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CICLO: XXX

**HERBICIDE RESISTANCE IN WEEDS
AND EFFECTS OF SALINITY**

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*«[...] a tratti, alzando il capo spaziavo lo sguardo e sentivo nello
spettacolo di quella campagna qualcosa di grande di bello,
struggendomi di non comprenderlo appieno e di non saperlo rendere in
parole. [...]»*

(Cesare Pavese)

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Abstract

Weeds are considered one of the main obstacle for crop production, cause of reduction in quality and crop yield.

The most effective and cost-efficient tool of weed management is represented by herbicide application. Nevertheless, the wide use of the same herbicides or the same mechanisms of action may result in the appearance of weed populations no longer controlled by them.

In addition to herbicide resistance issues, in recent years, saltwater intrusion phenomena are developing.

According to FAO estimates, about 20% of irrigated land is interested by the increase of salt levels. This condition is stressed by the competition for fresh water in agricultural and civil uses, consequence of climate changes, growing population, socio-economic development and contamination.

In Italy, salt water intrusion cases can be found in some areas; among which, we count the Po Plain and the Oristano Province, both characterized by rice cultivation.

The studies described in the dissertation were realized by considering two main objectives. The first one was to analyse the consequences of the repeated use of the same herbicide mode of actions on different weed species. The second one was to analyse the effect of saline stress on the biology of two weed species (barnyard grass and weedy rice) and of a crop (rice); furthermore, the studies were aimed to evaluate the potential relationship between saline stress and response to herbicide treatments.

In a first study, the appearance of herbicide resistance in various *Lolium multiflorum* and *Apera spica-venti* populations escaped to herbicide field applications through the use of ALS-inhibitors, ACCase inhibitors and PSII inhibitors has been investigated. In order to confirm the selection of resistance, the populations of both weed species not controlled by the herbicide application were subjected to genetic analysis. The results obtained at the end of the trials underlined the selection of herbicide resistance (target-site, non-target-site and multiple) in some of the tested *L. multiflorum* and *A. spica venti* populations.

The second and third study were aimed at evaluating the effect of salinity conditions on germination and first seedling growth (root and shoot length) of two weed species and a crop; the potential relationship between herbicide resistance and response to saline stress was also investigated. The second study was conducted on six *Echinochloa* spp. populations (three sensitive and three resistant to ALS-inhibitor herbicides) while the third study was performed on five weedy rice populations (four sensitive and one resistant to ALS-inhibitors) and on two rice varieties (Baldo and CL80). The results obtained at the end of the two studies showed that weed populations and rice varieties were affected by salinity. In general,

with the increase of salt concentration a decrease of seed germination capacity, speed of germination and root and shoot growth was found. The fourth study was carried out with the aim to evaluate the effect of salinity on germination and seedling growth of two weed species (barnyard grass and weedy rice) and a crop (rice), in combination with their response to herbicide applications. The physiological parameters considered were: seed germination capacity, speed of germination, plant height, shoot and root fresh weight, SPAD, content of chlorophyll *a*, chlorophyll *b* and carotenoids. The results obtained at the end of the study underlined the effect of salinity on all the physiological parameters, both for weed populations and for rice variety. In general, with the increase of salt level a progressive reduction of seed germination capacity, speed of germination, plant height, shoot and root fresh weight was observed. A variable trend in response to saline conditions was found in the case of SPAD, chlorophyll and carotenoids concentrations. Similarly, the response to the combination between salt stress and herbicide application varied among the tested weed population.

Riassunto

Le infestanti sono considerate uno dei principali fattori di limitazione delle produzioni agricole, causa di significative perdite sia dal punto di vista quantitativo che dal punto di vista qualitativo. La forma più efficace ed economicamente efficiente di controllo della flora infestante è rappresentata dall'impiego di erbicidi. Tuttavia, il ripetuto utilizzo dei medesimi erbicidi o di erbicidi con medesimo meccanismo di azione può tradursi nella comparsa di popolazioni di infestanti non più controllate dagli stessi.

