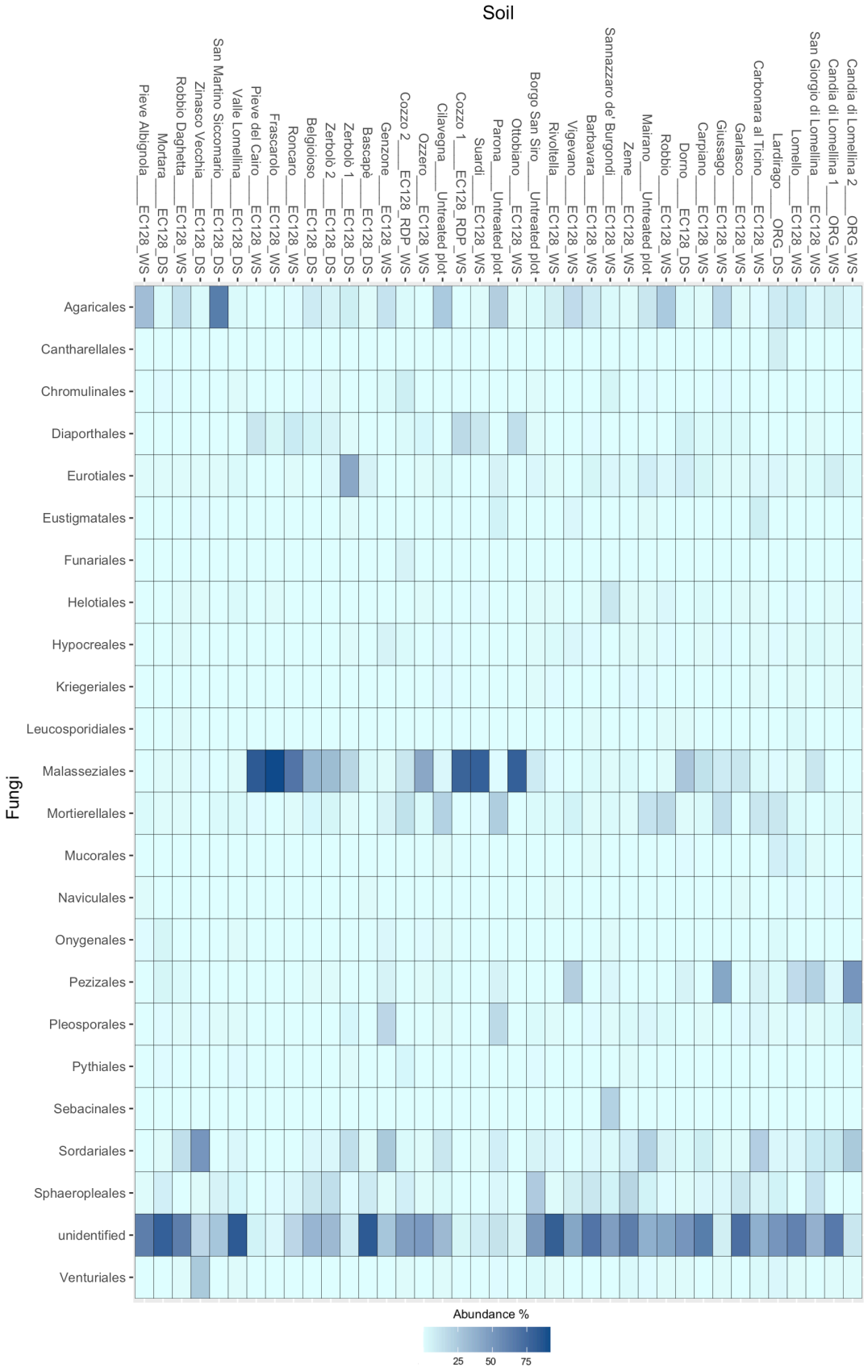

Figure 5.6: Heatmap of fungal orders in soils. Darker colors mean higher abundance. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

Agaricales is an ubiquitous order of *Basidiomycota*, distributed across all continents. Most of the strains are terrestrial. Their habitats including all types of woodland and grassland. Their higher presence was surveyed in San Martino Siccomario. *Malasseziales* is an order of *Basidiomycota* naturally found on the skin of many animals, including humans. *Mortirellales* are filamentous fungi that inhabit various environments. Some strains of this order belong to the plant growth-promoting fungi (PGPF) and are found in the bulk soil, rhizosphere and plants tissues (Ozimek et al., 2020). *Pezizales* are an order of the phylum *Ascomycota* and are saprobic, mycorrhizal, or parasitic on plants. Some species grow on soil, wood, leaves and dung. Soil-inhabiting strains often fruit in habitats with a high pH and low content of organic matter, including disturbed ground such as paddies (Hansen et al., 2006; Kirk et al., 2008). *Sordariales* is one of the most diverse taxonomic groups of *Ascomycota*. Most of them are commonly found on dung or decaying plant matter (Huhndorf et al., 2004).

5.5.3. Relationship between microbiota and edaphic chemical and physical characteristics

Heatmap in Figure 5.7 depicts a characterization of the analyzed soils on the basis of their chemical / physical features and the number of bacterial and fungal orders recorded. Each row shows the parameter analyzed, and the color palette takes on darker brown tones in soils where high values were recorded, lighter tones, tending toward ochre and white, where low values were recorded.

Hierarchical clustering splits soil samples in two main groups. The cluster A comprises soils poorer in macronutrients and organic matter (S_org), characterized by a sandy texture. The cluster B includes soil samples generally richer in macronutrients and organic matter (S_org), which possess a silt/clay texture. The number of fungal orders seems to be influenced by sandy texture, organic matter (S_org), carbon (C_org) and the presence of some macronutrients (Ca, Na, Mg and P_ass). In particular, sandy soils poor in organic matter, carbon and macronutrients host a major number of fungal strains.

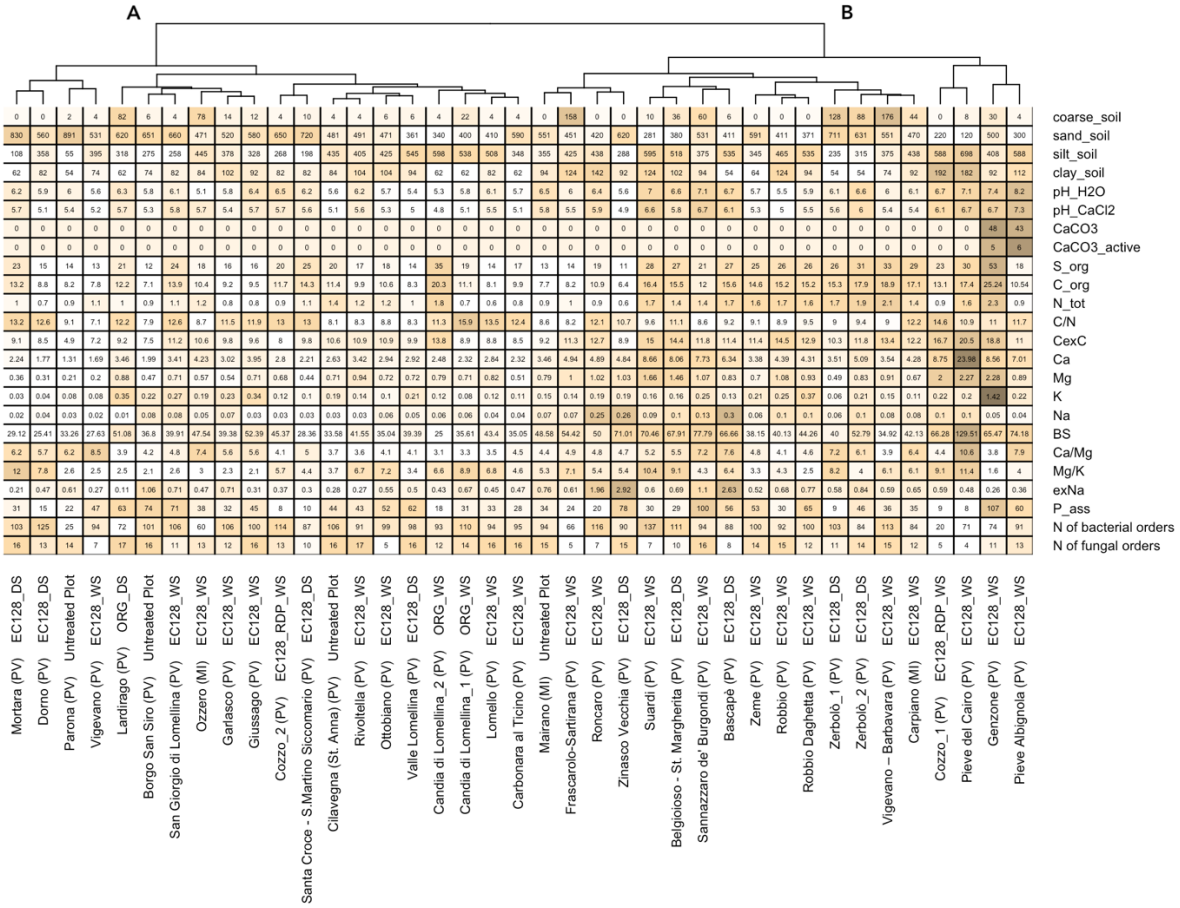

Figure 5.7: Double heatmap / cluster analysis of edaphic chemical / physical parameters and of the number of bacterial and fungal orders. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; ORG: organic; WS: wet seeding; DS: dry seeding.

Correlogram in Figure 5.8 shows the correlations existing between the analyzed parameters. Red eclipses are positive correlations ($R \geq 0.8$), blue eclipses are negative correlations ($R \leq -0.8$). Stars indicate significant correlations ($P < 0.05$).

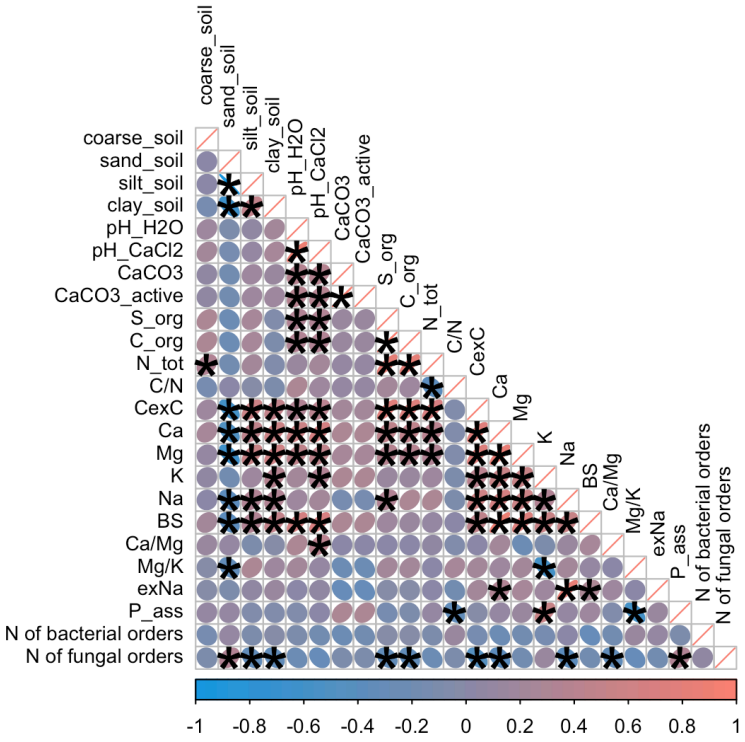

Figure 5.8: Correlogram of soils chemical and physical features with the number of bacterial and fungal orders.

There are significant correlations (positive and negative) between the number of fungal orders and the soils texture (in particular sand, silt and clay). Furthermore, significant correlations are recorded between the number of fungal orders and organic matter (S_org), carbon (C_org) and the presence of some macronutrients (Ca, Na, Mg and P_ass). Regarding the number of bacterial orders, no significant correlation was found with the edaphic variables analyzed. In any case, the greater or lesser presence of bacteria and fungi in soils seems to have an equivalent trend.

5.6 Conclusions

Results describe the diversity in the microbial composition of paddy soils.

Data obtained by means of conventional plate culture method demonstrate how fungal communities are heavily influenced by some soil chemical/physical features. In addition, the present work suggests how different microfungal actors may interact in the soil compartment, in a complex network of interactions that can on the one hand disfavor yields or affect the quality of production, as in the case of the genera *Fusarium* and *Penicillium*, and on the other hand be important resources for biocontrol (genus *Trichoderma*) and improvement/detoxification of toxic soil components (genus *Mucor* and *Aspergillus niger*).

Results obtained through metagenomic analysis of the bacterial component of the microbiota showed that extremophilic microorganisms of the *Archaea* kingdom (*Methanosarcinales* - methanogens) have been detected in many rice fields. These microorganisms are known to be widespread in the environment but are found with a high prevalence in environments where conditions such as acidity, temperature, and heavy metals make it difficult for the bacteria to proliferate. In addition to the presence of *Archea* and pathogenic organisms, rice fields also differ from each other in their content of nitrogen-fixing microorganisms. *Rhizobiales* and *Actinomycetales* are highly present in almost all paddy soils. The presence of bacteria capable of fixing nitrogen should provide the soils in which they are found with an additional supply of nutrients that can be exploited for the growth of plants grown there.

The metagenomic analysis of the fungal component of the microbiota allowed the characterization of a group of soils (Cozzo 1, Cozzo 2, Ottobiano, Pieve Del Cairo, and Zerbolò 2) that share a high prevalence of symbiotic fungi and opportunistic pathogens belonging to the order *Malasseziales.*

Unidentified sequences in this work do not yet appear to be recorded within reference databases, and studies of this type are scarce in the literature. Given the established influence of soil microbial composition on environmental balances and

agricultural crops, it is more necessary than ever to initiate new research to acquire more information about it.

In general, the paddy field environment is characterized by continuous imbalances and the constant presence of sources of disturbance due to agronomic practices. These factors affect bacterial and fungal communities of paddies soil. In general, all bacterial and fungal strains identified in this study are typical of highly impacted environments. The presence of *Archaea* (i.e. order *Methanosarcinales*) is indicator of an extremely disturbed environment.

5.7 References

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Chapter 6 - *Echinochloa* species identification

This chapter focuses on the identification of *Echinochloa* spp. specimens collected. Identification represents the first step in the analysis of herbicide resistance. Since the genetics of species is different, it is crucial to obtain a precise and correct classification of samples in order to proceed with further experiments.

6.1 Introduction

The genus *Echinochloa* (L.) P. Beauv. belongs to the tribe *Paniceae* R. Br., family *Poaceae* (or *Gramineae*), and includes about 50 annual or perennial species, many of which are weeds (Gould et al., 1972; Michael, 1983; Clayton & Renvoize, 1986; Bajwa et al., 2015, Vidotto et al., 2007). These species are generally cosmopolitan and therophyte native from Europe which have been distributed all over the world, from Asia to America, up to Australia (Carey et al., 1995; Van Devender et al., 1997; Gealy et al., 2003). The most common species in the South-Western Europe are *Echinochloa colona* (L.) Link, *Echinochloa crus-galli* (L.) Beauv, *Echinochloa hispidula* (Retz.) Nees ex Royle, *Echinochloa oryzoides* (Arduino), Fritsch, *Echinochloa oryzicola* (Vasing.) Vasing. and *Echinochloa phyllopogon* (Stapf) Stapf ex Kossenko.

Thanks to their ability to adapt to different ecological conditions and their ability to mimic the crop, *Echinochloa* species are very successful competitors (Barrett, 1983; Fischer et al., 1997; Danquah et al., 2002b; Gibson et al., 2002). *Echinochloa* species have C4 photosynthetic cycle (Patterson, 1984) and some of them (*E. crus-galli*, in particular) can grow both in dry and flooded soils (Benvenuti et al., 1997). These species are the major threat for agriculture, especially in Italian rice cropping, where represent the main weeds: yield losses caused by *Echinochloa* spp. infestations in rice can be very severe and variable in relation to the cultivar and the duration of competition (Holm et al., 1977; Sparacino et al., 1994; Ferrero et al., 2002). Different control practices, including intense chemical control, are applied to reduce infestation in rice cultivation and production systems in many countries (Gibson et al., 2003; Hoagland et al., 2004). Although these efforts, significant crop losses are recorded each year (Hassan et al., 1994; Pandey, 1996; Smith, 1988). Recent studies pointed out that *Echinochloa* spp. competition is strongly reduced when its germination is delayed and it becomes negligible if paddies are kept weed-free for 30 days or more after seeding (Gibson et al., 2002, Vidotto et al., 2007). Gibson and Fisher (2001) demonstrated that in Sri Lanka fields the competition of *E. crus-galli* with rice is mostly due to the interaction between roots. In fact, it has been notice that inhibition of rice growth increased with increasing weed density.

As a matter of fact, infestation of rice by *Echinochloa* species is increasing year after year due to the development of herbicide resistance. *Echinochloa* populations resistant to a variety of herbicide chemicals have been reported, including bispyribac-sodium, cyhalofop-butyl, molinate, propanil, and quinclorac (Garro et al., 1991; Fischer et al., 2000, Fischer et al., 1993; Busi et al., 2002, Iwakami et al, 2013; Cusaro et al., 2022a). Such a scenario has occurred as a result of constraints imposed by current regulations (EC Reg./1107/2009) that have reduced the range of herbicides that can be used, favouring the evolution of herbicide-resistant populations in many weeds, including *Echinochloa* spp.

Despite the great problem posed by these weeds against agriculture and their wide geographic distribution and adaptability, only little information is available on the genetic and morphological variability of them and how this characteristic may be related to herbicide sensitivity/resistance (Vidotto et al., 2004). A deep knowledge of *Echinochloa* species morphological and genetic characteristics would be crucial for their identification, leading to a better targeted management. As a matter of facts, the classification of *Echinochloa* species is often difficult and uncertain, due to the high degree of intraspecific polymorphism characterizing many species of this genus (Michael, 1983). In addition to morphological variability, it must be taken into account that a considerable genetic diversity has also been reported by several studies (Danquah et al., 2002a, Danquah et al., 2002b). It has been reported that the variations observable in some morphological characters (plant height, length of the spikelet, leaf area, number of seeds per spikelet) differ in the species depending on the geographical area (Tasrif et al., 2004). Some *Echinochloa* species also show wide variability in a number of morphological and competition-related traits, such as plant size, tillering ability, seed size, and germination behavior (Barrett and Wilson, 1983; Norris, 1996). For example, panicle shape, length and attitude can be very variable (Norris, 1992), such as seed production. This latter feature characterizes several factors, including competition pressure and timing of emergence (Holm et al., 1977; Norris, 2003). Therefore, proper identification and discrimination of species belonging to the genus *Echinochloa* represent complex and difficult tasks. Nevertheless, they would be of great importance either from an agronomic and economic point of view, either because systematics studies

on these genus are still ongoing. In fact, during the years many researches have been conducted focusing on European *Echinochloa* species identification by means of a morphometric approach (Carretero, 1981; Costea & Tardiff 2002; Tabacchi et al., 2006; Viggiani and Tabacchi, 2017; Hoste et al., 2022). Despite the efforts profuse, there is still a lot of uncertainty.

In the recent years, techniques of molecular biology such as polymerase chain reaction (PCR), polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and DNA barcodes studies have been developed and implemented, opening new possibilities for taxonomic studies on many extant species that are not well characterized morphologically. Since DNA can be extracted either from herbarium specimens or from fresh samples, identification through molecular biology techniques is more accurate than morphological approach. Yasuda et al. (2002) proposed a different methodology to identify *Echinochloa* species performing a polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis. This technique was based on a sequencing analysis that highlighted the presence of nucleotides substitutions within *Echinochloa* species in two non-coding regions of chloroplast DNA (cpDNA), an intergenic spacer region between trnT (UGU) and trnL (UAA), and an intron region of trnL (UAA). These mutation are useful to discriminate *Echinochloa* species throughout selective amplification and digestion of the interested cpDNA trait. Moreover, Yasuda et al. reported the presence of another difference among *Echinochloa* species in the intergenic spacer region between trnT and trnL. This trait measures 495 bp in length in *E. oryzicola* and *E. oryzoides* while 392 bp in length in *E. crus-galli*. The application of this latter methodology has resolved many disputes regarding *Echinochloa* genus systematics, allowing a more precise and less doubtful identification of its species. However, it must be considered that costs to apply this methodology are much higher than performing a morphometric analysis.

This chapter illustrates the protocol applied for the identification of resistant *Echinochloa* populations, based both on the observation of morphological characters and the analysis of intergenic spacer region between trnT (UGU) and trnL (UAA), and of an intron region of trnL (UAA) of the cpDNA.

6.2 Materials & Methods

6.2.1 Samples preparation

A portion of fresh tissue (~ 1 g) was collected from each sample and stored at –20°C for further genetic analysis. The whole samples with inflorescences were dried for morphometric analysis.

6.2.2 Morphometric identification

For each *Echinochloa* spp. sample, morphometric analysis was carried out considering morphological characters analyzed in Carrettero (1981), Costea & Tardif (2002) and Tabacchi et al. (2006). All the characters were observed under optical stereo-microscope (Zeiss) at 10X and 40X magnification.

Quantitative characters were measured using a digital caliper, with an accuracy of one hundred of millimeter. There were considered: (1) the length and width of spikelets; (2) the length of the lower and upper glumes; (3) the ratio lower glume length/spikelet length; (4) the ratio lower glume length/sterile lemma length; (5) the length of panicle; (6) the length and width of the caryopsis. Spikelets and caryopsis have been measured according to Brusoni (1994) sampling scheme, considering 15 spikelet per sample. Mean values were then computed for all quantitative morphological characters measured.

Qualitative characters recorded were: (1) the shape of the lower and upper glumes; (2) the shape of sterile lemma; (3) the presence/absence and arrangement of hairs on the sterile lemma and lower glume; (4) the presence/absence of awns; (5) the color of the caryopsis; (6) the shape of the caryopsis schutellar region; (7) the presence and shape of stigmas of the caryopsis; (8) the presence/absence of hairs in the collar region and on the leaves surface; (9) the color of the spikelets; (10) the color of the basal stem. Mode values were computed for all qualitative morphological characters recorded.

6.2.3 Identification by statistical analysis

The entire statistical analysis was carried out using R 3.6.3 software. Statistical elaboration was performed to group the samples by similarity of the most variable and discriminating morphological traits analyzed. Factor Analisys of Mixed Data (FAMD) was performed to evidence the contribute of each quantitative trait analyzed in order to differentiate *Echinochloa* samples (function "fviz_famd_var" - package factoextra (Kassambara et al., 2020)). Spearmann correlation was calculated in order to evidence correlation among measured quantitative traits and graphed, (function "cor.test" - packages GGally (Schloerke et al. 2021), Hmisc (Harrell Jr, 2021) and corrplot (Wei et al., 2021)). Hierarchical clustering was carried out attempting to discriminate the samples on the basis of their morphological similarity, referring to a sample of certain identification. The dendrogram was constructed using the function "HCPC" (package FactoMineR (Le et al., 2008) on the basis of the "Manhattan" distance, using the "complete" clustering algorithm. Hierarchical clustering was plotted using the function "fviz_dend" (package factoextra (Kassambara et al., 2020)).

6.2.4 Molecular identification

Frozen leaf material was disrupted using TissueRuptor II (Qiagen, Hilden, Germany). Total DNA was extracted using the DNeasy plant mini kit (Qiagen) according to manufacturer's recommendations. The two primer sets specific to a target regions of the intergenic spacer between trnT (UGU) and trnL (UAA), and the entire intron region of trnL (UAA) defined according to the sequence data of early watergrass (*Echinochloa oryzoides* (Arduino) Fritsch.) (AB223081), late watergrass (*Echinochloa oryzicola* (Vasing.) Vasing) (AB223068), and barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.) (AB223073) (http://www.ncbi.nlm.nih.gov/GenBank) were used in PCR amplifications (Figure 6.1). A target region of the intergenic spacer between trnT (UGU) and trnL (UAA), and the entire intron region of trnL (UAA) were amplified separately by using PCR with two primer sets

(Trn-a and Trn-b1; Trn-c and Trn-d – Figure 6.1, Table 6.1) according to Yasuda et al. (2002) and Mennan et al. (2012).

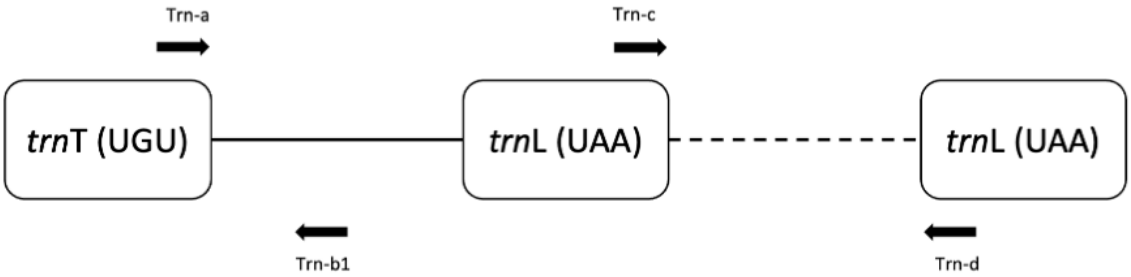

Figure 6.1: The location of primers for amplification and sequencing of the three non-coding regions on cp-DNA. Arrows indicate the direction of strand synthesis. Boxes indicate coding region, solid lines indicate spacer region, dashed lines indicate intron region

Table 6.1: Sequences of the primers for amplification of two noncoding regions of chloroplast DNA (cpDNA).

Primer	**Sequence 5′ – 3′**	**Strand**	**Reference**
Trn-a	CATTACAAATGCGATGCTCT	Sense	Taberlet et al. 1991
Trn-b1	AACGATCGAATGAAAATGCC	Antisense	Yamaguchi et al. 2005
Trn-c	CGAAATCGGTAGACGCTACG	Sense	Taberlet et al. 1991
Trn-d	GGGGATAGAGGGACTTGAAC	Antisense	Taberlet et al. 1991

PCR amplifications were performed in a total volume of 10 µl, containing 2µl of 20 ng μl^{-1} genomic DNA, 2.4µl (0.8U) of GoTaq® Hot Start Green Master Mix (Promega, Madison, Wisconsin, USA), 0.6 µl of each primer (1 µM), 0.5µl of MgCl2 (2mM) and 2 µl of sterile nuclease free water (Promega). The PCR amplification was conducted using a T100 Thermal Cycler (BioRad, Hercules, CA) programmed for 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 57°C, and 1 min extension at 68°C after initial denaturalization for 3 min at 95°C, followed by a final extension step of 10 min at 72° C.

Then, trn-a/trn-b1 amplicons were digested with restriction enzyme EcoRI (G*AATTC), while trn-c/trn-d amplicons were digested with restriction enzyme Alu I (AG*CT) and/or Dra I (TTT*AAA). The digestion reaction were performed in a total volume of 15 µl containing 1 µl of TAE buffer, 1µl (12 U) of enzyme (EcoRI, Alu I, Dra I - Promega),

10 µl of nuclease free water (Promega) and 3 µl of the PCR product (200 ng ~). Digestion was performed in a dry bath (Labnet, part of Aurogene srl, Italy) at 37°C for 24 hours.

RFLPs were finally checked by electrophoresis on 2% agarose gel in 1 x TAE buffer stained with ethidium bromide. PCR products were then visualized under UV light in Molecular Imager® Gel DocTM XR + (BIO-RAD, Hercules, CA, USA). Amplicon size was determined using 100 bp DNA Ladder (Promega). Identification was performed referring to Yasuda et al. (2002), Mennan et al. (2012) and Amaro-Blanco et al. (2021).

6.3 Results and Discussion

6.3.1 Morphometric identification

In Table 6.2 are listed mean and mode values for all quantitative and qualitative measured morphological characters referring to Carrettero (1981), Costea & Tardif (2002) and Tabacchi et al. (2006).

Up to 156 *Echinochloa* spp. samples, 110 were identified univocally throughout the morphological identification according to Carrettero (1981), Costea & Tardif (2002) and Tabacchi et al. (2006). Precisely, 48 samples were identified as *E. crus-galli*, 45 as *E. oryzicola* and 17 as *E. oryzoides*. The least 46 samples were not identified or identified as different species by the three keys. These samples were labelled with a "?" in ID column (Table 6.2).

As a matter of facts, plants which survived to herbicide application are damaged, underdeveloped or wilted and showed brown spots on leaves. Hence, the measurement of morphometric traits could likely be subjected to bias. For this reason, their identification might be very difficult.

Statistical analysis was performed to group specimens on the basis of morphological traits similarity.

Table 6.2: mean and mode values of measured quantitative and qualitative morphological characters. In the first, column is reported the municipalities where specimens were collected. In the second, third, fourth and fifth columns are reported species names assigned through identification keys. Specimens of doubt identification were marked with "?"