Nel corso degli ultimi anni, ai problemi legati alla selezione di popolazioni di infestanti resistenti agli erbicidi si associano quelli derivanti dall'insorgenza di fenomeni di intrusione salina nelle acque. Sulla base di quanto stimato dalla FAO, circa il 20% delle terre irrigate risulta essere interessato da un incremento dei livelli di salinità. Tale fenomeno è accentuato dalla competizione per l'impiego di acqua dolce in campo agricolo e civile derivante da più fattori, quali cambiamento climatico, crescita demografica, sviluppo socio-economico e contaminazione. In Italia, la presenza di casi di intrusione salina è stata riscontrata in vari areali, tra cui la pianura del Po e la Provincia di Oristano, entrambe interessate dalla coltivazione risicola.

Gli studi descritti nella tesi sono stati realizzati considerando due obiettivi principali: analizzare gli effetti derivanti dall'impiego ripetuto degli stessi meccanismi di azione su differenti specie infestanti e verificare gli effetti dello stress salino sulla biologia di due specie infestanti e di una coltura;

è stata, altresì, valutata la potenziale presenza di connessione tra stress salino e risposta al trattamento erbicida.

Il primo studio è stato condotto con l'obiettivo di verificare la comparsa di resistenza ad erbicidi in diverse popolazioni di *Lolium multiflorum* e *Apera spica-venti* sfuggite a trattamenti di campo basati sull'impiego di inibitori dell'ALS, dell'ACCasi e del PSII. Al fine di confermare l'effettiva insorgenza di resistenza, le popolazioni di entrambe le specie infestanti sfuggite alle applicazioni erbicide sono state sottoposte ad analisi genetica. I risultati ottenuti al termine dello studio hanno evidenziato lo sviluppo di fenomeni di resistenza (target-site, non target site e multipla) in alcune delle popolazioni di *L. multiflorum* e *A. spica venti* oggetto di studio.

Il secondo ed il terzo studio sono stati realizzati al fine di valutare l'effetto delle condizioni saline sulla germinazione e sulle prime fasi di sviluppo di due specie infestanti e di una coltura; nel corso degli studi è stata, inoltre, verificata la potenziale presenza di una relazione tra resistenza ad erbicidi e risposta allo stress salino.

Il secondo studio è stato condotto su sei popolazioni di *Echinochloa* spp. (tre sensibili e tre resistenti agli inibitori dell'ALS) mentre il terzo ha preso in esame cinque popolazioni di riso crodo (quattro sensibili e una resistente agli inibitori dell'ALS) e due varietà di riso (Baldo e CL80). I risultati ottenuti al termine dei due studi hanno mostrato l'influenzata esercitata dalla salinità su specie infestanti e coltura. In generale, al crescere della concentrazione salina è stato osservato un decremento di capacità germinativa, velocità di germinazione e sviluppo di radici e germogli.

Il quarto studio è stato realizzato con l'obiettivo di valutare l'effetto della salinità su germinazione e crescita di due specie infestanti (giavoni e riso crodo) e di una coltura (riso). È stato, altresì, analizzato l'effetto derivante dalla combinazione di salinità e trattamento erbicida. Nel corso dello studio sono stati considerati i seguenti parametri fisiologici: capacità di germinazione, velocità di germinazione, altezza delle piante, peso fresco di porzione epigea ed ipogea, SPAD, contenuto di clorofilla *a*, clorofilla *b* e carotenoidi. I risultati ottenuti hanno evidenziato la presenza di un effetto della salinità su ciascuno dei parametri fisiologici considerati, sia nel caso delle infestanti sia nel caso della coltura. In generale, all'aumentare della concentrazione salina è stata osservata una progressiva riduzione di capacità di germinazione, velocità di germinazione, altezza delle piante, peso fresco di porzione epigea ed ipogea. Un trend variabile in risposta alle condizioni saline è stato riscontrato nel caso di SPAD, contenuto in clorofilla e carotenoidi. Per quanto concerne la combinazione stress salino - applicazione erbicida, dai risultati ottenuti è emersa una risposta alle diverse concentrazioni saline differenziata per ciascuna delle popolazioni infestanti testate.

Introduction

1. Weed Science

“Weed science” is an integrative, applied scientific discipline (Radosevich *et al.*, 1997) that studies weeds. These are plants responsible for damaging the human activities or for limiting their functionality.

The biological and eco-physiological characters of weeds and their impact on human activities (especially on agriculture), their function on the agroecosystem and the control means are investigated by weed science. In the Anglophone culture, weed science is divided in two components: “Principles of weed science” and “Science and technology of weed control”. The first one studies the biology of weeds and their effect on functional plants and on the habitat in which they grow; the second one concerns the analysis of the various control tools (chemical, mechanical, biological) and their agronomic management.

In order to achieve a low economic and environmental impact on weed control, weed science combines knowledge deriving from biology, ecology, agronomy, chemistry, biochemistry, genetic, physiology and ecotoxicology (Catizone and Zanin, 2001).

2. Weed

Weeds are present as vegetation category as a result of human selection, among the numerous components of plant kingdom, as subjects with advisable characteristics. As well as some plants are positively considered for their usefulness or beauty, others are suppressed as a consequence of the apparent absence of the above mentioned positive characters. All around the world, weeds are identified as undesirable element and significant economic issue (Radosevich *et al.*, 1997).

However, the term “weed” has not an ecological base but it can be considered an anthropocentric concept. In fact, the term “weed” is not associated to a definition that is universally agreed. Some partial definitions are, therefore, proposed (Zanin *et al.*, 2001).

2.1. Weed definition

2.1.1. Ecological definition

According to Baker, 1965 “a plant is a weed if, in a determined geographical area, its population grows entirely or preponderantly in environments that are highly disturbed by human activities”. In this explanation, the word weed is associated with that of “adventitious”. Hamel and Dansereau (1949) affirm that “adventitious” are “the plants that

grow spontaneously in environments disturbed by human activities". Since the majority of adventitious are also weeds, this explanation has been widely adopted by the weed scientists.

Another definition of the term "weed" is that proposed by Bunting, 1960: *"weeds are pioneer plants of secondary ecological successions; of these, the plowed fields are a special case"*. In this case the term "ecological succession" is at the centre of the explanation: it refers the gradual process that leads to mutation of the plant community composition over time. It is possible to distinguish two forms of succession: primary and secondary. The first one is a succession that happens in an environment previously devoid of other organisms while the second one occurs after that a portion of the pre-existing community is removed by a disturbance. In plant ecology, any form of action responsible of destruction or damage of vegetal biomass is considered a "disturbance". As a consequence, the cultural practices, such as tillage, weed control, mowing, grazing, are considered disturbance that causes secondary successions (Zanin *et al.*, 2001).

2.1.2. Technical definition

The European Weed Research Society (EWRS) defines weed as *"every plant or vegetation, excluding the mushrooms, that interferes with the human objectives"*. In this case, the concept of noxiousness is associated with the term "weed" (Zanin *et al.*, 2001).

According to some authors, it is necessary to establish a criterion based on which a plant can be considered disagreeable or noxious. This criterion must simultaneously consider ecological and economical aspects and the decision must be the result of a balance of advantages and disadvantages. As a consequence, a plant must be considered a weed only starting from a certain threshold density.

2.1.3. Ecological and technical definition

Holzner, 1982 affirms that *"weeds are plants that are adapted to the habitat modified by human activities and that interfere with these"*. This explanation can be repeated as *"...weeds are undesired adventitious"*. Nevertheless, even this latest definition cannot be considered fully exhaustive; this because a negative connotation is attributed to the adventitious plant and the weeds are limited to the adventitious (Zanin *et al.*, 2001).

2.2. Weed classification

Based on the aims of the classification, various methods can be adopted to catalogue weeds.

Five partition modalities are proposed below (Zanin *et al.*, 2001).

2.2.1. Classification into biological groups

This classification was proposed by the Danish ecologist Raunkiaer (1905) and later adapted to weed science by Montegut (1982 and 1984). According to this classification, the biological groups in which the plants can be divided are the consequence of the mode with which they overcome the unfavourable period of the season (Table 1). In effect, this period can be overcome in form of seed or bud; in the case of bud, their position relative to the ground surface is also considered.