IDENTIFICATION_Costea&Tardif_2002	IDENTIFICATION_Carretero_1981	IDENTIFICATION_Tabacchi_etal_2006	Sample_ID	SU_ID	ID	Upper_Glume_Length_(mm)	Spikelet_Width_(mm)	Sterile_Lemma_Length_(mm)	Spikelet_Length_(mm)	Sterile_Lemma_Central_Area_Shape	Lower_Glume_Length_(mm)	N_of_tricomes_Sterile_Lemma	Awn_length_(mm)	Ratio_Lower_Glume/Lemma_Sterile	Ratio_Lower_Glume/Spikelet	Pannicle_Length_(mm)	Pannicle_Internodes_Length_(mm)	Caryopsis_Length_(mm)	Caryopsis_Width_(mm)	Caryopsis_Stiles	Scutellar_Shape	Caryopsis_Color	Tricome_Presence_Root_Crown	Tricome_Presence_Leaf	Inflorescence	Ear_Disposition	Spikelet_Color	Basal_Stem_Color
E. oryzicola	E. oryzicola	E. oryzicola	Daghetta1	Robbio_Daghetta_1	Eoryzicola	6.36	2.07	4.53	5.44	glabra	3	5	9.56	0.66	0.55	<20_cm	23.28	2.16	1.64	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	Daghetta2	Robbio_Daghetta_2	Eoryzicola	7.34	2.15	4.76	6.05	glabra	3.31	5	12.2	0.7	0.55	< 20 cm	21.56	2.16	1.75	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	Daghetta3	Robbio_Daghetta_3	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	Daghetta4	Robbio_Daghetta_4	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzoides	E. oryzoides	E. oryzoides	MI1.1	Mairano_1	Eoryzoides	6.61	2.21	3.8	5.2	tricomi	2.11	5	41.78	0.56	0.4	>20_cm	25.02	1.5	1.36	sì	S	NA	no	no	eretta	alterne	brune	rosso
E. oryzoides	E. oryzoides	E. oryzoides	MI1.2	Mairano_2	Eoryzoides	6.41	2.21	4.08	5.25	tricomi	2.25	5	34.84	0.55	0.43	>20_cm	48.64	1.58	1.31	sì	S	NA	no	no	eretta	alterne	brune	rosso
E. oryzoides	E. oryzoides	E. oryzoides	MI1.3	Mairano_3	Eoryzoides	5.5	2.32	3.91	4.71	tricomi	1.77	5	43.43	0.46	0.38	>20_cm	37.99	1.98	1.4	sì	S	NA	no	no	eretta	alterne	brune	rosso
E. oryzoides	E. oryzoides	E. oryzoides	MI1.4	Mairano_4	Eoryzoides	4.57	2.07	3.71	4.14	tricomi	1.58	5	43.8	0.43	0.38	>20_cm	31.27	1.87	1.45	sì	S	NA	no	no	eretta	alterne	brune	rosso
E. oryzicola	E. oryzicola	?	MI2.1	Ozzero_1	?	5.55	2.09	5.33	5.44	glabra	3.36	3	0	0.63	0.62	<20_cm	18.45	1.53	1.37	sì	S	NA	no	si	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	MI2.2	Ozzero_2	Eoryzicola	5.96	2.38	5.26	5.61	glabra	2.75	3	0	0.52	0.49	<20_cm	17.54	2	1.76	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	MI2.3	Ozzero_3	Eoryzicola	5.72	2.53	4.91	5.32	glabra	2.81	3	0	0.57	0.53	<20_cm	8.74	2	1.64	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	?	?	MI2.4	Ozzero_4	?	6.39	2.47	5.23	5.81	tricomi	2.97	3	0	0.57	0.51	<20_cm	7.98	2.35	1.87	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzoides	?	E. oryzoides	PV1.1	Ottobiano_1	?	6.87	2.36	5.02	5.95	tricomi	2.81	5	12.05	0.56	0.48	<20_cm	25	2.42	1.78	sì	S	NA	no	no	eretta	alterne	verdi	NA
E. oryzoides	?	E. oryzoides	PV1.2	Ottobiano_2	?	6.95	2.36	4.52	5.74	glabra	2.71	5	12.03	0.6	0.47	<20_cm	8.98	2.48	1.91	sì	S	NA	no	no	eretta	alterne	verdi	NA
E. oryzoides	?	?	PV1.3	Ottobiano_3	?	5.32	2.31	4.38	4.85	glabra	2.43	5	11.13	0.56	0.5	<20_cm	9.77	2.4	1.9	sì	S	NA	no	no	eretta	alterne	verdi	NA
E. oryzoides	?	E. oryzoides	PV1.4	Ottobiano_4	?	6.39	2.41	4.59	5.49	glabra	2.32	5	7.04	0.51	0.42	<20_cm	7.67	2.57	1.89	sì	S	NA	no	no	eretta	alterne	verdi	NA
E. oryzicola	E. oryzicola	E. oryzicola	PV10.1	Rivoltella_1	Eoryzicola	6.36	2.07	4.53	5.44	glabra	3	5	9.56	0.66	0.55	<20_cm	23.28	2.16	1.64	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV10.2	Rivoltella_2	Eoryzicola	7.34	2.15	4.76	6.05	glabra	3.31	5	12.2	0.7	0.55	< 20 cm	21.56	2.16	1.75	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV10.3	Rivoltella_3	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV10.4	Rivoltella_4	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
?	E. oryzoides	E.oryzoides	PV11.1	Vigevano_Barbavara_1	?	5.95	2.41	4.39	5.17	glabra	2.1	3	4.14	0.48	0.41	<20_cm	5.96	2.57	1.9	sì	S	NA	sì	no	eretta	opposte	verdi	verde
E. oryzicola	E. oryzicola	E.oryzicola	PV11.2	Vigevano_Barbavara_2	Eoryzicola	6.39	2.4	4.15	5.27	glabra	2.95	5	10.5	0.71	0.56	<20_cm	7.92	2.35	1.75	sì	R	NA	sì	no	eretta	opposte	verdi	verde
?	E. oryzicola	E.oryzicola	PV11.3	Vigevano_Barbavara_3	?	4.88	2.34	4.15	4.51	glabra	2.32	3	2.51	0.56	0.51	<20_cm	8.46	2.62	1.86	sì	R	NA	sì	no	eretta	opposte	verdi	verde
E.oryzicola	E. oryzicola	E. oryzicola	PV11.4	Vigevano_Barbavara_4	Eoryzicola	5.7	2.44	4.47	5.08	glabra	2.99	3	6.87	0.67	0.59	<20_cm	6.77	2.49	1.85	sì	R	NA	sì	no	eretta	opposte	verdi	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV12.1	Bascape_1	Ecrusgalli	3.37	1.92	3.26	3.31	tricomi	1.55	5	0	0.48	0.47	<20_cm	22.46	1.85	1.44	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV12.2	Bascape_2	Ecrusgalli	3.35	1.92	3.26	3.3	tricomi	1.52	5	0	0.47	0.46	<20_cm	22.46	1.83	1.45	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
?	?	E. oryzoides	PV12.3	Bascape_3	?	4.95	2.12	3.6	4.28	tricomi	1.83	5	3.87	0.51	0.43	<20_cm	17.99	1.75	1.42	no	S	NA	no	no	eretta	alterne	verdi/brune	verde
?	?	?	PV12.4	Bascape_4	?	4.75	1.98	3.48	4.12	tricomi	1.63	5	21.34	0.46	0.39	<20_cm	17.92	1.71	1.42	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. crus-galli ?	?	E. crus-galli	PV13.1	Valle_Lomellina_1	?	3.66	1.69	3.1	3.38	glabra	1.4	3	0	0.45	0.41	<20_cm	9.86	1.66	1.21	sì	S	NA	no	si	natante	alterne	brune	rosso
E. crus-galli	E. crus-galli	E. crus-galli	PV13.2	Valle_Lomellina_2	Ecrusgalli	3.61	1.83	3.44	3.52	glabra	1.4	3	0	0.41	0.4	<20_cm	9.86	1.73	1.28	sì	S	NA	no	si	natante	alterne	brune	rosso
E. crus-galli	?	?	PV13.3	Valle_Lomellina_3	?	3.89	1.92	3.46	3.67	glabra	1.53	3	0	0.44	0.42	<20_cm	9.86	1.75	1.46	sì	S	NA	si	si	eretta	alterne	verdi/brune	rosso
E. crus-galli	?	E. crus-galli	PV13.4	Valle_Lomellina_4	?	3.65	1.77	3.66	3.66	glabra	1.39	3	0	0.38	0.38	<20_cm	9.86	1.74	1.28	sì	S	NA	si	no	eretta	alterne	verdi/brune	rosso
?	E. oryzicola	E. oryzicola	PV14.1	Frascarolo_Sartirana_1	?	5.44	2.25	4.05	4.74	glabra	2.44	3	6.04	0.6	0.51	<20_cm	15.08	2.54	1.87	sì	S	NA	sì	no	eretta	alterne	verdi	verde
?	E. oryzoides	?	PV14.2	Frascarolo_Sartirana_2	?	4.49	2.1	3.28	3.89	tricomi	1.46	5	30.34	0.45	0.38	<20_cm	14.76	1.94	1.35	sì	S	NA	sì	sì	natante	alterne	verdi/brune	verde
E. oryzoides	E. oryzoides	E. oryzoides	PV14.3	Frascarolo_Sartirana_3	Eoryzoides	5.54	2.04	3.54	4.54	tricomi	1.95	5	13.05	0.55	0.43	>20_cm	13.65	1.82	1.37	sì	S	NA	no	no	natante	opposte	verdi/brune	verde
?	E. oryzoides	E. oryzoides	PV14.4	Frascarolo_Sartirana_4	?	6.35	2.11	3.46	4.9	tricomi	1.92	5	43.23	0.56	0.39	>20_cm	13.65	1.82	1.36	sì	S	NA	no	no	natante	alterne	verdi/brune	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV15.1.1	Zerbolo1_1	Eoryzicola	4.1	2.14	4.5	4.3	glabra	2	3	3.96	0.45	0.47	<20_cm	37.25	2.47	1.91	sì	S	NA	sì	no	eretta	alterne	verdi	NA
E. oryzicola	E. oryzicola	E. oryzicola	PV15.1.2	Zerbolo1_2	Eoryzicola	4.4	2.05	3.85	4.13	glabra	1.91	3	1.69	0.5	0.46	<20_cm	23.65	2.34	1.75	sì	S	NA	sì	no	natante	alterne	verdi	NA
E. oryzicola	E. oryzicola	E. oryzicola	PV15.1.3	Zerbolo1_3	Eoryzicola	5.13	2.28	4.04	4.58	glabra	2.27	3	4.34	0.56	0.5	<20_cm	30.25	2.49	1.92	sì	S	NA	sì	no	natante	alterne	verdi	NA
E. oryzicola	E. oryzicola	E. oryzicola	PV15.1.4	Zerbolo1_4	Eoryzicola	4.87	2.31	3.85	4.36	glabra	2.15	5	2.46	0.56	0.49	<20_cm	27.07	2.42	1.78	sì	S	NA	sì	no	natante	alterne	verdi	NA
E. oryzicola	E. oryzicola	E. oryzicola	PV15.2.1	Zerbolo2_1	Eoryzicola	6.36	2.07	4.53	5.44	glabra	3	5	9.56	0.66	0.55	<20_cm	23.28	2.16	1.64	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV15.2.2	Zerbolo2_2	Eoryzicola	7.34	2.15	4.76	6.05	glabra	3.31	5	12.2	0.7	0.55	< 20 cm	21.56	2.16	1.75	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV15.2.3	Zerbolo2_3	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV15.2.4	Zerbolo2_4	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV16.1	Belgioioso_StMargherita_1	Eoryzicola	4.37	2.19	4.37	4.37	glabra	2.23	3	0	0.52	0.51	<20_cm	9.86	2.46	1.79	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzoides	E. oryzoides	?	PV16.2	Belgioioso_StMargherita_2	?	5.27	2.54	4.59	4.93	glabra	2.48	3	0	0.54	0.5	<20_cm	9.86	2.76	1.98	sì	R	NA	no	no	eretta	alterne	verdi	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV16.3	Belgioioso_StMargherita_3	Ecrusgalli	3.06	2	2.89	2.98	glabra	1.23	3	reste	0.43	0.41	<20_cm	9.86	1.59	1.39	sì	S	NA	sì	sì	eretta	alterne	verdi/brune	rosso
E. oryzicola	E. oryzicola	E. oryzicola	PV16.4	Belgioioso_StMargherita_4	Eoryzicola	4.51	2.17	3.97	4.24	glabra	2.2	3	0	0.55	0.52	<20_cm	9.86	2.37	1.7	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV17.1.1	Cozzo1_1	Eoryzicola	4.46	2.02	4.18	4.32	glabra	2.31	2	2.56	0.55	0.53	<20_cm	3.87	2.22	1.6	no	R	NA	sì	no	eretta	alterne	verdi/brune	rosso
E. oryzicola	E. oryzicola	E. oryzicola	PV17.1.2	Cozzo1_2	Eoryzicola	4.21	2.28	3.7	3.95	glabra	2.02	2	2.52	0.54	0.51	<20_cm	5.82	2.39	1.85	sì	R	NA	sì	no	eretta	alterne	verdi/brune	rosso
E. oryzicola	E. oryzicola	E.oryzicola	PV17.1.3	Cozzo1_3	Eoryzicola	4.3	2.16	4.37	4.33	glabra	2.2	2	0	0.5	0.51	<20_cm	6.91	2.16	1.73	sì	R	NA	sì	no	eretta	alterne	verdi	rosso
E. oryzoides	E.oryzoides	?	PV17.1.4	Cozzo1_4	?	4.26	2.03	3.73	3.99	glabra	1.88	2	1.85	0.5	0.47	<20_cm	6.03	2.22	1.61	no	R	NA	sì	no	eretta	alterne	verdi	rosso
E. oryzicola	E. oryzicola	E. oryzicola	PV17.2.1V	Cozzo2_1V	Eoryzicola	4.46	2.02	4.18	4.32	glabra	2.31	2	2.56	0.55	0.53	<20_cm	3.87	2.22	1.6	no	R	NA	sì	no	eretta	alterne	verdi/brune	rosso
E. oryzicola	E. oryzicola	E. oryzicola	PV17.2.2V	Cozzo2_2V	Eoryzicola	4.21	2.28	3.7	3.95	glabra	2.02	2	2.52	0.54	0.51	<20_cm	5.82	2.39	1.85	sì	R	NA	sì	no	eretta	alterne	verdi/brune	rosso
E. oryzicola	E. oryzicola	E.oryzicola	PV17.2.3V	Cozzo2_3V	Eoryzicola	4.3	2.16	4.37	4.33	glabra	2.2	2	0	0.5	0.51	<20_cm	6.91	2.16	1.73	sì	R	NA	sì	no	eretta	alterne	verdi	rosso
E. oryzoides	E.oryzoides	?	PV17.2.4V	Cozzo2_4V	?	4.26	2.03	3.73	3.99	glabra	1.88	2	1.85	0.5	0.47	<20_cm	6.03	2.22	1.61	no	R	NA	sì	no	eretta	alterne	verdi	rosso
?	?	E.crus-galli	PV18.1	Lardirago_1	?	2.81	1.69	2.68	2.75	glabra	1.04	1	0	0.78	0.38	<20_cm	15.51	1.7	1.26	no	S	NA	no	no	eretta	alterne	verdi/brune	rosso
E.crus-galli	?	?	PV18.2	Lardirago_2	?	2.51	1.65	2.27	2.39	glabra	0.94	1	0	0.42	0.39	<20_cm	46.32	1.7	1.35	sì	S	NA	no	no	eretta	alterne	verdi	rosso
E. crus-galli	E. crus-galli	E.crus-galli	PV18.3	Lardirago_3	Ecrusgalli	2.94	1.66	2.41	2.68	glabra	1.09	1	0	0.45	0.41	<20_cm	31.6	1.58	1.2	no	S	NA	no	no	eretta	alterne	verdi	rosso
E. crus-galli	E. crus-galli	E.crus-galli	PV18.4	Lardirago_4	Ecrusgalli	2.89	1.74	2.84	2.87	glabra	1.13	1	0	0.4	0.4	<20_cm	46.76	1.6	1.22	no	S	NA	no	no	eretta	alterne	verdi/brune	rosso
?	E. oryzoides	E. oryzoides	PV2.1	Sannazzaro_deBurgondi_1	?	5.09	2.23	3.35	4.22	tricomi	1.85	5	10.17	0.55	0.44	>20_cm	23.75	1.58	1.35	sì	S	NA	no	no	natante	alterne	verdi	verde
?	E. oryzoides	E. oryzoides	PV2.2	Sannazzaro_deBurgondi_2	?	5.98	2.11	3.74	4.86	tricomi	1.83	5	28.23	0.49	0.38	>20_cm	21.48	1.42	1.21	sì	S	NA	no	no	natante	alterne	verdi	verde
?	?	?	PV2.3	Sannazzaro_deBurgondi_3	?	4.27	1.94	3.22	3.74	tricomi	1.4	5	10.95	0.43	0.37	>20_cm	19.95	1.82	1.41	sì	S	NA	no	no	natante	alterne	verdi	verde
E. oryzoides	E. oryzoides	E. oryzoides	PV2.4	Sannazzaro_deBurgondi_4	Eoryzoides	5.85	2.2	3.87	4.86	tricomi	1.81	5	55.32	0.47	0.37	>20_cm	13.69	2.03	1.46	sì	S	NA	no	no	natante	alterne	verdi	verde
E. oryzicola	E. oryzicola	E.oryzicola	PV21.1	Roncaro_1	Eoryzicola	5.07	2.15	3.73	4.4	glabra	2.28	2	4.44	0.61	0.52	<20_cm	7.98	2.47	1.75	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzicola	E. oryzicola	E.oryzicola	PV21.2	Roncaro_2	Eoryzicola	4.04	2.25	3.94	3.99	glabra	2.12	2	0	0.54	0.53	<20_cm	7.54	2.37	1.96	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E.oryzicola	PV21.3	Roncaro_3	Eoryzicola	5.25	2.26	3.76	4.5	glabra	2.5	2	5.11	0.66	0.55	<20_cm	3.45	2.36	1.88	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzicola	E. oryzicola	E.oryzicola	PV21.4	Roncaro_4	Eoryzicola	4.81	2.21	4.27	4.54	glabra	2.47	2	4.24	0.58	0.54	<20_cm	4.97	2.46	1.8	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E crus-galli	E crus-galli	E crus-galli	PV22.1	Zinasco_Vecchia_1	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	opposte	gialle	NA
E crus-galli	E crus-galli	E crus-galli	PV22.2	Zinasco_Vecchia_2	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	opposte	gialle	NA
E crus-galli	E crus-galli	E crus-galli	PV22.3	Zinasco_Vecchia_3	Ecrusgalli	3.65	1.86	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	opposte	gialle	NA
E crus-galli	E crus-galli	E crus-galli	PV22.4	Zinasco_Vecchia_4	Ecrusgalli	3.64	1.8	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	opposte	gialle	NA
?	?	?	PV23.1	Pieve_Albignola_1	?	4.5	2.16	3.88	4.19	glabra	2.14	4	1.56	0.55	0.51	<20_cm	10.87	NA	NA	NA	NA	NA	no	no	eretta	alterne	verdi	verde
?	E. oryzicola	E.oryzicola	PV23.2	Pieve_Albignola_2	?	4.52	2.4	4.13	4.32	glabra	2.25	4	0	0.55	0.52	<20_cm	7.52	2.55	1.92	no	R	NA	no	no	eretta	alterne	verdi	verde
?	?	E.oryzicola	PV23.3	Pieve_Albignola_3	?	4.49	2.19	4.21	4.35	glabra	2.18	4	0	0.52	0.5	<20_cm	5.9	2.41	1.88	no	R	NA	no	no	eretta	alterne	verdi	verde
E. crus-galli	E. crus-galli	E.crus-galli	PV23.4	Pieve_Albignola_4	Ecrusgalli	4.01	2.19	4.02	3.85	glabra	1.98	4	0	0.49	0.51	<20_cm	7.22	2.37	1.87	sì	R	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzicola	E. oryzicola	E.oryzicola	PV24.1	SanMartino_Siccomario_1	Eoryzicola	5.07	2.49	4.31	4.69	glabra	2.69	5	5.24	0.62	0.57	<20_cm	4.98	2.41	1.93	sì	R	NA	no	no	eretta	alterne	verdi/brune	verde
?	?	E.oryzicola	PV24.2	SanMartino_Siccomario_2	?	4.09	2.36	3.88	3.98	glabra	2.02	5	0	0.52	0.51	<20_cm	3.99	2.45	1.91	sì	R	NA	no	no	eretta	alterne	verdi/brune	verde
E. crus-galli	E. crus-galli	E.crus-galli	PV24.3	SanMartino_Siccomario_3	Ecrusgalli	2.9	1.61	2.92	2.91	tricomi	1.2	4	6.92	0.41	0.41	<20_cm	8.94	1.41	1.16	sì	S	NA	sì	sì	eretta	alterne	verdi/brune	rosso
E.oryzoides	?	?	PV24.4	SanMartino_Siccomario_4	?	4.43	2.26	4.94	4.69	glabra	2.25	2	0	0.45	0.48	<20_cm	3.76	2.52	1.94	sì	R	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzoides	E. oryzicola	E. oryzicola	PV26.1	SanGiorgio_Lomellina_1	?	5.71	2.29	4.44	5.11	glabra	2.88	3	4.85	0.65	0.56	<20_cm	20.76	2.72	1.94	sì	S	NA	no	no	eretta	alterne	brune	rosso
E. oryzoides ?	E. oryzoides	E. oryzicola	PV26.2	SanGiorgio_Lomellina_2	?	4.76	2.14	3.91	4.17	glabra	2.23	3	1.57	0.57	0.54	<20_cm	19.54	2.68	1.89	sì	S	NA	no	no	eretta	alterne	brune	verde
E. oryzoides ?	E. oryzoides	E. oryzicola	PV26.3	SanGiorgio_Lomellina_3	Eoryzoides	4.74	2.13	3.93	4.17	glabra	2.28	3	2.04	0.58	0.55	<20_cm	19.54	2.67	1.87	sì	S	NA	no	no	eretta	alterne	brune	verde
E. oryzoides ?	E. oryzoides	E. oryzicola	PV26.4	SanGiorgio_Lomellina_4	Eoryzoides	4.74	2.13	3.93	4.17	glabra	2.28	3	2.04	0.58	0.55	<20_cm	19.54	2.67	1.87	sì	S	NA	no	no	eretta	alterne	brune	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV27.1	Lomello_1	Eoryzicola	6.36	2.07	4.53	5.44	glabra	3	5	9.56	0.66	0.55	<20_cm	23.28	2.16	1.64	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV27.2	Lomello_2	Eoryzicola	7.34	2.15	4.76	6.05	glabra	3.31	5	12.2	0.7	0.55	< 20 cm	21.56	2.16	1.75	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV27.3	Lomello_3	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV27.4	Lomello_4	Eoryzicola	7.18	2.15	4.82	6	glabra	3.34	5	11.95	0.69	0.56	< 20 cm	21.56	2.2	1.77	sì	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzoides	E. oryzicola	E. oryzicola	PV28.1	Dorno_1	Eoryzoides	5.03	2.2	3.99	4.47	glabra	2.6	4	5.54	0.65	0.58	>20_cm	36.79	2.6	1.71	sì	S	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola ?	E. oryzicola	E. oryzicola	PV28.2	Dorno_2	Eoryzicola	5.16	2.36	4.48	4.82	glabra	2.74	5	3.59	0.61	0.57	<20_cm	27.06	1.95	1.5	NA	NA	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola ?	E. oryzicola	E. oryzicola	PV28.3	Dorno_3	Eoryzicola	5.3	2.38	4.56	4.93	glabra	2.81	5	3.77	0.62	0.57	<20_cm	27.06	2.14	1.59	NA	NA	NA	no	no	eretta	alterne	verdi	verde
E. oryzicola ?	E. oryzicola	E. oryzicola	PV28.4	Dorno_4	Eoryzicola	5.3	2.38	4.56	4.93	glabra	2.81	5	3.77	0.62	0.57	<20_cm	27.06	2.14	1.59	NA	NA	NA	no	no	eretta	alterne	verdi	verde
E. oryzoides	E. oryzoides	E.oryzoides	PV3.1	Suardi_1	Eoryzoides	5.63	2.3	4.58	5.11	glabra	2.29	5	7.54	0.5	0.45	<20_cm	4.76	2.53	1.9	no	R	NA	sì	no	eretta	opposte	verdi/brune	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV3.2	Suardi_2	Eoryzicola	4.75	2.17	3.81	4.28	glabra	2.24	2	3.36	0.59	0.52	<20_cm	2.78	2.37	1.77	sì	R	NA	sì	no	eretta	opposte	verdi/brune	verde
E. oryzoides	E.oryzoides	?	PV3.3	Suardi_3	?	4.73	2.3	3.32	4.03	glabra	1.82	2	1.74	0.58	0.46	<20_cm	3.86	2.48	1.88	sì	R	NA	sì	no	eretta	opposte	verdi/brune	verde
?	?	?	PV3.4	Suardi_4	?	4.8	2.13	3.77	4.28	glabra	2.08	2	4.94	0.55	0.49	<20_cm	6.93	2.35	1.62	sì	R	NA	sì	no	eretta	opposte	verdi/brune	verde
E. oryzoides ?	E. oryzicola	E. oryzicola	PV30.1	Giussago_1	Eoryzoides	7.1	2.3	4.84	5.97	glabra	3.3	5	10.31	0.69	0.55	<20_cm	31.36	2.65	1.87	no	S	NA	sì	no	eretta	alterne	verdi	verde
E. oryzicola	E.oryzicola	E. oryzicola	PV30.2	Giussago_2	Eoryzicola	4.82	2.09	3.98	4.4	glabra	2.81	5	1.71	0.71	0.64	<20_cm	19.84	1.61	0.89	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzicola	E.oryzicola	E. oryzicola	PV30.3	Giussago_3	Eoryzicola	5.16	2.1	4.2	4.68	glabra	2.96	5	3.38	0.7	0.63	<20_cm	19.84	1.66	1.06	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzicola	E.oryzicola	E. oryzicola	PV30.4	Giussago_4	Eoryzicola	5.16	2.1	4.2	4.68	glabra	2.96	5	3.38	0.7	0.63	<20_cm	19.84	1.66	1.06	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV31.1	Carbonara_alTicino_1	Ecrusgalli	3.02	1.58	3.3	3.16	glabra	1.26	4	NA	0.38	0.4	<20_cm	39.24	1.65	1.19	no	S	NA	no	no	natante	alterne	verdi/brune	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV31.2	Carbonara_alTicino_2	Ecrusgalli	3.76	1.68	3.15	3.46	glabra	1.51	4	1.39	0.48	0.44	<20_cm	22.04	1.84	1.35	no	S	NA	no	no	natante	alterne	verdi/brune	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV31.3	Carbonara_alTicino_3	Ecrusgalli	3.73	1.63	3.1	3.42	glabra	1.51	4	1.15	0.49	0.44	<20_cm	22.04	1.82	1.35	no	S	NA	no	no	natante	alterne	verdi/brune	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV31.4	Carbonara_alTicino_4	Ecrusgalli	3.73	1.63	3.1	3.42	glabra	1.51	4	1.15	0.49	0.44	<20_cm	22.04	1.82	1.35	no	S	NA	no	no	natante	alterne	verdi/brune	verde
E. oryzoides	?	E. oryzoides	PV32.1.1	Candia_diLomellina1_1	?	4.5	2.21	4.71	4.6	tricomi	1.79	5	0	0.38	0.39	<20_cm	14.69	2.56	1.77	sì	S	NA	no	no	eretta	alterne	rosse	rosso
?	?	E. oryzoides	PV32.1.2	Candia_diLomellina1_2	?	3.81	2.13	3.8	3.81	tricomi	1.64	5	0	0.43	0.43	<20_cm	15.34	1.92	1.69	sì	S	NA	no	no	eretta	alterne	rosse	rosso
E. oryzoides	E. oryzoides	E. oryzoides	PV32.1.3	Candia_diLomellina1_3	Eoryzoides	4.33	2.19	4.94	4.63	tricomi	2.01	3	0	0.41	0.43	<20_cm	14.79	2.53	1.81	no	S	NA	no	no	eretta	alterne	verdi/rosse	rosso
E. oryzicola	E. oryzicola	E. oryzicola	PV32.1.4	Candia_diLomellina1_4	Eoryzicola	5.04	2.45	4.02	4.53	tricomi	2.59	3	4.23	0.64	0.57	<20_cm	15.82	2.64	2.03	no	S	NA	no	no	eretta	alterne	verdi/rosse	rosso
E. oryzoides	E. oryzoides	E. oryzoides	PV32.2.1	Candia_diLomellina2_1	Eoryzoides	4.19	2.08	4.32	4.26	glabra	1.77	3	0	0.42	0.42	<20_cm	13.77	2.35	1.63	sì	S	NA	no	no	eretta	alterne	verdi	verde
?	?	E. oryzicola	PV32.2.2	Candia_diLomellina2_2	?	4.66	2.06	4.56	4.61	glabra	2.13	3	0	0.47	0.46	<20_cm	12.26	2.16	1.57	sì	S	NA	no	no	eretta	alterne	verdi	verde
?	?	E. oryzicola	PV32.2.3	Candia_diLomellina2_3	?	4.43	2.17	4.41	4.42	glabra	2.06	3	0	0.47	0.47	<20_cm	15.67	2.35	1.66	sì	S	NA	no	no	eretta	alterne	verdi	verde
?	?	?	PV32.2.4	Candia_diLomellina2_4	?	4.4	1.89	4.65	4.53	glabra	2.06	3	0	0.44	0.45	<20_cm	16.87	1.87	1.32	sì	S	NA	no	no	natante	alterne	verdi	verde
?	E. oryzicola	E. oryzicola	PV4.1	Borgo_SanSiro_1	?	5.44	2.25	4.05	4.74	glabra	2.44	3	6.04	0.6	0.51	<20_cm	15.08	2.54	1.87	sì	S	NA	sì	no	eretta	alterne	verdi	verde
?	E. oryzoides	?	PV4.2	Borgo_SanSiro_2	?	4.49	2.1	3.28	3.89	tricomi	1.46	5	30.34	0.45	0.38	<20_cm	14.76	1.94	1.35	sì	S	NA	sì	sì	natante	alterne	verdi/brune	verde
E. oryzoides	E. oryzoides	E. oryzoides	PV4.3	Borgo_SanSiro_3	Eoryzoides	5.54	2.04	3.54	4.54	tricomi	1.95	5	13.05	0.55	0.43	>20_cm	13.65	1.82	1.37	sì	S	NA	no	no	natante	opposte	verdi/brune	verde
?	E. oryzoides	E. oryzoides	PV4.4	Borgo_SanSiro_4	?	6.35	2.11	3.46	4.9	tricomi	1.92	5	43.23	0.56	0.39	>20_cm	13.65	1.82	1.36	sì	S	NA	no	no	natante	alterne	verdi/brune	verde
E. crus-galli	E. crus-galli	E. crus-galli	PV5.1	Cilavegna_1	Ecrusgalli	4.6	1.95	3.29	3.95	tricomi	1.78	5	17.55	0.54	0.45	>20_cm	39.29	1.87	1.4	sì	S	NA	no	no	natante	alterne	brune	NA
E. crus-galli	E. crus-galli	E. crus-galli	PV5.2	Cilavegna_2	Ecrusgalli	4.31	2.11	3.54	3.93	tricomi	2.15	5	36.98	0.61	0.55	>20_cm	46.02	1.88	1.52	sì	S	NA	no	no	eretta	alterne	verdi	NA
E. crus-galli	E. crus-galli	E. crus-galli	PV5.3	Cilavegna_3	Ecrusgalli	3.64	1.7	3.07	3.36	tricomi	1.53	5	3.69	0.5	0.46	<20_cm	18.72	1.71	1.23	sì	S	NA	no	no	eretta	alterne	verdi	NA
E. crus-galli	E. crus-galli	E. crus-galli	PV5.4	Cilavegna_4	Ecrusgalli	3.72	1.71	3.03	3.38	glabra	1.45	5	5.15	0.48	0.43	>20_cm	50	1.62	1.28	sì	S	NA	no	no	eretta	opposte	gialle	NA
?	E. oryzoides	E. oryzoides	PV6.1	Zeme_1	?	4.57	1.89	3.44	4	tricomi	1.52	5	27.97	0.44	0.38	>20_cm	22.34	1.83	1.46	sì	S	NA	no	no	eretta	alterne	brune	NA
?	E. oryzoides	E. oryzoides	PV6.2	Zeme_2	?	4.62	2.09	3.42	4.02	tricomi	1.46	5	25.95	0.43	0.36	<20_cm	32	1.83	1.38	sì	S	NA	no	no	eretta	opposte	brune	NA
?	E. oryzoides	E. oryzoides	PV6.3	Zeme_3	?	4.62	2.04	3.39	4.01	tricomi	1.51	5	36.35	0.44	0.38	>20_cm	35	1.9	1.43	sì	S	NA	no	no	eretta	opposte	brune	NA
?	E. oryzoides	E. oryzoides	PV6.4	Zeme_4	?	4.45	1.89	3.39	3.92	tricomi	1.55	5	35.45	0.46	0.39	>20_cm	40	1.86	1.34	sì	S	NA	no	no	eretta	opposte	brune	NA
E. oryzoides	E. oryzoides	E. oryzoides	PV7.1	Robbio_1	Eoryzoides	5.06	2.26	4.07	4.57	glabra	1.95	4	4.78	0.48	0.43	<20_cm	23.96	2.62	2.05	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzoides	E. oryzoides	?	PV7.2	Robbio_2	?	5.43	2.35	3.86	4.65	glabra	2.31	4	8.02	0.6	0.5	<20_cm	22.12	2.63	2.1	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzoides	E. oryzoides	E. oryzoides	PV7.3	Robbio_3	Eoryzoides	5.23	2.23	3.7	4.47	glabra	1.9	4	3.32	0.51	0.42	<20_cm	19.64	2.54	1.87	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. oryzoides	E. oryzoides	E. oryzoides	PV7.4	Robbio_4	Eoryzoides	5.23	2.23	3.7	4.47	glabra	1.9	4	3.32	0.51	0.42	<20_cm	19.64	2.54	1.87	sì	S	NA	no	no	eretta	alterne	verdi/brune	verde
E. crus-galli	E.crus-galli	E. crus-galli	PV8.1	Parona_1	Ecrusgalli	3.1	1.91	2.89	3	glabra	1.25	3	0	0.44	0.42	<20_cm	14.87	1.7	1.37	sì	S	NA	sì	no	natante	alterne	rosse	rosso
E. crus-galli	E. crus-galli	E. crus-galli	PV8.2	Parona_2	Ecrusgalli	3.09	1.72	2.83	2.96	glabra	1.3	3	0	0.46	0.44	<20_cm	11.24	1.67	1.33	sì	S	NA	sì	no	natante	alterne	rosse	rosso
E. crus-galli	E. crus-galli	E. crus- galli	PV8.3	Parona_3	Ecrusgalli	3.24	1.87	2.82	3.03	glabra	1.19	3	0	0.42	0.39	<20_cm	13.21	1.56	1.35	sì	S	NA	sì	no	natante	alterne	rosse	rosso
E. crus-galli	E. crus-galli	E. crus-galli	PV8.4	Parona_4	Ecrusgalli	3.64	2.09	3.36	3.5	glabra	1.55	3	0	0.46	0.44	<20_cm	13.21	1.68	1.4	sì	S	NA	sì	no	natante	alterne	rosse	rosso
E. crus-galli	E. crus-galli	E. crus-galli	PV9.1	Vigevano_1	Ecrusgalli	3.04	1.54	3.28	3.16	pelosa	1.42	0	0	0.44	0.45	<20_cm	33.81	1.49	1.19	no	S	ocra	no	no	eretta	alterne	verdi	NA
E. crus-galli	E. crus-galli	E. crus-galli	PV9.2	Vigevano_2	Ecrusgalli	3.22	1.62	3.34	3.28	pelosa	1.66	0	5.99	0.5	0.5	<20_cm	27.36	1.83	1.34	sì	S	ocra	no	no	eretta	alterne	verdi	NA
E. crus-galli	E. crus-galli	E. crus-galli	PV9.3	Vigevano_3	Ecrusgalli	3.06	1.66	3.36	3.21	pelosa	1.7	0	0	0.51	0.53	<20_cm	21.98	1.95	1.46	sì	S	ocra	no	no	eretta	alterne	verdi	NA
E. crus-galli	E. crus-galli	E. crus-galli	PV9.4	Vigevano_4	Ecrusgalli	3.14	1.75	3.8	3.47	pelosa	1.78	0	0	0.47	0.51	<20_cm	46.66	1.9	1.44	sì	S	ocra	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV19.1	Pieve_delCairo_1	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV19.2	Pieve_delCairo_2	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV19.3	Pieve_delCairo_3	Ecrusgalli	3.65	1.86	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV19.4	Pieve_delCairo_4	Ecrusgalli	3.64	1.8	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E. oryzicola	E. oryzicola	E. oryzicola	PV20.1	Genzone_1	Eoryzicola	5.16	3.9	2.6	5.31	3 file	2.28	0	0	0.65	0.59	<20_cm	18.99	2.37	1.67	sì	S	giallo	no	no	eretta	alterne	gialle	verde
E.oryzicola	E.oryzicola	E.oryzicola	PV20.2	Genzone_2	Eoryzicola	5.16	3.9	2.6	5.31	4 file	3.28	0	0	0.64	0.59	<20_cm	19.84	2.43	1.82	sì	S	giallo	no	no	eretta	alterne	verdi/brune	verde
E. oryzicola	E. oryzicola	E. oryzicola	PV20.3	Genzone_3	Eoryzicola	4.1	2.14	4.5	4.3	glabra	2	0	3.96	0.45	0.47	<20_cm	37.25	2.47	1.91	sì	S	NA	sì	no	eretta	alterne	verdi	NA
E. oryzicola	E. oryzicola	E. oryzicola	PV20.4	Genzone_4	Eoryzicola	4.4	2.05	3.85	4.13	glabra	1.91	0	1.69	0.5	0.46	<20_cm	23.65	2.34	1.75	sì	S	NA	sì	no	natante	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV25.1	Mortara_1	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV25.2	Mortara_2	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV25.3	Mortara_3	Ecrusgalli	3.65	1.86	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV25.4	Mortara_4	Ecrusgalli	3.64	1.8	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV29.1	Garlasco_1	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV29.2	Garlasco_2	Ecrusgalli	3.74	1.86	3.13	3.43	glabra	1.97	0	4.92	0.64	0.58	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV29.3	Garlasco_3	Ecrusgalli	3.65	1.86	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	PV29.4	Garlasco_4	Ecrusgalli	3.64	1.8	3.13	3.39	glabra	1.97	0	4.92	0.64	0.59	<20_cm	21.32	1.88	1.33	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	MI3.1	Carpiano_1	Ecrusgalli	3.66	1.69	3.1	3.38	glabra	1.4	3	0	0.45	0.41	<20_cm	9.86	1.66	1.21	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	MI3.2	Carpiano_2	Ecrusgalli	3.61	1.83	3.44	3.52	glabra	1.4	3	0	0.41	0.4	<20_cm	9.86	1.73	1.28	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	MI3.3	Carpiano_3	Ecrusgalli	3.89	1.92	3.46	3.67	glabra	1.53	3	0	0.44	0.42	<20_cm	9.86	1.75	1.46	sì	S	NA	no	no	eretta	alterne	verdi	NA
E.crus-galli	E.crus-galli	E.crus-galli	MI3.4	Carpiano_4	Ecrusgalli	3.65	1.77	3.66	3.66	glabra	1.39	3	0	0.38	0.38	<20_cm	9.86	1.74	1.28	sì	S	NA	no	no	eretta	alterne	verdi	NA

FAMD shows which of the quantitative measured traits possess the higher rate of variation among samples and better contribute to discriminate species, while corrplot which of the quantitative traits are correlated (Figure 6.2). From FAMD resulted that awn length, upper glume length, spikelet length, lower glume length and caryopsis length and width are the most variable traits among samples (Figure 6.2 A – longest arrows). Moreover, upper glume length, spikelet length, lower glume length better contribute in discrimination among the analyzed *Echinochloa* specimens (Figure 6.2 A – red arrows). Spearman correlation evidenced that spikelet length is significantly correlated with upper glume length, spikelet width, sterile lemma length, ratio lower glume/lemma sterile, ratio lower glume/spikelet, panicle length and caryopsis dimensions. Moreover, caryopsis length and width are strongly correlated. Strong and significant correlations are labelled with the star and thin ellipse.

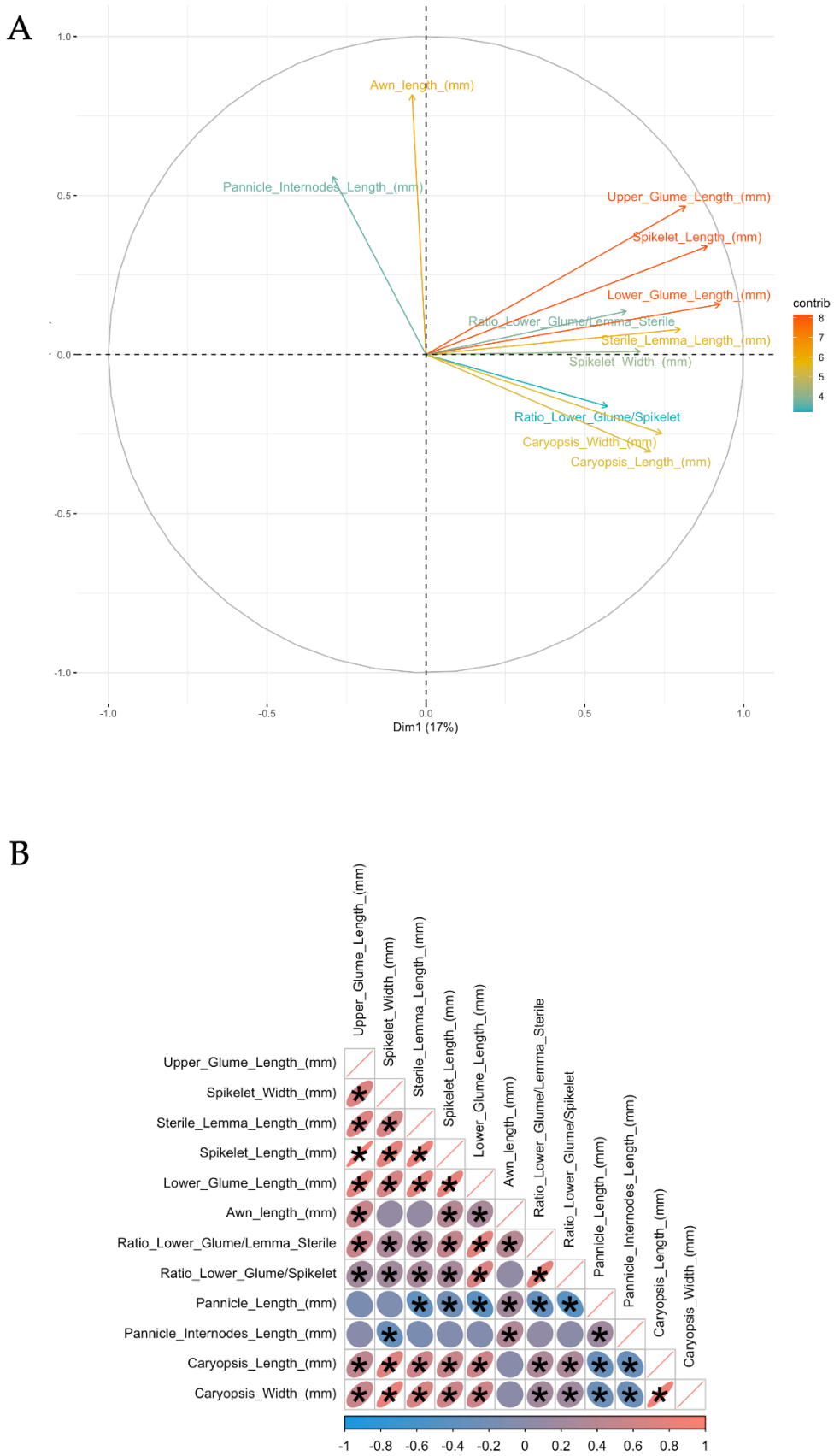

Figure 6.2: FAMD (A) and corrplot (B) of quantitative measured variables. In the corrplot, strong and significant correlations are labelled with the star and thin ellipse. Red ellipses mark positive correlations, blue ellipses negative ones.

Considering contributions and correlations between quantitative morphological traits analyzed, only upper glume length, spikelet length, lower glume length, awn length and caryopsis length and width were considered for the following analysis, in order to reduce the number of variables producing bias and allow a more precise clustering.

Hierarchical clustering separates the samples within two main clusters: A and B (Figure 6.3). Cluster A (black) is divided in two sub-clusters. The sub-cluster A.1 comprises mainly *E. crus-galli* specimens and few *E. oryzicola*. The sub-cluster A.2 is more branched and includes samples of *E. oryzicola*, *E. oryzoides* and *E. crus-galli.* Cluster B is divided in two sub-clusters. Sub-cluster B.1 (green) includes all samples of *E. crus-galli*, while sub-cluster B.2 (blue) comprises samples of *E. oryzoides*. Specimens marked with "?" are those whose morphometric identification was doubtful.

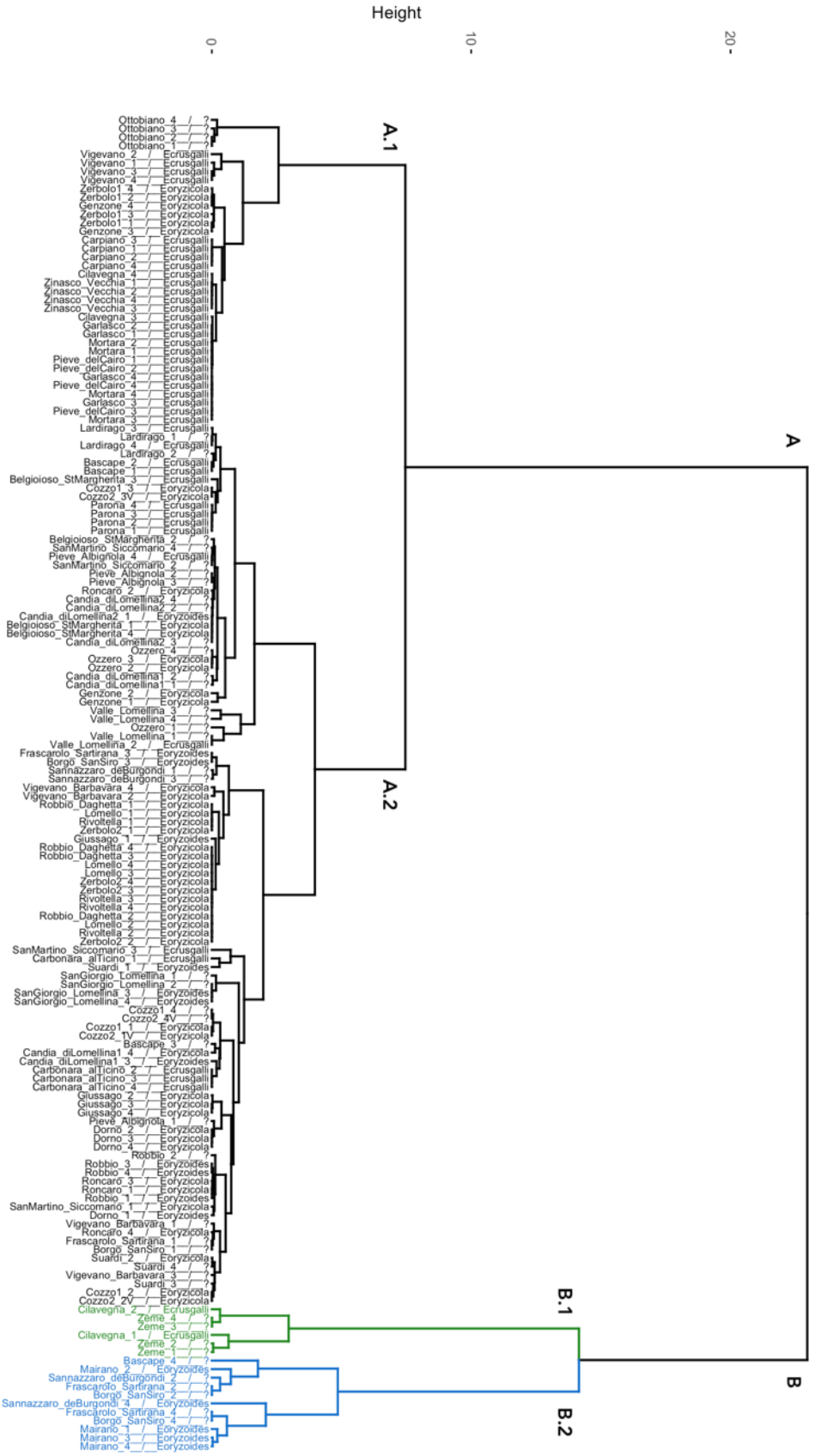

Figure 6.3: Hierarchical clustering of *Echinochloa* spp. samples on the basis of measured morphological traits. Specimens marked with "?" are those whose morphometric identification was doubtful.

6.3.2 Molecular identification

Identification was performed referring to Yasuda et al. (2002), Mennan et al. (2012) and Amaro-Blanco et al. (2021).

Specifically, in *E. oryzicola* (late watergrass) and *E. oryzoides* (early watergrass) trn-a / trn-b1 region is 481 bp length, whereas in *E. crus-galli* (barnyardgrass) is shorter, 449-bp length. Moreover, since trn-a / trn-b1 region owns a restriction site in *E. crus-galli*, when digested with EcoRI it resulted in two fragments of 271 and 178 bp. This feature allows discrimination simply throughout PCR amplification (Figure 6.4 A) or digestion (Figure 6.4 B).

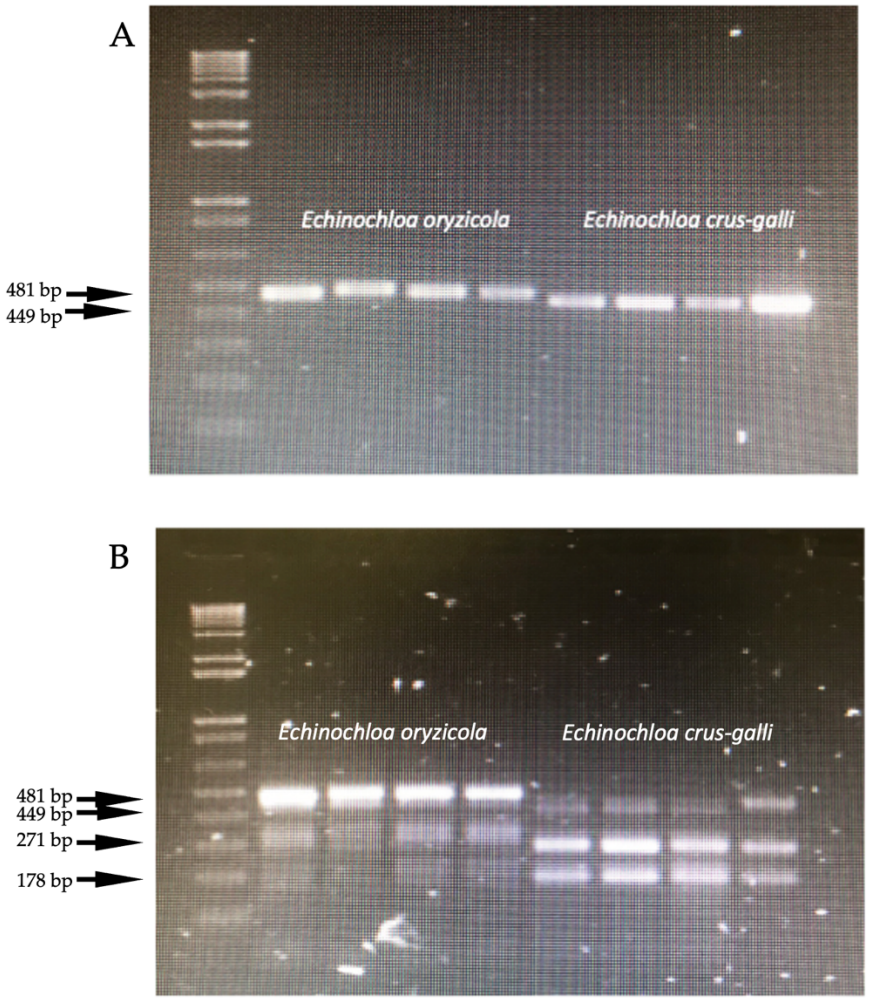

Figure 6.4: amplification of trn a / b1 region (A) and digestion of trn a / b1 region with EcoRI (B).

Differently, trn-c / trn-d region length is 620 bp and is equal in *E. crus-galli*, *E. oryzicola* and *E. oryzoides*. However, this region possess one restriction sites in *E. oryzicola* (digestion with Alu I, resulting in two fragments of 178 and 447 bp – digestion with Dra I, resulting in two fragments of 120 and 500 bp) and one in *E. crus-galli* (digestion with Dra I, resulting in two fragments of 120 and 500 bp), but none in *E. oryzoides* (Figure 6.5).

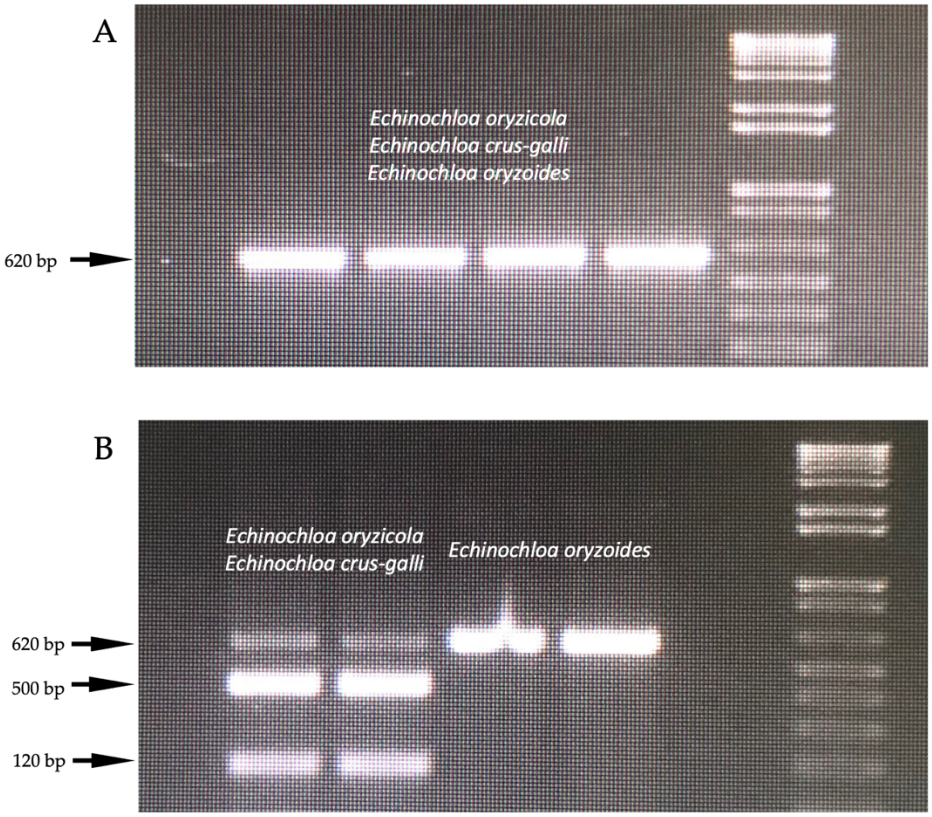

Figure 6.5: amplification of trn c / d region (A) and digestion of trn c / d region with Dra1 (B). Amplicons length does not allow to discriminate *Echinochloa* species. Trn c / d region digestion allow to discriminate *E. oryzoides*, *E. oryzicola* and *E. crus-galli*.

Samples identification through RFLP allows to discriminate unambiguously all the doubtful samples classified by means of cross-use of the three identification keys or statistical analysis (Table 6.3).

All the 46 samples of doubt identification were classified as *E. crus-galli* or *E. oryzicola*. Moreover, all the samples identified through morphometric / statistical analysis as *E. oryzoides* possess a RFLP profile in the trn c / d region typical of *E. oryzicola.* In any case,

all 156 collected specimens were checked by means of RFLP analysis. To sum up, 84 were classified as *E. oryzicola* and 72 as *E. crus-galli.*

Echinochloa crus-galli (L.) P. Beauv. represents one of the most widely distributed and problematic weeds in agriculture (Barret and Wilson, 1983; Bajwa et al., 2015). This plant generally reaches 80 cm in height, but can be up to 150 cm tall. It has completely glabrous, flat lamina leaves that are dark green in color and sometimes with purplish streaks on the collar or laminar edges, 5-15 mm wide, sharply sheathed, and rough on the edges and upper page. The leaf sheath is devoid of ligules and auricles at all stages of development, which is a useful character for the recognition of this species. The inflorescences characterized by a digitate panicle, up to 20 cm long, consisting of a central, slightly incurved or nearly erect rachis, from which small, alternating, more or less aristate racemes branch off, often purplish in color. The awns can vary in length and shape: straight, sinuous or curved. Spikelets are pedunculate, bi-flowered. The glumes, 2 per spikelet, are different from each other: keeled and of different length. The fertile lemma, under the upper glume, is mutic and shiny. The sterile aristate lemma, located under the lower glume, is generally variable in length. The upper flower is hermaphrodite, fertile and gives rise to the caryopsis, the other, lower, is sterile. This species is characterized by high phenotypic plasticity, a key factor in both its distribution and its inherent adaptive capacity (Bajwa et al., 2015).

Echinochloa oryzicola (Vasing) Vasing is another problematic weed that can reach up to 50 cm in height. It is an annual plant typical of flooded rice fields. It possess a tuft of brownish hairs in the leaf collar region. The inflorescence is normally erect and spreading, but occasionally may be horizontal or pendulous, and the color ranges from green to red. The spikelets are ovate-elliptical in shape (not more than 2 cm long). The caryopsis is yellowish, nearly round and measures 2-2.4 mm. The stigmas are often persistent. This species is especially common in flooded rice fields (Yamasue; 2001). Despite *E. oryzicola* shows a relatively limited diversity of its morphological characteristics, its spikelets could shape a "C" form, with convex and glossy lemmas, and a "F" form, with flat and coarse lemmas (Yasuda et al., 2002; Aoki et al., 2008).

Table 6.3: samples identification through cross-use of the three identification keys (Carretero, 1981; Costea & Tardiff, 2002; Tabacchi et al., 2006), statistical analysis and RFLP analysis.

ID	IDENTIFICATION_Carretero	IDENTIFICATION_Costea&Tardif	IDENTIFICATION_Tabacchi	IERARCHICAL CLUSTERING IDENTIFICATION	RFLP IDENTIFICATION
Candia_diLomellina1_1	*?*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*	*E. oryzicola*
Candia_diLomellina1_2	*?*	*?*	*E. oryzoides*	*E. oryzicola*	*E. oryzicola*
Ozzero_1	*E. oryzicola*	*E. oryzicola*	*?*	*E. crus-galli*	*E. oryzicola*
Ozzero_4	*?*	*E. oryzicola*	*?*	*E. oryzicola*	*E. oryzicola*
Ottobiano_1	*?*	*E. oryzoides*	*E. oryzoides*	*E. crus-galli*	*E. crus-galli*
Ottobiano_2	*?*	*E. oryzoides*	*E. oryzoides*	*E. crus-galli*	*E. crus-galli*
Ottobiano_3	*?*	*E. oryzoides*	*?*	*E. crus-galli*	*E. crus-galli*
Ottobiano_4	*?*	*E. oryzoides*	*E. oryzoides*	*E. crus-galli*	*E. crus-galli*
Vigevano_Barbavara_1	*E. oryzoides*	*?*	*E.oryzoides*	*E.oryzicola*	*E. oryzicola*
Vigevano_Barbavara_3	*E. oryzicola*	*?*	*E.oryzicola*	*E.oryzicola*	*E. oryzicola*
Valle_Lomellina_1	*?*	*E. crus-galli ?*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
Valle_Lomellina_3	*?*	*E. crus-galli*	*?*	*E. crus-galli*	*E. crus-galli*
Valle_Lomellina_4	*?*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
Frascarolo_Sartirana_1	*E. oryzicola*	*?*	*E. oryzicola*	*E. oryzicola*	*E. oryzicola*
Frascarolo_Sartirana_2	*E. oryzoides*	*?*	*?*	*E. oryzoides*	*E. oryzicola*
Frascarolo_Sartirana_4	*E. oryzoides*	*?*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Belgioioso_StMargherita_2	*E. oryzoides*	*E. oryzoides*	*?*	*E. oryzicola*	*E. oryzicola*
Pieve_Albignola_1	*?*	*?*	*?*	*E. oryzicola*	*E. oryzicola*
Pieve_Albignola_2	*E. oryzicola*	*?*	*E.oryzicola*	*E. crus-galli*	*E. oryzicola*
Pieve_Albignola_3	*?*	*?*	*E.oryzicola*	*E. crus-galli*	*E. oryzicola*
SanMartino_Siccomario_2	*?*	*?*	*E.oryzicola*	*E. oryzicola*	*E. oryzicola*
SanMartino_Siccomario_4	*?*	*E.oryzoides*	*?*	*E. oryzicola*	*E. oryzicola*
Suardi_3	*E.oryzoides*	*E. oryzoides*	*?*	*E. oryzicola*	*E. oryzicola*
Suardi_4	*?*	*?*	*?*	*E. oryzicola*	*E. oryzicola*
Zeme_1	*E. oryzoides*	*?*	*E. oryzoides*	*E. crus-galli*	*E. crus-galli*
Zeme_2	*E. oryzoides*	*?*	*E. oryzoides*	*E. crus-galli*	*E. crus-galli*
Zeme_3	*E. oryzoides*	*?*	*E. oryzoides*	*E. crus-galli*	*E. crus-galli*
Zeme_4	*E. oryzoides*	*?*	*E. oryzoides*	*E. crus-galli*	*E. crus-galli*
Borgo_SanSiro_1	*E. oryzicola*	*?*	*E. oryzicola*	*E. oryzicola*	*E. oryzicola*
Borgo_SanSiro_2	*E. oryzoides*	*?*	*?*	*E. oryzoides*	*E. oryzicola*
Borgo_SanSiro_3	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Borgo_SanSiro_4	*E. oryzoides*	*?*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Sannazzaro_deBurgondi_1	*E. oryzoides*	*?*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Sannazzaro_deBurgondi_2	*E. oryzoides*	*?*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Sannazzaro_deBurgondi_3	*?*	*?*	*?*	*E. oryzoides*	*E. oryzicola*
Sannazzaro_deBurgondi_4	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Robbio_1	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Robbio_2	*E. oryzoides*	*E. oryzoides*	*?*	*E. oryzicola*	*E. oryzicola*
Robbio_3	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
Robbio_4	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzoides*	*E. oryzicola*
SanGiorgio_Lomellina_1	*E. oryzicola*	*E. oryzoides*	*E. oryzicola*	*E. oryzoides*	*E. oryzicola*
SanGiorgio_Lomellina_2	*E. oryzoides*	*E. oryzoides ?*	*E. oryzicola*	*E. oryzoides*	*E. oryzicola*
Cozzo1_4	*E.oryzoides*	*E. oryzoides*	*?*	*E. oryzicola*	*E. oryzicola*
Cozzo2_4V	*E.oryzoides*	*E. oryzoides*	*?*	*E. oryzicola*	*E. oryzicola*
Vigevano_Barbavara_1	*E. oryzoides*	*?*	*E.oryzoides*	*E. oryzicola*	*E. oryzicola*
Vigevano_Barbavara_3	*E. oryzicola*	*?*	*E.oryzicola*	*E. oryzicola*	*E. oryzicola*
Suardi_3	*E.oryzoides*	*E. oryzoides*	*?*	*E. oryzicola*	*E. oryzicola*
Suardi_4	*?*	*?*	*?*	*E. oryzicola*	*E. oryzicola*
Bascape_3	*?*	*?*	*E. oryzoides*	*E. oryzicola*	*E. oryzicola*
Bascape_4	*?*	*?*	*?*	*E. oryzoides*	*E. oryzicola*
Lardirago_1	*?*	*?*	*E.crus-galli*	*E. crus-galli*	*E. crus-galli*
Lardirago_2	*?*	*E.crus-galli*	*?*	*E. crus-galli*	*E. crus-galli*
Pieve_Albignola_1	*?*	*?*	*?*	*E. oryzoides*	*E. oryzicola*
Pieve_Albignola_2	*E. oryzicola*	*?*	*E.oryzicola*	*E. oryzoides*	*E. oryzicola*
Pieve_Albignola_3	*?*	*?*	*E.oryzicola*	*E. oryzoides*	*E. oryzicola*
Candia_diLomellina2_2	*?*	*?*	*E. oryzicola*	*E. oryzicola*	*E. oryzicola*
Candia_diLomellina2_3	*?*	*?*	*E. oryzicola*	*E. oryzicola*	*E. oryzicola*
Candia_diLomellina2_4	*?*	*?*	*?*	*E. oryzicola*	*E. oryzicola*

6.3.3 Identification keys comparison and analyzed traits description

Carretero (1981) focuses mainly on spikelet length, lower glumes/spikelet length ratio, presence of rests, arrangement and bearing of leaves and inflorescence, as well as the characteristics of the panicle. Costea & Tardif (2002) focus heavily on details, such as the presence of tufts of hairs in the leaf collar region or on the leaf sheath, the color and shape of the caryopses, their size, and the environment hosting such weed species (exclusive or not of rice fields). Tabacchi et. al (2006) mainly consider the color of the culm base and the length of the spikelet, so it can also be used in the field as an initial species identification approach. This key also focuses on the ratio of the lower glume to the spikelet length (≥ or ≤ 0.45), a discriminating character to classify *E. oryzoides* and *E. oryzicola* species.

Among the characters analyzed, the morphology and size of the spikelets, glumes and caryopses turn out to be among the most discriminating; in fact, it can be seen that there is a marked difference between the spikelets of *E. crus-galli*, which are smaller and brownish / reddish in color, compared to those of *E. oryzicola*, which are larger and green in color (Figure 6.6).

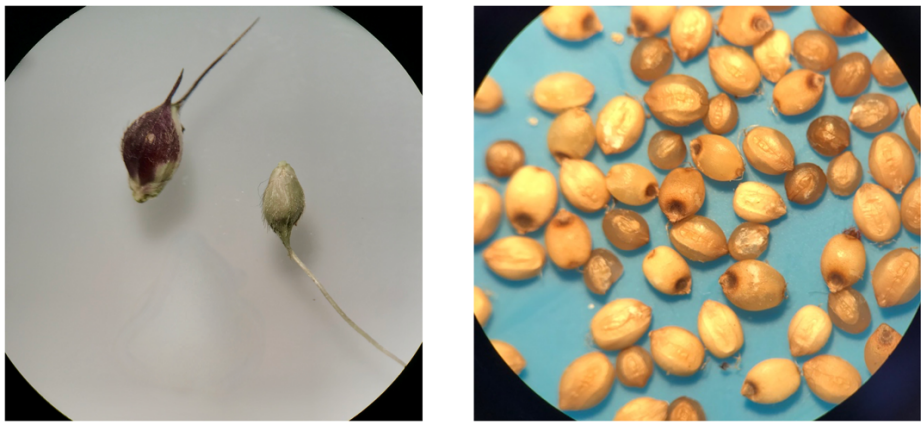

Figure 6.6: morphometric traits comparison. A: spikelets of *E. crus-galli* (smaller and green) and *E. oryzicola* (bigger and brune/green. B: caryopsis of *E. crus-galli* (smaller and darker) and *E. oryzicola* (bigger and lighter).

For a correct, though uncertain, species identification it would be necessary to cross-use the three aforementioned identification keys in order to obtain a more precise and unbiased result. Nevertheless, *Echinochloa* species identification through morphometric analysis results to be a very difficult task due to the high morphological variability of these species (Brusoni, 1991; Altop et al., 2010; Claerout et al., 2015; Cusaro et al. 2022). In addition, when tissue damages are present due to herbicide application, the plant could be underdeveloped, slightly wilted or dried up. Therefore, morphological features collection might be biased and morphometric analysis could lead to unprecise results. It is therefore more reliable to combine this method with statistical analysis or a DNA analysis approach.

6.4 Conclusions

This study made it possible to highlight the morphological characters most discriminating the species of the genus *Echinochloa*, outlining an effective and precise protocol for identification. From the results obtained, it can be deduced that only the joint use of the three determination keys allows accurate identification of *Echinochloa* species, except in some cases, where identification is doubtful because discordance was found in the ability to discriminate between *E. oryzoides* and *E. oryzicola*.

For more objective and accurate identification, species identification using RFLP markers should be employed.

6.5 References

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Chapter 7 – Analysis of intraspecific variability of *Echinochloa* species

This chapter describes the assessment of intraspecific variability in *E. crus-galli* and *E. oryzicola* through the analysis of microsatellites loci.

In general, *Echinochloa* species are characterized by high genetic variability that may favors adaptability and influences the variety of response to environmental stressors, such as herbicides. Thus, analyzing this characteristic for each of the identified *Echinochloa* species could provide important information regarding the behavior of these organisms toward herbicide application and the development of resistance phenomena.

The first part of this chapter is devoted to the analysis of intraspecific variability in *E. crus-galli* and to the setting-up of an analytical methodology that could provide a result as precise and detailed as useful for subsequent analyses focused on herbicide resistance. For these reasons, specimens of *E. crus-galli* collected in 2019 and 2020 in Lomellina for PhD research aims, were flanked by some specimens sampled in non-weeded paddies in 2017 and 2018. The results obtained were published in an article titled *An Improved Method for Assessing Simple Sequence Repeat (SSR) Variation in Echinochloa crus-galli (L.) P. Beauv (Barnyardgrass)* in Diversity Journal (IF 3.031 - MDPI https://www.mdpi.com/1424-2818/14/1/3).

The second part of this chapter is devoted to the analysis of intraspecific variability in *E. oryzicola*. The same analytical methodology used for *E. crus-galli* was applied.

7.1 Introduction

The genus *Echinochloa* (L.) P. Beauv. (*Poaceae*) includes over 50 annual and perennial species, many of which are considered among the most economically important and most problematic weeds in agriculture. These species are widespread globally and are able to colonize different habitats, including cereal fields, but also the banks of ditches and ruderal and urban environments. Their wide distribution is a consequence of their remarkable competitive and adaptive ability. In fact, thanks to their broad resilience and their biologic characteristics (i.e. genetic variability, ability to imitate culture, allelopathy and C4 metabolism), these plants are very successful competitors (Tabacchi et al., 2006; Vidotto et al., 2007; Aoki et al., 2008; Bajwa et al., 2015; Lee et al., 2016; Lim et al., 2021; Wu et al., 2022). As a matter of facts, intraspecific genetic variability that characterizes *Echinohcloa* species represents a key factor for their survival in environments subject to high disturbance levels. Furthermore, the wide morphologic polymorphism of these species is direct consequence of this trait.

7.2 Analysis of intraspecific variability in *E. crus-galli*

The results obtained from this part of the research work were published in the following article:

An Improved Method for Assessing Simple Sequence Repeat (SSR) Variation in Echinochloa crus-galli (L.) P. Beauv (Barnyardgrass)

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Abstract: *Echinochloa crus-galli* (L.) P. Beauv. (barnyardgrass) is one of the most noxious weeds infesting Italian rice fields. It is characterized by high genetic intraspecific variability and has developed resistance to several classes of herbicides. The aim of our study was to assess, for the first time in Italy, the genetic diversity in *E. crus-galli* from differently managed rice fields in the Lombardy region (Northern Italy) using eight specific SSR markers. To this purpose, an amplification protocol was optimized, testing different DNA concentrations, PCR mixtures, and temperatures. A total of 48 alleles were identified in 144 samples. SSR fingerprint analysis using R 3.6.3 software (poppr, polysat, and StAMPP) allowed us to handle SSRs as codominant and polyploid data. The results suggested that genetic richness and diversity were high. The analysis of molecular variance (AMOVA) indicated that genetic variation exists mainly between agronomic managements (47.23%) and among populations (37.01%). Hierarchical clustering and PCoA were in concordance with the identification of four distinct genetic groups. Our results confirm that SSR markers represent a valuable and affordable tool for the assessment of *E. crus-galli* genetic diversity and would grant useful information to plan more targeted, effective, and sustainable control strategies against barnyardgrass. The improved methodology applied here allowed us to assess the genetic variability of an allo-hexaploid species without information loss and biased results.

Keywords: *Echinochloa crus-galli*; SSR markers; PCR optimization; polyploid data; genetic intraspecific variability

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1. Introduction

Genetic variability plays a fundamental role in the adaptive response of organisms to varied environmental conditions. Weeds have evolved a genetic and phenetic plasticity that allows them to colonize very different ecosystems and to survive under the most adverse ecological stresses [1,2]. Weed intraspecific biodiversity assessment is fundamental for good weed control, including problematic cases of herbicide resistance [1,3]. Agricultural management systems influence weed biodiversity. The repeated use of herbicides that have the same mechanism of action favors the rapid evolution of herbicide resistance in weeds, aided by European

regulatory constraints on the use of plant protection products (Reg. EC/1107/2009) and the practice of monoculture.

The genus *Echinochloa* (P.) Beauv. (*Poaceae*) is one of the most widespread weeds and is composed of around 50 species, mostly located in minor cereal fields and rice paddies, and therefore represents a major agricultural and economic problem [4]. In the Italian rice farming regions, the most commonly found species of this genus are *Echinochloa colonum* (L.) Link, *Echinochloa crus-galli* (L.) P. Beauv., *Echinochloa crus-pavonis* (Kunth) Schult., *Echinochloa erecta* (Pollacci) Pignatti, *Echinochloa oryzicola* (Vasinger) Vasinger, *Echinochloa hostii* (M. Bieb.) Link, and *Echinochloa phyllopogon* (Stapf) Stapf ex Kossenko [4,5].

E. crus-galli (barnyardgrass) is an allo-hexaploid (2n = 6x = 54) difficult to control annual weed with worldwide distribution [6–8]. It represents one of the most problematic weed species in rice fields due to its competitive abilities such as mimicking rice, exhibiting rapid germination and growth, and producing seeds in high abundance [9].

E. crus-galli is characterized by high genetic variability and intraspecific polymorphism [4,9–13] making the morphological identification of this weed very difficult. In addition, the different *E. crus-galli* biotypes exhibit differential herbicide susceptibility as a result of high genetic variability, allowing herbicide resistance to develop. Several studies have also demonstrated that agricultural managements, including herbicide application, could affect the genetic variability and adaptability of many species within the genus *Echinochloa*, including *E. crus-galli* [14–18]. Therefore, the assessment of genetic intraspecific diversity is very important and useful and may provide valuable information for the improvement of agricultural management practices, with particular regard to the *E. crus-galli* species. Hence, there is a need for a reproducible, rapid, and affordable methodology to analyze such variability [4,9,18–20].

For this task there are many molecular markers available to detect genetic polymorphisms of orphan plants (i.e., organisms without a publicly available reference genome sequence), such as weeds [21–27]. The majority of studies have been carried out using RAPDs and AFLPs, although these markers are not reliable due to poor reproducibility. Moreover, dominant markers may become problematic and lead to a significant loss of information when applied to polyploid species [4,9,18–20,28].

Microsatellites, or Simple Sequence Repeats (SSRs), appear to be the most appropriate markers to study genetic variability as they are highly informative and powerful tools for plant genetic analysis, being codominant, multiallelic, highly mutable, and polymorphic [29–31]. Since SSR alleles differ in length by many base pairs, SSR markers are well resolved on agarose gel [25,26]. SSRs are often recorded and analyzed as dominant markers [32–38], but this leads to loss of information about allelic variance and the presence of heterozygosity, as they are codominant [39]. However, their scoring as codominant markers in polyploid species (such as *E. crus-galli*) presents several challenges, as almost all population genetics software has been developed for haploid and/or diploid genotype analysis [40].

Only a few studies have been conducted to assess the genetic diversity of *E. crus-galli* using SSRs, due to the limited number of SSRs that have been developed specifically for this species. In fact, Lee et al. (2016) studied the genetic diversity of *Echinochloa* spp. with SSR markers from related species (*Poaceae*) [25]. Recently, new SSR markers have been identified in *E. crus-galli* through Restriction Site-Associated DNA (RAD) sequencing [22] and New Generation Sequencing (NGS) technology (Illumina) [41].

In this study, we aimed to assess the genetic diversity in *E. crus-galli* from differently managed rice fields in the Lombardy region (Northern Italy) using SSR markers developed by Chen et al. (2017) [22], scoring and analyzing them as codominant. The improved methodology applied here allowed us to assess the genetic variability of this allo-hexaploid species, without incurring a loss of information. To obtain a highly reliable, reproducible, rapid, and affordable methodology, it was necessary to optimize the whole analytic procedure. To the best of our knowledge, this study is the first to use specific SSR markers and to score them as codominant in the assessment of the genetic variability of Italian barnyardgrass populations.

Intraspecific variability assessment provides information on the ecological tolerance and competitive ability of *E. crus-galli,* which is useful for establishing effective and sustain- able weed management strategies, especially in rice fields where barnyardgrass herbicide-resistant populations can cause serious problems.

2. Materials and Methods

2.1. Sample Collection

Samples were collected from 39 rice fields in the Lombardy region of Northern Italy, where a high frequency of herbicide resistance has been reported. Rice fields were managed using two different rice farming practices: conventional or pre-emergent weed control. In each paddy, sample collection was carried out in a 3 m × 6 m experimental parcel (plot) (Figure 1). We collected the maximum number of samples present (4/5 specimens) within each experimental parcel, with a total of 150 samples. Paddies with less than 4 samples per plot were excluded from the final analysis. This reduced the total number of samples to 146 collected from a total of 36 plots (Table S1). Leaf material was stored at −5 °C until DNA extraction.

Figure 1. Picture of: (**a**) *Echinochloa crus-galli* panicle; (**b**) experimental parcel with *Echinochloa crus-galli.*

2.2. DNA Extraction and Quality Analysis

DNA was extracted using the DNeasy Plant Kit (QIAGEN spa, Hilden, Germany) protocol. Tissues were previously crushed using 2% CTAB buffer [42].

DNA concentration and purity were checked using Nanodrop (ThermoFisher s.p.a., Waltham, MA, USA). The absorbance ratio of the extracted genomic DNA at 260/280 nm ranged from 1.34 to 2.01, while at 230/260 nm it ranged from 0.70 to 2.27. DNA concentrations ranged from 50 ng/µL to 163 ng/µL. The quality of DNA was observed by running 4 µL of crude extracted DNA in 0.8% agarose gel. The DNA samples giving smear in the gel were re-extracted.

2.3. Molecular Characterization of Species

Species identification was carried out using PCR-RFLP methodology. Chloroplast DNA (cpDNA) intergenic spacer region between trnT and trnL genes was amplified with primers trn-a and trn-b1 and digested with endonuclease EcoRI (G*AATTC), whereas the entire intron region of trnL was amplified with primers trn-c and trn-d and digested with endonucleases AluI (AG*CT) and DraI (TTT*AAA) according to Amaro-Blanco et al. (2021) [43] (Table S2). The reaction–restriction mixtures were incubated overnight at 37 °C. The digested products were separated on a 2% agarose gel in 1 X TBE buffer stained with ethidium bromide and visualized under UV rays with Molecular Imager® Gel DocTM XR + (BIO-RAD, Hercules, CA, USA). The digested product size was determined by making a comparison with a 100 bp DNA Ladder (Promega, Madison, WI, USA).

2.4. SSR Loci Amplification and Protocol Optimization

The SSR amplification was conducted according to the protocol described by Chen et al. (2017). The names of the SSR loci identified by Chen et al. (2017) with the corresponding repeated motifs and the primer sequences are listed in Table 1 [22]. In order to obtain reproducible results, PCR conditions (DNA template and reagent concentrations/temperatures of denaturation and annealing steps) were investigated. A series of gradient PCRs was performed to determine the most effective annealing temperatures of primers. Chen et al.'s (2017) protocol (a) and our optimized protocol (b) are compared in Table 2.

Table 1. *Echinochloa crus-galli* Simple Sequence Repeats. Locus name, motif, and primer sequences.

Locus Name	SSR Motif	Primer Sequences (5′—3′)
EG1	(TG)7	F: GCTCCTGAACTGTGTACATTCTTGC R: TCGATTCACCCTTCAGCTTCTC
EG2	(TA)6	F: CATCGGATTCAGATTGAAAGGG R: GGTCGTAGGTCTATAGTCCGTAGAGTCA
EG301	(AT)5	F: GCGTCGTCAAGTCGTTCTTCTA R: TGTATTCAGCTGTCGTGCATGT
EG302	(ATTT)8	F: ATTCGAACACCCATCAACCAAC R: GAAACAGAAGGGAGGTGTGCTG
EG305	(AT)4	F: AGCCGTTCCTCTAGTCGGATTTCT R: TATTCAGCTGCCGTGCATGTAGTA
EG306	(CT)8	F: TAAAACAAAACGACCGGCGTAA R: TCAATCATTTCAGCCTTCGGAT
EG307	(ATC)11	F: AACATTGTCATCACAAATATCATCATCA R: AATCAAGGAAGCCCCTTCACTC
EG320	(TA)5	F: CAACTCATAAGACAATTCAAAGGGTTT R: GCATCATTTAAGCATCAAAATGACA

Table 2. Protocol comparison.

(a) Chen et al.'s (2017) Protocol	(b) Optimized Protocol
PCR Mixture (in a Total Volume = 10 μL)	**PCR Mixture (in a Total Volume = 10 μL)**
0.2 μL of crude DNA (6–8 ng) extract	2 μL of diluted DNA from crude extract (10 ng/μL)
0.4 μL of each primer (0.4 μM)	1 μL of each primer (10 μM)
5 μL of Taq polymerase Ready Mix (0.27 UI) (Dongsheng Biotech)	5.3 μL of Taq polymerase Ready Mix (0.4 UI) KAPA 2X Taq Extra Hot Start Ready-mix PCR Kit (Resnova S.r.l.)
($MgCl_2$ total concentration = 1.6 mM)	Addition of 0.5 μL of $MgCl_2$ ($MgCl_2$ total concentration = 2.5 mM)
nuclease-free H_2O—ad volume	nuclease-free H_2O—ad volume
PCR program	**PCR program**
initial denaturation step at 94 °C for 4 min	initial denaturation step at 95 °C for 5 min
35 cycles of:	35 cycles of:
94 °C for 30 s	95 °C for 30 s
relative annealing temperatures for 30 s	relative annealing temperatures for 30 s
72°C for 1 min	72°C for 1 min
final extension step at 72 °C for 10 min	final extension step at 72 °C for 10 min

DNA amplification was performed with KAPA 2X Taq Extra Hot Start Ready-mix PCR Kit (Resnova S.r.l. Genzano, Roma, Italy) through a T100 Thermal Cycler (BIO-RAD, Hercules, California, USA). Optimization

of the SSR amplification protocol was performed on a small number of the samples collected and tested in triplicate. Once improved, the protocol was extended to the analysis of all samples collected.

2.5. DNA Fingerprinting Analysis

The total volume of PCR amplicons was loaded on 2% agarose gel in 1X TBE buffer, stained with ethidium bromide, and separated at 100 V for 60 min. Molecular markers were visualized with Molecular Imager® Gel DocTM XR + (BIO-RAD, Hercules, California, USA). Amplicon size was determined by making a comparison with an E-Gel® 1 Kb Plus DNA Ladder (ThermoFisher s.p.a.). For each SSR primer pair, amplicons of the same size across different isolates were considered to be the same allele. For each SSR listed in Table 1, the reproducibility of the test was validated by three replicates in which the same experimental conditions were applied and with each replicate producing a similar result. Amplicon fragment sizes were determined using the software Molecular Imager® Gel DocTM XR + (BIO-RAD, Hercules, California, USA). A matrix of codominant data was then constructed.

2.6. Statistical Analysis

The number of observed alleles per locus (Na) was computed using R 3.6.3 software (poppr 2.9.3) [44–46]. The polymorphism information content (PIC) values were calculated using the formula of Liu et al. (2011) [47]:

$$\mathrm{PIC} = 1 - \sum_{j=1}^{n} P_{ij}^2 \quad (1)$$

where P_{ij} is the frequency of j_{th} allele for i_{th} locus and summation extends over n alleles, scored for each SSR locus, according to Prevost et al. (1999) [48] and Tiwari et al. (2016) [49].

Genotypic richness (the number of multilocus genotypes observed per population—MLG); genotypic diversity (percentage of polymorphism detected by each population—%P; Shannon–Wiener Index of MLG diversity per population—H [50]; Stoddart and Taylor's Index of MLG diversity per population—G [51]; Simpson's Index per population—lambda [52]; Evenness index per population—E.5 [53]; expected heterozygosity or Nei's unbiased gene diversity per population—He [54]; observed heterozygosity per population—Ho) were analyzed using R 3.6.3 software (poppr 2.9.3, pegas 1.0-1) [44–46,55].

Analysis of molecular variance (AMOVA) for each hierarchical comparison (between agricultural management, among and within populations) was run with 10,000 per-mutations by R 3.6.3 software (poppr 2.9.3, StAMPP 1.6.3) [45,46,56]. Pairwise Fst values between populations were determined with 10,000 permutations by R 3.6.3 software (polysat 1.7-5) [57,58] and plotted as a levelplot.

Genetic similarity was calculated using Nei's unbiased genetic distance with R 3.6.3 software (poppr 2.9.3) [38]. Hierarchical clustering was performed based on Ward's method to maximize the between-cluster variance with R 3.6.3 software (dendextend 1.15.2) [59]. Genotypes were sorted by PCoA (Ward's method), showing their distributions in a scatter plot, using R 3.6.3 software (poppr 2.9.3, FactoMineR 2.4, ggplot2 3.3.5) [60,61].

3. Results

3.1. Molecular Characterization of Species

Chloroplast DNA (cpDNA) intergenic spacer region nucleotide length between trnT and trnL genes differs in *Echinochloa* spp. (481 bp) and *E. crus-galli* (449 bp). Moreover, this region has an EcoRI restriction site only in *E. crus-galli*. In this method, species identification was first performed by electrophoresis of PCR products and then validated by digestion of intergenic spacer region between trnT and trnL genes with EcoRI endonuclease. For higher accuracy, the trnL intron region was amplified and digested with AluI and DraI endonucleases, producing the same results. Among the 146 analyzed samples, 144 specimens were identified as *E. crus-galli*.

3.2. SSR Protocol Optimization

Figure 2 shows EG302 fingerprints obtained from a comparison of SSR amplification protocols on a small number of samples. It is possible to observe on the left (a) the DNA fingerprints obtained with the application of Chen et al.'s (2017) protocol and on the right (b) those obtained applying our modified conditions [22]. The optimized protocol allowed us to better observe the presence of multiple allelic variants (from ~180 bp to ~240 bp) due to the different sequence lengths of the SSR locus. These results proved to be reproducible in triplicate analysis. All the samples were processed by means of the optimized methodology.

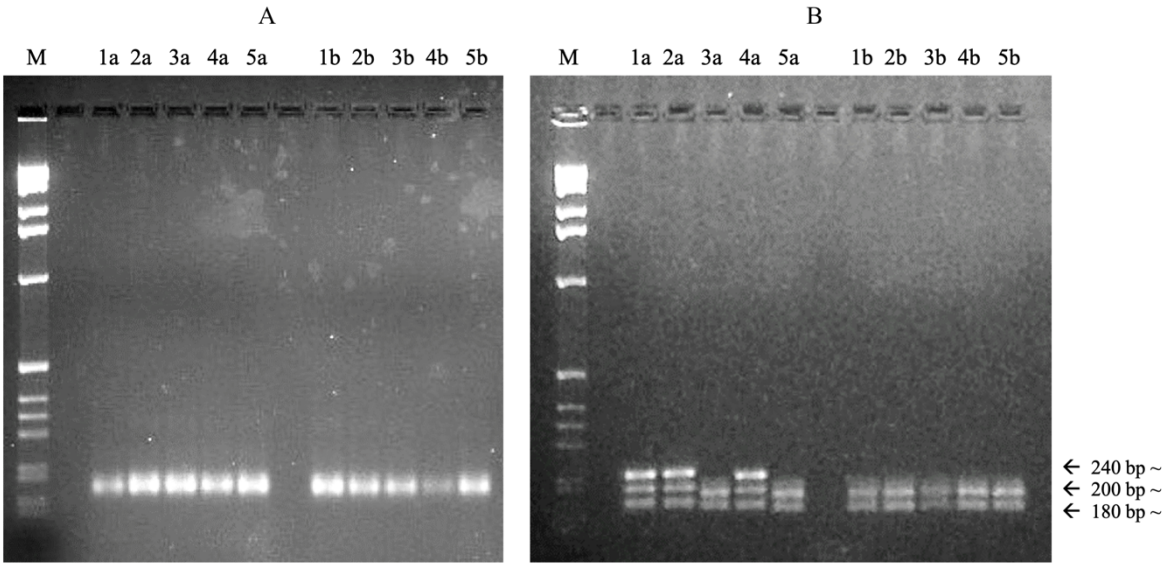

Figure 2. Fingerprint comparison. (a) Results obtained according to Chen et al. (2017) [22]. M: E-Gel® 1 Kb Plus DNA Ladder (ThermoFisher s.p.a.); 1a,2a,3a,4a,5a: different *Echinochloa crus-galli* samples from rice field "a"; 1b,2b,3b,4b,5b: different *Echinochloa crus-galli* samples from rice field "b"; (b) Results obtained after PCR optimization. M: E-Gel® 1 Kb Plus DNA Lad-der (ThermoFisher s.p.a.); 1a,2a,3a,4a,5a: different *Echinochloa crus-galli* samples from rice field "a"; 1b,2b,3b,4b,5b: different *E. crus-galli* samples from rice field "b".

The results obtained from the different annealing temperatures tested following a gradient PCR, in comparison with the temperatures applied by Chen et al. (2017) with the same primer set [22], are reported in Table 3.

Table 3. Comparison between the annealing temperature (AT) of Simple Sequence Repeat markers in *Echinochloa crus-galli* according to Chen et al. (2017) and annealing temperature determined by post-gradient PCR results.

Locus Name	AT According to Chen et al. (2017)	AT According Post-Gradient PCR Results
EG1	49 °C	40.6 °C
EG2	51.5 °C	50 °C
EG301	57 °C	43.3 °C
EG302	57 °C	48 °C
EG305	57 °C	55 °C
EG306	57 °C	43.2 °C
EG307	57 °C	55.6 °C
EG320	57 °C	46.5 °C

3.3. Genetic Richness and Diversity Analysis

We analyzed 144 individuals of *E. crus-galli* from 36 paddies in the Lombardy region of Northern Italy. A total of 48 different alleles were detected using 8 SSR markers. Allele number (Na) ranged from 2 (EG307) to 12 (EG301), with an average of 6 alleles per locus. Polymorphic information content (PIC) ranged from 0.76 (EG307) to 0.98 (EG320, EG301), with an average of 0.92 per locus (Table 4).

Table 4. Estimates parameters of allele numbers and polymorphic information content (PIC) in the 8 pairs of Simple Sequence Repeat markers analyzed in *Echinochloa crus-galli.*

Locus	Na	PIC
EG1	5	0.93
EG2	3	0.88
EG302	8	0.97
EG305	5	0.96
EG306	4	0.94
EG307	2	0.76
EG320	9	0.98
EG301	12	0.98
Mean	6	0.92

Na = average number of alleles per locus, *PIC* = polymorphic information content.

The analysis of the genetic richness and diversity parameters per population recorded a high diversity for the majority of plots (Table 5). The average percentage of polymorphic loci (%P) was 34.46%. The overall number of multilocus genotypes (MLG) observed was 78 and values ranged between 1 and 4. In 5 populations out of 36, an MLG value of 1 was recorded. The overall Shannon–Wiener Index of MLG diversity (H) was 29.80, with an average value of 0.82 [42]. The overall Stoddart and Taylor's Index of MLG diversity (G) was 36.64, with an average value of 2.40 [43]. The overall Simpson's Index (lambda) was 0.97, with an average value of 0.50 [44]. The overall Evenness (E.5) was 28.21, with an average value of 0.91 [45]. The overall expected heterozygosity or Nei's unbiased gene diversity (He) was 0.96, with an average value of 0.03 [46]. The overall observed heterozygosity (Ho) was 1.10, with an average value of 0.03.

Table 5. Genetic diversity parameters in *Echinochloa crus-galli.*

Population ID	N	%P	MLG	H	G	Lambda	E.5	He	Ho
EcgP01	4	41.15	3	1.04	2.67	0.63	0.91	0.33	0.67
EcgP02	4	39.06	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP03	4	41.15	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP04	4	39.06	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP05	4	36.46	2	0.69	2.00	0.50	1.00	0.01	0.01
EcgP06	4	33.33	1	0.00	1.00	0.00	--	0.01	0.00
EcgP07	4	38.02	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP08	4	38.54	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP09	4	34.90	4	1.39	4.00	0.75	1.00	0.03	0.02
EcgP10	4	32.29	4	1.39	4.00	0.75	1.00	0.03	0.02
EcgP11	4	37.50	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP12	4	33.33	4	1.39	4.00	0.75	1.00	0.03	0.02
EcgP13	4	38.54	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP14	4	38.54	4	1.39	4.00	0.75	1.00	0.03	0.02
EcgP15	4	35.42	1	0.00	1.00	0.00	--	0.01	0.00
EcgP16	4	35.94	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP17	4	35.42	1	0.00	1.00	0.00	--	0.01	0.00
EcgP18	4	32.81	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP19	4	32.81	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP20	4	33.33	1	0.00	1.00	0.00	--	0.01	0.00
EcgP21	4	33.33	1	0.00	1.00	0.00	--	0.01	0.00
EcgP22	4	31.77	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP23	4	31.77	3	1.04	2.67	0.63	0.91	0.02	0.01

EcgP24	4	36.98	4	1.39	4.00	0.75	1.00	0.03	0.02
EcgP25	4	28.13	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP26	4	29.17	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP27	4	31.25	2	0.69	2.00	0.50	1.00	0.01	0.01
EcgP28	4	28.65	2	0.56	1.60	0.38	0.79	0.01	0.01
EcgP29	4	31.25	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP30	4	28.13	2	0.69	2.00	0.50	1.00	0.01	0.01
EcgP31	4	27.08	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP32	4	30.73	4	1.39	4.00	0.75	1.00	0.03	0.02
EcgP33	4	34.38	4	1.39	4.00	0.75	1.00	0.03	0.02
EcgP34	4	35.94	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP35	4	39.06	3	1.04	2.67	0.63	0.91	0.02	0.01
EcgP36	4	35.42	3	1.04	2.67	0.63	0.91	0.02	0.01
Total	144	----	78	29.8	36.64	0.97	28.21	0.96	1.10
Mean	4	34.46	2.61	0.82	2.40	0.50	0.91	0.03	0.03

N = number of individuals per population, *%P* = percentage of polymorphism detected in each population, *MLG* = number of multilocus genotypes observed per population, *H* = Shannon–Wiener Index of MLG diversity per population, *G* = Stoddart and Taylor's Index of MLG diversity per population, *lambda* = Simpson's Index per population, *E.5* = Evenness index per population (in populations where lambda is equal to 0, E.5 values could not be scored), *He* = expected heterozygosity per population, *Ho* = observed heterozygosity per population.