Table 1. Life forms in which plants can be categorized on the basis of the propagation system. (Modified from Zanin *et al.*, 2001).

Propagation only by seed		
<i>Therophytes species</i> - Annual cycle		<i>Biennials species</i> - Biennial cycle
Propagation by seed and buds little under earth		
<i>Hemicryptophytes species</i> - Multi-year cycle		
Propagation by seed and buds placed on profound vegetative organ		
<i>Geophyte species</i> - Vivid plans		
Propagation by seed and buds placed on ground surface on structure with variable lignification		
<i>Camephyte species</i>	<i>Nanophanerophytes species</i>	<i>Phanerophytes species</i>

2.2.2. Classification into eco-physiological groups

This classification was proposed by Montegut in 1975. According to it, weeds originate from seed grown in specific period of the season based on determined eco-physiological requirements. As a consequence of this, weeds can be divided into the following five groups: indifferent species, autumn, winter, spring and summer species (Table 2).

Table 2. Eco-physiological groups in which weeds can be divided. (Modified from Zanin *et al.*, 2001).

Indifferent species	
Totally indifferent	Germination can occur from 0 to 30-33°C. * <i>Senecio vulgaris</i> , <i>Stellaria media</i> , <i>Poa annua</i>
Partially indifferent	Germination stops with temperature less than 5°C. * <i>Conyza candensis</i> , <i>Fumaria officinalis</i> , <i>Veronica persica</i>
Apparently indifferent	Growth occurs during the whole year. * <i>Raphanus raphanistrum</i> , <i>Sinapis arvensis</i>
Autumn species	
Narrow autumn germination	Species indifferent to the photoperiod with bloom at the end of winter. <u>Temperate regions*</u> : <i>Arabidopsis thalina</i> , <i>Cardamine hirsute</i> <u>Mediterranean region*</u> : <i>Diploaxis erucoides</i> , <i>Vicia</i> spp.
Preferential autumn or pre-spring germination	Species that combine the need for cold for vernalization with that of long day. Flowering occurs at the end of spring. <u>Temperate region*</u> : <i>Alopecurus myosuroides</i> , <i>Gallium aparine</i> <u>Mediterranean region*</u> : <i>Avena barbata</i> , <i>Myagrum perfoliatum</i>
Preferential post-winter or occasionally autumn germination	Species with preferably post-winter germination and occasionally autumnal. Biennial species in the strict sense. * <i>Carduus nutans</i> , <i>Cirsium vulgare</i> , <i>Daucus carota</i> *
Winter species	
Species with seeds characterized by dormancy and able to germinate at 0-5°C. Germination induced by low temperature. * <i>Apera spica-venti</i> , <i>Papaver</i> spp., <i>Ranunculus arvensis</i>	
Spring species	
Narrow spring species	Species characterized by strict springtime germination period. * <i>Bifora radians</i> , <i>Fallopia convolvulus</i> , <i>Polygonum aviculare</i>
Prolonged spring species	Species that prolong germination even at the beginning of the summer. * <i>Abutilon theophrasti</i> , <i>Bisens</i> spp., <i>Polygonum persicaria</i>
Summer species	
Sub-thermophilic species	Distinction in the two groups according to thermal needs. * <i>Digitaria sanguinalis</i> , <i>Setaria viridis</i> , <i>Solanum nigrum</i>
Thermophilic species	* <i>Amaranthus</i> spp., <i>Echinochloa crus-galli</i> , <i>Sorghum halepense</i>

*some of the species included in the group.

2.2.3. Ecological classification (or Grime classification)

Based on this classification, plants adopt various strategies in order to acquire the resources and to lessen the extinction risk. According to Grime, plant growth is limited by two main factors: the stress and the disturbance. The stress occurs when a fundamental factor such as light, water, mineral salts, temperature, CO₂ is provided in a not optimal amount. The disturbance occurs when the plant biomass is reduced due to interventions such as mowing, tillage, grazing or fire.