3.4. Analysis of Molecular Variance (AMOVA)

The analysis of molecular variance (AMOVA) was carried out considering the 36 populations studied, calculating the molecular variation attributable to the differentiation between agricultural managements and among and within the populations. High percentages of variation (%V) were found between agricultural managements (%V = 47.23%) and among populations (%V = 37.01%). A lower proportion was found within populations (%V = 15.74%) (Table 6). Pairwise Fst values between populations were plotted in a levelplot and ranged between 0.000 and 0.310 (Figure S1, Table S3).

Table 6. Analysis of molecular variance (AMOVA) based on Simple Sequence Repeats in *Echinochloa crus-galli.*

Source	DF	SS	MS	Est. Var.	%	*p*
Between agricultural managements	1	8.20	8.20	0.11	47.23%	<0.001
Among populations	34	18.66	0.54	0.12	37.01%	<0.001
Within populations	108	4.24	0.03	0.04	15.74%	<0.001
Total	143	31.10	0.21	0.27	100%	

df = degree of freedom, *SS* = sum of squares, *MS* = mean squares, *Est. var*. = estimate of variance, % = percentage of total variation, *p* = *p*-value based on 10,000 permutations.

3.5. Hierarchical Clustering and Principal Coordinates Analysis

Hierarchical clustering identified two main genetic groups, corresponding to clusters I and II (Figure 3). Cluster I included samples from experimental parcels where only pre-emergent weed control was applied. It was divided into two subclusters (red and yellow). Cluster II comprised of samples from experimental parcels where conventional weed control was applied. It was divided into two subclusters (blue and green). Overall, four different genetic groups of accessions were identified (red, yellow, blue, green).

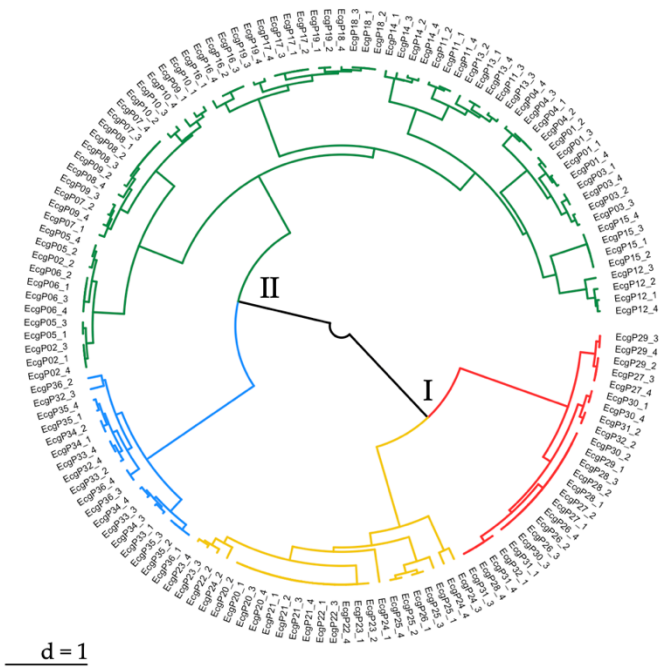

Figure 3. Ward.D2 hierarchical clustering based on Simple Sequence Repeats in *Echinochloa crus-galli*. *Cluster I (red and yellow)* = specimens collected from experimental parcels where only pre-emergent weed control was applied. *Cluster II (blue and green)* = specimens collected from experimental parcels where conventional weed control was applied.

Likewise, Principal Coordinates Analysis identified four genetic groups (red, yellow, blue, and green) on the first three coordinates, explaining 53.67% (cumulative values) of the total variability (Figure 4).

Figure 4. Principal Coordinates Analysis based on Simple Sequence Repeats in *Echinochloa crus-galli*. *Groups red and yellow* = specimens collected from experimental parcels where only pre-emergent weed control was applied. *Groups blue and green* = specimens collected from experimental parcels where conventional weed control was applied.

4. Discussion

Currently, there is little information in the literature on genetic variability studies carried out using molecular markers within the genus *Echinochloa*, and more specifically within the allo-hexaploid species *E. crus-galli*. Previously published studies had either focused on the analysis of morphological and phenological characteristics of this species or had studied genetic variability using molecular markers, which are less reliable and more subject to reproducibility problems. Only recently, some studies have approached this issue by developing and testing specific SSR markers for *Echinochloa* species, which are useful for analyzing the genetic diversity and adaptive evolution of these weeds [22,41]. Chen et al. (2017) developed specific and exclusive SSR markers for *E. crus-galli* and tested them as dominant [22], whereas Lee et al. (2019) developed cross-specific SSR markers for *Echinochloa* spp. and tested them as codominant [41]. Although this weed is particularly widespread in rice-growing areas and has, over the years, developed resistance to many classes of herbicide, no analysis of genetic variability has been conducted so far by SSR markers on Italian populations of *E. crus-galli*. In the present study, the eight SSR markers developed by Chen et al. (2017) [22] were used to assess the genetic diversity in *E. crus-galli* from rice fields in the Lombardy region (Northern Italy), scoring and analyzing them as codominant.

Molecular markers are useful tools to assay the genetic variability of plant genomes that have not been sequenced (i.e., *Echinochloa* spp.). SSRs are the most widely used molecular markers because of their high reliability, reproducibility, and affordability. However, it is often necessary to optimize and standardize the PCR mixture and profile in the procedure of DNA amplification and analysis. The dilution of the DNA template, the application of an annealing temperature lower than those applied by Chen et al. (2017), and the adjustment of Taq polymerase and $MgCl_2$ concentrations provided more successful results that were able to obtain well-defined *E. crus-galli* SSR DNA fingerprints on agarose gel [22]. In fact, the presence of contaminants in crude DNA extract could affect the PCR outcome. Moreover, at high annealing temperatures, the PCR efficiency is reduced be-cause only a portion of the primer molecules is able to initiate polymerization due to the high instability of their pairing with the template [23,62–65]. In addition, high Taq polymerase concentration in the PCR mixture reduces replication slippage [23,66], while a high MgCl2 concentration favors more successful base pairing [67]. In the present study, the optimum Taq polymerase concentration was found to be 0.4 IU and the optimum MgCl2 concentration was 2.5 mM, allowing us to obtain more defined, accurate, reproducible, and reliable DNA SSR fingerprints on agarose gel.

SSRs are commonly used in studies of population genetic diversity and structure since they are highly variable, reproducible, codominant markers for which mutational relationships between alleles can be inferred. However, their usefulness is compromised in polyploid organisms because it is difficult, or impossible, to determine allele copy number in partially heterozygous genotypes, and because inheritance patterns are complex [57,58]. To overcome this problem, many researchers have resorted to coding each allele as a dominant marker [32–38], missing part of SSRs informativeness [39,40,57,58]. In fact, it would be more appropriate to treat them as codominants [39]. For the analysis of polyploid SSR data, there are only a few computer programs available [57]. Most of the software deals with haploids and/or diploids and does not work for polyploid data. Additionally, format conversion of such data is limited [27,28,32,68]. Recently, a few statistical programs have been developed for polyploid/codominant data without in-formation loss.

The results of this study showed that the polymorphism of SSR loci is manifested as the presence of a different number of alleles (bands on agarose gel) at each locus in the different samples. Hence, the analysis of such SSR polymorphisms was very difficult. In order to solve this problem, an extensive literature search was performed to find out useful information on the analysis of SSR markers recorded as codominant in allo-polyploid organisms. This issue was figured out using the libraries poppr 2.9.3 (Genetic Analysis of Populations with Mixed Reproduction) [45,46], polysat 1.7-5 (Tools for Polyploid Microsatellite Analysis) [57,58], and StAMPP 1.6.3 (Statistical Analysis of Mixed-Ploidy Populations) [56] implemented in the R statistical software [44]. These R packages allowed us to correctly import, read, and analyze all of our SSR hexaploid data [45,46], with particular regard to the number of alleles scored at each locus in the samples [56–58]. This allowed us to calculate parameters such as the index of genetic differentiation (Fst) and the degree of heterozygosity expected and observed in each population. Therefore, it was crucial to apply the appropriate methodology to

study SSR markers as codominant for the assessment of genetic variability of the allo-hexaploid *E. crus-galli*, in order to maximize the genetic polymorphism information available.

The analysis of 144 *E. crus-galli* samples collected from 36 rice fields in the Lombardy region (Northern Italy) using the eight polymorphic SSR markers identified by Chen et al. (2017), recorded high values of genetic richness and diversity parameters per population, mostly where chemical control was applied [22]. In general, we noted that the proportion of multilocus genotypes (MLG), and thus the richness in genotypes, was higher in experimental parcels where conventional weed control was applied (MLG > 1). In contrast, we found that the proportion of MLG is commonly lower in experimental parcels where only pre-emergent weed control was applied (MLG = 1). We also found that the Shannon–Wiener (H) and Stoddart and Taylor's (G) indexes of diversity in multilocus genotypes, linked to the genotype richness, showed the same outcome, with higher values in those experimental parcels where conventional chemical control was applied. Simpson dominance index (lambda), ranging from 0 (no genotypes are different) to 1 (all genotypes are different) provides an estimate of the probability that two randomly selected genotypes are different, and it is linked to the proportion of MLG. In general, we found high values in conventional weeded experimental parcels. The Evenness index (E.5), which provides a measure of the distribution of genotype abundances, recorded a value closer to 1 in experimental parcels with equally abundant genotypes (mostly in conventional weeded rice paddies), while a value closer to 0 was found in experimental parcels dominated by a single genotype (mostly in pre-emergent weeded rice paddies). Expected (He) and observed (Ho) heterozygosity, which are fundamental measures of genetic variation that describe the proportion of heterozygous genotypes expected under the Hardy–Weinberg equilibrium, showed high values in one population (EcgP01) [54].

According to Wright [69], if the coefficient of genetic differentiation (Fst) is less than 0.25, the level of genetic differentiation among populations is low. Our results showed different levels of genetic differentiation among *E. crus-galli* populations (Fst ranging from 0.00 to 0.31). In general, these values were high in pairwise comparisons of the weeded rice fields but were low in pre-emergent weeded rice fields. AMOVA results showed that 37.01% of the total genetic variation occurred among populations, consistently with the biology of therophytes.

Hierarchical clustering, which provides a genetic differentiation of the analyzed samples, confirmed the AMOVA results. It suggested that different agricultural practices seem to play a role in the genetic differentiation of samples into two main clusters (cluster I = experimental parcels with only pre-emergent weed control application; cluster II = experimental parcels with conventional weed control application). Clusters I and II sub-divided the analyzed samples into two subclusters, based on their genetic variability. Hierarchical clustering and Principal Coordinates Analysis were in concordance with the identification of four distinct genetic groups (red, yellow, blue, and green).

The high genetic variability of *E. crus-galli* highlighted in this study, especially in conventionally weeded rice fields, might be the result of the selective pressure induced by the herbicide control. In fact, it has been reported that high levels of genetic diversity are associated with high disturbance. Genetic diversity is closely related to the adaptive capacity of a species and guarantees, both to the individual and to the progeny, the possibility to better adapt when the ecological conditions are less stable and the evolutionary pressures more intense [70]. Intensive, single-crop farming, together with the constant application of the same herbicides over time, favored the survival and development of resistant individuals and consequently caused the progressive fragmentation and local genetic differentiation of the surviving populations [17]. In any case, this fact could also be due to the biology of this species. In general, annual weed species (therophytes) that are pollinated by wind have higher levels of variation among populations [7]. Nybom (2004) showed that the genetic variability of perennial species is mostly conserved within populations, while that of annual species is mostly conserved among populations [71,72].

Such analysis could be a useful tool for preliminary screening, to obtain information on the possible risk of herbicide resistance evolution in this weed, and to predict distribution patterns of susceptible/resistant populations [41].

5. Conclusions

Our findings confirmed that SSR markers represent a reliable, rapid, and affordable tool to assess the genetic variability in *E. crus-galli*. The optimized protocol provided more reproducible and reliable DNA SSR fingerprints. In addition, the application of suitable software to score SSR data as codominant in polyploid species avoided biased results. High genetic intraspecific diversity was found. AMOVA revealed that there was a higher genetic diversity among (37.01%) than within (15.74%) populations. Genetic variability was found to be higher in conventional weeded paddies than in pre-emergent weeded paddies, highlighting that this weed exhibits a high adaptive capacity in response to selective pressures driven by chemical herbicide control. The results obtained from this study represent a basis for a fast-track assessment of *E. crus-galli* genetic variability that is useful for more targeted, effective, and sustainable control of this weed.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Summary of population ID and agronomic managements, Table S2: Sequences of the primers for amplification of two noncoding regions of chloroplast DNA (cpDNA), Table S3: Pairwise Fst values between populations, Figure S1: levelplot of pairwise Fst between populations.

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7. Supplementary material

Table S1. Summary of population ID and agronomic managements.

Plot	N	Agronomic management	Population ID
1	4	Conventional weed control	EcgP01
2	4	Conventional weed control	EcgP02
3	4	Conventional weed control	EcgP03
4	4	Conventional weed control	EcgP04
5	4	Conventional weed control	EcgP05
6	4	Conventional weed control	EcgP06
7	4	Conventional weed control	EcgP07
8	4	Conventional weed control	EcgP08
9	4	Conventional weed control	EcgP09
10	4	Conventional weed control	EcgP10
11	4	Conventional weed control	EcgP11
12	4	Conventional weed control	EcgP12
13	4	Conventional weed control	EcgP13
14	4	Conventional weed control	EcgP14
15	4	Conventional weed control	EcgP15
16	4	Conventional weed control	EcgP16
17	4	Conventional weed control	EcgP17
18	4	Conventional weed control	EcgP18
19	4	Conventional weed control	EcgP19
20	4	Pre-emergent weed control	EcgP20
21	4	Pre-emergent weed control	EcgP21
22	5	Pre-emergent weed control	EcgP22
23	4	Pre-emergent weed control	EcgP23
24	4	Pre-emergent weed control	EcgP24
25	5	Pre-emergent weed control	EcgP25
26	4	Pre-emergent weed control	EcgP26
27	4	Pre-emergent weed control	EcgP27
28	4	Pre-emergent weed control	EcgP28
29	4	Pre-emergent weed control	EcgP29
30	4	Pre-emergent weed control	EcgP30
31	4	Pre-emergent weed control	EcgP31
32	4	Conventional weed control	EcgP32
33	4	Conventional weed control	EcgP33
34	4	Conventional weed control	EcgP34
35	4	Conventional weed control	EcgP35
36	4	Conventional weed control	EcgP36

Plot = experimental parcel in rice fields where *Echinochloa* samples have been collected, *N* = number of individuals collected per plot, *Agronomic management* = chemical control applied in plots, *Population ID* = Population identification code.

Table S2. Sequences of the primers for amplification of two noncoding regions of chloroplast DNA (cpDNA).

Primer	Sequence 5′ --- 3′
Trn-a	F: CATTACAAATGCGATGCTCT
Trn-b1	R: AACGATCGAATGAAAATGCC
Trn-c	F: CGAAATCGGTAGACGCTACG
Trn-d	R: GGGGATAGAGGGACTTGAAC

Table S3. Pairwise Fst values between populations.

Populations	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35	P36
P1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P2	0.087	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P3	0.055	0.050	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P4	0.055	0.063	0.029	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P5	0.087	0.003	0.052	0.062	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P6	0.094	0.045	0.090	0.104	0.043	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P7	0.065	0.046	0.085	0.090	0.053	0.112	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P8	0.059	0.051	0.087	0.080	0.058	0.101	0.006	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P9	0.087	0.010	0.054	0.060	0.017	0.063	0.037	0.039	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P10	0.096	0.039	0.063	0.056	0.043	0.089	0.059	0.051	0.025	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P11	0.057	0.099	0.071	0.048	0.099	0.137	0.091	0.082	0.098	0.093	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P12	0.110	0.106	0.113	0.103	0.111	0.177	0.100	0.099	0.109	0.114	0.076	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P13	0.051	0.104	0.087	0.064	0.101	0.137	0.074	0.065	0.101	0.095	0.027	0.128	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P14	0.060	0.100	0.090	0.078	0.097	0.146	0.074	0.073	0.105	0.094	0.052	0.112	0.029	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P15	0.093	0.133	0.112	0.109	0.138	0.150	0.150	0.141	0.151	0.156	0.081	0.128	0.119	0.123	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P16	0.084	0.093	0.076	0.062	0.098	0.138	0.100	0.092	0.090	0.084	0.082	0.133	0.094	0.102	0.081	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P17	0.043	0.099	0.076	0.061	0.104	0.146	0.063	0.054	0.097	0.090	0.058	0.113	0.059	0.068	0.117	0.052	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P18	0.051	0.108	0.084	0.059	0.113	0.126	0.089	0.067	0.101	0.089	0.066	0.133	0.072	0.092	0.115	0.054	0.014	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P19	0.060	0.127	0.103	0.083	0.133	0.155	0.094	0.076	0.124	0.107	0.080	0.145	0.080	0.098	0.113	0.075	0.025	0.022	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P20	0.216	0.234	0.241	0.242	0.238	0.281	0.202	0.197	0.236	0.249	0.216	0.223	0.218	0.216	0.260	0.235	0.203	0.218	0.225	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P21	0.216	0.234	0.241	0.242	0.238	0.281	0.202	0.197	0.236	0.249	0.216	0.223	0.218	0.216	0.260	0.235	0.203	0.218	0.225	0.000	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P22	0.223	0.236	0.243	0.248	0.240	0.296	0.203	0.203	0.241	0.256	0.222	0.224	0.224	0.217	0.270	0.241	0.209	0.233	0.237	0.007	0.007	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P23	0.230	0.238	0.245	0.254	0.242	0.310	0.204	0.209	0.245	0.262	0.226	0.223	0.230	0.217	0.277	0.246	0.216	0.247	0.249	0.018	0.018	0.009	--	--	--	--	--	--	--	--	--	--	--	--	--	--
P24	0.168	0.160	0.168	0.179	0.159	0.239	0.125	0.140	0.163	0.180	0.152	0.150	0.152	0.128	0.188	0.150	0.142	0.183	0.182	0.123	0.123	0.103	0.091	--	--	--	--	--	--	--	--	--	--	--	--	--
P25	0.193	0.223	0.227	0.228	0.232	0.261	0.200	0.187	0.235	0.238	0.209	0.230	0.208	0.219	0.250	0.239	0.206	0.208	0.191	0.172	0.172	0.171	0.205	0.173	--	--	--	--	--	--	--	--	--	--	--	--
P26	0.131	0.147	0.138	0.153	0.152	0.236	0.120	0.131	0.164	0.182	0.142	0.162	0.141	0.120	0.195	0.165	0.128	0.169	0.159	0.163	0.163	0.150	0.147	0.075	0.129	--	--	--	--	--	--	--	--	--	--	--
P27	0.114	0.142	0.126	0.137	0.148	0.219	0.117	0.124	0.157	0.177	0.128	0.166	0.127	0.105	0.176	0.149	0.111	0.145	0.148	0.165	0.165	0.162	0.158	0.092	0.169	0.016	--	--	--	--	--	--	--	--	--	--
P28	0.150	0.169	0.151	0.173	0.177	0.279	0.139	0.157	0.189	0.214	0.158	0.195	0.156	0.129	0.222	0.182	0.147	0.201	0.199	0.208	0.208	0.195	0.182	0.089	0.232	0.024	0.017	--	--	--	--	--	--	--	--	--
P29	0.108	0.141	0.126	0.131	0.146	0.204	0.117	0.118	0.154	0.164	0.122	0.165	0.121	0.100	0.167	0.143	0.105	0.129	0.130	0.158	0.158	0.160	0.162	0.105	0.146	0.024	0.006	0.037	--	--	--	--	--	--	--	--
P30	0.140	0.158	0.141	0.165	0.170	0.272	0.136	0.155	0.189	0.208	0.158	0.189	0.154	0.117	0.218	0.183	0.149	0.202	0.196	0.199	0.199	0.187	0.174	0.084	0.221	0.020	0.016	0.005	0.030	--	--	--	--	--	--	--
P31	0.159	0.180	0.161	0.185	0.192	0.295	0.147	0.165	0.198	0.220	0.161	0.211	0.156	0.139	0.238	0.190	0.156	0.210	0.207	0.223	0.223	0.212	0.199	0.103	0.249	0.043	0.037	0.012	0.054	0.026	--	--	--	--	--	--
P32	0.104	0.127	0.109	0.122	0.129	0.188	0.095	0.095	0.128	0.123	0.103	0.113	0.102	0.091	0.145	0.124	0.086	0.113	0.114	0.176	0.176	0.175	0.175	0.114	0.181	0.086	0.080	0.098	0.080	0.100	0.104	--	--	--	--	--
P33	0.181	0.214	0.190	0.208	0.220	0.262	0.186	0.178	0.219	0.209	0.194	0.192	0.194	0.186	0.215	0.205	0.173	0.188	0.198	0.276	0.276	0.282	0.288	0.223	0.291	0.238	0.227	0.266	0.223	0.260	0.282	0.068	--	--	--	--
P34	0.212	0.244	0.223	0.235	0.246	0.284	0.211	0.202	0.240	0.231	0.218	0.217	0.221	0.214	0.233	0.224	0.194	0.208	0.217	0.302	0.302	0.308	0.313	0.249	0.311	0.257	0.244	0.281	0.241	0.271	0.293	0.088	0.007	--	--	--
P35	0.151	0.182	0.161	0.171	0.181	0.218	0.149	0.140	0.178	0.166	0.154	0.151	0.157	0.154	0.175	0.164	0.129	0.142	0.153	0.236	0.236	0.242	0.248	0.193	0.245	0.197	0.187	0.223	0.185	0.214	0.235	0.053	0.015	0.024	--	--
P36	0.162	0.192	0.172	0.182	0.191	0.227	0.160	0.150	0.187	0.172	0.165	0.160	0.168	0.162	0.181	0.177	0.143	0.157	0.155	0.252	0.252	0.258	0.264	0.205	0.242	0.195	0.189	0.225	0.183	0.214	0.237	0.049	0.021	0.025	0.019	--

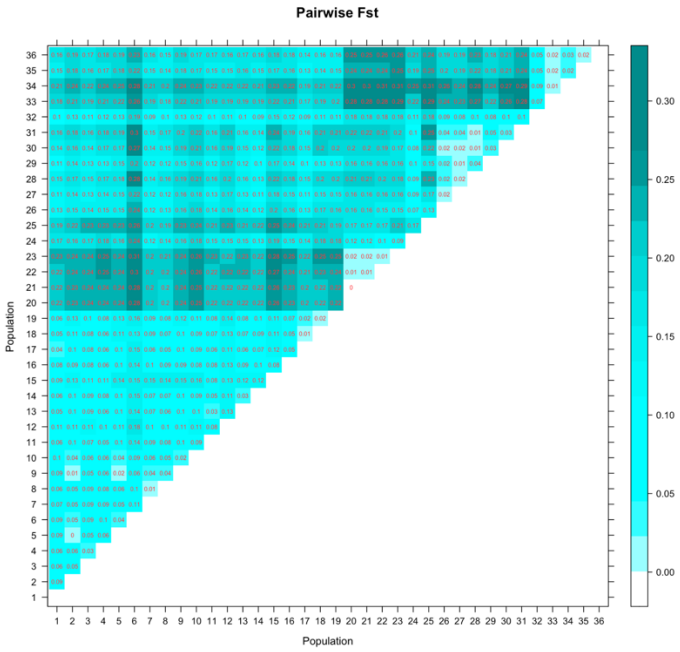

Figure S1. Levelplot of pairwise Fst values between populations.

7.3 Analysis of intraspecific variability in *E. oryzicola*

7.3.1 Introduction

Among the most noxious species infesting paddies there is *Echinochloa oryzicola* (Vasinger) Vasinger (late watergrass) (Figure 7.1 A). *E. oryzicola* is an annual allo-tetraploid weed (2n = 4X = 36) native to Asia that has now spread all over the world, from tropical to temperate regions (Ye et al., 2020). This weed generally reaches 50 cm in height, but can also be up to 150 cm tall with fibrous roots and erect and robust culms. It has leaves often characterized by a tuft of brown hairs in the collar region, greenish leaves 5-12 mm wide, hairless on the edge. The inflorescence is an erect or slightly drooping panicle of 8-20 cm formed by racemes commonly arranged alternately on the rachis (Figure 7.1 B). The spikelets are mutic or mucronate, and measure about 2.2 / 2.7 mm in length. The fruit is a caryopsis, usually of yellowish color, that can reach 2 to 2.4 mm in length (Carratero, 1981; Pignatti, 1982; Lopez-Martinez et al., 1999; Costea&Tardif, 2002; Sparacino et al., 2007; Banfi & Galasso, 2010; Hoste et al., 2022).

Despite the high phenotypic plasticity that characterizes the genus *Echinochloa*, *E. oryzicola* shows a relatively limited diversity of its morphological characteristics, although it has spikelets with two morphs, the "C" form, with convex and glossy lemmas, and the "F" form, with flat and coarse lemmas (Yasuda et al., 2002; Aoki et al., 2008). In general, late-watergrass could be easily distinguished from the congeners normally present in crops, in particular for the strictly erect inflorescence, the size of the spikelets and the kernels.

Figure 7.1: *Echinochloa oryzicola*. A: *E. orizicola* in paddy field. B: Particular of *E. oryzicola* panicle.

While adapting to a wide range of natural environments, *E. oryzicola* prefers flooded rice fields, where it has developed a very complex survival strategy. Seeds located in the soil have annual cycles between the quiescent and non-quiescent state. Non-dormant seeds have unique characteristics, metabolically adapted to submerged conditions to germinate and grow by anaerobic respiration and alcoholic fermentation. The expression of the gene encoding the enzyme that catalyzes ATP synthesis through mitochondrial oxidative phosphorylation is higher in dormant seeds, which have greater oxygen uptake and enzymatic aerobic respiration activity than non-dormant seeds, allowing them to remain viable in flooded soil by conventional aerobic breathing, from the autumn period to the beginning of the following spring. Moreover, in the early stages of development, late-watergrass possesses almost perfect camouflage with rice plants, thus escaping manual weeding. The various adaptive survival strategies of *E. oryzicola* in submerged rice fields derive from the genetic traits inherited from the progenitors and from those that have been selected by agronomic practices, included chemical control (Nakatani et al., 1998; Yamasue, 2001; Lim et al., 2021; Panozzo et al., 2021).

Cases of multiple resistance have been reported for *E. oryzicola*, due to its adaptability, resulting from a high genetic variability (Neve et al. 2009; Jia et al., 2020;

Amaro-Blanco et al., 2021). In fact, several recent studies have shown that crop characteristics, agricultural management, including herbicide application, could influence the genetic diversity and adaptability of many *Echinochloa* species, including *E. oryzicola* (Altop et al., 2011; Kaloumenos et al., 2013; Claerhout et al., 2015; Altop et al., 2018; Mascanzoni et al., 2018). Despite the great threat represented by these weeds, the role of intraspecific variability and its relation with the adaptability of these species towards ecological stressors is a poorly investigated topic. As a matter of facts, only a limited number of researches conducted on *Echinochloa* species have focused on herbicide resistance also considering genetic variability (Vidotto et al., 2007; Lee et al., 2019). The analysis of intraspecific genetic diversity and population structure of resistant *Echinochloa* species are essential to obtain valuable information useful to monitor the spread of resistant populations, to develop adequate management plans and design new strategies of weed control (Jia et al., 2020; Kaloumenos et al., 2013; Chauhan et al., 2022).

Among the PCR-based techniques to analyze genetic variability, microsatellites (or SSR) markers represent the better choice to study this feature in plants, due to their high reliability and reproducibility. Since they are codominant, multiallelic, highly changeable and polymorphic, they are preferred to many other molecular markers (Nybom, 2004; Ruiz-Santella et al., 2006; Pfeiffer et al., 2011; Stift et al., 2019; Wu et al., 2019; Kurian et al., 2020; Cusaro et al., 2022). Nevertheless, as *E. oryzicola* is an orphan species (i.e., organisms without a publicly available reference genome sequence), the knowledge of microsatellites loci useful to assess its genetic variability is limited and only a few number of SSR markers is available. Recently, Lee et al. (2019), using sequencing technology (NGS) (Illumina), identified 13 cross-specific SSR markers, useful for studying the genetic diversity of *Echinochloa* spp., including late watergrass.

Due to the difficulties related to regulatory and agronomic reasons, there is an increasingly urgent need to carry out molecular investigations about adaptability of *Echinochloa* species, in order to deepen the knowledge about factors linked with herbicide resistance occurrence. Findings will contribute to develop more targeted and less impactful weed control strategies, safeguarding the environment and the human health.

The aim of this study-section was to evaluate the genetic intraspecific diversity of herbicide-resistant *E. oryzicola* specimens collected from a series of rice fields in Lombardy by analyzing the variability of microsatellite loci identified by Lee et al. (2019).

7.3.2 Materials & Methods

In this study-section, only specimens identified as *E. oryzicola* from the entire collection were considered. A total of 84 samples were analyzed: 72 samples survived to herbicide treatment (presumed to be "resistant") and 12 samples (considered as "susceptible") collected in organic paddies.

Specifically for this analysis, specimens collected in the same SU were considered as a group of individuals belonging to a population.

To each individual, and identification code was assigned on the basis of membership population (see Table 7.1).

Table 7.1: *Echinochloa oryzicola* specimens listed by ID, population and municipality / agricultural practice.

ID	Population	Municipality of the farm / Management
Eory_PV3_1	PV3	Suardi__EC128/2009
Eory_PV3_2	PV3	Suardi__EC128/2009
Eory_PV3_3	PV3	Suardi__EC128/2009
Eory_PV3_4	PV3	Suardi__EC128/2009
Eory_PV7_1	PV7	Robbio__EC128/2009
Eory_PV7_2	PV7	Robbio__EC128/2009
Eory_PV7_3	PV7	Robbio__EC128/2009
Eory_PV7_4	PV7	Robbio__EC128/2009
Eory_PV10_1	PV10	Rivoltella__EC128/2009
Eory_PV10_2	PV10	Rivoltella__EC128/2009
Eory_PV10_3	PV10	Rivoltella__EC128/2009
Eory_PV10_4	PV10	Rivoltella__EC128/2009
Eory_PV11_1	PV11	Vigevano-Barbavara__EC128/2009
Eory_PV11_2	PV11	Vigevano-Barbavara__EC128/2009
Eory_PV11_3	PV11	Vigevano-Barbavara__EC128/2009
Eory_PV11_4	PV11	Vigevano-Barbavara__EC128/2009
Eory_PV14_1	PV14	Frascarolo-Sartirana__EC128/2009
Eory_PV14_2	PV14	Frascarolo-Sartirana__EC128/2009
Eory_PV14_3	PV14	Frascarolo-Sartirana__EC128/2009
Eory_PV14_4	PV14	Frascarolo-Sartirana__EC128/2009
Eory_PV15_1_1	PV15_1	Zerbolò1__EC128/2009
Eory_PV15_1_2	PV15_1	Zerbolò1__EC128/2009
Eory_PV15_1_3	PV15_1	Zerbolò1__EC128/2009
Eory_PV15_1_4	PV15_1	Zerbolò1__EC128/2009
Eory_PV15_2_1	PV15_2	Zerbolò2__EC128/2009
Eory_PV15_2_2	PV15_2	Zerbolò2__EC128/2009
Eory_PV15_2_3	PV15_2	Zerbolò2__EC128/2009
Eory_PV15_2_4	PV15_2	Zerbolò2__EC128/2009
Eory_PV16_1	PV16	Belgioioso-StantaMargherita__EC128/2009
Eory_PV16_2	PV16	Belgioioso-StantaMargherita__EC128/2009
Eory_PV16_3	PV16	Belgioioso-StantaMargherita__EC128/2009
Eory_PV16_4	PV16	Belgioioso-StantaMargherita__EC128/2009
Eory_PV18_1	PV18	Lardirago__ Organic
Eory_PV18_2	PV18	Lardirago__ Organic
Eory_PV18_3	PV18	Lardirago__ Organic
Eory_PV18_4	PV18	Lardirago__ Organic
Eory_PV20_1	PV20	Genzone__EC128/2009
Eory_PV20_2	PV20	Genzone__EC128/2009

Eory_PV20_3	PV20	Genzone__EC128/2009
Eory_PV20_4	PV20	Genzone__EC128/2009
Eory_PV21_1	PV21	Roncaro__EC128/2009
Eory_PV21_2	PV21	Roncaro__EC128/2009
Eory_PV21_3	PV21	Roncaro__EC128/2009
Eory_PV21_4	PV21	Roncaro__EC128/2009
Eory_PV23_1	PV23	PieveAlbignola__EC128/2009
Eory_PV23_2	PV23	PieveAlbignola__EC128/2009
Eory_PV23_3	PV23	PieveAlbignola__EC128/2009
Eory_PV23_4	PV23	PieveAlbignola__EC128/2009
Eory_PV24_1	PV24	SanMartinoSiccomario__EC128/2009
Eory_PV24_2	PV24	SanMartinoSiccomario__EC128/2009
Eory_PV24_3	PV24	SanMartinoSiccomario__EC128/2009
Eory_PV24_4	PV24	SanMartinoSiccomario__EC128/2009
Eory_PV26_1	PV26	Lomello__EC128/2009
Eory_PV26_2	PV26	Lomello__EC128/2009
Eory_PV26_3	PV26	Lomello__EC128/2009
Eory_PV26_4	PV26	Lomello__EC128/2009
Eory_PV27_1	PV27	SanGiorgioLomellina__EC128/2009
Eory_PV27_2	PV27	SanGiorgioLomellina__EC128/2009
Eory_PV27_3	PV27	SanGiorgioLomellina__EC128/2009
Eory_PV27_4	PV27	SanGiorgioLomellina__EC128/2009
Eory_PV28_1	PV28	Dorno__EC128/2009
Eory_PV28_2	PV28	Dorno__EC128/2009
Eory_PV28_3	PV28	Dorno__EC128/2009
Eory_PV28_4	PV28	Dorno__EC128/2009
Eory_PV30_1	PV30	Giussago__EC128/2009
Eory_PV30_2	PV30	Giussago__EC128/2009
Eory_PV30_3	PV30	Giussago__EC128/2009
Eory_PV30_4	PV30	Giussago__EC128/2009
Eory_PV32_1_1	PV32_1	CandiaLomellina1__Organic
Eory_PV32_1_2	PV32_1	CandiaLomellina1__ Organic
Eory_PV32_1_3	PV32_1	CandiaLomellina1__ Organic
Eory_PV32_1_4	PV32_1	CandiaLomellina1__ Organic
Eory_PV32_2_1	PV32_2	CandiaLomellina2__ Organic
Eory_PV32_2_2	PV32_2	CandiaLomellina2__ Organic
Eory_PV32_2_3	PV32_2	CandiaLomellina2__ Organic
Eory_PV32_2_4	PV32_2	CandiaLomellina2__ Organic
Eory_MI2_1	MI2	Ozzero__EC128/2009
Eory_MI2_2	MI2	Ozzero__EC128/2009
Eory_MI2_3	MI2	Ozzero__EC128/2009
Eory_MI2_4	MI2	Ozzero__EC128/2009
Eory_Daghetta_1	Daghetta	Robbio_Daghetta__EC128/2009
Eory_Daghetta_2	Daghetta	Robbio_Daghetta__EC128/2009
Eory_Daghetta_3	Daghetta	Robbio_Daghetta__EC128/2009
Eory_Daghetta_4	Daghetta	Robbio_Daghetta__EC128/2009

EC 128/2009: Directive 128/2009 of European Community.

7.3.2.1 DNA extraction

DNA was extracted using the DNeasy Plant Kit (QIAGEN spa, Hilden, Germany), following manufacturer's instructions. Extracted DNA was quantified using QBIT (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

7.3.2.2 Microsatellites loci amplification

The SSR loci identified by Lee et al. (2019) analyzed in this study are shown in Table 7.2, together with the number of repeats of the corresponding nucleotide unit and its primers.

Table 7.2: *Echinochloa oryzicola* SSR loci listed with repeated unit and primer sequences.

Locus	SSR unit	Primer sequence 5' - 3'
KEC5	$(AAT)_{12}$	P: TTCTCAGGTCTAGCAGGATGTT P: ATGGTTTAGTCCAATTTGCATC
KEC38	$(GCT)_{10}$	P: ATCCAAGTCTTCAAGCACTCAT P: TCGATGTTTTTTAATCTCGTCT
KEC42	$(TTA)_{17}$	F: GTTATTGGTCCCTCAGATGGTA P: GGAACTAAATTAAAAAATGGGC
KEC48	$(TAG)_{10}$	F: CGTACAATTCATCACAGGGTTA P: GTTTATTCCATGGTTGGGACT
KEC59	$(TGC)_{12}$	F: CATCCAGACAACCATACATCTG P: AGAACATGCTGGAATCAAACTT
KEC125	$(GCT)_{8}$	P: CTTCTTTATCTCCAACGCAAAC P: CTATAAGCTCTCCCATTGATCG
KEC136	$(ACA)_{11}$	F: TCCATTCTTCACGATCATCATA P: TAGCAGGAGCTGTTTTTTTTTTTC
KEC160	$(TTC)_{8}$	P: TTAATCTTGAAGGTACGGTGCT P: CATCAGTATGATGAACTGGCAC
KEC171	$(TGC)_{9}$	F: GAAGATGAAGGGGAAAGAATTG P: TGCCATCTCATTTTGTGTTTTA
KEC195	$(GCC)_{8}$	F: ACGTCTCTTTACAGAAAACCCC P: TTGCTCATACCTATCCAATTCC
KEC205	$(CTG)_{9}$	F: TCATGGTACGTGTAGTAGGCTG P: TATAGCGACCCTTTTGACCTTA
KEC217	$(GCC)_{8}$	P: ATCAGAAGACGTCATATGGGAG P: AGAAGACGCAGCAGAAGAAAG
KEC157	$(CAA)_{7}$	P: AACCGTGGTGGAAATCGCAG P: ACCACGAGTTGTCGATGTTGT

PCR amplifications were performed in a total volume of 10 µl, containing 2 µl of genomic DNA (20/30 ng), 5 µl (1X) of GoTaq® Hot Start Green Master Mix (Promega, Madison, Wisconsin, USA), 1 µl of each primer (1 µM), 0.5µl of MgCl2 (2.5 mM) and 2 sterile nuclease free water (Promega) ad volume.

In order to obtain reproducible SSR fingerprints, the PCR protocol has been optimized as reported in Cusaro et al. (2022). The PCR amplification was conducted using a T100 Thermal Cycler (BioRad, Hercules, CA) as follow: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at temperatures estimated by Gradient PCR, for each primers, pair for 30 s, extension at 68°C for 1 min. Final extension step at 72° C for 10 min.

Total volume amplified was loaded into a 2% agarose gel (BIO-RAD™, Hercules, California, USA) with 1X TBE (BIO-RAD™) stained with ethidium bromide. Electrophoresis was performed at 100 V for 60'. SSR fingerprints were observed using a trans-illuminator Molecular Imager® Gel Doc™ XR+ (BIO-RAD). The fingerprints were obtained by electrophoretic travel at 100 V for 60'. Amplicon fragments size was determined using a 100 bp DNA ladder (Promega).

For each SSR primer pair, amplicons of the same size were considered to be the same allele. For each SSR listed in Table 2, the reproducibility of the test was validated by three replicates in which the same experimental conditions were applied and with each replicate producing a similar result. A matrix of codominant data was then constructed.

7.3.2.3 Statistical analysis

The number of observed alleles per locus (Na) was computed using R 3.6.3 software (poppr 2.9.3 (Kamvar et al., 2014)). The polymorphism information content (PIC) values were calculated using the formula of Liu et al. (2011):

$$PIC = 1 - \sum (P_{ij})^2$$

P_{ij} corresponds to the allelic frequency of the i-th sample in the j-th primer, recorded for each of the 13 primers that were used – according to Prevost et al. (1999) and Tiwari et al. (2016). Genotypic richness (the number of multilocus genotypes observed per population— MLG); genotypic diversity (percentage of polymorphism detected by each population— %P); Shannon–Wiener Index (H) of MLG diversity per population (Shannon, 2001); Stoddart and Taylor's Index (G) of MLG diversity per population (Stoddart et al., 1988); Simpson's Index (λ) per population (Simpson, 1949); Evenness index (E.5) per population (Pielou, 1975); expected heterozygosity (He) or Nei's unbiased gene diversity per population (Nei, 1978); observed heterozygosity (Ho) per population were analyzed using R 3.6.3 software (function "poppr" – packages poppr 2.9.3 (Kamvar et al., 2014), pegas 1.0-1 (Paradis, 2010)). Analysis of molecular variance (AMOVA) among and within populations was run with 10.000 permutations by R 3.6.3 software (function "poppr.amova" - packages poppr 2.9.3 (Kamvar et al., 2014), StAMPP 1.6.3 (Pembleton et al., 2013)). Pairwise Fst values between populations were determined with 10.000 permutations by R 3.6.3 software (function "calcPopDiff" – package polysat 1.7-5 (Clark et al., 2017)) and plotted as a levelplot (function "levelplot" – package lattice 0.10-10 (Sarkar et al., 2008)). Genetic similarity was calculated using Nei's unbiased genetic distance with R 3.6.3 software (poppr 2.9.3 (Kamvar et al., 2014)). Hierarchical clustering was performed based on "Ward.D2"

method to maximize the between-cluster variance and "Canberra" distance algorithm with R 3.6.3 software (function "circlize_dendrogram" – package dendextend 1.15.2 (Galili, 2015)). Genotypes were sorted by PCoA ("Ward.D2" method), showing their distributions in a scatter plot, using R 3.6.3 software (function "fviz_cluster" – packages factoextra (Alboukadel et al., 2020), FactoMineR 2.4 (Le et al., 2008), ggplot2 3.3.5 (Wickham, 2016)).

7.3.3 Results & Discussion

7.3.3.1 Genetic Richness and Diversity Analysis

We analyzed 84 resistant specimens of *E. oryzicola:* 72 survived weed control and presumed to be "resistant" and 12 collected from organic rice fields and considered as "susceptible". A total of 41 different alleles were detected using 13 SSR markers. Allele number (Na) ranged from 2 (EG307) to 6 (EG301), with an average of 3.15 alleles per locus. Polymorphic information content (PIC) ranged from 0.80 (KEC157) to 0.99 (KEC195 and KEC217), with an average of 0.91 per locus (Table 7.3).

Table 7.3: Estimated parameters of allele numbers and polymorphic information content (PIC) in the 8 pairs of Simple Sequence Repeat markers analyzed in *Echinochloa oryzicola*.

Locus	Na	PIC
KEC5	2	0.84
KEC38	5	0.98
KEC42	3	0.93
KEC48	5	0.97
KEC59	2	0.85
KEC125	2	0.86
KEC136	3	0.90
KEC160	4	0.95
KEC171	2	0.87
KEC195	2	0.99
KEC205	3	0.93
KEC217	6	0.99
KEC157	2	0.80
Mean	3.15	0.91

The analysis of genetic richness parameters by population has generally recorded a high diversity (Table 7.4). The mean percentage of polymorphic loci (%P) was 28.57%. The total number of multilocus genotypes (MLGs) observed was 58 and values ranged from 1 to 4. In 2 out of 21 populations, an MLG value of 1 was recorded. The Shannon-Wiener (H)

index was 3.88, with an average value of 0.18. The total Stoddart and Taylor index (G) was 37.13, with an average value of 1.77. The Simpson index (Lambda) resulted in 0.97, with an average value of 0.05. The calculation of the equity index (E.5) gave a value of 0.76, with an average value of 0.04. The expected total heterozygosity or gene diversity of Nei (He) was 0.43, with a mean value of 0.02. The observed total heterozygosity (Ho) was 0.399, with a mean value of 0.019.

Table 7.4: Genetic diversity parameters in *Echinochloa oryzicola*.

Population ID	Management	N	MLG	%P	H	G	Lambda	E.5	He	Ho
Eory_PV3	EC 128/2009	4	3	53.84	1.04	2.66	0.63	0.91	0.28	0.0182
Eory_PV7	EC 128/2009	4	3	23.07	1.04	2.66	0.63	0.91	0.14	0.0182
Eory_PV10	EC 128/2009	4	3	15.38	1.04	2.66	0.63	0.91	0.08	0.0182
Eory_PV11	EC 128/2009	4	3	15.38	1.04	2.66	0.63	0.91	0.08	0.0242
Eory_PV14	EC 128/2009	4	4	38.46	1.38	4	0.75	1	0.21	0.0242
Eory_PV15_1	EC 128/2009	4	4	23.07	1.38	4	0.75	1	0.18	0.0121
Eory_PV15_2	EC 128/2009	4	2	7.69	0.56	1.6	0.37	0.79	0.04	0.0182
Eory_PV16	EC 128/2009	4	3	84.71	1.04	2.66	0.63	0.91	0.44	0.0242
Eory_PV18	Organic	4	4	46.15	1.38	4	0.75	1	0.27	0.0182
Eory_PV20	EC 128/2009	4	3	15.38	1.04	2.66	0.63	0.91	0.08	0.0182
Eory_PV21	EC 128/2009	4	3	23.07	1.04	2.66	0.63	0.91	0.12	0.0061
Eory_PV23	EC 128/2009	4	1	7.69	0	1	0	-	0	0.0121
Eory_PV24	EC 128/2009	4	2	53.84	0.56	1.6	0.37	0.79	0.27	0.0121
Eory_PV26	EC 128/2009	4	2	7.69	0.56	1.6	0.37	0.79	0.04	0.0061
Eory_PV27	EC 128/2009	4	1	7.69	0	1	0	-	0	0.0182
Eory_PV28	EC 128/2009	4	3	15.38	1.04	2.66	0.63	0.91	0.08	0.0121
Eory_PV30	EC 128/2009	4	2	23.07	0.69	2	0.5	1	0.16	0.0242
Eory_PV32_1	Organic	4	4	46.15	1.38	4	0.75	1	0.24	0.0242
Eory_PV32_2	Organic	4	4	30.76	1.38	4	0.75	1	0.22	0.0182
Eory_MI2	EC 128/2009	4	3	30.76	1.04	2.66	0.63	0.91	0.16	0.0242
Eory_Daghetta	EC 128/2009	4	4	30.76	1.38	4	0.75	1	0.27	0.0485
Total	---	84	58	---	3.88	37.13	0.97	0.76	0.43	0.3999
Average	---	4	2.76	28.57	0.18	1.77	0.05	0.04	0.02	0.0190

N = number of individuals per population, %P = percentage of polymorphism detected in each population, MLG = number of multilocus genotypes observed per population, H = Shannon–Wiener Index of MLG diversity per population, G = Stoddart and Taylor's Index of MLG diversity per population, lambda = Simpson's Index per population, E.5 = Evenness index per population (in populations where lambda is equal to 0, E.5 values could not be scored), He = expected heterozygosity per population, Ho = observed heterozygosity per population.

7.3.3.2 Analysis of Mulecular Variance (AMOVA)

The analysis of molecular variance (AMOVA) was carried out considering the 21 populations studied, calculating the degree of genetic variability among the different populations and within them, based on the SSR loci investigated. The results of the AMOVA analysis show a proportion of inter-population genetic variability (65.5 %) higher than intra-population genetic variability (34.5 %), as shown in Table 7.5. Pairwise Fst values

between populations have been shown in Table 7.6 and in the levelplot below. Fst ranges from 0.21 to 1.00 (Table 7.6, Figure 7.2).

Table 7.5: Analysis of molecular variance (AMOVA) based on Simple Sequence Repeats in *Echinochloa oryzicola*

AMOVA	**Df**	**SS**	**MS**	**Est.Var.**	**%**	***p***
Among the populations	20	21338.13	1066.95	235.70	65,5 %	< 0.001
Within populations	63	7821.66	124.15	124.15	34,5%	< 0.001
Total	83	29160.80	351.33	359.85	100%	

Table 7.6: Pairwise Fst values between populations of *Echinochloa oryzicola*

Fst	Eory_PV3	Eory_PV7	Eory_PV10	Eory_PV11	Eory_PV14	Eory_PV15_1	Eory_PV15_2	Eory_PV16	Eory_PV18	Eory_PV20	Eory_PV21	Eory_PV23	Eory_PV24	Eory_PV26	Eory_PV27	Eory_PV28	Eory_PV30	Eory_PV32_1	Eory_PV32_2	Eory_MI2	Eory_Daghetta
Eory_PV3	0,00																				
Eory_PV7	0,21	0,00																			
Eory_PV10	0,50	0,44	0,00																		
Eory_PV11	0,47	0,49	0,57	0,00																	
Eory_PV14	0,46	0,56	0,61	0,48	0,00																
Eory_PV15_1	0,49	0,56	0,65	0,58	0,58	0,00															
Eory_PV15_2	0,61	0,71	0,83	0,76	0,67	0,56	0,00														
Eory_PV16	0,35	0,41	0,52	0,47	0,44	0,33	0,29	0,00													
Eory_PV18	0,35	0,45	0,54	0,59	0,45	0,59	0,61	0,36	0,00												
Eory_PV20	0,54	0,68	0,78	0,78	0,59	0,69	0,85	0,52	0,34	0,00											
Eory_PV21	0,57	0,69	0,75	0,74	0,56	0,68	0,80	0,47	0,35	0,36	0,00										
Eory_PV23	0,72	0,82	0,88	0,89	0,71	0,77	0,95	0,59	0,50	0,74	0,23	0,00									
Eory_PV24	0,49	0,61	0,70	0,65	0,45	0,57	0,68	0,34	0,32	0,45	0,38	0,58	0,00								
Eory_PV26	0,59	0,73	0,81	0,77	0,57	0,68	0,88	0,46	0,39	0,66	0,51	0,88	0,39	0,00							
Eory_PV27	0,63	0,78	0,85	0,81	0,60	0,71	0,94	0,51	0,46	0,76	0,60	1,00	0,49	0,14	0,00						
Eory_PV28	0,58	0,71	0,76	0,72	0,55	0,65	0,80	0,44	0,40	0,62	0,49	0,78	0,44	0,23	0,26	0,00					
Eory_PV30	0,50	0,61	0,67	0,59	0,48	0,62	0,73	0,38	0,44	0,62	0,55	0,76	0,46	0,49	0,54	0,46	0,00				
Eory_PV32_1	0,49	0,61	0,69	0,65	0,54	0,64	0,70	0,39	0,39	0,48	0,37	0,58	0,38	0,41	0,46	0,41	0,22	0,00			
Eory_PV32_2	0,59	0,63	0,70	0,68	0,62	0,69	0,71	0,44	0,50	0,54	0,43	0,56	0,46	0,58	0,62	0,56	0,42	0,28	0,00		
Eory_MI2	0,57	0,63	0,67	0,60	0,56	0,66	0,72	0,46	0,50	0,63	0,59	0,73	0,51	0,54	0,57	0,51	0,28	0,31	0,36	0,00	
Eory_Daghetta	0,56	0,64	0,66	0,65	0,50	0,64	0,74	0,44	0,44	0,60	0,50	0,65	0,39	0,50	0,53	0,46	0,32	0,29	0,30	0,22	0,00

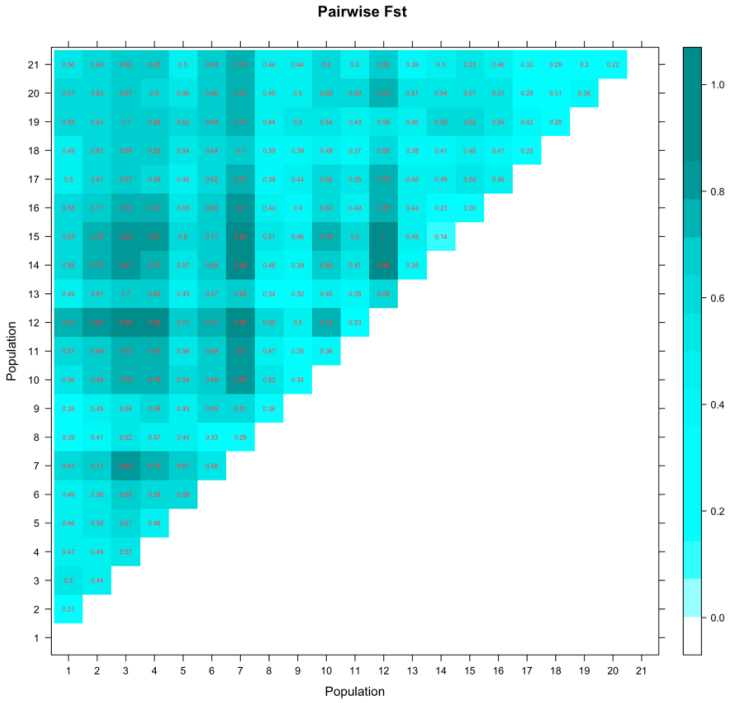

Figure 7.2: Levelplot of pairwise Fst values between populations of *Echinochloa oryzicola*.

7.3.3.3 Hierarchical Clustering and Principal Coordinates Analysis

Hierarchical clustering identified two main genetic groups, corresponding to clusters I and II (Figure 7.3 A). Cluster I is divided into two subgroups (red and yellow). Cluster II is divided into two subgroups (blue and green). Altogether, four different genetic groups (red, yellow, blue, green) have been identified. Similarly, principal coordinate analysis identified four genetic groups (red, yellow, blue, and green) on the first three coordinates, explaining about the 35 % (cumulative values) of total variability (Figure 7.3 B). Overlapping of blue and green sub-clusters indicates genetic similarity among specimens.

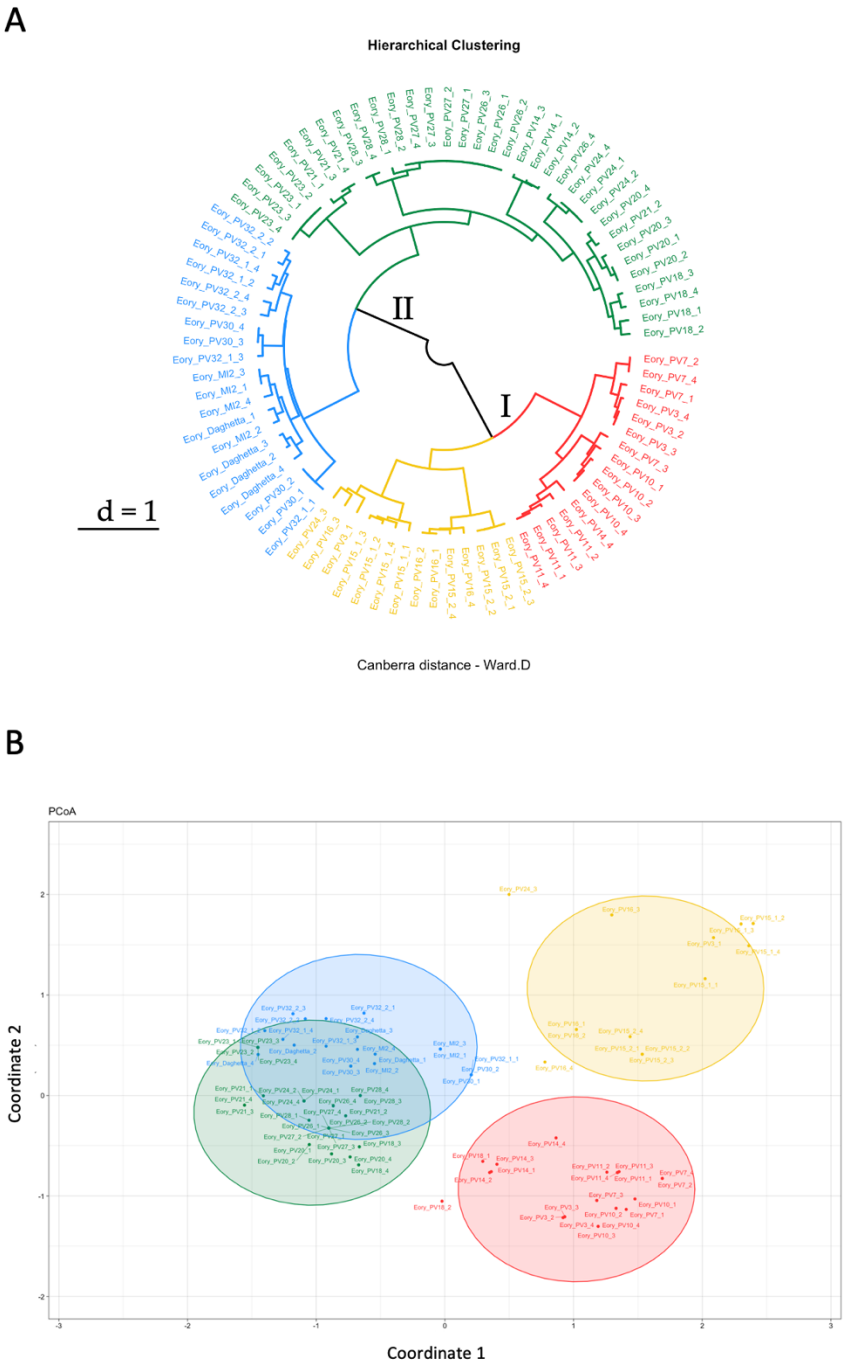

Figure 7.3: A: Hierarchical clustering. B: Principal Coordinate Analysis.

The results obtained in this work highlighted the presence of a different number of alleles for each SSR locus in the different samples. From the analysis of the 13 SSR cross-specific loci discovered by Lee et al. (2019) in 84 *E. oryzicola* specimens, varying values of genetic richness and genetic diversity were obtained for the examined populations, from both weeded and organic rice fields. Furthermore, no appreciable difference of genetic

richness and genetic diversity values was recorded between specimens collected from weeded and organic paddy fields.

In general, the proportion of multilocus genotypes (MLGs) and the richness in genotypes recorded high values in most of the populations analyzed (MLG > 1), except for Eory_PV23 and Eory_PV27 populations where the proportion of MLG is lower (MLG = 1). These two late-watergrass populations were collected in weeded paddies of Pieve Albignola and San Giorgio di Lomellina. A MLG value = 1 means that individuals in the same population have no appreciable genetic differences in the microsatellite loci analyzed. Hierarchal clustering highlighted this result since the individuals of these populations are grouped together at the same level, suggesting a minimal SSR loci variability among them.

The results obtained from the analysis of Shannon-Wiener (H) and Stoddart and Taylor (G) indices confirmed such a result, with high values in almost all populations except for Eory_PV23 and Eory_PV27. The Simpson dominance index (λ) represents a measure of diversity that incorporates both genotypic richness and genotype abundance per population. It provides an estimate of the probability that two randomly selected genotypes are different and ranges from 0 (no genotype is different) to 1 (all genotypes are different). All the populations investigated possess high λ values. In Eory_PV23 and Eory_PV27 λ measures 0, suggesting that all specimens possess similar alleles in the SSR loci analyzed. In population where λ values are comprised between 0 and 1, there is evidence of some individuals with the same alleles at SSR loci. The equitability index (E.5), which measures the distribution of genotypic abundances in a group of organisms, recorded a value equal to 1 in rice fields where the population possessed genotypes with equal abundance (Eory_PV14, Eory_PV15_1, Eory_PV18, Eory_PV30, Eory_PV32_1, Eory_PV32_2, Eory_Daghetta), while a value equal to 0 in rice fields where a single genotype is present (Eory_PV23 and Eory_PV27). Expected (He) and observed heterozygosity (Ho) are fundamental measures of genetic variability in a population and describe the proportion of heterozygous genotypes expected under Hardy-Weinberg equilibrium (Nei, 1978). These parameters recorded different values in each of the populations: specifically a maximum of 0.44 in the Eory_PV16 population and a minimum of 0 in Eory_PV23 and Eory_PV27

populations. To sum up, the results obtained show that there is no clear difference in genetic richness between specimens collected from weeded paddies and specimens from organic paddies, unlike that found for *E. crus galli* (Cusaro et al., 2022): as a matter of fact, high genetic richness was recorded in almost all individuals of *E. oryzicola* populations.

According to Wright (Wright, 1965; Tong et al., 2020), if the pairwise Fst value between populations is less than 0.25, the level of genetic differentiation between populations is low. The results obtained showed Fst values ranging from 0.21 to 1.00, but the majority recorded values higher than 0.25. AMOVA showed high genetic variability among populations (65.5 %) and less variability among specimens of the same population (34.5 %). This assessment shows their great genetic variability of them, key feature for their adaptation towards different ecological conditions.

As a matter of facts, herbicide application represents a selective pressure that favors specimens that better adapt (Nakatani et al., 1998; Yamasue, 2001; Lim et al., 2021; Panozzo et al., 2021). On the other hand, polyploidy is a very common condition in plants and is one of the main drivers of plant species diversification, playing an important role in their genome evolution (Soltis et al., 2015; Van de Peer et al., 2017). For this reason, polyploidy could be an important factor that enhanced weeds genetic variability and adaptive plasticity (Te Beest et al., 2012; Freeling et al., 2015; Ye et al., 2020).

With regard to the evolution of herbicide resistance, genetic variability and ecological adaptability play a key role in weed survival. Therefore, it is useful to conduct studies on genetic diversity and population structure in noxious weed species, in order to prevent herbicide resistance occurrence and to better manage weeds spread, favoring a more sustainable and less impactful agriculture.

7.3.4 Conclusions

The results obtained highlighted that *E. oryzicola* specimens collected from rice fields in the Lombardy region is characterized by high intraspecific genetic diversity: inter-population higher than intra-population variability.

7.4 Conclusions

The results of these experiments showed that both species of *Echinochloa* are characterized by high intraspecific variability. Specifically, greater genetic diversity was recorded in resistant *E. crus-galli* populations than in susceptible ones, while no difference was detected between presumed-resistant and susceptible *E. oryzicola* populations. These results suggest that the relationship between genetic variability and herbicide resistance occurrence in *Echinochloa* species appears to be weak, supporting the findings of Claerhout et al. (2015).

The need to conduct investigations about the variability of *Echinochloa* species derives from the rising spread of the resistance to herbicide phenomena in the species of this genus. Nevertheless, this is expected to be a difficult task due to the limited number of genomic sequences (i.e microsatellites) available for the species of the genus *Echinochloa*. It will be necessary to sequence new microsatellite loci in order to deepen the analysis of *Echinochloa* spp. genetic diversity.

7.5 References

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Chapter 8 – Analysis of herbicide resistance cases in *Echinochloa* species

This chapter describes the analysis of herbicide resistance in *Echinochloa crus-galli* (L.) P. Beauv and *Echinochloa oryzicola* (Vasinger) Vasinger.

The first section of the chapter is devoted to the analysis of target site resistances (TSRs).

The second section is focused on the analysis of non-target site resistances (NTSRs) and of epigenetic mechanisms (miRNAs and DNA methylation) affecting herbicide resistance occurrence and regulation. In particular, findings obtained from the analysis of miRNAs and relative target genes involved in herbicide detoxification against bispyribac-sodium in *E. crus-galli* were the subject of a publication titled *Involvement of miRNAs in Metabolic Herbicide Resistance to Bispyribac-Sodium in Echinochloa crus-galli (L.) P. Beauv.* in Plants Journal (IF 4.658 - MDPI) - Collection *New Trends in Plant Science in Italy* (https://www.mdpi.com/2223-7747/11/23/3359)

The last part of this chapter is devoted to the analysis of the relation between herbicide epigenetic resistances incidence in *Echinochloa* spp. specimens and ecological biotic and abiotic factors characterizing sites in which they were collected.

8.1 Introduction

Herbicide resistance can be linked either to the occurrence of a DNA mutation of genes expressing herbicide target proteins that causes a reduction in the affinity and efficacy of the herbicide for the target itself (Target Site Resistances – TSR), or to metabolic processes of detoxification that decrease the dosage of herbicide reaching their target in the plant (Non Target Site Resistances - NTSR). NTSR represents the most common mechanism that allows plants to overcome chemical control (Yuan et al., 2006). Compared with target-site resistance, non-target-site herbicide resistance poses a greater threat to agriculture due to the involvement of a gene network and the unpredictability of the phenomenon. Indeed, non-target-site resistance mechanisms are myriad because of the underlying apparent genomic plasticity among weeds, especially those with high intraspecific variability, such as *Echinochloa* species (Yuan et al., 2006).

In *Echinochloa* species TSR cases have been widely analyzed towards the most popular ALS, ACCase and EPSPS[15] inhibiting herbicides.

For example, Riar et al. (2012) have analyzed the physiological and molecular mechanisms leading to resistance towards ALS inhibiting herbicides in American *E. crus-galli* specimens. This study showed that mutations occurring in Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654} codons of the ALS gene resulted in cross-resistance against several herbicides. In Italy, Panozzo et al. (2017) have discovered a DNA mutation in the codon 122, which generally encodes alanine (Ala), leading to asparagine (Asn) expression in the ALS gene of *E. crus-galli* specimens. Such a result corroborates Riar et al. (2012) findings. Furthermore, Kaloumenos et al. (2012) discovered a missense mutation in the codon 574 of the ALS gene in Greek *E. oryzicola* specimens associated with cross-resistance

[15] 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase is an enzyme produced by plants and microorganisms which catalyzes the chemical reaction:

phosphoenolpyruvate (PEP) + 3-phospho shikimate (S3P) ⇌ phosphate + 5-enolpyruvylshikimate-3-phosphate (EPSP)

This enzyme is typical of plants and represents a key enzyme in the shikimic acid pathway, which is involved in the synthesis of the aromatic amino acids. EPSPS inhibition leads to depletion of the aromatic amino acids tryptophan, tyrosine, and phenylalanine that are needed for protein synthesis.

to ALS-inhibiting herbicides (i.e. penoxsulam, bispyribac-sodium, imazamox, foramsulfuron, nicosulfuron and rimsulfuron).

TSR to ACCase-inhibiting herbicide are caused by missense mutation occurring in Ile_{1781}, Trp_{1999}, Trp_{2027}, Ile_{2041}, Asp_{2078}, Cys_{2088} and Gly_{2096} codons on the acetyl-CoA-carboxylase gene. TSR to EPSPS-inhibiting herbicide could be linked with a missense mutation in the Pro_{106} codon of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase gene (Yuan et al., 2006). However, it should be considered that each different herbicide molecule binds the respective target enzyme at a specific active site. Hence, the manifestation of resistance to a particular chemical is associated with a missense mutation occurred exactly at the specific substrate's binding site (Figure 8.1). Since most herbicides target specific enzyme, TSR is mostly mono-genic and involves a single mutated target enzyme. It is therefore relatively easy to study the molecular mechanisms of target site resistance. Furthermore, recent technological developments in molecular biology have made it possible to obtain 3-D models of protein structure: this has allowed further study of target site resistance. (Yuan et al., 2006).

In any case, this is an evolving and human-driven phenomenon itself: the creation of new herbicide molecules interacting with their respective substrates at certain active sites will lead to the evolution of new cases of TSR due to novel mutations.

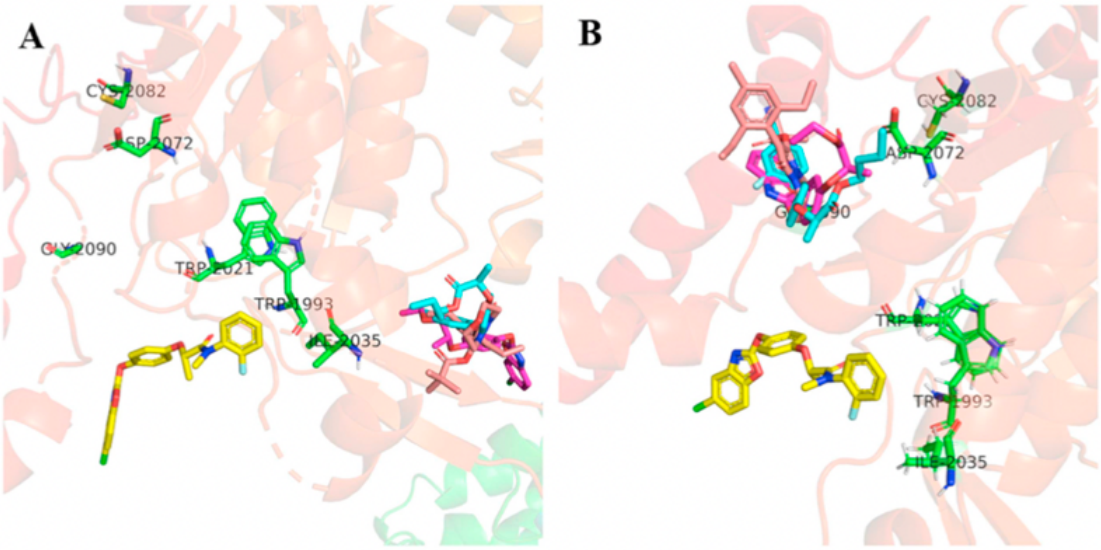

Figure 8.1: Molecular-docking of different ACCase protein (A: Wild type; B: Ile-1781-Leu) with two ACCase inhibitors. Cyhalofop- butyl was colored in blue; fenoxaprop-P-ethyl was colored in pink; metamifop was colored in yellow; pinoxaden was colored in rose gold. Picture taken from Fang et al. (2020) https://doi.org/10.1016/j.pestbp.2020.104634

To analyze the presence of missense mutations leading to TSRs, *restriction fragment length polymorphism* (RFLP) analysis represents a valuable tool because of its reproducibility, reliability and low cost if compared to sequencing. RFLP is a technique that uses the presence of mutations (polymorphisms) in DNA sequences to distinguish individuals. In RFLP analysis, a DNA sample is digested into fragments by one or more endonucleases at restriction sites on the nucleotide sequence. The resulting restriction fragments are separated by gel electrophoresis and recognized according to their size (Saiki et al., 1985). RFLP assay has been largely and is still applied to analyze TSR occurrence (Ng et al., 2003; Kaloumenos et al., 2012; Ghanizadeh et al., 2021).

More complex to study are non-target site resistance (NSTR) phenomena, since they involve a multiplicity of interconnected events. It should be considered that herbicides are chemicals that do not normally provide a natural substrate for the enzymes involved in resistance. Therefore, in response to a constantly changing environment, plants have developed sophisticated detoxification systems for xenobiotics (Shimabukuro et al., 1971).

NTSR is related to a plant detoxification process that follows a four-step scheme, in each of which a number of specific proteins are involved (Sandermann, 2004). Phase I

and II represent the detoxification proper. In phase I, herbicide molecules are activated to expose certain functional groups for phase II enzymes. Oxidation is a typical phase I detoxification reaction. It is generally carried out by cytochrome monooxygenases belonging to the P450 family or by mixed-function oxidases (Schuler et al., 2003). Phase II generally involves the conjugation of a bulky hydrophilic molecule by thiols or sugars (glutathione-S-transferase) to the previously activated herbicide molecule: this allows the final product of phase II detoxification to be recognized by enzymes active in phase III (Bowles et al. 2005). It follows phase III, which involves the active transport of the conjugated molecule into the vacuole or extracellular space. ABC transporters are the most common group of transporters involved in this phase (Klein et al., 2006). Finally, phase IV involves further degradation of the conjugated molecule in the vacuole or extracellular spaces, but is still an undiscovered topic. To sum up, plant proteins involved in herbicide detoxification might be numerous and there is still much to be studied and discovered about them. To date, it is known that mainly cytochromes P450, glutathione-S-transferases and ABC transporters participate in NTSR (Yuan et al., 2006).

Quantitative relative polymerase chain reaction (qRT-PCR) is a useful tool for analyzing the level of gene expression associated with enzyme activity. Relative quantification relates the PCR signal of the unknown target gene in a treatment group to that of another sample belonging to the control (untreated) group (Livak el at. 2001). Although this technique cannot provide absolute quantification, but quantification based on comparison with the control sample, it represents one of the most widely used techniques to analyze the expression of genes involved in the detoxification of herbicides (Ywakami et al, 2013; Dalazen et al., 2018).

Recent studies on species of the genus *Echinochloa* have shown that the expression of genes involved in herbicide detoxification can be regulated by epigenetic mechanisms (Pan et al., 2022). In addition, Pan et al. (2016) showed that some miRNAs are involved in regulation of genes conferring resistance to fenoxaprop-P-ethyl in *Beckmannia syzigachne* (Steud.) Fernald.

Epigenetics studies changes in DNA expression that are not attributable to the presence of mutations in the nucleotide sequence. In fact, DNA and the proteins that interact with it can undergo chemical changes that alter the expression level of genes. Depending on the type, some epigenetic modifications can be passed down through the generations, while others cannot. The totality of all epigenetic modifications in a genome is called the *epigenome* (NIH[16], 2022).

Among the epigenetic mechanisms that act before DNA transcription to mRNA, and therefore heritable, there are histone modifications such as DNA methylation. It is a biological process by which methyl groups are added to the DNA molecule and is triggered by imbalance factors. DNA methylation has two main roles in plants: defending the genome against selfish DNA elements and regulating gene expression (Liu et al., 2015). Moreover, DNA methylation changes the activity of a DNA without changing the sequence: when occurs at a gene promoter, it typically repress mRNAs transcription, hence genes expression (Feng et al., 2010).

In 2022, Pan et al. demonstrated that in Chinese *E. crus-galli* specimens the expression of a cytochrome P450 (CYP81A68) was affected by DNA methylation. It has been highlighted that a lower methylation rate in the promoter region is likely to be related to a higher level of CYP81A68 expression in resistant versus susceptible plants (Pan et al., 2022).

To analyze the level of global DNA methylation, *coupled restriction enzyme digestion - random amplification* (CRED-RA) analysis represent a reliable and low-cost technique. DNA is initially digested by two methyltransferases (HpaII and MspI) that recognize the presence of methyl groups (CH^{3-}) on DNA cytosines and selectively cut the molecule. Next, the digested DNA is amplified by the technique *random amplification of polymorphic DNA* (RAPD). Hence, the analysis of the electrophoretic fingerprints allows the overall DNA methylation level to be obtained (Bolukbasi et al., 2016).

Among the epigenetic mechanisms that are not heritable, because post-transcriptional, there are small-non-coding RNAs (sncRNAs), including microRNAs

[16] National Human Research Genome Institute - https://www.genome.gov/

(miRNAs). MicroRNAs are small, endogenous, non-coding RNAs (sncRNAs), 20–24 nucleotides in length that are conserved across species and kingdoms. They function by pairing with the 3′ UTR of target mRNAs and repressing their translation, or by targeting the mRNA for its degradation: in this way gene expression is repressed. Cleavage of mRNA appears to be the predominant mechanism of miRNA-driven regulation in plants (Mallory et al., 2006).

In a research conducted on resistant lines of *E. colona* in western Australia was hypothesized that, in addition to DNA mutations or indels in TSR- or NTSR- related genes, herbicide resistance might also be influenced by epigenetic processes such as DNA and histone modifications, and various non-coding RNAs, particularly miRNAs (Goh et al., 2018).

To analyze miRNA expression, the qRT-PCR technique could be used, as for gene expression (Pan et al., 2016).

It has been extensively analyzed and it is nowadays well known that epigenetics plays an important role in the adaptation of plants to stressful ecological factors (i.e. cold, heat, dehydration, presence of toxic agents) by activating pathways that regulate adaptive responses that restore the cellular homeostasis of organisms during sudden environmental changes (Wang et al., 2015; Li et al., 2019; Shi et al., 2021). Little is known, however, about how plant adaptation toward the chemical stress represented by herbicides is related to epigenetic responses. However, since herbicides are xenobiotics producing chemical alterations in plants cells, similarly to other molecules (i.e., ROS, high concentrations of heavy metals or reactive substances, etc...), it is likely to be assumed that epigenetic adaptation of weeds toward chemical control is comparable to that of other biotic and abiotic stressors.

Consequently, it would be useful to identify and analyze which ecological stressors are present in the agricultural environment that can trigger adaptive epigenetic responses (i.e., miRNA and methylation) in weeds.

The aims of this study-section are:

1. to distinguish herbicide resistances related to target site or non-target site mechanisms;
2. analyzing epigenetic adaptive mechanisms that regulate herbicide resistance occurrence;
3. analyzing ecological stressors trigging epigenetic adaptive responses that could be related with herbicide resistance occurrence regulation.

8.2 Analysis of Target Site Resistances (TSR) in *Echinochloa* species

8.2.1 Materials & Methods

8.2.1.1 DNA extraction, amplification and digestion

Total DNA was extracted from resistant specimens using the DNeasy plant mini kit (QIAGEN, Hilden, Germany) following manifacturer's instruction and quantified on a Qbit fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Amplification of the ALS (X51514.1), ACCase (AJ310767.1) and EPSPS (AT2G45300) genes was performed in order to obtain DNA sequences including codons where the occurrence of specific mutations is attributable to TSR onset against a particular herbicide (Déyle et al., 2005; Powles et al., 2010; Kaloumenos et al., 2012; Riar et al., 2012; Morran et al, 2018).

Only mutations reported to be related with TSR occurrence in *E. crus-galli* and *E. oryzicola* toward the herbicides molecules administered "in field" were considered. Specifically:

ALS gene → Ala_{122}, Pro_{197} and Trp_{574} codons
ACCase gene → Ile_{1781} and Ile_{2041} codons
EPSPS gene → Pro_{106}

Amplification was carried out on a T-100 (BIO-RAD, Hercules, California, USA) thermal cycler as follows: initial denaturation step at 95°C for 5 min, 35 cycles including denaturation at 95°C for 45 sec, annealing at the specific primer pair temperature for 45 sec and extension at 72°C for 45 sec, followed by final extension at 72°C for 10 min. PCR amplifications were performed in a total volume of 10 µl, containing 2µl of genomic DNA (20 ng), 2.4µl (0.8U) of GoTaq® Hot Start Green Master Mix (Promega, Madison, Wisconsin, USA), 0.6 µl of each primer (1 µM), 0.5µl of MgCl2 (2mM) and 2 µl of sterile nuclease free water (Promega). Primer pairs sequences are listed in Table 8.1.

Table 8.1: Sequences of the primers for amplification of ALS, ACCase and EPSPS genes in *Echinochloa* spp.

Gene	*Sequence 5′ – 3′*	*Annealing T*	*Reference*
ALS-forward*	CTGGYGCYKCTGTGGCYAAC	59°C	Kaloumenos et al., 2012
ALS-reverse*	CWGGRGTBTCRAGCATCTTC		
ACCase-forward*	CAGCYTGATTCCCAYGAGCGYTC	61°C	Déyle et al., 2005
ACCase-reverse*	CCATGCAYTCTTYGAGYTCCTCTGA		
EPSPS-forward	AAGGACGCCAAAGAGGAAGT	60°C	Morran et al., 2018
EPSPS-reverse	ATCCCCTTGACACGAACAGG		
*degenerate primer *N: all nucleotides; R: guanine / adenine (purine); Y: cytosine / thymine (pyrimidine); B: guanine / thymine / cytosine; W: adenine / thymine*			

Then, ALS, ACCase and EPSPS amplicons were respectively digested with a specific endonuclease at a particular restriction site. This assay was performed in relation to the herbicide administered in field, since each different herbicide chemical family binds the respective substrate (enzyme) in a specific position of its aminoacidic sequence.

In Table 8.2 are listed, for ALS, ACCase and EPSPS genes, the codons where the occurrence of a mutation is related with herbicide resistance in *Echinochloa* species, together with the respective restriction site sequence and endonuclease.

Table 8.2: list of codons in ALS, ACCase and EPSPS genes where the occurrence of mutations is attributable to TSR onset in *Echinochloa* spp. For each codon, restriction site sequence and respective endonuclease is provided.

Gene	**Codon**	**Restriction site (5′ – 3′)**	**Endonuclease**
ALS	Ala_{122}	GG*CGCC	NarI
	Pro_{197}	ACTGGN*	Bse1I
	Trp_{574}	CAGTG*G	BtsI
ACCase	Ile_{1781}	R*AATTY	XapI
	Ile_{2041}	G*AATTC	EcoRI
EPSPS	Pro_{106}	NGG*CCN	HaeIII
N: all nucleotides; R: guanine / adenine (purine); Y: cytosine / thymine (pyrimidine)			

The restriction site digestion reaction was performed in a total volume of 15 µl containing 1 µl of TAE buffer, 1µl (12 U) of enzyme, 10 µl of nuclease free water (Promega) and 3 µl of the PCR product (200 ng ~). Digestion was performed in a dry bath (Labnet, part of Aurogene srl, Italy) at 37°C for 24 hours. RFLPs were finally checked by electrophoresis on 2% agarose gel in 1 x TAE buffer stained with ethidium bromide. PCR products were then visualized under UV light in Molecular Imager® Gel DocTM XR + (BIO-RAD). Amplicon size was determined using 100 bp DNA Ladder (Promega). RFLP profiles allows to discriminate wild type (susceptible) biotypes from mutated (resistant) biotypes.

8.2.2 Results & Discussion

In Table 8.3 are listed SU where resistant *Echinochloa* specimens were collected with each specific herbicide molecule applied.

Table 8.3: List of collection sites of resistant *Echinochloa* spp. specimens accounted with the administered herbicide and its HRAC identification code.

MUNICIPALITY	FARM	HERBICIDE ADMINISTERED	HRAC
Ottobiano (PV)	Baino (InnovaTech)	Clethodim (ACCase)	A
Sannazzaro de' Burgondi (PV)	Bellone (InnovaTech)	Bispyribac-sodium + Biopower (ALS) / Clethodim (ACCase)	AB
Suardi (PV)	Magnani (InnovaTech)	Imazamox + DASH HC (ALS) / Profoxydim + DASH HC (ACCase)	AB
Zeme (PV)	Braggio (InnovaTech)	Clethodim (ACCase)	A
Robbio (PV)	Greppi (InnovaTech)	Bispyribac-sodium + Biopower (ALS)	B
Vigevano (PV)	Marchesani (InnovaTech)	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Rivoltella (PV)	Costanzo (InnovaTech)	Imazamox + DASH HC (ALS)	B
Vigevano – Barbavara (PV)	Rabellotti (InnovaTech)	Penoxsulam (ALS)	B
Ozzero (MI)	Tacconi (InnovaTech)	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Robbio Daghetta (PV)	Daghetta Giovanni	Imazamox (ALS)	B
Bascapè (PV)	Bazzini Pietro	Profoxydim + DASH HC (ACCase)	A
Valle Lomellina (PV)	Gatti Massimo	Imazamox (ALS)	B
Frascarolo-Sartirana (PV)	Rossignano Paolo	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Zerbolò (PV)	Sala Pietro	Imazamox / Profoxydim / Glyphosate (ALS / ACCase / EPSPS)	ABG
Zerbolò (PV)	Sala Pietro	Imazamox / Profoxydim / Glyphosate (ALS / ACCase / EPSPS)	ABG
Belgioioso - St. Margherita (PV)	Foletti Angelo	Imazamox / Profoxydim / Glyphosate (ALS / ACCase / EPSPS)	ABG
Cozzo (PV)	Cascina Buscaiolo	Imazamox / Profoxydim (ALS / ACCase)	AB
Cozzo (PV)	Cascina Buscaiolo	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Cozzo (PV)	Cascina Buscaiolo	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Pieve del Cairo (PV)	Maccagno	Imazamox (ALS)	B
Genzone (PV)	Manzi	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Roncaro (PV)	Gestioni Agricole	Imazamox (ALS)	B
Zinasco Vecchia (PV)	Il Chicco	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Pieve Albignola (PV)	Az. Ag. Genagricola	Cyhalofop-butyl (ACCase)	A
Santa Croce - S.Martino Siccomario (PV)	Carnevale Baraglia Francesco	Imazamox (ALS)	B
Mortara (PV)	Ferraris	Imazamox (ALS)	B
Lomello (PV)	Pistone (InnovaTech)	Cyhalofop-butyl (ACCase)	A
San Giorgio di Lomellina (PV)	Gilardi (InnovaTech)	Cyhalofop-butyl (ACCase)	A
Dorno (PV)	Bellone	Cyhalofop-butyl (ACCase)	A
Garlasco (PV)	Spina	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Giussago (PV)	Collivasone (InnovaTech)	Cyhalofop-butyl / Profoxydim + DASH HC (ACCase)	A
Carpiano (MI)	Cascina Pojago	Imazamox (ALS)	B
Carbonara al Ticino (PV)	Cascina Cavallera	Profoxydim + DASH HC (ACCase)	A

In relation to the ALS-inhibiting herbicides administered, the presence of mutations in the ALS gene conferring TSR to herbicide belonging to the chemical families of triazolopyrimidine (penoxulam), imidazolinone (imazamox) and pyrimidinyl(thio)benzoate (bispyribac-sodium) was surveyed through selective digestion with endonuclease listed in Table 8.2. Furthermore, the presence of mutations in the ACCase gene, which confer TSR towards ACCase-inhibiting herbicides of the chemical families of aryloxyphenoxy-propionate (cyhalofop-butyl) and cyclohexanedione (chletodim and

profoxydim), was surveyed through selective digestion with endonucleases listed in Table 8.2. Finally, the presence of Pro-Ser mutation at the Pro106 codon of the EPSPS gene conferring TSR against glyphosate was assessed with selective digestion with HaeIII endonuclease.