Considering the extremes of these two conditions, defined as “high” and “low”, and connecting them, four combinations are obtained. Three of these represent potential strategies adopted by plants in order to ensure an adequate resource storage and can be defined as ruderal species (R), stress-tolerant species (S), and competitor species (C).

Stress-tolerant species (S) are suitable for developing in environments characterized by the limited presence of one or more factors. They show: small, enduring and often needle-shaped leaves with cuticle equipped with high amounts of wax. Furthermore, these species are characterized by: prolonged seedling stage; blooming occurring several times during life cycle but without a close dependence on the season; limited amounts of energy used for seed production; slow growth; limited response (in physiological terms) to stress; accumulation of photosynthetic products in leaves, stems and roots; palatability by specialized herbivores reduced or absent.

Competitor (C) are species that can maximize the exploitation of resources in relatively fertile and unmanaged environments. They are characterized by: dense and highly branched leafy and radical structure; short-lived leaves with growth peaks that occur during the period of maximum potential productivity of the environment; blooming after the period of maximum potential productivity. These species also show: limited energy allocated for seed production; seeds that often present dormancy; rapid growth and morphological response to stress, accumulation of photosynthetic products preferably in vegetative structures; palatability by herbivores variable.

Ruderal species (R) show: low development; limited branching; very short seedling stage; early and abundant flowering. Moreover, they are characterized by: use of large amount of energy for seed production; seeds numerous and characterized by dormancy; rapid growth, response to stress through the use of more resources for seed production; photosynthetic products accumulated only in seeds, palatability by herbivores high.

According to Grime classification, weeds can be defined as competitor-ruderal species, as they are characterized by an evolutionary strategy intermediate between the type Competitor and the type Ruderal (Zanin *et al.*, 2001).

2.2.4. Botanical classification

The knowledge of botanical classification of a weed species as well as the systematic relationship with the cultivated plants are of fundamental importance. It allows to explain the effect of the cultural practices on the evolution of weed communities and to understand the extent of the competitive relationship between weed and crop. In addition, the botanical similarity/dissimilarity between weeds and crop may influence the selectivity of herbicides (Zanin *et al.*, 2001).

A generic classification system is the one proposed by Ross and Lembi (1985), which includes the division into dicotyledonous and monocotyledonous species. These two groups are commonly shortened in “dicots” and “monocots”, respectively.

Dicots are characterized by the presence of two cotyledons or seed leaves. Commonly, they are identified with term “broadleaved plants”.

Monocots are plants that present only one cotyledon. The majority of monocot weeds belong to two families, *Poaceae* and *Cyperaceae*, commonly defined with the terms “grasses” and “sedges”, respectively. The first one includes the monocots whose leaves generally show a ligule or occasionally an auricle; the leaf sheaths are split around the stem, with the stem being round or flattened in cross section with hollow internodes. The second group includes monocots with leaves lacking of ligules and auricles; the leaf sheaths are continuous around the stem, which is triangular in section in many species and with solid internodes (Radosevich *et al.*, 1997).

3. Weed management

Weeds widespread in an agricultural environment can be considered as the ecological and evolutionary reaction exhibited by indigenous and introduced flora of a specific area to opportunities and challenges represented by crop cultivation (Jordan and Jannink, 1997; Mortimer, 1997; Clements *et al.*, 2004).

Different studies have attempted the assessment of economic and environmental costs resulting from the presence of weeds (Neve *et al.*, 2009). According to Oerke (2006), weeds represent, among all pests affecting crops, those that are able to cause the highest production losses (34%). It was estimated that the average potential losses due to weeds are equal, globally, to 23% in wheat, 37% in rice, 40% in maize, 30% in potatoes, 37% in soybean and 36% in cotton (Oerke, 2006).

In order to contain the adverse effects on development and yield of crops resulting from the presence of weed species, it is possible to apply different control means.

The strategies for weed management can be divided in non-chemical means and chemical means.