In Figure 8.2 are shown examples of RFLP profiles obtained from the analysis of TSR in *Echinochloa* spp. specimens. White arrows indicate the presence of mutations occurring at the ALS, ACCase and EPSPS genes, conferring resistance towards a particular herbicide chemical family.

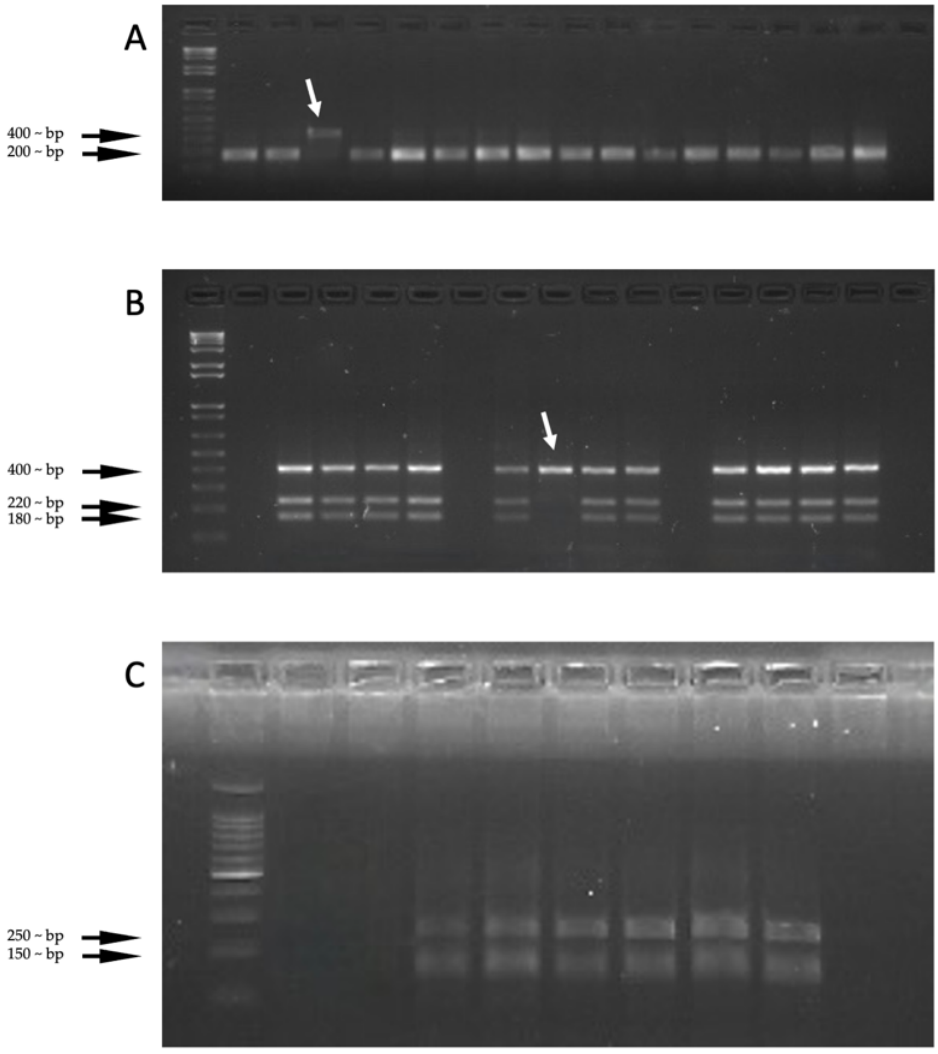

Figure 8.2: RFLP profiles of TSR analysis obtained on 2% agarose gel. A: Digestion of ALS gene in Trp_{574} codon with BtsI endonuclease. B: Digestion of ACCase gene in Ile_{2041} codon with EcoRI endonuclease. C: Digestion of EPSPS gene in Pro_{106} codon with HaeIII endonuclease.

In particular, the white arrow in figure 8.2 A shows the RFLP pattern of one resistant *E. oryzicola* specimen collected at the farm of Zerbolò (PV) where is present a mutation $Trp_{574}Leu$ in the ALS gene. In this case, the enzymatic digestion by BstI occurred only in wild type specimens. In figure 8.2 B is shown the RFLP pattern of a mutation occurred at codon $Ile_{2041}Asn$ in the gene ACCase in a resistant *E. crus-galli* specimen collected at the farm of Zinasco Vecchia (PV). In figure 8.2 C is shown the RFLP pattern of wild type resistant specimens of *E. crus-galli* in which there is no mutation at $Pro_{106}Leu/Thr$ codon in the EPSPS gene. Out of 160 *Echinochloa* spp. specimens, only 1 individual of *E. oryzicola* and 1 of *E. crus-galli* possess a mutation which could be related with TSR against ALS and ACCase-inhibiting herbicides respectively.

Interestingly, in fifteen samples digestion of codon Trp_{574} with BtsI produced RFLP profiles attributable to heterozygous individuals. Such *E. crus-galli* and *E. oryzicola* specimens were collected in Zerbolò (PV), Vigevano (PV), Rivoltella (PV) and Belgioioso (PV) (Figure 8.3).

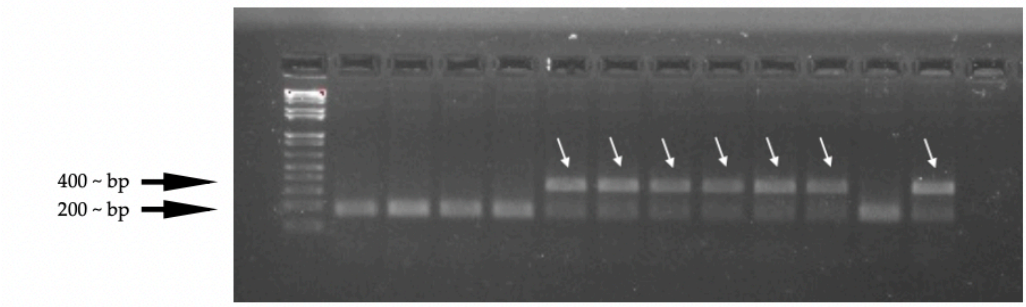

Figure 8.3: RFLP pattern of heterozigous *Echinochloa* spp. specimens in Trp_{574} codon of the ALS gene. It could be noticed the presence of a 400 ~ bp undigested fragment and of a 200 ~ bp digested fragment in heterozygous specimens (white arrows).

In those cases, probably due to the karyotype of *Echinochloa* species (*E. crus-galli* – allo-hexaploid; *E. oryzicola* – allo-tetraploid), the co-presence of a mutated allele and a wild type allele (double band presence – see white arrows) could induce a higher tolerance[17]

[17] http://gire.mlib.cnr.it/index.php?sel=descrizione - *Tolerance* is the innate ability of a species to survive and reproduce after herbicide treatment. This implies that there was no artificial selection that made the species tolerant, but it is in a natural way.

towards herbicide. In any case, since this represents a diagnostic analysis, further investigations in this regard will need to be carried out.

To sum up, in 17 specimens up to 128 were identified mutations attributable to TSR occurrence, about 13% of the whole samples collection.

The evolution of TSR resistance phenomena in weeds has already been extensively investigated. Acetohydroxyacid synthase (AHAS), also called acetolactate synthase (ALS), is the primary enzyme involved in the biosynthesis of branched-chain amino acids (valine, leucine, and isoleucine). ALS catalyzes the formation of aceto-hydroxybutyrate and acetolactate, It is the target site of a large number of herbicide families (sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine, pyrimidinyl-thiobenzoate and sulfonyl-aminocarbonyl-triazolinone). These herbicides block the synthesis of branched-chain amino acids, resulting in plant death. These herbicides have low mammalian toxicity and are selective in the world's major crops. These favorable qualities have ensured their global and intensive use in many different crops and over large areas. As a matter of facts, the most of herbicides useful to control *Echinochloa* species are ALS-inhibitors (Powles et al., 2010). It was understood that AHAS herbicide-resistant plants could have a mutant AHAS enzyme that was not recognized by the herbicide molecule and thus conferred resistance. Among the most observed mutations there are Pro_{197} and Trp_{574}. In Pro_{197}, substitution with Ser is a particularly common mutation, while the Trp_{574}Leu mutation is known to confer a broad spectrum of resistance (Powles et al., 2010).

Acetyl-CoA carboxylase A is a key enzyme in lipid biosynthesis. It catalyzes the formation of malonyl-CoA from the carboxylation of acetyl-CoA. ACCase herbicides were introduced in 1978 and have been widely used for weed control. In response to the widespread and intensive use of ACCase herbicides, many weeds have developed resistance phenomena, often due to mutations. Among mutations occurring in the codons of the ACCase gene, Leu_{1781}Ile is the most common in many weeds. In addition, the Ile_{2041}Asn

and Asp2078Gly mutations are known to confer high-level resistance to many ACCase herbicides (Powles et al., 2010).

Glyphosate is the most widely used herbicide in the world since it controls a broad spectrum of annual and perennial weeds. Glyphosate is a specific and potent inhibitor of the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPSP). Inhibition of EPSPS activity by glyphosate disrupts the shikimate pathway and inhibits the production of aromatic amino acids, causing plant death. Glyphosate TSR was first identified in a mutated biotype of *Eleusine indica*, where a proline substitution to serine at codon 106 of a highly conserved region of the EPSPS gene had been documented (Powles et al., 2010).

As a matter of facts, it is increasingly recognized that the level and spectrum of TSR is either determined by the particular mutation or by the plants' homozygosity/heterozygosity for the mutation or, most importantly, by the herbicide chemical and dosage applied. However, it is incorrect to simply assume that all resistance mutations at the target site confer high-level resistance. The reality is that, on a case-by-case basis, particular target-site mutations confer high-level or rather low-level of resistance. It is therefore important to consider the herbicide dose for the evaluation of TSR phenomena. Indeed, it should be taken into account that the presence of heterozygous resistant specimens could evolve into homozygous resistant populations due to the fixation of the mutation that confers TSR toward a particular herbicide in subsequent generations, thus leading to rapid spread of the phenomenon. Furthermore, all detoxification mechanisms that occur in plant cells after herbicide administration should not be neglected (Powles et al., 2010).

8.3 Analysis of epigenetic mechanisms (miRNA and DNA methylation) involved in herbicide resistance in *Echinochloa* species

The analysis of mutations at codons of ALS, ACCase and EPSPS genes allowed to exclude specimens survived towards chemical control due to TSR. Only wild-type *Echinochloa* spp. specimens were considered for the analysis of NTSR and epigenetic mechanisms.

Controlled growth and herbicide application tests were performed in growth chamber at Agricola 2000 ScpA, in order to maintain controlled experimental conditions ad to exclude any biotic and abiotic factor of stress that could affect epigenetic adaptive responses in tested plants, with the exception of the herbicide stress only. Not treated specimens were used as "control/susceptible samples".

Seeds of *E. crus-galli* and *E. oryzicola* were sown in separate 100 mL pots containing a universal organic compound (Vigorplant Italia Srl, Fombio, Italy). Plants were maintained in a growth chamber with a mean temperature of 20 °C and 70% relative humidity and a photoperiod of 14/10 h (day/night). At the three-leaf germination stage, the same herbicide molecule applied in field was sprayed at the label dose on plants. Chemical treatment was applied to the entire plant collection using a Honda WJR 2525 ET® backpack sprayer (Honda Motor Co., Ltd., Minato, Tokyo, Japan) with a spray pressure of 4 bar and speed of 43 m/s, resulting in a spray volume of ~ 300/400 l/ha. Three biological replicates were used for each treatment.

Plant tissue was collected before (BT) and after treatment (AT), within 21 days from herbicide administration. Leaves were stored at −40 °C.

Herbicide sensitivity was tested 21 days after treatment, following European and Mediterranean Plant Protection Organization (EPPO) standards (EPPO, 2011). Hence, susceptible (S) and resistant (R) biotypes were identified.

In Table 8.4 are summarized tests of herbicide administration, with seeds collection sites, treatment applied and tested sensitivity.

Table 8.4: Summary of herbicide administration tests, with seeds collection sites, treatment applied and tested sensitivity.

Species	Seeds collection site	Treatment	Label dose sensitivity (EPPO)	Biotype ID
E. crus-galli	Weeded paddy	Bispyribac-sodium + Biopower (ALS)	ALS **Resistant**	*E. crus-galli - R*
E. crus-galli	Not treated parcel	Bispyribac-sodium + Biopower (ALS)	ALS **Susceptible**	*E. crus-galli - S*
E. oryzicola	Weeded paddy	Imazamox + DASH HC (ALS)	ALS **Resistant**	*E. oryzicola - R*
E. oryzicola	Organic paddy	Imazamox + DASH HC (ALS)	ALS **Susceptible**	*E. oryzicola - S*
E. crus-galli	Weeded paddy	Chletodim (ACCase)	ACCase **Resistant**	*E. crus-galli - R*
E. crus-galli	Not treated parcel	Chletodim (ACCase)	ACCase **Susceptible**	*E. crus-galli - S*
E. oryzicola	Weeded paddy	Profoxydim + DASH HC (ACCase)	ACCase **Resistant**	*E. oryzicola - R*
E. oryzicola	Organic paddy	Profoxydim + DASH HC (ACCase)	ACCase **Susceptible**	*E. oryzicola - S*

8.3.1 Involvement of miRNAs in Metabolic Herbicide Resistance to Bispyribac-Sodium in Echinochloa crus-galli (L.) P. Beauv.

The results obtained from this part of the research work were published in the following article:

Involvement of miRNAs in Metabolic Herbicide Resistance to Bispyribac-Sodium in *Echinochloa crus-galli* (L.) P. Beauv. (https://www.mdpi.com/2223-7747/11/23/3359)

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Abstract: Several mechanisms involved in weed herbicide resistance are unknown, particularly those acting at the epigenetic level, such as the capacity of small-non-coding RNAs (sncRNAs) to target messenger RNAs of genes involved in herbicide detoxification. The transcription of these sncRNAs is stimulated by epigenetic factors, thereby affecting gene expression. This study was carried out in order to evaluate, for the first time in *Echinochloa crus-galli* (L.) P. Beauv. (barnyardgrass), the capacity of miRNAs to regulate the expression of genes associated with bispyribac-sodium detoxification. The expression profiles of eight miRNAs with a high degree of complementarity (≥80%) with mRNAs of genes involved in herbicide detoxification (CYP450, GST and eIF4B) were determined by qRT-PCR before and after herbicide spraying. Five of the miRNAs studied (gra-miR7487c, gma-miR396f, gra- miR8759, osa-miR395f, ath-miR847) showed an increased expression after herbicide application in both susceptible and resistant biotypes. All the miRNAs, except gra-miR8759, were more highly expressed in the herbicide-resistant biotypes. In specimens with increased expression of miRNAs, we observed reduced expression of the target genes. The remaining three miRNAs (ata-miR166c-5p, ath-miR396b-5p and osa-miR5538) showed no over-expression after herbicide treatment, and no difference in expression was recorded between susceptible and resistant biotypes. Our results represent a first overview of the capacity of miRNAs to regulate the expression of genes involved in bispyribac-sodium detoxification in the genus *Echinochloa*. Further research is required to identify novel miRNAs and target genes to develop more focused and sustainable strategies of weed control.

Keywords: herbicide resistance; bispyribac-sodium (ALS-inhibitor); *Echinochloa crus-galli* (L.) P. Beauv.; epigenetic regulation; miRNAs; Cytochrome P450; glutathione-S-transferase; eIF4B

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1. Introduction

Herbicide resistance (HeR) is a major threat to worldwide agricultural systems. HeR is an example of the adaptive evolution of weeds in response to human selective pressures, resulting in the evolution of global resistance to a wide range of herbicides in many weed species [1,2]. Generally, weed resistance to herbicides

consists of two main mechanisms: target site resistance (TSR) and non-target site resistance (NTSR). TSR involves DNA mutation of genes expressing herbicide target proteins, causing a reduction in affinity and efficacy of the herbicide for its target site. NTSR mechanisms involve metabolic processes of detoxification that are able to decrease the amount of herbicide that can reach target organelles in the plant (i.e., vacuolar sequestration or enzymatic degradation of herbicide molecules) [1–7]. Chemical control in the form of herbicides has so far represented the most effective tool for managing weeds.

As a result of strict European regulations on the use of plant protection products (Reg EC/1107/2009), the repeated use of an increasingly narrow range of herbicides targeting the same metabolic pathways has led to the evolution of herbicide-resistant populations [1–12].

Furthermore, artificial selection of agronomic traits in rice (*Oryza sativa* L.) has unintentionally promoted the evolution of crop-like weed biotypes. As a result, the weeds can evade chemical control and eradication from fields, allowing them to spread throughout the agroecosystem (*Vavilovian mimicry*) [13,14].

Echinochloa species are the most prevalent weeds infesting crop cultivations and paddy fields globally due to their wide ecological success and ability to mimic the crop. Among them, *Echinochloa crus-galli* (L.) P. Beauv. (barnyardgrass) is one of the most problematic and widespread species in agriculture. It is listed as a major weed species in Italian rice fields and has developed resistance to several classes of herbicides [15–17].

E. crus-galli is an allo-hexaploid (2n = 6x = 54) annual plant characterized by high genetic variability and intraspecific polymorphism, making its morphological identification difficult. It produces a large number of seeds, is highly competitive, has a large adaptive capacity and is resistant to several herbicide classes, all features that can lead to a reduction in agricultural productivity and make it difficult to control [18–26]. Worldwide losses in rice yield due to *E. crus-galli* are estimated to be around 35% of the total crop [15,16,27]. Hence, understanding the mechanisms concerning the adaptability and occurrence of herbicide resistance in this weed is essential for establishing adequate, effective and sustainable weed management strategies.

Although both TSR and NTSR have been widely studied, NTSR mechanisms are more complex to explain and investigate [28–33]. Previous works on *E. crus-galli* have mainly focused on the mechanisms underlying NTSR, but not on the epigenetic processes that regulate the genes involved in herbicide detoxification [28–31]. These latter mechanisms have been poorly studied in this species [15].

A recent study conducted on resistant lines of *Echinochloa colona* (L.) Link in Western Australia suggested there are unknown mechanisms of herbicide resistance [34]. It has been hypothesized that, in addition to DNA mutations or indels in TSR- or NTSR- related genes, herbicide resistance might also be influenced by epigenetic processes such as DNA and histone modifications, and various non-coding RNAs, particularly microRNAs (miRNAs) [32,33].

MicroRNAs are small, endogenous, non-coding RNAs (sncRNAs), 20–24 nucleotides in length, that have been shown to regulate post-transcription gene expression. They function by pairing with the 3′ UTR of target mRNAs and repressing translation, or by targeting the mRNA for degradation [35–42]. If it is partially complementary to the miRNA, the mRNA is targeted for translational inhibition. In this way, an individual miRNA can post-transcriptionally affect the expression of hundreds of mRNAs, profoundly altering both qualitative and quantitative gene expression [42]. Cleavage of mRNA appears to be the predominant mechanism of miRNA-driven regulation in plants. Furthermore, miRNAs are conserved across species and kingdoms. For example, it has been reported that plants and animals share miRNAs of the miR854 family, indicating a common origin as regulators of transcriptional mechanisms. Trans-kingdom miRNA conservation has also been highlighted between fungal miRNA-like RNAs (milRNAs) and plant and animal miRNAs, which show many similarities [43–45]. These small RNAs play an important regulatory role in various biological processes of plants. Their spectrum of action is extremely wide and most miRNAs do not function independently but are involved in overlapping regulatory networks. They act as epigenetic regulators to control gene expression of key enzymes involved in multiple plant metabolic pathways. For example, by regulating proteins critical for development and growth, including those involved in xenobiotic detoxification, they negatively regulate the target mRNA at the post-transcriptional level without modifying the gene sequences [35–45]. It is known that miRNAs also play a crucial role in regulating plant adaptive responses to biotic and abiotic stresses and help restore cell homeostasis upon sudden environmental changes. Biotic or abiotic stresses cause plants to over- or under-express miRNAs or to synthesize new miRNAs, which in turn control the expression of genes involved in various stress response pathways [46–50].

NTSR due to herbicide detoxification represents the most common mechanism that allows plants to overcome chemical control. Cytochrome P450 monooxygenases (also called CYP or P450) and glutathione S-transferases (GST) represent the main enzymes acting in these processes. The family of Cytochromes P450 encodes heme-dependent enzymes that normally catalyze oxygen and NADPH-dependent monooxygenation reactions. The P450 family includes multiple genes which facilitate the denaturation of a wide range of chemicals. The GST gene family includes multifunctional enzymes that catalyze the conjugation of glutathione into various substrates to form a polar S-glutathionylated (R-SG) product. The R-X substrates that are conjugated are often hydrophobic and electrophilic toxic chemicals, including herbicides. The diversity of the GST gene family allows them to detoxify a wide range of chemicals and to play a role in the synthesis of several secondary metabolites. The involvement of both gene families in response to herbicide application and resistance has been widely documented. The expression and regulation of these genes play fundamental roles in herbicide resistance [5,7,51].

Nevertheless, the regulation of enzymes involved in herbicide detoxification by miRNAs remains unclear and is an under-researched area in *E. crus-galli*. To the best of our knowledge, there have been no studies on the regulatory mechanisms of herbicide resistance mediated by miRNAs, although the role of miRNAs in regulating plant responses to abiotic and biotic stresses is well understood [46–50]. The only study which has analyzed the regulation of genes involved in resistance to fenoxaprop-P-ethyl by miRNAs was conducted by Pan et al. (2016) on *Beckmannia syzigachne* (Steud.) Fernald [52].

This research was carried out as a part of the EpiResistenze research project (https://epiresistenze.unipv.it/, accessed on 6 November 2022) aimed at analyzing the epigenetic mechanisms involved in herbicide resistance in the genus *Echinochloa* P. Beauv., in order to support more effective weed prevention and control programs. In this study, a set of eight genes (cytochrome P450 monooxygenase, glutathione-S-transferase and eIF4B translation initiation factor), which have previously been found to be involved in the herbicide detoxification network in many plant species, was selected to be analyzed. Some of these genes have already been described in *E. crus-galli* (CYP81A68, GSTF1, EcGST and eIF4B), while others have been described in *Echinochloa phyllopogon* (Stapf) Stapf ex Kossenko (CYP72A122, CYP72A254, CYP71AK2 and CYP81A22) [3,4,30,53,54]. The eight miRNAs used in this study were identified *in silico* by bioinformatic analysis, based on a high degree of complementarity with the mRNA sequences of the genes considered. The expression profile of miRNAs and their gene target was assessed in herbicide-susceptible and -resistant barnyardgrass biotypes before and after herbicide administration by means of quantitative RT-PCR (qRT-PCR).

The purpose of this research was to assess the role of miRNAs in the regulation of the expression of genes involved in bispyribac-sodium detoxification in *E. crus-galli* from rice fields in the Lombardy region of northern Italy. Furthermore, we evaluated if transcription of miRNAs is triggered by herbicide administration.

2. Results

The sensitivity and resistance of plants to bispyribac-sodium were tested three weeks after herbicide treatment, and resistant (R) and susceptible (S) biotypes were identified.

Figure 1 shows an example of a susceptible and a resistant barnyardgrass biotype three weeks after treatment with bispyribac-sodium.

Figure 1. *Echinochloa crus-galli* susceptible and resistant biotypes observed three weeks after treatment with bispyribac-sodium. Wilting of the susceptible plant leaves is clearly visible.

The expression levels of eight genes known to be involved in herbicide detoxification and of eight miRNAs selected for their ability to target the same genes were analyzed. The miRNAs were first subjected to extensive bioinformatic analysis using the psRNATarget: A Plant Small RNA Target Analysis Server [55,56]. This tool allowed us to determine the degree of complementarity between the miRNA and the mRNA of putative target genes: miRNAs with a proportion of nucleotide correspondence ≥80% were selected for this study.
The miRNAs selected have not previously been tested in *E. crus-galli*.
Table 1 lists the miRNAs and genes considered.

Table 1. miRNAs analyzed in *Echinochloa crus-galli* and their target genes.

miRNAs		**Target Genes**	
Name	**a.n. (miRbase)**	**Name**	**a.n. (NCBI)**
ata-miR166c-5p	MIMAT0037248	CYP72A122	AB734013.1
ath-miR396b-5p	MIMAT0000945	CYP81A22	AB872310.1
osa-miR395f	MIMAT0000974	CYP81A68	OK483200.1
ath-miR847	MIMAT0004278	CYP71AK2	AB733990.1
gra-miR7486c	MIMAT0034235	CYP72A254	AB755796.1
gma-miR396f	MIMAT0021069	GSTF1	HF548530.1
osa-miR5538	MIMAT0022174	EcGST1	JX518596
gra-miR8759	MIMAT0034189	eIF4B1	AB720070.1

ata—Aegilops tauschii Coss.*; ath—Arabidopsis* thaliana (L.) Heynh.*; gma—Glycine max* (L.) Merr.*; gra—Gossypium raimondii* Ulbr.*; osa—Oryza sativa* L.*; a.n.*—accession number.

The expression analysis of both miRNAs and their target genes was assessed by quantitative REAL-TIME PCR (qRT-PCR) (see Section 4) [57]. Results are reported in Figure 2. The expression levels of miRNAs and their target genes are compared before (BT) and after (AT) bispyribac-sodium application in susceptible (S) and resistant (R) specimens of *E. crus-galli*.

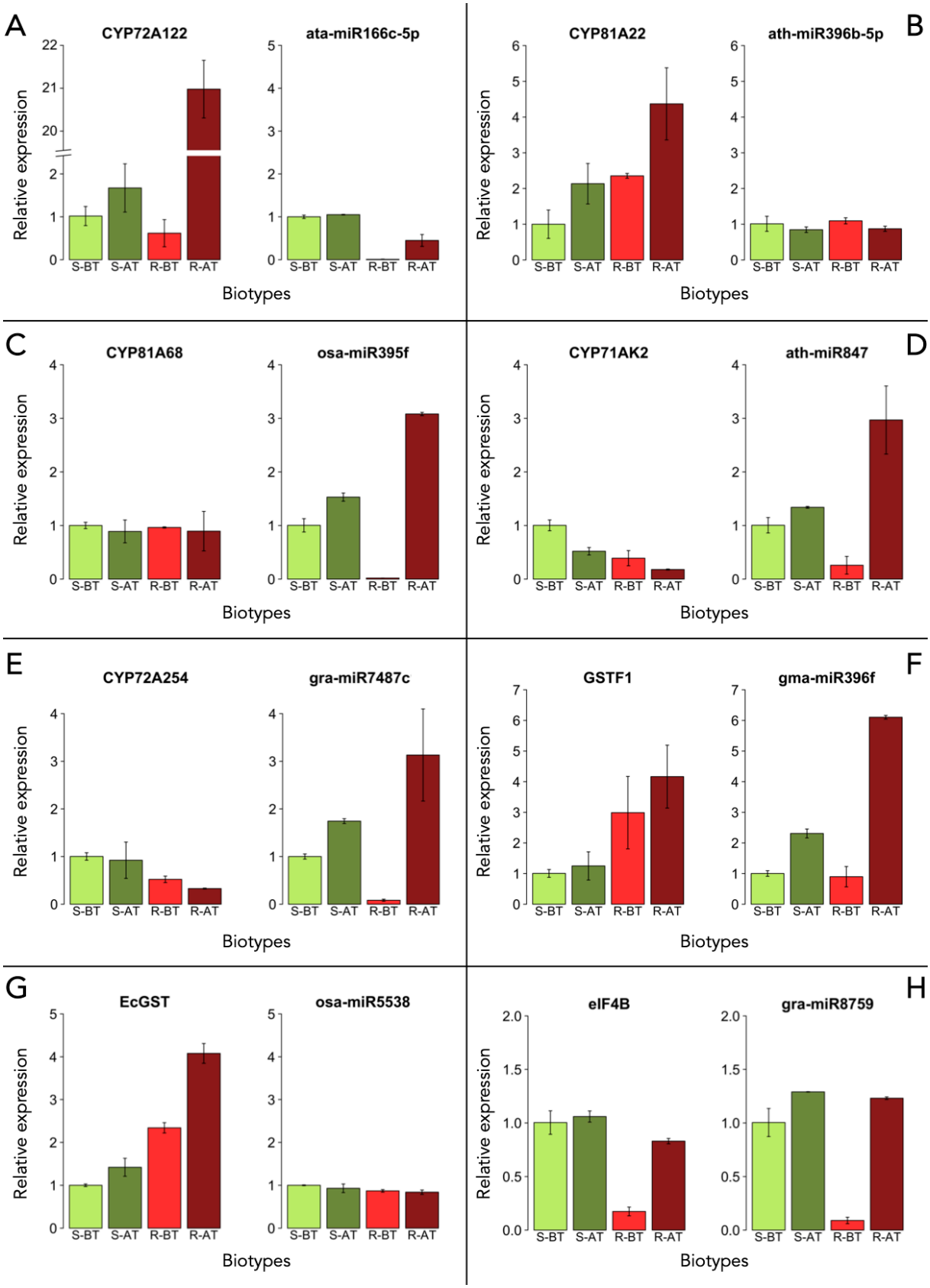

Figure 2. Expression levels of the miRNAs studied and the mRNA of their target genes in susceptible (S) and resistant (R) specimens of *Echinochloa crus-galli* before (BT) and after (AT) bispyribac-sodium application. (**A**) CYP72A122 and ata-miR166c-5p; (**B**) CYP81A22 and ath-miR396b-5p; (**C**) CYP81A68 and osa-miR395f; (**D**) CYP71AK2 and ath-miR847; (**E**) CYP72A254 and gra-miR7487c; (**F**) GSTF1 and gma-miR396f; (**G**) EcGST and osa-miR5538; (**H**) eIF4B and gra-miR8759. Error bars indicate the standard deviation of three replicates.

In Figure 2A, the values of expression of the CYP72A122 gene and ata-miR166C-5p are reported. The expression of CYP72A122 increased after treatment with bispyribac-sodium in both susceptible and resistant biotypes. The highest expression value was recorded in resistant biotypes, with a value 20 times higher than the untreated susceptible specimens (1.01 ± 0.22 vs. 20.97 ± 1.67; $p < 0.05$). The expression of ata-miR166C-5p showed a slight increase in resistant biotypes after treatment (0.01 ± 0.006 vs. 0.45 ± 0.13; $p < 0.05$); however, it was found to be under-expressed when compared to the susceptible biotypes.

Figure 2B shows the values of expression of the CYP81A22 gene and of ath-miR396b-5p. In resistant biotypes, CYP81A22 expression was significantly higher ($p < 0.05$) than in the sensitive biotypes both before and after treatment, as expected due to the detoxifying role of the protein. Moreover, we observed that bispyribac-sodium application was able to trigger the expression of CYP81A22. In both susceptible and resistant biotypes, CYP81A22 expression doubled after herbicide administration (from 1.00 ± 0.39 to 2.13 ± 0.56; from 2.35 ± 0.06 to 4.37 ± 1.00; $p < 0.05$). No difference in the expression of ath-miR396b-5p was recorded either before or after bispyribac-sodium application for both susceptible and resistant biotypes.

Figure 2C highlights that the CYP81A68 gene had similar expression values in both susceptible and resistant biotypes before and after treatment. osa-miR395f expression appeared to be induced by bispyribac-sodium in susceptible specimens (S-AT) with an increase of around 50% (from 1.00 ± 0.12 to 1.53 ± 0.07; $p < 0.05$). In resistant biotypes before treatment (R-BT), the miRNA expression was negligible (0.02 ± 0.001), but after herbicide application (R-AT), expression significantly increased (3.08 ± 0.03) compared to the reference sample (S-BT) ($p < 0.05$).

Figure 2D shows that CYP71AK2 expression appeared to be affected by bispyribac-sodium application, as observed by a reduction in the expression values of around half in both the susceptible and resistant biotypes ($p < 0.05$). The transcription of ath-miR847 appeared to be stimulated by herbicide application, with a slight increase in the susceptible biotypes (from 1.00 ± 0.14 to 1.34 ± 0.01) and a larger increase in the resistant biotypes (from 0.25 ± 0.16 to 2.96 ± 0.63; $p < 0.05$).

The same trend was observed in the CYP72A254 gene and gra-miR7487c, as shown in Figure 2E. For susceptible and resistant biotypes, higher gene expression values were recorded before treatment and there was a further reduction after herbicide application. For gra-miR7487c expression, herbicide treatment stimulated the expression of this miRNA, with a significant increase in the resistant biotypes (from 0.08 ± 0.02 to 3.13 ± 0.96; $p < 0.05$), tripling in expression when compared to the susceptible specimens before treatment (S-BT).

In Figure 2F, GSTF1 was over-expressed by three times in resistant biotypes compared to susceptible biotypes before bispyribac-sodium application, and over-expressed by four times in resistant biotypes after herbicide spraying ($p < 0.05$). The expression of gma-miR396f significantly increased only after treatment in both susceptible and resistant biotypes, with the highest increase observed in resistant biotypes, from 0.89 ± 0.33 to 6.10 ± 0.05 ($p < 0.05$).

Figure 2G shows that EcGST expression was higher in resistant biotypes before bispyribac-sodium administration (R-BT) in comparison to susceptible biotypes. The application of herbicide induced a further stimulation of EcGST expression in both susceptible and resistant biotypes. A significant increase was observed in the resistant biotype (from 2.34 ± 0.12 to 4.08 ± 0.23; $p < 0.05$), about four times higher compared to the reference untreated specimen (S-BT). The expression of osa-miR5538 was the same across all specimens before and after herbicide application.

In Figure 2H, the expression of the translation initiation factor eIF4B was significantly lower in the resistant biotypes compared to the susceptible biotypes before bispyribac-sodium administration ($p < 0.05$). After treatment, there was no significant increase in eIF4B expression in the susceptible biotypes (S-AT). In contrast, in the resistant biotypes, expression increased fourfold upon treatment (from 0.17 ± 0.04 to 0.83 ± 0.02); however, this expression value was lower than that observed in the reference untreated susceptible biotypes (S-BT). Before herbicide application, the expression of gra-miR8759 was around eight times lower in resistant (R-BT) compared to susceptible (S-BT) biotypes. Herbicide treatment induced an increase in the expression of gra-miR8759 in both biotypes, with comparable values (1.29 ± 0.002 in S-AT and 1.23 ± 0.01 in R-AT).

3. Discussion

In this study, we investigated the role of miRNAs in the regulation of genes involved in the detoxification of bispyribac-sodium (Nominee®). The expression of a set of miRNAs (ata-miR166c-5p, ath-miR396b-5p, osa-miR395f, ath-miR847, gra-miR7486c, gma-miR396f, osa-miR5538 and gra-miR8759), targeting five different cytochrome P450 genes (CYP72A122, CYP81A22, CYP81A68, CYP71AK2 and CYP72A254), two glutathione-S-transferase genes (GSTF1 and EcGST) and the eIF4B translation initiation factor, was evaluated in susceptible and resistant *E. crus-galli* biotypes from rice fields in the Lombardy region of northern Italy. These genes have previously been found to be involved in herbicide resistance [3,4,15,30,54]. The set of miRNAs was selected based on their ability to pair with mRNA sequences of the above-mentioned genes, following a bioinformatic analysis using the psRNATarget: A Plant Small RNA Target Analysis Server [55,56]. This study was conducted as part of a research project (*EpiResistenze*) which aimed to investigate the role of epigenetic mechanisms in the occurrence and regulation of herbicide resistance.

To date, studies have primarily analyzed the role of DNA mutations (TSR) or genes that detoxify herbicides (NTSR) in resistant weeds [9,10,28–31,34,51,54], but the role of epigenetic factors is still poorly understood.

Pan et al. (2022) demonstrated that different expression values of CYP81A68 in resistant and susceptible *E. crus-galli* Chinese plants are related to different levels of methylation in the promoter region of the gene [15].

Limited information is available on the role of miRNAs in the adaptation of weeds against chemical control and in the occurrence of herbicide resistance. Pan et al. (2016) analyzed differential regulation of some miRNAs in fenoxaprop-P-ethyl-resistant *B. syzigachne*, highlighting the regulatory role of bsy-miR160a-5p, bsy-miR164a, bsy-miR408-3p, novel-bsy-miR-12, novel-bsy-miR-15, novel-bsy-miR-19 and novel-bsy-miR-29 on stress response genes related to NTSR [52].

The only study on barnyardgrass which considered miRNAs was carried out by Fang et al. (2015), who found an increase in the expression of some miRNAs in response to phenolic acids produced by *O. sativa* (rice allelopathy) [58].

To our knowledge, this study is the first focusing on the role of miRNAs in the regulation of genes involved in herbicide resistance in the genus *Echinochloa*.

The miRNAs we considered in this study have previously been identified in other plant species as playing a role in hybridization [59], growth regulation [60–63] and in response to various biotic and abiotic stresses including nutrient deficiency, drought, cold and salinity [64–70]. Moreover, miRNAs of the miR396 family are known to target oxidases, including cytochromes involved in xenobiotic detoxification [71].

Through bioinformatic analysis using the psRNATarget tool, the ability to couple mRNAs of the selected target genes and to down-regulate their expression was verified *in silico* (see Table 1).

From our results, five of the miRNAs analyzed (osa-miR395f, ath-miR847, gra-miR7487c, gma-miR396f and gra-miR8759) showed an increased expression after herbicide treatment in both susceptible and resistant biotypes. Except gra-miR8759, these miRNAs recorded the highest degree of expression in the resistant biotypes, and we observed a reduced expression of the corresponding target proteins. The remaining three miRNAs (ata-miR166c-5p, ath-miR396b-5p and osa-miR5538) showed no over-expression after herbicide treatment and no differences in expression between susceptible and resistant biotypes.

These results indicate that the miRNAs analyzed in this study play a role in the regulation of plant response to bispyribac-sodium treatment. Herbicide application triggered the transcription of miRNAs which down-regulated the expression of target genes, reducing their detoxification ability. In instances when herbicide spraying did not stimulate the transcription of miRNAs, the target gene mRNAs could be translated into proteins, leading to herbicide detoxification.

These findings suggest there is involvement of the selected miRNAs in the occurrence of Nominee® resistance/susceptibility in *E. crus-galli* from Italian rice paddy fields. Figure 3 summarizes how miRNAs regulate the adaptive response of plants to bispyribac-sodium.

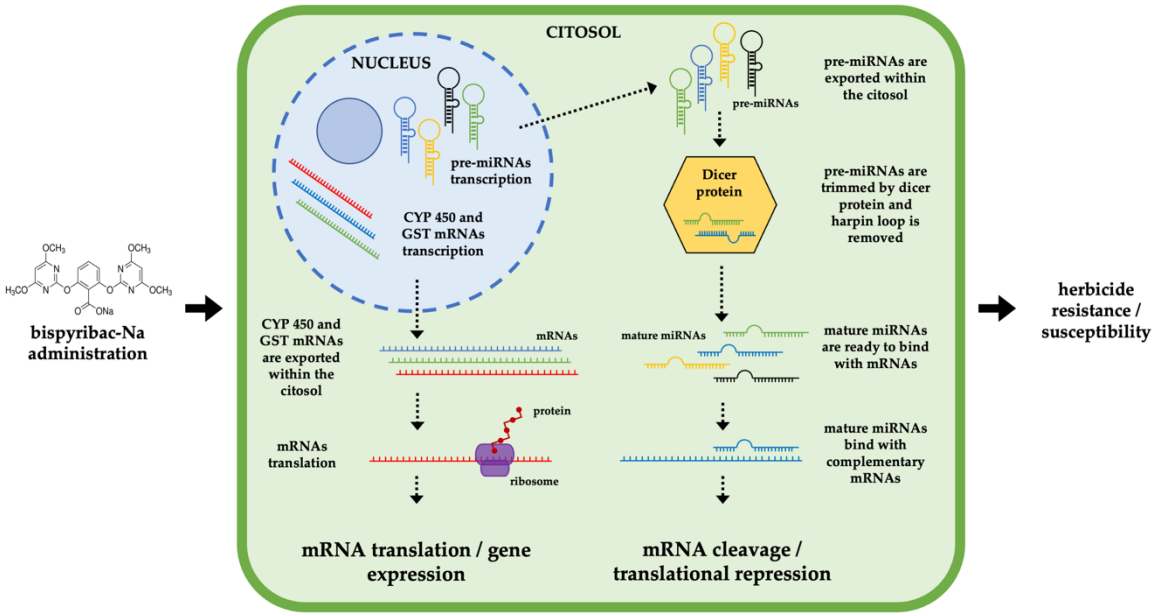

Figure 3. The role of miRNAs in the adaptive response of plants to bispyribac-sodium.

The results of this work are of great interest as they represent the first report on the regulation of herbicide resistance by miRNAs in the genus *Echinochloa*. They also verify the expression of miRNAs in *E. crus-galli*, as has been previously described in other species. This latter observation corresponds with recent reports that these molecules are conserved not only within a species but also across kingdoms, influencing gene expression even in phylogenetically unrelated organisms [72–76].

The topic of regulatory networks in miRNAs has been scarcely investigated. Considering that epigenetic mechanisms are triggered by various abiotic and biotic ecological factors, plant response to stresses (including herbicides) may involve complex physiological pathways where environmental signals stimulate many responses, some of which can affect the expression of genes involved in herbicide metabolism.

It is important to underline that translational down-regulation due to miRNAs occurs at the post-transcriptional level and represents a further step in the control of gene expression that can lead to a reduction in, or lack of, the gene product. The assessment of the entire miRNAome of *E. crus-galli* is critical to better understand the regulation of proteins involved in herbicide resistance and the interaction with ecological factors in this noxious weed. Moreover, it would be useful to identify the ecological factors able to regulate miRNAs in the agricultural environment. A thorough understanding of miRNA regulation of detoxifying genes, taking into account epigenetic–environment interactions, will help to optimize precision weed management (PWM) technologies. More targeted and sustainable strategies for controlling herbicide resistance could help to reduce chemical inputs, improving food health and protecting the environment.

4. Materials and Methods

4.1. Plant Materials, Growth Conditions and Herbicide Treatment

Seeds were collected from adult plants of *E. crus-galli* in experimental parcels from paddy fields in the Lombardy region of northern Italy. Resistant (R) biotypes that had survived chemical control were identified in a parcel in which bispyribac-sodium (Nominee®) had been applied. Susceptible (S) biotypes were identified from a non-treated parcel and were therefore used as the reference susceptible line.

Seeds from each biotype were sown in separate 100 mL pots containing a universal organic compound (Vigorplant Italia Srl, Fombio, Italy). Plants were maintained in a growth chamber with a mean temperature of 20 °C and 70% relative humidity and a photoperiod of 14/10 h (day/night). Three biological replicates were used for each treatment.

Bispyribac-sodium (Nominee®) was sprayed at the label dose of 60–75 mL/ha with the addition of adjuvant (Biopower® 1 L/ha). Chemical treatment was applied to the entire plant collection at the three-leaf stage using a Honda WJR 2525 ET® backpack sprayer (Honda Motor Co., Ltd., Minato, Tokyo, Japan) with a spray pressure of 4 bar and speed of 43 m/s, resulting in a spray volume of 300/400~L/ha.
The sensitivity to bispyribac-sodium herbicide was tested three weeks after treatment following European and Mediterranean Plant Protection Organization (EPPO) standards (EPPO, 2011) [77]. Plant tissue was collected before treatment at the three-leaf stage from susceptible (S-BT) and resistant (R-BT) biotypes. After herbicide administration, tissues were re-collected from susceptible (S-AT) and resistant (R-AT) biotypes. Leaves were stored at −40 °C until RNA extraction.
The presence of mutations in the acetolactate synthetase gene known to be involved in Target Site Resistances (TSRs) against bispyribac-sodium was tested through selective amplification and digestion of the ALS gene in order to select only wild-type barnyardgrass plants [28].

4.2. RNA Extraction

Total RNA was extracted from leaf tissues of susceptible (S) and resistant (R) biotypes before and after herbicide spraying using the RNeasy Plant Kit (QIAGEN SpA, Hilden, Germany) according to the manufacturer's instructions.

4.3. Candidate NTSR Gene Selection and Candidate miRNA Prediction

The metabolic genes to be tested were selected on the basis of previously published studies and by using a specific scoring database (Table 2). The expressions of cytochrome P450 monooxygenase (CYP81A68, CYP71AK2, CYP72A122, CYP72A254, CYP81A22) and gluthatione-S-transferase (EcGST, GSTF1) genes were analyzed. In addition, the expression of the eIF4B gene was assessed. This gene is present in eukaryotic organisms and is involved in the detoxification of the xenobiotics pathway [30].

Table 2. Nucleotide sequences of the primers used for qRT-PCR analysis of CYP450, GST and eIF4B genes' expression in *Echinochloa crus-galli*.

Gene ID	NCBI a.n.	Primer Sequence (5′-3′)	Reference
CYP71AK2	AB733990.1	F: acgtgtgggacaagttcctg R: ggctttgatgcgatcgtctg	Iwakami et al. 2013 [3]
CYP72A254	AB755796.1	F: ttacgaggtactccggctgt R: gtcagggtcgtggtgaatgt	Iwakami et al. 2013 [3]
CYP72A122	AB734013.1	F: agttcaagccggagaggttc R: catcttggcttcaagcagcg	Iwakami et al. 2013 [3]
CYP81A68	OK483200.1	F: gactattcaacccgggcgat R: caagttctgcacggcaagag	Pan et al. 2022 [15]
CYP81A22	AB872310.1	F: cggcgcgctggtccagtt R: tgacatgagcagttccatcg	Iwakami et al. 2014 [4]
EcGST1	JX518596	F: gccgaggaggacctgaagaac R: gtgactcacagataggcttaccgt	Li et al. 2013 [54]
GSTF1	HF548530.1	F: tgcctcttcaaccccatgat R: aggtactcgtgctgggagag	Dalazen et al. 2018 [30]
eIF4B1	AB720070.1	F: cgagcagcttacaagggact R: gtggttccataccaccacga	Dalazen et al. 2018 [30]
b-actin	HQ395760.1	F: gtgctgttccagccatcgttcat R: ctccttgctcatacggtcagcaata	Li et al. 2013 [54]

The primer sequences of candidate non-target site resistance (NTSR) genes were designed using the program Primer BLAST (accessed on 8 May 2022 - *https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi*) from the most conserved region of each sequence obtained in GenBank (accessed on 4 May 2022 - http://www.ncbi.nlm.nih.gov/genbank). For each gene, primer pairs were designed based on an annealing temperature of around 60 °C, with a size of approximately 20 bp and an expected amplification fragment of 100–150 bp (Table 2).

A set of miRNAs able to target the genes selected for the study were searched on the basis of complementarity scoring and secondary structure analysis between the sequence of mRNA target genes and the sequence of putative miRNA, using the psRNATarget: A Plant Small RNA Target Analysis Server tool (accessed on 1 June 2022 - https://www.zhaolab.org/psRNATarget/home) [55,56]. miRNAs with complementarity ≥80% were chosen for the analyses.
The expression of genes and miRNAs was assessed through quantitative real-time PCR (qRT-PCR) [57].
In Tables 2 and 3, the genes and miRNAs, together with respective primer sequences, are listed.

Table 3. Sequences of mature miRNAs selected for the expression analysis in *Echinochloa crus-galli*.

Name	miRbase a.n.	miRNA Sequence (5′-3′)	Reference
ata-miR166c-5p	MIMAT0037248	ggaacguuggcuggcucgagg	Jia et al. 2013 [59]
ath-miR396b	MIMAT0000945	uuccacagcuuucuugaacuu	John-Rohades et al. 2004 [66]
ath-miR847	MIMAT0004278	ucacuccucuucuucuugaug	Rajagopalan et al. 2006 [67]
gma-miR396f	MIMAT0021069	agcuuucuugaacuucuuaugccua	Radwan et al. 2011 [68]
gra-miR7486c	MIMAT0034235	uuuguccacgugaacagaaaacgc	Xue et al. 2013 [62]
gra-miR8759	MIMAT0034189	ugguggaaguauugugcccgg	Xue et al. 2013 [62]
osa-miR395f	MIMAT0000974	gugaauuguuuguggggaacuc	John-Rohades et al. 2004 [66]
osa-miR5538	MIMAT0022174	acugaacucaaucacuugcugc	Wei et al. 2011 [69]
U6 snRNA	NR141593.1	cttcggggacatccgataaaattg	Salanoubat et al. 2000 [70]

ata—Aegilops tauschii Coss., ath—Arabidopsis thaliana (L.) Heynh, gma—Glycine max (L.) Merr., gra—Gossypium raimondii Ullbr., osa—Oryza sativa L., a.n.—accession number

4.4. cDNA Synthesis and qRT-PCR Analysis

cDNA was obtained following a reverse transcription reaction with the miRCURY LNA RT Kit (QIAGEN SpA). The reaction mixture contained 10 µL of RNA template (5 ng/µL), 4 µL of 5 × miRCURY RT reaction buffer, 2 µL of 10 × miRCURY RT enzyme mix and 10 µL of nuclease-free H_2O. The reverse transcription reaction was performed at 37 °C for 60 min, followed by 95 °C for 10 min.
The expression profiles of cytochromes P450, gluthatione-S-transferase and eIF4B genes were determined using qRT-PCR. The amplification was carried out using the SYBR Green® kit (Takara Holdings Inc., Shimogyō-ku, Kyoto, Japan), with the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, US) on 96-well plates (PCR-96M2-HS-C®, Axygen Scientific – part of Thermo Fisher Scientific, Waltham, MA, US). For each sample, amplification was carried out in a total volume of 10 µL containing 2 µL of DNA sample (~5 ng/µL) and 8 µL of master mix composed of 5 µL of TB Green Prmix Ex Taq (Tli RNaseH Plus, Takara Holdings Inc., Shimogyō-ku, Kyoto, Japan), 0.5 µL (10 µM) of forward and reverse primers, 0.5 µL of ROX Reference Dye and 1.5 µL of nuclease-free H_2O. The amplification reactions were run in a three-step program with an initial incubation at 95 °C for 30 s, followed by 40 cycles of amplification (95 °C for 5 s, 60 °C for 30 s). A dissociation cycle was carried out at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s then increasing the temperature stepwise by 0.3 °C.
The expression analysis of miRNAs was carried out in a total volume of 10 µL containing 2 µL of 1:80 diluted cDNA sample and 8 µL of master mix composed of 5 µL of TB Green Prmix Ex Taq (Tli RNaseH Plus) (Takara), 1 µL of miRCURY LNA miRNA (QIAGEN SpA) and 2 µL of nuclease-free water. The amplification reactions were run in a three-step program including melting curve analysis with an initial incubation at 95 °C for 30 s, followed by 40 amplification cycles (95 °C for 5 s, 60 °C for 31 s). A dissociation cycle was carried out at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, then increasing the temperature stepwise by 0.3 °C.
The threshold values (Ct) were determined by the 7300 Real-Time PCR System on-board software. The comparative Ct method ($2^{-\Delta\Delta Ct}$ method) was used to calculate the expression levels of candidate genes and of miRNAs [78]. Each sample were tested in triplicate.
The gene-specific primers and miRNAs used for real-time PCR are listed in Tables 2 and 3.
The determination of relative expression was performed considering b-actin housekeeping gene (HQ395760.1) as an internal reference for protein expression and U6 small nuclear RNA (AT3G14735.1) as an internal reference for miRNAs.

4.5. Statistical Analysis

The calculation of relative expression levels was carried out using the ΔΔCt method [78].

The relative expression was calculated by the ΔCt method using Equation (1):

$$\Delta\Delta Ct = (Ct_{target} - Ct_{reference}) - (Ct_{calibrator} - Ct_{reference}), \quad (1)$$

where susceptible biotypes (EcgS) were considered as a calibrator [78].

The expression levels of genes and miRNAs calculated for susceptible (S) and resistant (R) barnyardgrass biotypes were presented as means and standard deviations calculated from three replicates.

The relative expression values (fold change) and standard deviations of candidate genes and miRNAs were graphed as bar plots in R 3.6.3 software [79].

Significant differences in expression levels of candidate metabolic genes and relative miRNAs before and after treatment were analyzed using a t-test in R 3.6.3 software [79].

4. Conclusions

Our findings highlight the post-transcriptional regulation of cytochromes P450, glutathione-S-transferase and eIF4B genes by miRNAs triggered by bispyribac-sodium application in *E. crus-galli* Italian biotypes. When the miRNA is over-expressed, it exhibits a negative regulatory function towards the gene target, inducing herbicide susceptibility. Otherwise, the under-expression of the miRNA leads to the occurrence of resistance due to herbicide detoxification. Increased expression after herbicide administration in susceptible and resistant biotypes was recorded for five of the miRNAs studied (gra-miR7487c, gma-miR396f, gra-miR8759, osa-miR395f, ath-miR847). These miRNAs, with the exception of gra-miR8759, were more highly expressed in the herbicide-resistant biotypes. There was no over-expression after herbicide treatment and no differences in expression between susceptible and resistant biotypes for the remaining three miRNAs (ata-miR166c-5p, ath-miR396b-5p and osa-miR5538). In the specimens with high expression values of miRNAs, reduced expression of the target genes was observed.

MicroRNAs previously described in other plant species were selected on the basis of having a high complementarity with target mRNAs of proteins known to be involved in bispyribac-sodium detoxification and previously untested in *E. crus-galli.* The results obtained here represent a preliminary step to better understand the role of epigenetic regulation driven by miRNAs in herbicide resistance. Further analysis will be necessary to expand the known number of miRNAs involved in these metabolic pathways. Despite growing evidence of a central regulatory role by miRNAs in gene expression, these small molecules and their functions are still poorly understood. A deeper knowledge of the plant miRNAome could be useful to understand how the resistance/susceptibility of weeds to chemical control is influenced by the complex network in which genes and miRNAs synergistically act.

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8.3.2 Involvement of miRNAs in Metabolic Herbicide Resistance to Chletodim in Echinochloa crus-galli (L.) P. Beauv.

8.3.2.1 Materials & Methods

The experiment was conducted with reference to the same experimental design used in the analysis of bispyribac-sodium resistance.

Resistances (TSRs) against Chletodim was tested through selective amplification and digestion of the ACCase gene in order to select only wild-type barnyardgrass plants (Riar et al, 2013).

8.3.2.2 Results & Discussion

In Figure 8.4 are reported the values of expression recorded for the analyzed NTSR genes and relative miRNAs in resistant and susceptible *E. crus-galli* biotypes before and after Chletodim (ACCase) administration.

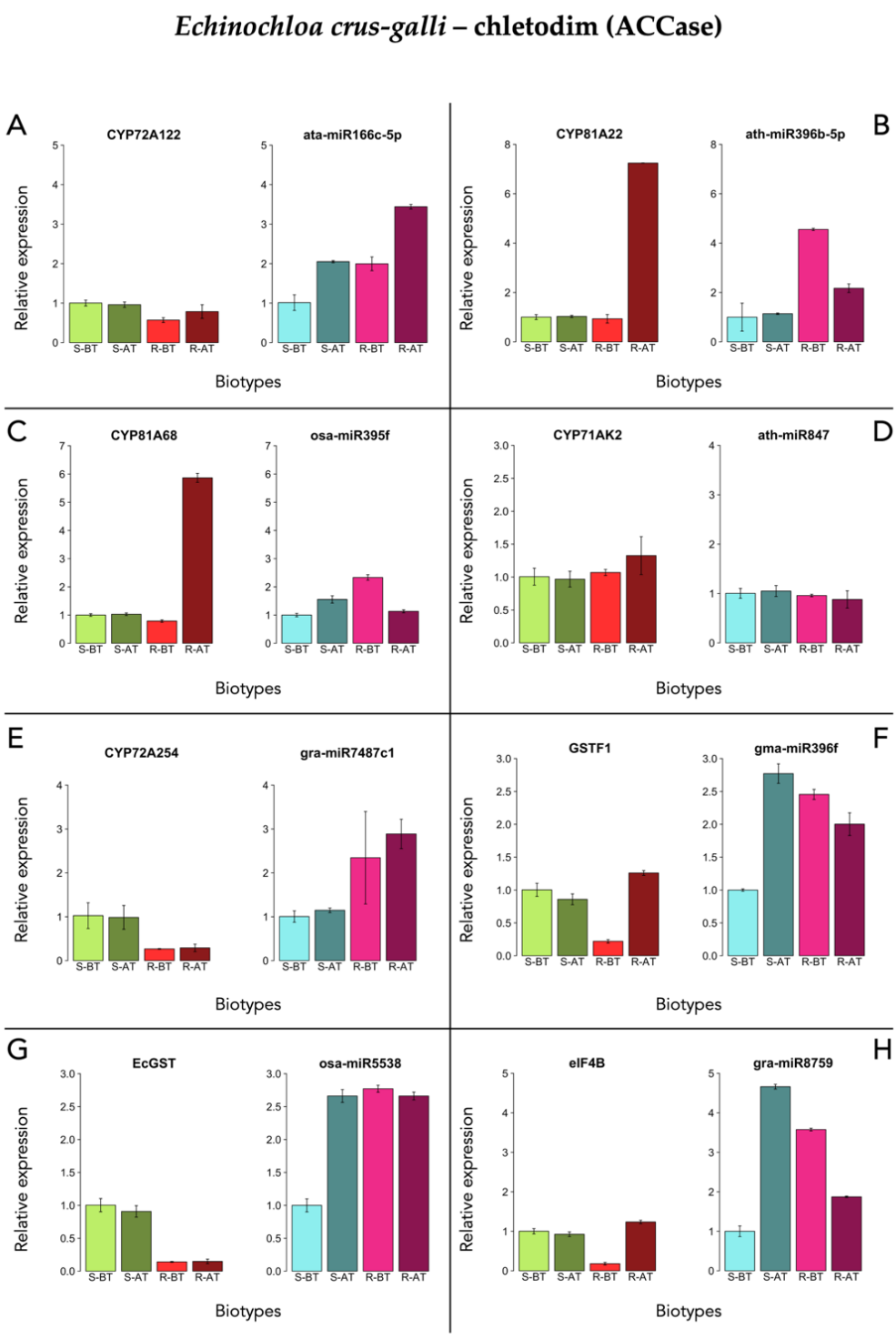

Figure 8.4: Expression levels of the miRNAs studied and the mRNA of their target genes in susceptible (S) and resistant (R) specimens of *Echinochloa crus-galli* before (BT) and after (AT) chletodim application. (A) CYP72A122 and ata-miR166c-5p; (B) CYP81A22 and ath-miR396b-5p; (C) CYP81A68 and osa-miR395f; (D) CYP71AK2 and ath-miR847; (E) CYP72A254 and gra-miR7487c; (F) GSTF1 and gma-miR396f; (G) EcGST and osa-miR5538; (H) eIF4B and gra-miR8759. Error bars indicate the standard deviation of three replicates.

High expression values were recorded for CYP81A22 and CYP81A68 genes in resistant biotypes after Chletodim administration ($p < 0.05$). In parallel, the expression of related miRNAs ath-miR396b-5p and osa-miR395f were recorded declining after herbicide treatment in resistant biotypes (Figure 7B and 7C). Chletodim seemed not to trigger these miRNAs expression, allowing the two cytochromes of CYP81A sub-family to express and act in the herbicide detoxification.

Otherwise, CYP72A122, CYP72A254 and EcGST genes recorded an expression declining after herbicide spraying in resistant biotypes, while respective miRNAs ata-miR166c-5p, gra-miR7487c and osa-miR5538 raised up, triggered by herbicide application ($p < 0.05$) (Figure 7A, 7E and 7G). GSTF1 and eIF4B genes recorded a low increase after herbicide administration in resistant biotypes, however resulting in an under-expression due to the repressive action of related miRNAs gra-miR7487c and gra-miR8759 triggered by Chletodim application (Figure 7F and 7H).

In general, under-expression of the eight analyzed genes involved in herbicide detoxification was recorded in susceptible biotypes both before and after Chletodim administration, while over-expression of the two cytochromes P450 (CYP81A22 and CYP81A68) was recorded in resistant biotypes after weeding.

8.3.3 Involvement of miRNAs in Metabolic Herbicide Resistance to Imazamox in Echinochloa oryzicola (Vasinger) Vasinger.

8.3.3.1 Materials & Methods

The experiment was conducted with reference to the same experimental design used in the analysis of bispyribac-sodium resistance.

Resistances (TSRs) against Imazamox was tested through selective amplification and digestion of the ALS gene in order to select only wild-type late- watergrass plants (Riar et al, 2013).

8.3.3.2 Results & Discussion

In Figure 8.5 are reported the values of expression recorded for the analyzed NTSR genes and relative miRNAs in resistant and susceptible *E. oryzicola* biotypes before and after Imazamox (ALS) administration.

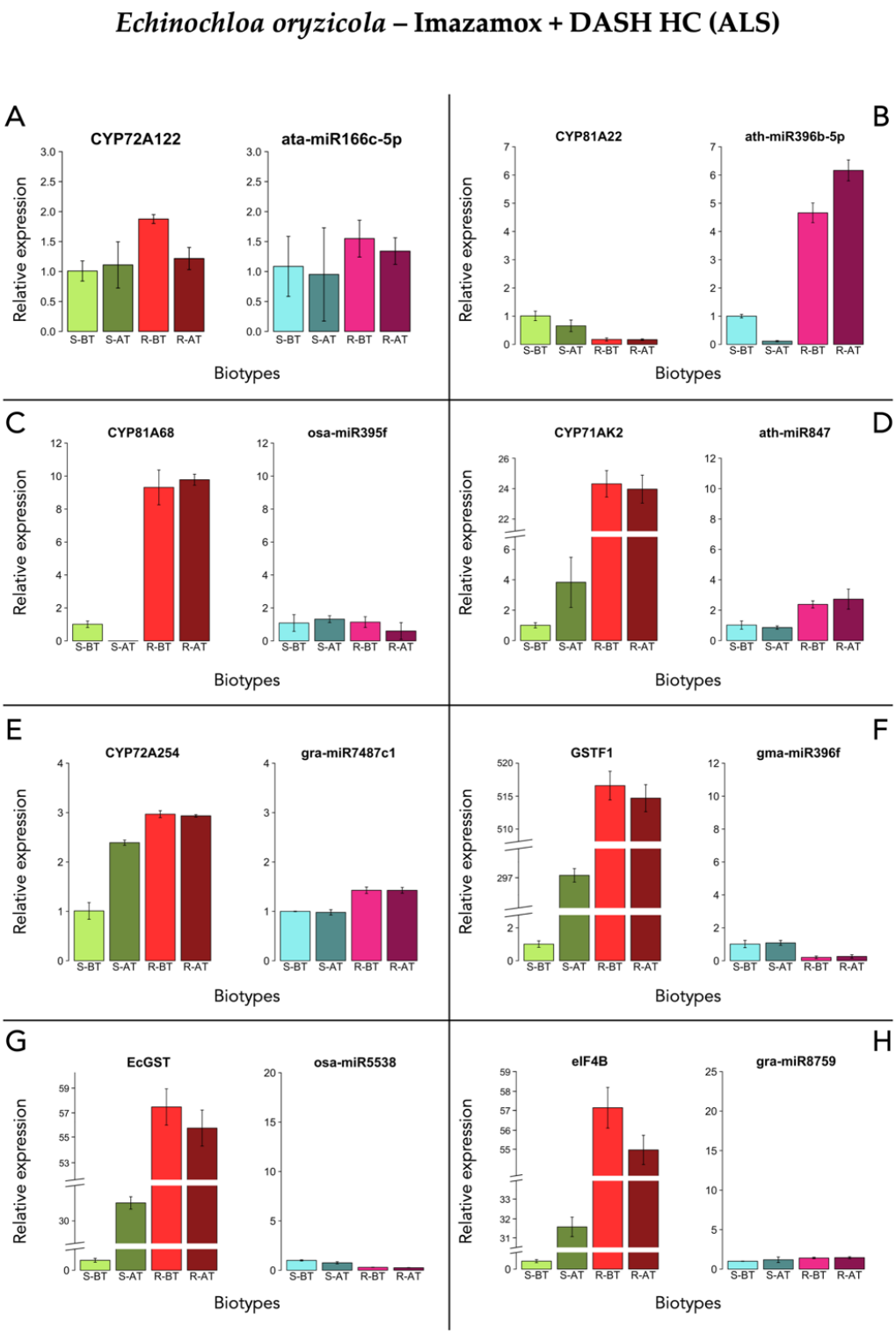

Figure 8.5: Expression levels of the miRNAs studied and the mRNA of their target genes in susceptible (S) and resistant (R) specimens of *Echinochloa oryzicola* before (BT) and after (AT) Imazamox application. (A) CYP72A122 and ata-miR166c-5p; (B) CYP81A22 and ath-miR396b-5p; (C) CYP81A68 and osa-miR395f; (D) CYP71AK2 and ath-miR847; (E) CYP72A254 and gra-miR7487c; (F) GSTF1 and gma-miR396f; (G) EcGST and osa-miR5538; (H) eIF4B and gra-miR8759. Error bars indicate the standard deviation of three replicates.

High expression values were recorded for CYP81A68, CYP71AK2, CYP72A254, GSTF1, EcGST and eIF4B genes after Imazamox administration, especially in resistant biotypes ($p < 0.05$). In parallel, low expression levels were recorded for related miRNAs osa-miR395f, ath-miR847, gra-miR7487c, gma-miR396f, osa-miR5538 and gra-miR8759 after herbicide treatment in both susceptible and resistant biotypes (Figure 8C, 8D, 8E, 8F, 8G and 8H). CYP81A22 was under expressed in resistant biotypes while ath-miR396b-5p overexpressed either before or after treatment ($p < 0.05$). This high expression and increase upon Imazamox treatment of ath-miR396b-5p in resistant biotypes repress the translation of mRNAs encoding CYP81A22 (Figure 8B). The expression of GSTF1, EcGST and eIF4B genes recorded in susceptible biotypes after Imazamox administration was generally high. However, they resulted significantly less expressed if compared with those of resistant biotypes upon herbicide treatment ($p < 0.05$). To sum up, high levels of expression of genes involved in detoxification against Imazamox were recorded in resistant individuals, especially after treatment.

8.3.4 Involvement of miRNAs in Metabolic Herbicide Resistance to Profoxydim in Echinochloa oryzicola (Vasinger) Vasinger.

8.3.4.1 Materials & Methods

The experiment was conducted with reference to the same experimental design used in the analysis of bispyribac-sodium resistance.

Resistances (TSRs) against Profoxydim was tested through selective amplification and digestion of the ACCase gene in order to select only wild-type late- watergrass plants (Riar et al, 2013).

8.3.4.2 Results & Discussion

In Figure 8.6 are reported the values of expression recorded for the analyzed NTSR genes and relative miRNAs in susceptible and resistant *E. oryzicola* biotypes before and after Profoxydim (ACCase) administration.

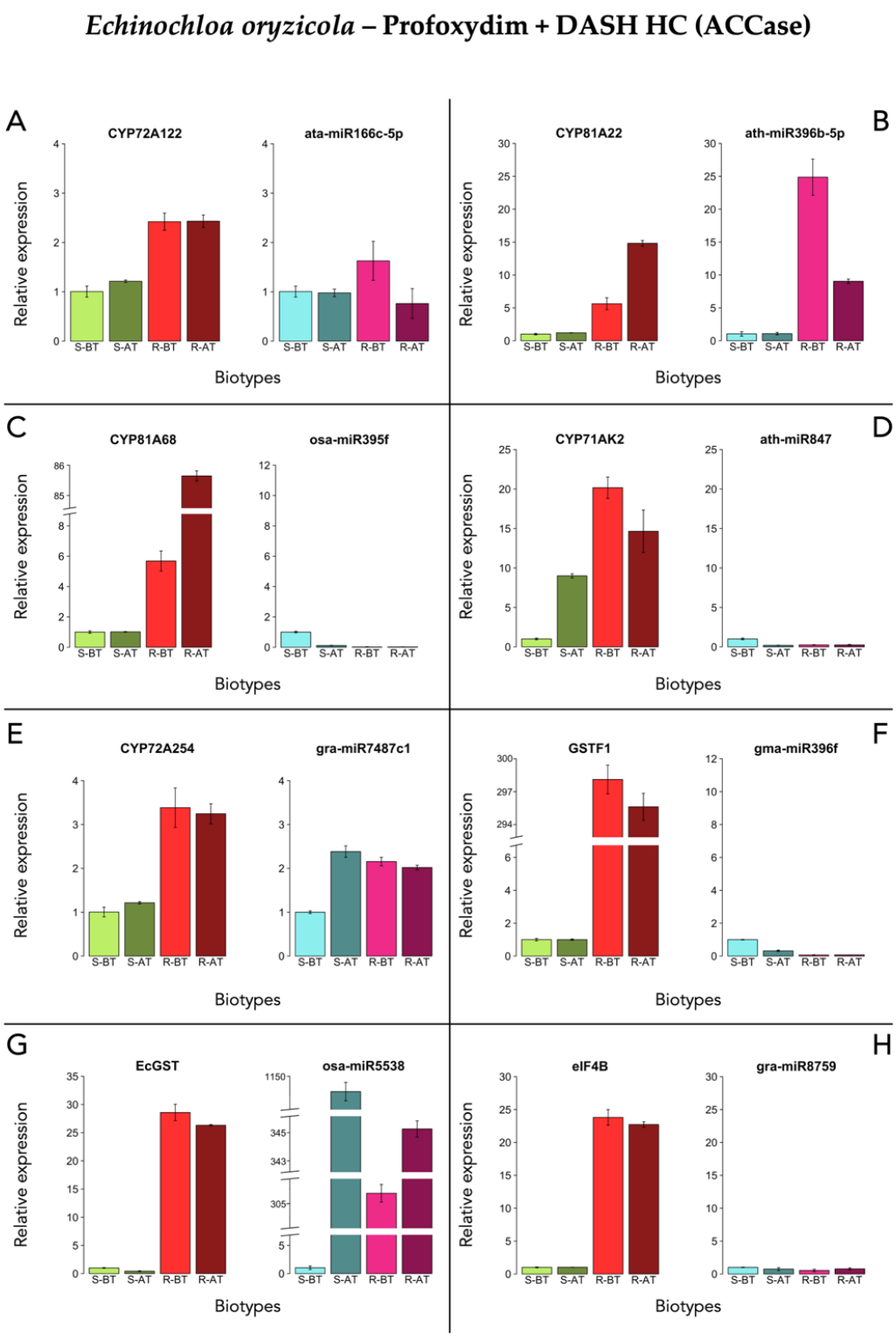

Figure 8.6: Expression levels of the miRNAs studied and the mRNA of their target genes in susceptible (S) and resistant (R) specimens of *Echinochloa oryzicola* before (BT) and after (AT) Profoxydim application. (A) CYP72A122 and ata-miR166c-5p; (B) CYP81A22 and ath-miR396b-5p; (C) CYP81A68 and osa-miR395f; (D) CYP71AK2 and ath-miR847; (E) CYP72A254 and gra-miR7487c; (F) GSTF1 and gma-miR396f; (G) EcGST and osa-miR5538; (H) eIF4B and gra-miR8759. Error bars indicate the standard deviation of three replicates.

Generally, high expression values were recorded for all the genes analyzed in resistant biotypes either before and after Profoxydim administration ($p < 0.05$). In CYP81A22 and CYP81A68 genes, expression level raised upon herbicide treatment, while in other genes recorded a slight but not significant decrease ($p > 0.05$). MicroRNA ath-miR396b-5p was overexpressed in resistant biotypes before herbicide spraying and significantly decrease after treatment, allowing the CYP81A22 gene to express. MicroRNA osa-miR5538 targeting EcGST resulted to be significantly triggered by Profoxydim application in susceptible and resistant biotypes, particularly in susceptible ones ($p < 0.05$), where repress the expression of its target gene. Instead, in resistant biotypes it counteracted the rising of EcGST expression, which was high before weeding and still remained overexpressed after treatment. Again, high levels of expression of genes involved in detoxification against Profoxydim were recorded in resistant individuals after treatment.

8.3.5 Involvement of miRNAs in Metabolic Herbicide Resistance in Echinochloa species – final considerations

The results obtained from the analyses of genes and relative miRNAs showed how the expression of certain genes and miRNAs appears to be stimulated by herbicide treatment, which acts as a stressor for weeds and triggers a network of epigenetic adaptive responses. In addition, it could be inferred how miRNAs act as regulators of the expression of their target genes, effectively counteracting the translation of the mRNAs they encode into proteins. Indeed, we note that, in general, when a miRNA is highly expressed, its target gene resulted under expressed. It should be considered, however, that a specific miRNA targets not just one specific messenger RNA, but many. It is therefore likely to assume that the expression of a particular miRNA may not always have an inhibitory effect only on a single specific gene.

It is well known that miRNAs play important functional roles towards many environmental stressors. The miRNAs considered in this work have been previously identified in other plants as playing a role in hybridization (Jia et al. 2013), growth-

regulation (Glazińska et al. 2013; Wang et al. 2015; Xue et al., 2013; Shi et al., 2021) and against various biotic and abiotic stresses including nutrient deficiency, drought, cold and salinity (Li et al., 2019; Matthewman et al., 2012; Jones-Rhoades et al., 2004; Rajagopalan et al, 2006; Radwan et al., 2011; Xue et al., 2013; Wei et al., 2011; Salanoubat et al., 2000). Moreover, miRNAs of the miR396 family are known to target oxidases, including cytochromes involved in xenobiotic detoxification (Kulcheski et al., 2011).

These results demonstrate that the miRNAs analyzed in this study play a role in the regulation of plant response to herbicide treatment in both *Echinochloa* species considered and for both ALS and ACCase inhibition mechanism.

Herbicide application triggers the transcription of miRNAs which down-regulates the expression of target genes, reducing their detoxification ability. Alternatively, when herbicide spraying does not stimulate the transcription of miRNAs, the target gene mRNAs could be translated in proteins leading to herbicide detoxification.

In relation to the analyzed genes, they are known to be involved in phases I and II of herbicide detoxification. In particular, recent work by Iwakamy et al. (2013; 2014) showed that CYP72A122, CYP71AK2, and CYP72A254 genes are involved in the detoxification of bispyribac-sodium in *E. phyllopogon.* Beside, Dalazen et al. (2018) showed that CYP81A6, GSTF1 and eIF4B genes are involved in the network of genes active in resistance against Imazethapyr in *E. crus-galli.* As a matter of facts, cytochromes belonging to CYP81A P450s subfamily induce resistance in *E. phyllopogon* against diverse and several herbicides, leading also to *cross-resistance.* However, it should be considered that each gene involved in herbicide detoxification presents affinity for a particular herbicide molecule (Dimaano et al., 2020).

The study conducted in the PhD project is limited to only a few herbicide formulations and a portion of the genes involved in the herbicide detoxification network. In the future, it could be considered to extend the research to other important enzyme families and to assess the entire miRNAome of *E. crus-galli* and *E. oryzicola,* in order to better understand the regulation of proteins involved in herbicide resistance.

Since this is a new and still under-investigated topic, further analysis will be needed to investigate for the role played by miRNAs and genes of herbicide-detoxification in the occurrence of herbicide resistance, also considering the role of the environment in affecting epigenetic adaptation in *Echinochloa* species.

8.4 Analysis of DNA methylation

8.4.1 Materials & Methods

8.4.1.1 DNA extraction and quantification

Total DNA (genomic, cpDNA and mtDNA) was extracted from leaf tissues of susceptible (S) and resistant (R) *Echinochloa* spp. biotypes before and after herbicides spraying using DNesay plant kit (QIAGEN) following manifacturer's instruction. Quantification was performed with QBIT (Thermo Scientific) following manifacturer's instruction.

8.4.1.2 CRED-RA

The level of global DNA methylation was analyzed by means of *coupled restriction enzyme digestion - random amplification* (CRED-RA) analysis.

DNA was firstly digested with HpaII and MspI methyl-endonucleases (Thermo Scientific) separately. Digestion reaction was performed in a T100 thermal cycler (BIO-RAD) as follows: digestion at 37°C for 4 hour, enzymes inactivation a 65°C for 20 min. Digestion reaction was carried out for each enzyme in a total volume of 30 μL including 10 μL of DNA (≤ 300 ng), 3μL of Buffer, 3 μL of SAM (1600 μM) and 0.5 μL of enzyme (0.5 U) and nuclease free water to volume.

Then digested DNA was randomly amplified, using primers listed in Table 8.5, in a T100 Thermal cycler (BIO-RAD). Random amplification reaction of polymorphic DNA (RAPD) was performed as follows: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 45 sec, annealing at 36°C for 45 sec and extension at 72°C for 2 min, followed by a final extension at 72°C for 15 min. Reaction was carried out in a total volume of 20 μl, including 12,5 μL (1,25U) of GoTaq® Green Master Mix (Promega), 2 μL of primer (1 μM) and 5 μL of DNA (~ 5 ng) and nuclease free water to volume.

Table 8.5: Nucleotide sequences of primers were used for RAPD reactions

Primers	Nucleotide sequence (5´- 3´)
OPC-01	TTCGAGCCAG
OPC-02	GTGAGGCGTC
OPC-04	CCGCATCTAC
OPC-05	TGGACCGGTG
OPC-08	TGGACCGGTG
OPC-11	AAAGCTGCGG

The total volume of RAPD amplicons was loaded on 2% agarose gel in 1X TAE buffer, stained with ethidium bromide, and separated at 100 V for 70 min. Molecular markers were visualized with Molecular Imager® Gel DocTM XR + (BIO-RAD).

8.4.1.3 Methylation pattern analysis

Table 8.6 was used as a reference to evaluate the data gathered by the CRED-RA analysis. Restriction enzymes of HpaII and MspI have different digestion abilities based on the cytosine status in the methylation pattern. All the amplified bands obtained from the CRED-RA analysis were divided into four methylation patterns based on the presence or absence of groups as indicated by Li et al. (2005), Wang et al. (2011), Pan et al. (2011) and Karan et al. (2012). Methylation profile was scored as bands presence (1) or absence (0).

CRED-RA fingerprints obtained from susceptible and resistant specimens before herbicide administration were compared with CRED-RA fingerprints obtained from the same specimens after treatment at the label dose.

Table 8.6: Methylation patterns produced by HpaII and MspI restriction enzymes according to their digestion abilities.

Methylation Type	Methylation Patterns	HpaII	MspI	Score of Band Profile			Methylation Description
				not digested	Hpa II	Msp I	
type I	CCGG GGCC **or** CC-CH3 GG	digestion	digestion	band	x	x	**No methylated** cytosine on double strands of DNA **or** **Inner methylated** cytosine on a single strand.
type II	CH3-CCGG	digestion	x	band	x	band	Outer methylated cytosine on a single DNA strand (**Hemi-methylation**)
type III	CC-CH3 GG GG CH3-CC	x	digestion	band	band	x	Inner methylated cytosine on a double DNA strands (**Full-methylation**)
type IV	CH3-CCGG GGCC-CH3 GGCC CCGG	x	x	band	band	band	Outer methylated cytosine on a double DNA strands (**Full-methylation**)

After analysis of the CRED-RA profiles, genomic template stability (%) was calculated with the following formula: GTS = (1-*a*/*n*) x 100, where letter of *a* refers to polymorphic band number of each sample and letter of *n* refers to the total band number in the control.

According to classification of Table 9, bands indicating each methylation type (I, II, III, and IV) were analyzed. The sum of the band numbers from the four methylation types gives the number of the total amplified bands. The percent ratio of each of the methylation type was calculated as follows (Karan et al., 2012; Bolukbasi et al., 2016):

- Non-methylated bands ratio (%) = [(I)/(I+II+III+IV)]x100
- Hemi-methylated bands ratio (%) = [(II)/(I+II+III+IV)]x100
- Full-methylated bands ratio (%) = [(III+IV)/(I+II+III+IV)]x100
- Total methylated bands ratio (%) = [(II+III+IV)/(I+II+III+IV)]x100

Percentage frequencies of methylation type were graphed as bar plots using Microsoft excel (Office 365).

Significant differences in DNA methylation level were analyzed by t-test using R 3.6.3 software.

8.4.2 Results & Discussion

Obtained results showed that primers adopted were useful to analyze the global DNA methylation level under the effects of herbicide administration in *Echinochloa* spp.

Figure 8.7 shows an example of CRED-RA fingerprint in which could be discriminated methylation patterns of the I, II, III and IV type.

- Light blue arrow indicates the presence of I type methylation (no methylation).
- Red arrow indicates the presence of II type methylation (hemi methylation).
- Green arrows indicate the presence of III type methylation (full methylation).
- White arrows indicate the presence of IV type methylation (full methylation).

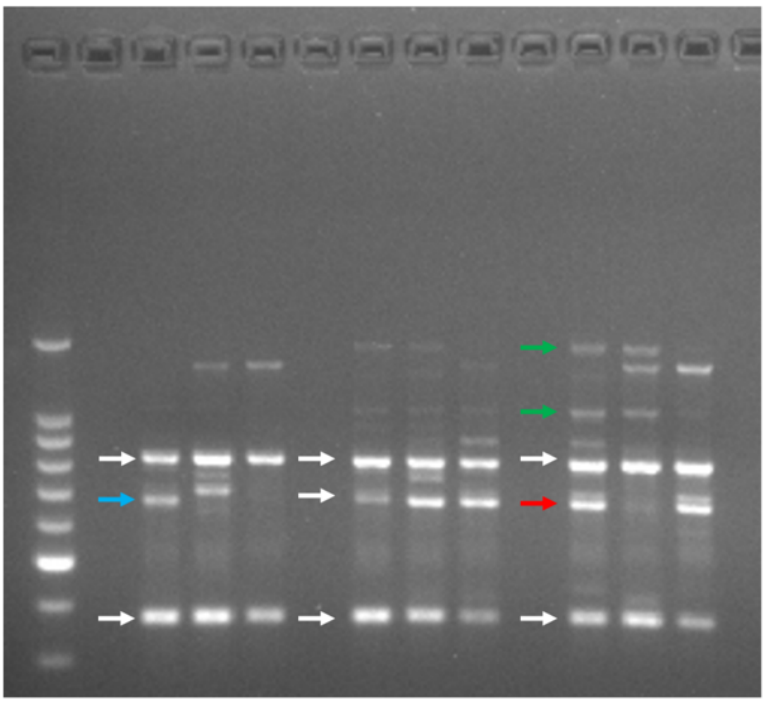

Figure 8.7: Methylation patterns produced by CRED-RA. Light blue arrow indicates the presence of I type methylation (no methylation). Red arrow indicates the presence of II type methylation (hemi methylation). Green arrow indicates the presence of III type methylation (full methylation). White arrows indicates the presence of IV type methylation (full methylation).

8.4.2.1 GTS – genomic template stability

The GTS % rate, a qualitative measure reflecting changes in CRED-RA fingerprints, hence of global DNA methylation level, were calculated for each of the six primers tested. In Table 8.7 are listed GTS rates recorded for susceptible and resistant *E.*

crus-galli biotypes before and after herbicide administration at the label dose. In Table 8.8 are listed GTS rates recorded for susceptible and resistant *E. oryzicola* biotypes before and after herbicide administration at the label dose.

Table 8.7: GTS rates (%) in susceptible and resistant *E. crus-galli* biotypes

Herbicide	Biotype	GTS (%) before treatment	GTS (%) after treatment
Bispyribac-sodium (ALS)	Resistant	55,56	63,15
Bispyribac-sodium (ALS)	Susceptible	51,32	67,79
Chletodim (ACCase)	Resistant	58,33	48,13
Chletodim(ACCase)	Susceptible	53,73	54,37

Table 8.8: GTS rates (%) in susceptible and resistant *E. oryzicola* biotypes

Herbicide	Biotype	GTS (%) before treatment	GTS (%) after treatment
Imazamox (ALS)	Resistant	54,27	48,89
Imazamox (ALS)	Susceptible	65,56	66,67
Profoxydim (ACCase)	Resistant	26,75	45,56
Profoxydim (ACCase)	Susceptible	49,44	55,56

The GTS rate in both *E. crus-galli* and *E. oryzicola* specimens recorded lower values in resistant biotypes than in susceptible ones after herbicide administration. Moreover, the GTS level recorded different values before and after herbicide treatment in both biotypes (Table 8.7 and 8.8). These findings showed that herbicide administration directly affected global DNA methylation level (Altunkaynak et al., 2016) and that resistant biotypes are less sensitive to these alterations.

8.4.2.2 Analysis of DNA methylation level

In Tables 8.9 and 8.10 are listed DNA methylation parameters recorded for susceptible and resistant *E. crus-galli* biotypes before and after herbicide administration at the label dose. In Figures 8.8 and 8.9 the same parameters are plotted as barplots.

Table 8.9: Data obtained from CRED-RA analysis of susceptible *E. crus-galli* biotypes.

Specimens	Susceptible - BT	Susceptible - AT	Susceptible - BT	Susceptible - AT
Herbicide	--	Bispyribac-sodium (ALS)	--	Chletodim (ACCase)
Total amplified bands	24	28	29	34
Total methylated bands	20,00	26,00	24,00	32,00
Total methylated bands ratio (%)	83,33	92,86	82,76	94,12
Fully methylated bands	18,00	23,00	21,00	31,00
Fully methylated bands ratio (%)	75,00	82,14	72,41	91,18
Hemi methylated bands	2,00	3,00	3,00	1,00
Hemi methylated bands ratio (%)	8,33	10,71	10,34	2,94
Non methylated bands ratio (%)	16,67	7,14	17,24	5,88

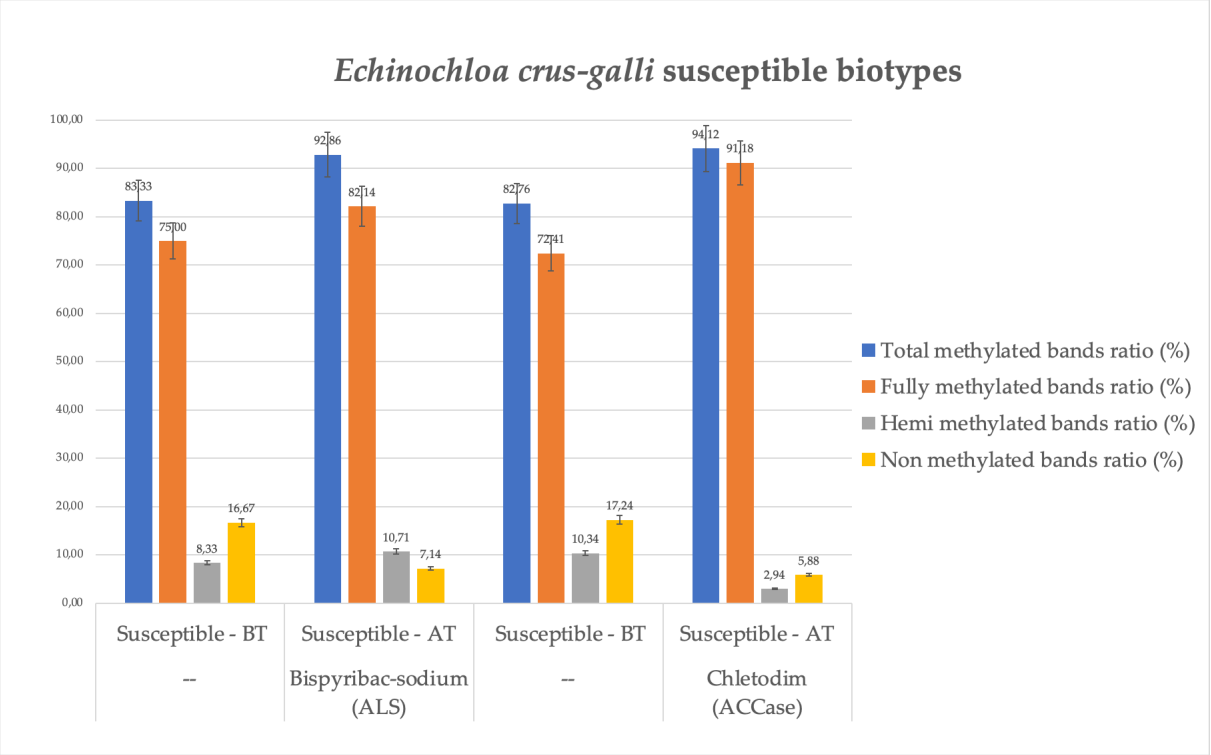

Figure 8.8: Global DNA methylation changes in susceptible *E. crus-galli* biotypes recorded before (BT) and after (AT) herbicide treatment. Error bars indicate the standard deviation.

Table 8.19: Data obtained from CRED-RA analysis of resistant *E. crus-galli* biotypes

Specimens	Resistant - BT	Resistant - AT	Resistant - BT	Resistant - AT
Herbicide	**--**	**Bispyribac-sodium (ALS)**	**--**	**Chletodim (ACCase)**
Total amplified bands	39	35	12	25
Total methylated bands	36,00	34,00	10,00	22,00
Total methylated bands ratio (%)	92,31	97,14	83,33	88,00
Fully methylated bands	35,00	34,00	8,00	21,00
Fully methylated bands ratio (%)	89,74	97,14	66,67	84,00
Hemi methylated bands	1,00	0,00	2,00	1,00
Hemi methylated bands ratio (%)	2,56	0,00	16,67	4,00
Non methylated bands ratio (%)	7,69	2,86	16,67	12,00

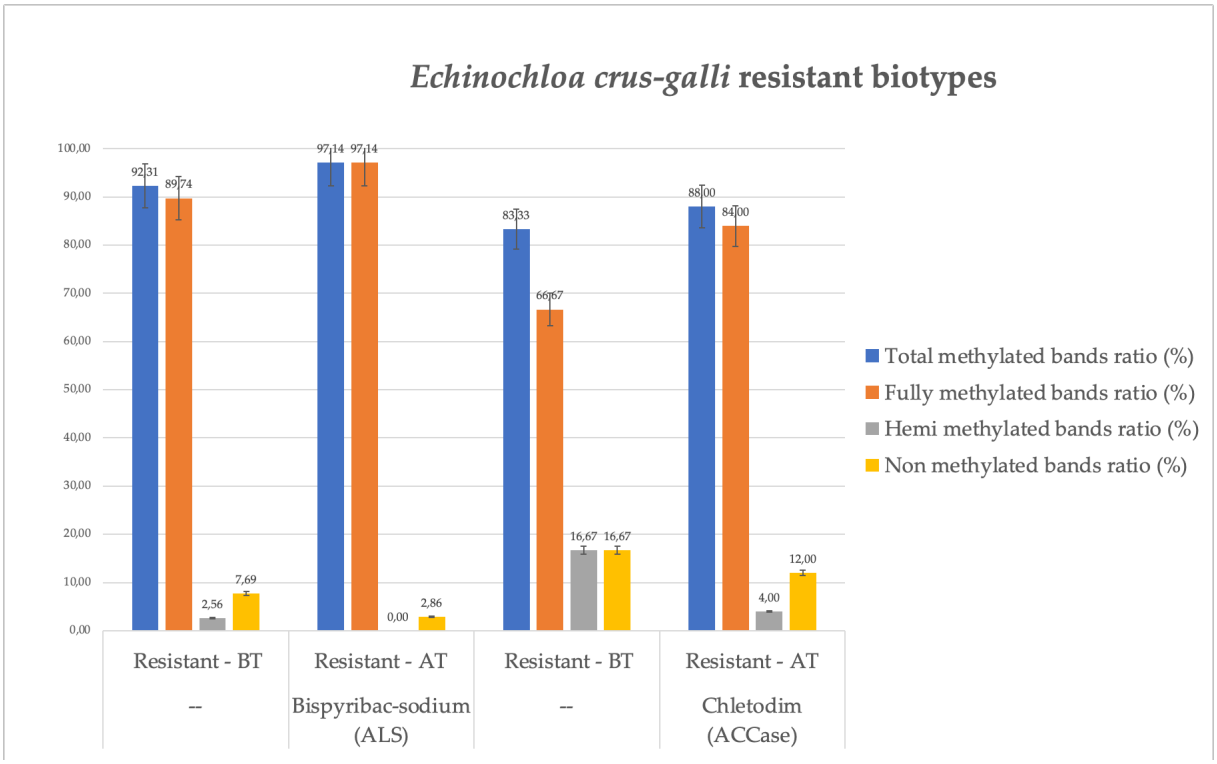

Figure 8.9: Global DNA methylation changes in resistant *E. crus-galli* biotypes recorded before (BT) and after (AT) herbicide treatment. Error bars indicate the standard deviation.

In Tables 8.11 and 8.12 are listed DNA methylation parameters recorded for susceptible and resistant *E. oryzicola* biotypes before and after herbicide administration at the label dose. In Figures 8.10 and 8.11 the same parameters are plotted as barplots.

Table 8.11: Data obtained from CRED-RA analysis of susceptible *E. oryzicola* biotypes

Specimens	Susceptible - BT	Susceptible - AT	Susceptible - BT	Susceptible - AT
Herbicide	--	Imazamox (ALS)	--	Profoxydim (ACCase)
Total amplified bands	20	22	22	20
Total methylated bands	19,00	22,00	19,00	20,00
Total methylated bands ratio (%)	95,00	100,00	86,36	100,00
Fully methylated bands	19,00	21,00	18,00	18,00
Fully methylated bands ratio (%)	95,00	95,45	81,82	90,00
Hemi methylated bands	0,00	1,00	1,00	2,00
Hemi methylated bands ratio (%)	0,00	4,55	4,55	10,00
Non methylated bands ratio (%)	5,00	0,00	13,64	0,00

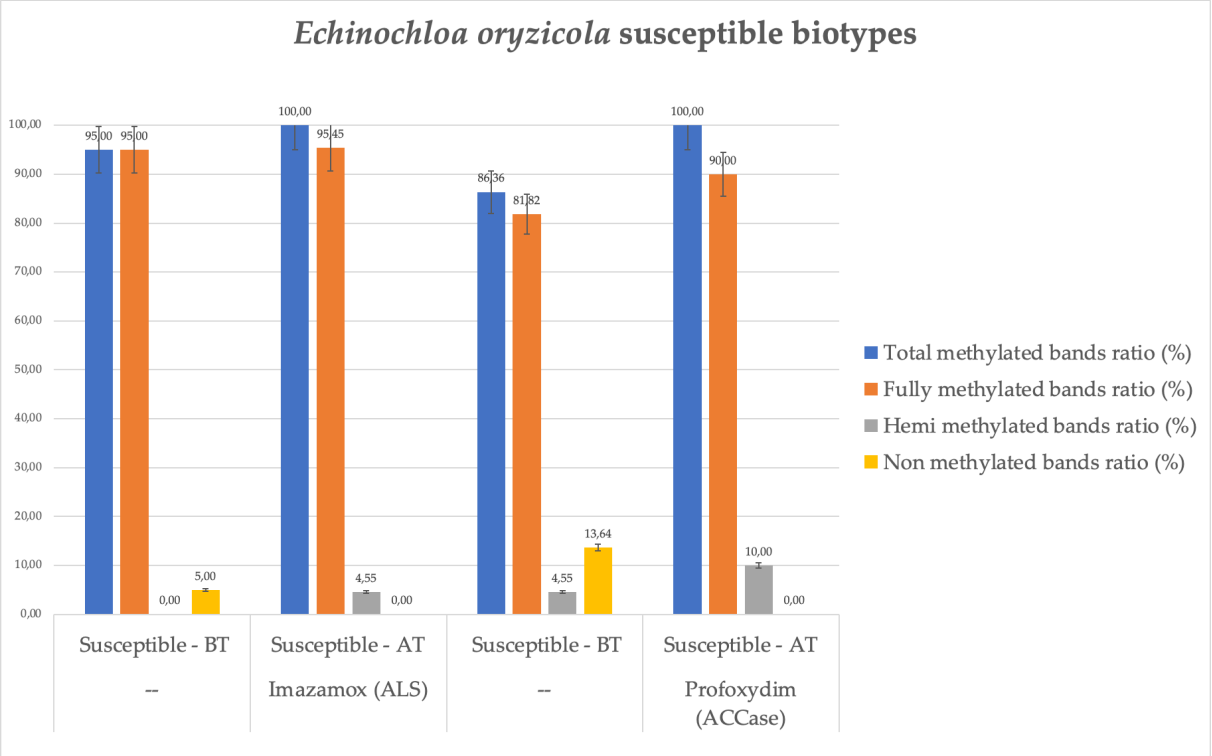

Figure 8.10: Global DNA methylation changes in susceptible *E. oryzicola* biotypes recorded before (BT) and after (AT) herbicide treatment. Error bars indicate the standard deviation.

Table 8.12: Data obtained from CRED-RA analysis of resistant *E. oryzicola* biotypes

Specimens	Resistant - BT	Resistant - AT	Resistant - BT	Resistant - AT
Herbicide	--	Imazamox (ALS)	--	Profoxydim (ACCase)
Total amplified bands	28	24	29	18
Total methylated bands	25,00	21,00	24,00	16,00
Total methylated bands ratio (%)	89,29	87,50	82,76	88,89
Fully methylated bands	23,00	19,00	24,00	14,00
Fully methylated bands ratio (%)	82,14	79,17	82,76	77,78
Hemi methylated bands	2,00	2,00	0,00	2,00
Hemi methylated bands ratio (%)	7,14	8,33	0,00	11,11
Non methylated bands ratio (%)	10,71	12,50	17,24	11,11

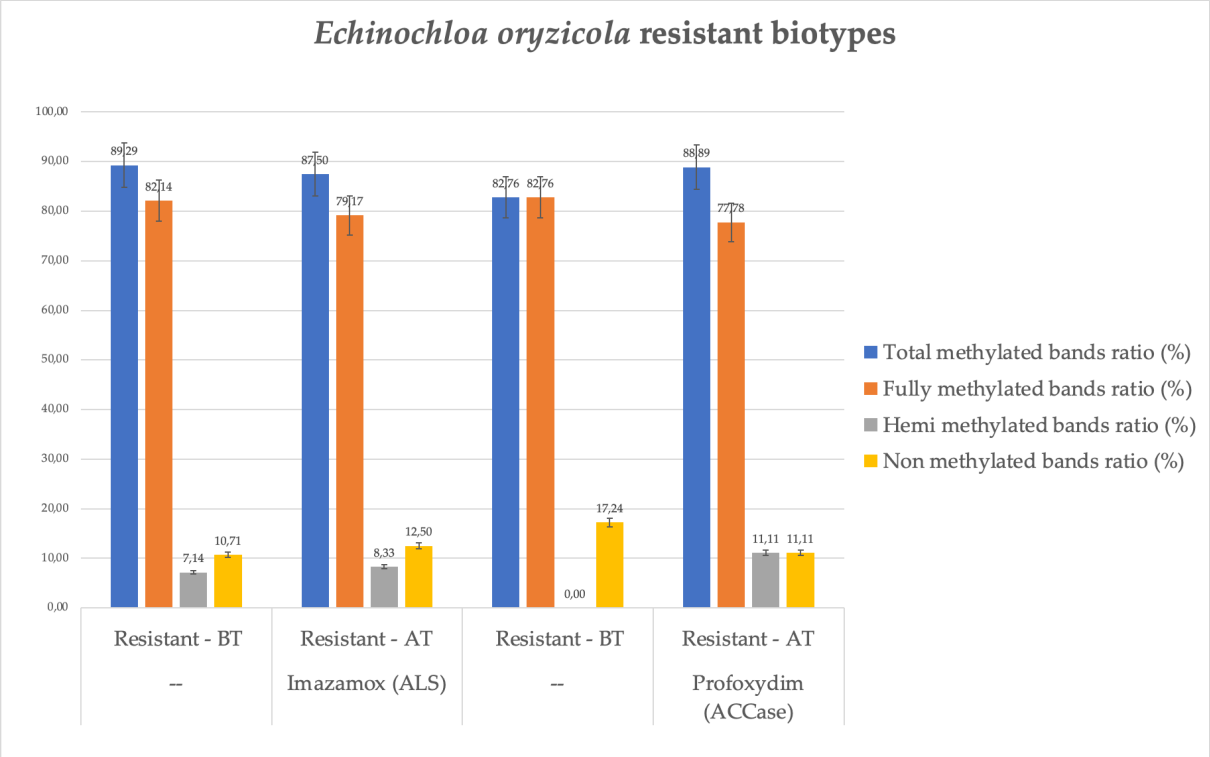

Figure 8.11: Global DNA methylation changes in resistant *E. oryzicola* biotypes recorded before (BT) and after (AT) herbicide treatment. Error bars indicate the standard deviation.

These analyses were conducted on *E. crus-galli* and *E. oryzicola* susceptible and resistant biotypes, before and after herbicide treatment, in order to evaluate the influence of herbicide stress on changes in global DNA methylation.

Generally, high levels of total DNA methylation were recorded in both susceptible and resistant specimens, either before and after treatment. For example, in *E. crus-galli* susceptible specimens total methylation level ranges from 82% before Chletodim application to 94% after treatment, while in resistant specimens treated ranges from 83%

before to 88% after weeding. In *E. oryzicola* susceptible specimens total methylation level ranges from 86% before weeding to 100% after Profoxydim spraying, while in resistant specimens ranges from 82% before to 88% after treatment. This findings highlight a greater extent of increase in total and full DNA methylation level in susceptible specimens following herbicide application compared to the increase recorded in resistant specimens.

In a recent study by Tyczewska et al. (2021) on maize was shown how glyphosate induced changes in global DNA methylation levels, which in turn affected (increasing or decreasing) the expression of some genes involved in herbicide detoxification. Hence, those results suggested how alterations in DNA methylation levels might play a role in resistance to glyphosate-based herbicide stress in *Zea mays* L. Findings of the current study corroborates Tyczewska et al. (2012) observations. In fact, similar results were obtained, before and after herbicide treatment in susceptible and resistant *Echionochloa* spp. specimens.

The present analysis showed that stress produced by herbicide administration caused different changes in the levels and types of DNA methylation in the resistant and susceptible biotypes of *Echinochloa* spp. tested, depending on their natural susceptibility or resistance to herbicides. The level and type of methylation can be reflected in changes in the expression of particular genes that may serve a more or less important function in the adaptive mechanisms of organisms in response to stress conditions. High changes in the level and profile of DNA methylation have been noted in susceptible biotypes of *Echinochloa* spp. after herbicide application that may lead to deregulation of the expression of genes involved in xenobiotic detoxification (such as cytochromes P450 and gluthatione-S-transferase), resulting in plant death. In contrast, minor changes in DNA methylation level were found for resistant biotypes of *Echinochloa* spp. after herbicide application. These findings support the hypothesis that DNA methylation may induce sensitivity/resistance to herbicide treatments in *Echinochloa* species through repression or stimulation of the expression of genes involved in herbicide detoxification (Tyczewska et al., 2012). In Figure 8.12 is summarized the role of DNA methylation in the regulation of herbicide resistance in *Echinochloa* spp.

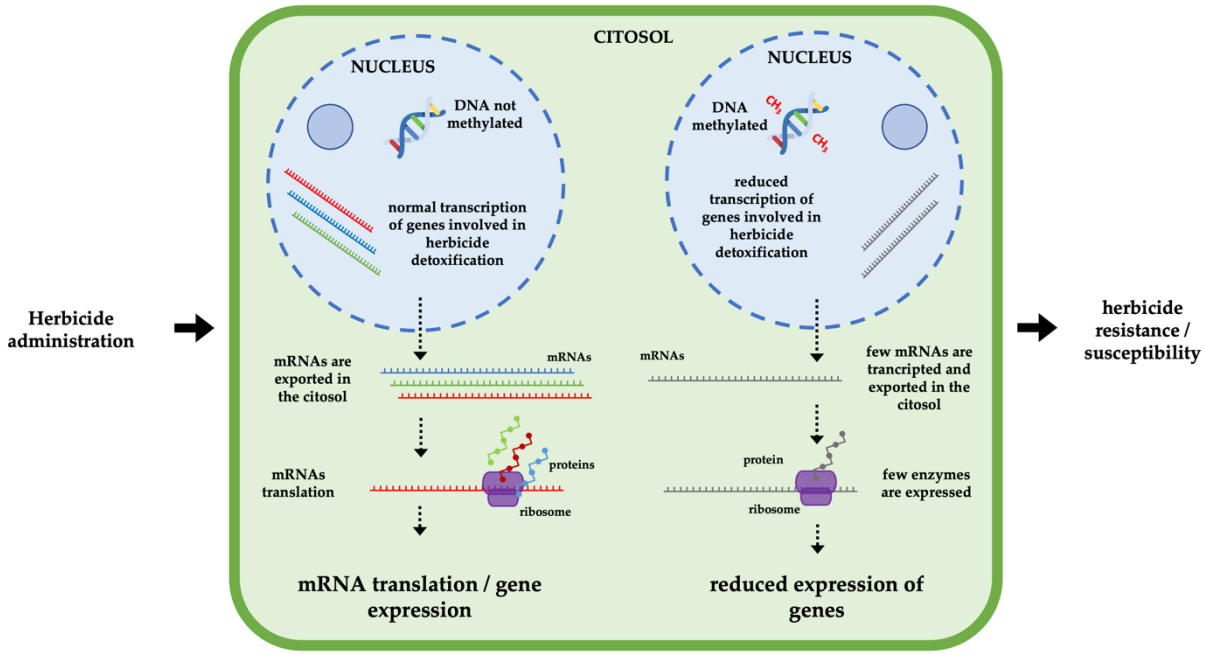

Figure 8.12: role of DNA methylation in the regulation of herbicide resistance in *Echinochloa* spp.

In conclusion, the results obtained showed how herbicide resistance occurs as a synergistic action of genetic traits, physiological factors and epigenetic mechanisms that play a regulatory action on gene expression, triggered by the application of the stress represented by the herbicide.

8.5 Analysis of the incidence of epigenetic resistance in relation to ecological variables

8.5.1 Introduction

Rice-growing areas are characterized by soils that are managed for rice cultivation. During the growing season, management practices include field preparation, plowing, flooding, and maintaining a layer of standing water during cultivation, which is followed by drainage and field drying. For these reasons, rice soils are often found in landscapes where surface and internal soil drainage is poor, thus in deltas and adjacent floodplains, valleys and coastal plains.

The chemical composition of rice soils is variable precisely because of these continuous tillage processes that cause imbalance. For example, during the period of flooding or submergence of the soil, the oxygen supply to the soil is zero. Within a day or two after flooding, aerobic microbes consume the trapped oxygen and render the soil anoxic except for a thin surface layer. Drainage and drying reverse these changes, rebalancing the oxygen concentration in the soil (Ponnamperuma, 1981). As a matter of facts, paddy soils are characterized by anaerobic conditions that persist throughout the agricultural season. These soils occupy an important niche in the biosphere and their importance is widely recognized. Anaerobic soils are primary sources of nutrients for plants grown in rice fields or wetlands. The decomposition process of organic matter in the absence of molecular oxygen leads to the production of bioavailable nitrogen and phosphorus, which promote plant productivity (ENR[18], 2020). Furthermore, pesticides (herbicides, insecticides, fungicides, etc…) application in paddy fields during the flooding season can easily cause soils pollution due to run-off or drainage of the chemicals and also due to their persistence in paddies. Therefore, it is crucial to know the chemical-physical and adsorption characteristics typical of each soil in order to predict the persistence of herbicides, and of pesticide in general, in rice fields and thus prevent their toxicity (Kibe et al., 2000)

[18] Ente Nazionale Risi - https://www.enterisi.it/servizi/notizie/notizie_homepage.aspx

As a matter of facts, crops and weeds, as well as microorganisms that inhabit this environment, are well adapted to live, survive and reproduce in presence of several stressors typical of agricultural lands (i.e. tillage, anaerobiosis, poor or rich micronutrients availability, presence of pesticides in the substrate, etc…). If we consider that epigenetics (miRNAs, DNA methylation, etc…) acts in regulating plant adaptive responses to biotic and abiotic stresses and helps restore cell homeostasis upon sudden environmental changes, it would be useful to analyze soil chemical-physical and biotic characteristics of each sampling unit (SU) in order to understand a possible relation between them, the adaptive responses of plants against such stressors and the onset of herbicide resistance.

The aim of this study was to obtain information about the relation between the incidence of herbicide resistances in which epigenetic mechanisms have been recognized and the ecologic variables (chemical/physical features, microbiota and meteorological variables) surveyed in each SU.

8.5.2 Materials & Methods

Analysis of soils chemical/physical features was performed as described in Chapter 4. Analysis of soils microbiota was carried out as described in Chapter 5.

The meteorological variables (average air temperature in °C, average humidity in % and average precipitation rate in mm/24h) characterizing the sites where SUs were identified were surveyed in ARPA database referring to recording stations located at Pavia, Mortara, Castello d'Agogna and Novara.

The incidence of *epiresistances* (resistances regulated by epigenetic mechanisms) was calculated as the % abundance coverage recorded by resistant *Echinochloa* spp. in each SUs. Only resistant wild-type specimens were considered for the analysis.

8.5.2.1 Epiresistances Incidence Analysis

The average air temperature (in °C), the average relative humidity (in %) and the average rate of precipitations (in mm/24h) recorded by ARPA stations during the summers of 2019 and 2020, together with soil chemical and physical traits and with the number of Bacteria and Fungi's orders surveyed in each visited SU were considered for the following analysis.

Spearman test was calculated in order to assess correlations between the analyzed chemical/physical parameters (function "cor.test" – package stats (R Core Team, 2019)). Only some of them were considered, excluding those that were correlated, in order to obtain a more precise and reliable analysis. Correlations were plotted as corrplot (function "corrplot" – packages ggplot2 (Wickham, 2016), Hmisc (Harrell Jr, 2021) and corrplot (Wei et al., 2021)).

Principal component analysis (PCA) was performed to assess the relationship between the ecological variables measured and the incidence of herbicide resistances regulated by epigenetic mechanisms in each SU (function "autoplot" – package ggplot2 (Wickham, 2016)). The ecological variables significantly correlated were excluded.

The entire statistical analysis was conducted using R 3.6.3 software (R Core Team, 2019).

8.5.3 Results and Discussion

Correlogram in Figure 8.13 shows the correlations existing between the analyzed chemical/physical soil features. Red eclipses are positive correlations ($R \geq 0.8$), blue eclipses are negative correlations ($R \leq -0.8$). Stars indicate significant correlations ($P < 0.05$).

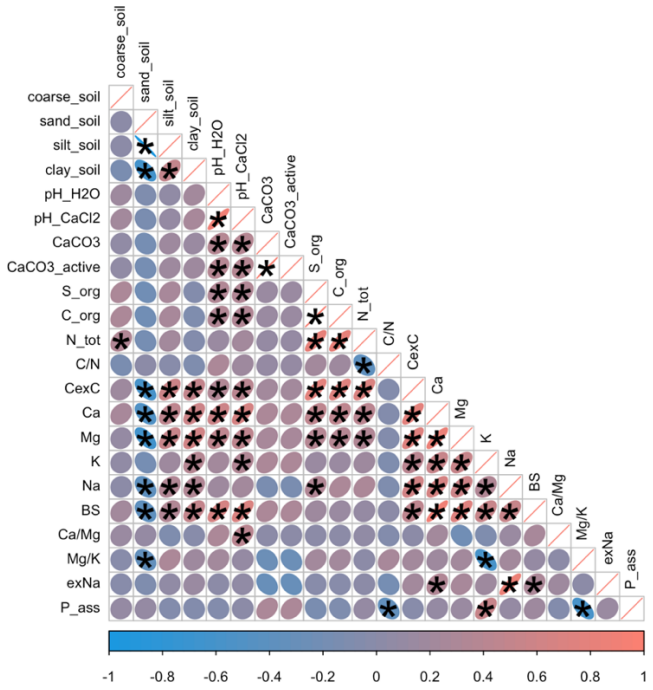

Figure 8.13: Correlogram of soils chemical and physical considered variables

There are significant strong correlations (positive and negative) between particles size (sand, silt and clay) and Ca, Mg, K, Na and degree of saturation in bases (GSB). In general, fine soils better retain macronutrients than coarse ones. Significant strong correlations resulted also between pH of H_2O and of $CaCO_3$ and C, N organic matter, Ca, Mg and K. Only total organic carbon (C_org), organic matter (S_org), total nitrogen (N_ tot), cationic exchange capacity (CexC), some macronutrients (Mg, K, Ca, Na and P_ass) and degree of saturation in bases (GSB) were considered for the following analysis.

Principal component analysis (PCA) in Figure 8.14 distributes the various farms visited (SU) according to the ecological variables surveyed and the incidence of epigenetic resistance recorded at each of them.

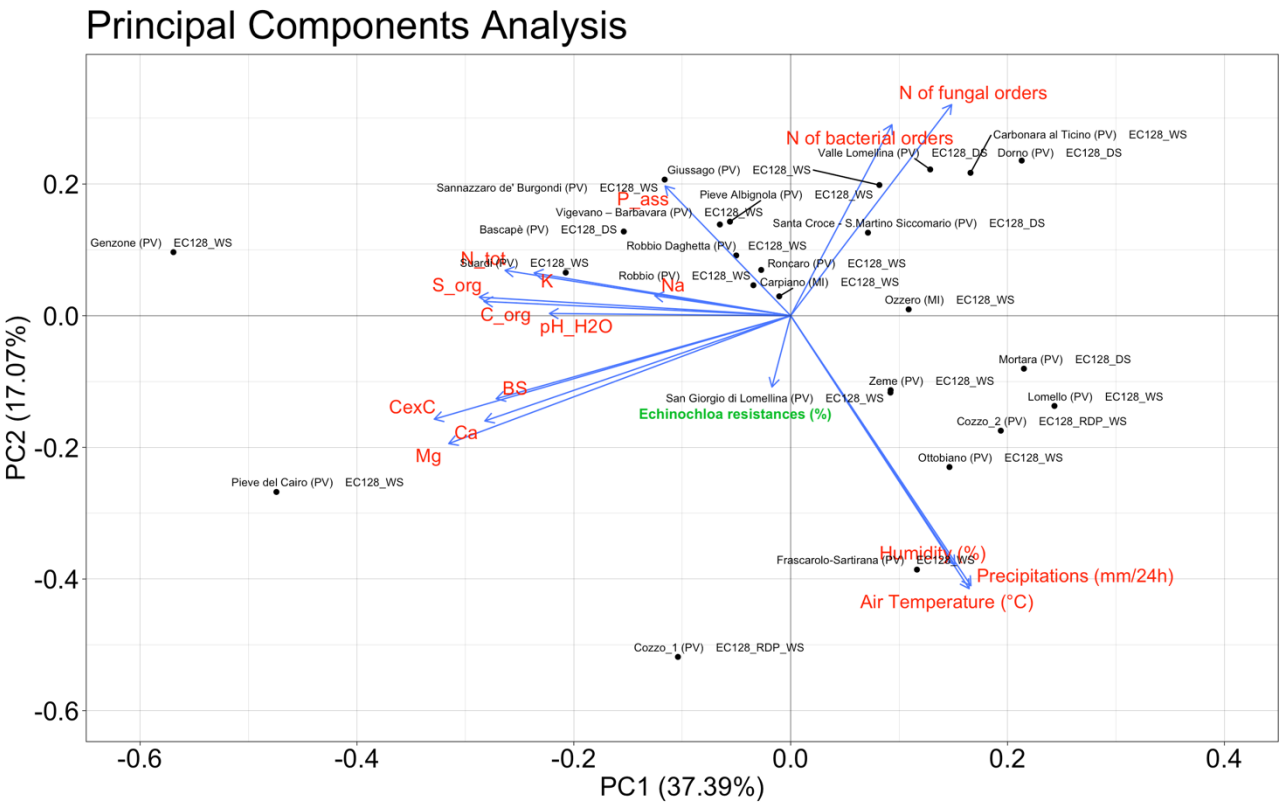

Figure 8.14: Principal components analysis indicating the relation between herbicide resistances regulated by epigenetic mechanisms incidence (*Echinochloa* resistances (%)) and ecological variables analyzed. EC128: Directive EC128/2009; RDP: Operation 10.1.01 of Rural Development Program; WS: wet seeding; DS: dry seeding.

The results obtained showed that there is a higher incidence of resistances, in which the regulatory action of epigenetic mechanisms is recognized, on farms located in sites characterized by higher rainfalls and where higher air average temperature and humidity values were recorded. Moreover, paddy soils are characterized by a low microbial biodiversity (Fungi and Bacteria) .

Considering that a recent experiment by Fang et al. (2015) has shown that the presence of bacterial strains of the genus *Myxococcus* acts as a stimulus for miRNA transcription in *E. crus-galli*, the results obtained from our analysis could be a starting point

for future analysis. Actually, the preservation and enhancement of soil biodiversity, through improved agronomic management implementing minimum or no tillage, organic fertilization, cover crops (conservative agriculture) and the application of bio-stimulant fertilizers, can help control resistance occurrence. This would make it possible to manage this harmful phenomenon in a more sustainable way, conserving biodiversity, using fewer herbicides and having less impact on the agroecosystem.

8.6 Conclusions

The results of this study conducted on resistant and susceptible biotypes of *E. crus-galli* and *E. oryzicola* showed that the mechanisms involved in the manifestation of herbicide resistance, genetic, metabolic and epigenetic, are multiple and synergistic. The occurrence of non-target site resistance has been shown to be closely related to epigenetic mechanisms acting in the regulation of DNA expression, such as miRNAs and methylation. Indeed, it has been shown how the transcription of certain miRNAs, which act by counteracting the expression of enzymes involved in the detoxification of certain herbicide molecules, is triggered by the stress represented by the herbicide. Furthermore, it was demonstrated how the level of global DNA methylation was affected by herbicide application and how its increase was greater in susceptible individuals than in resistant ones. In fact, weed management through more precise and less impactful use of herbicides is one of the great challenges facing humans in the near future.

To date, few studies have focused on the analysis of epigenetic adaptive mechanisms against herbicides and their role in the manifestation of herbicide resistance. In fact, most researches have focused on these important regulatory mechanisms of plant-environment interactions, but mainly associated with stress adaptation. Ecologic conditions influence plant physiology by triggering epigenetic responses. It has been noted how these adaptation can affect herbicide resistance through the regulation of the expression of genes involved in xenobiotic degradation–(Tyczewska et al., 2012; Pan et al. 2016; 2022). In the scenario where an increasingly narrow range of commercially available herbicide chemicals

favors the evolution of weed species that are increasingly adapted to survive against chemical control, and considering herbicides in the same way as other abiotic environmental stressors, it will be crucial to thorough understand the interactions between genetics, epigenetics, and the environment to correctly predict and monitor the evolution of herbicide resistance.

Developing knowledge of weed biology and advancing studies on epigenetics related to herbicide resistance will open a new avenue for a better comprehension of weed adaptation to chemical control. Understanding the epigenetic mechanisms triggered following herbicide treatment would enable more precise and sustainable weed monitoring actions to be planned.

Further, a broader understanding of chemical, physical and micro-biological characteristics would enable to obtain a better view of the ecology of paddy field ecosystem. This would allow to deepen the study of phenomena that regulate plant epigenetic mechanisms that lead to the manifestation of herbicide resistance, and to understand which are the most relevant ecological variables in this context.

8.7 References

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Chapter 9 – Final considerations and future perspectives

The action of epigenetic mechanisms triggered in response to herbicide application on the onset of resistance in weeds is still a poorly understood topic that only recently has been investigated. To date, only few works have been conducted on this subject, especially for species in the genus *Echinochloa*.

The study conducted in this dissertation is the first investigating this phenomena in species of the genus *Echinochloa* through a multidisciplinary approach. In fact, both the role of genetics and epigenetics was considered for the analysis of herbicide resistance occurrence, also taking into account the influence of ecological variables such as soil chemistry and physics, soil microbial biodiversity and meteorological and climate conditions.

What has emerged from the results obtained from the various experiments described in this doctoral dissertation, is that herbicide resistance is a constantly evolving phenomenon: in fact, it has been shown that resistant *Echinochloa* populations are a well-established and dynamic presence in the rice-growing territory of the Lombardy region. By comparison with GIRE maps, it was also noted how the resistance phenomenon is often underestimated.

The analysis of DNA sequence shows that only a small portion (~13%) of *Echinochloa* spp. specimens possess a mutation leading to the occurrence of target site resistances (TSRs). Furthermore, among these resistant mutant specimens there is a fair presence of resistant heterozygous individuals that will potentially generate new mutant populations able to survive against chemical control.

Considering the high intraspecific genetic variability that characterizes species of the genus *Echinochloa*, their allo-polyploid karyotype and their ability to hybridize, it can be assumed that the presence of resistant heterozygous individuals is a potential real threat to

rice farming. In the future, further analysis on this topic will be useful in order to obtain information to manage this problem.

Furthermore, it has been shown how, in the case of wild type *Echinochloa* spp. specimens, the manifestation of herbicide resistance represents the result of multiple factors. Findings obtained from investigations conducted on genes involved in NSTR resistance and on epigenetic mechanisms regulating their expression, show how miRNAs and DNA methylation are affected by herbicide as stressor and are involved in resistance/susceptibility occurrence. When herbicide does not stimulate miRNAs transcription and DNA methylation, the target genes could be expressed, leading to herbicide detoxification, hence resistance. Otherwise, when herbicide triggers these epigenetic mechanisms, they down-regulate the expression of target genes reducing their detoxification ability.

To summarize, it has been shown that herbicide resistance depends also on the synergistic action of enzymes involved in herbicide detoxification and epigenetic mechanisms that regulate their expression. It would be of great interest to deepen such analysis, sequencing the miRNAome of *Echinochloa* species and analyzing in detail the level of methylation of particular DNA genes.

Epigenetic mechanisms are known to be affected by the environment. Findings about the incidence of herbicide resistance show that, in addition to the stress represented by chemical control, ecological factors has an important influence on the evolution of *epiresistances*. In fact, results showed that soil microbial biodiversity, air temperature, air humidity and precipitations were the variables most related to the incidence of resistant *Echinochloa* populations.

It will be useful to conduct future investigations about the combined effect of herbicide and these ecological variables on epigenetic resistance, also considering climate changes affecting herbicide efficacy.

Findings show that genetic intraspecific variability in the genus *Echinochloa* does not appear to be related to the manifestation of resistance. Considering results of miRNAs, DNA methylation, and *epiresistances* incidence analyses, it could be assumed that the

variability of epigenetic mechanisms, influenced by ecological factors, plays a very important role in the manifestation of resistance.

A deep understanding of how the adaptive epigenetic responses may regulate the expression of genes involved in the herbicide detoxification pathway, and thus in the onset of resistance, represents an important step that would enable optimization of precision weed management (PWM) technologies.

More targeted and sustainable weed management strategies considering the preservation and enhancement of soil biodiversity, through conservative agriculture practices (CAP) adoption, can help herbicide resistance control, reducing chemical-inputs, improving food health and protecting the environment and human health.

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