3.1. Non-chemical control means

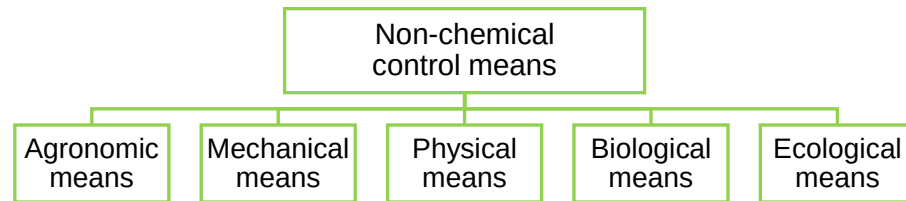


Figure 1. Non-chemical means applied for weed management (modified from Ferrero and Casini, 2001a; Pannacci *et al.*, 2017).

3.1.1. Agronomic means

Agronomic means of weed control comprise: prevention, crop rotation, tillage, varieties selection, crop establishment, fertilization and irrigation (Pannacci *et al.*, 2017).

- **Prevention.** Preventing the spread of weeds species and avoiding the introduction of new ones are the principal aims of this strategy. These objectives can be achieved through: the utilization of high purity seeds; the cleaning of machineries if they were used in a field where perennial weeds were present; the filtration of irrigation water; the recourse to an efficient hydraulic system to escape water stagnation, diffusion of some perennial weed species, limited crop development rate; the removal, before they disseminate, of weeds problematic to control (Pannacci *et al.*, 2017).
- **Crop rotation.** This strategy has a high influence on weeds community and is able to prevent the appearance of a dominant weed species (Hosseini *et al.*, 2014). With rotation, therefore, it contributes to the maintenance of weed community in which there are no species that have developed herbicide resistance, with a balanced presence of the different biological groups and where there is no species similar to crop. All of these elements contribute to the selection of a weed community characterized by less competitive capacity and easier to control (Eyre *et al.*, 2011; Bonciarelli *et al.*, 2016).
- **Tillage.** Soil seedbank and weed emergence are highly affected by tillage. It affects depth of seeds distribution and environmental characteristics to which seeds themselves are exposed in the soil and, consequently, their germination ability (Chauhan and Johnson, 2009b).
- **Varieties selection.** As well as the quantity and quality of production and resistance capacity to cope with biotic and abiotic stress, crop varieties should also be selected based on their competitive ability

against the weeds. Such capacity can be achieved by selecting characters such as radical growth, faster initial development, high leaf surface and production of allelopathic substances (Pannacci *et al.*, 2017). To those, agronomic strategies such as plant spatial pattern, sowing time and reduction of the distance between crop rows can be added (Mahajan and Chauhan, 2011, 2013).

- **Crop establishment.** A regular emergence and a well-established crop are key elements in achieving high competitive ability of crops against weed species. These characters are determined by: high vigour of crop's seeds, correct preparation of the seedbed and appropriate sowing depth (Pannacci *et al.*, 2017).
- **Fertilization.** In general, weeds are characterized by a nutrient use efficiency higher than crops. For this reason, it is hoped that strategies (such as the changing of timing, placement and source) that can favour a better availability of resources for crop are adopted (Nichols *et al.*, 2015).
- **Irrigation.** Opportunities of weed species management can, in irrigated environment, result from the spatial and temporal variation in soil moisture. In presence of a top layer of dry soil, planting large-seeded crops deeper into moister soil can favour crop germination and emergence, providing the crop with an starting advantage over weeds (Nichols *et al.*, 2015).

3.1.2. Mechanical means

Mechanical control represents, in agricultural production, one of the weed management tools alternative to the use of chemical applications. These methods of weed control can be adopted both as indirect / preventive measures in order to decrease the growth and the seed dispersal both as direct / curative means aimed at eliminating the weeds present (Ferrero and Casini, 2001b).

3.1.3. Physical means

Physical means of weed management include: mulching, solarization, flooding and thermal actions (Marenco and Lustosa, 2000; Bond and Grundy, 2001; Ferrero and Casini, 2001c; Virbickaite *et al.*, 2006; Rask and Kristoffersen, 2007).

- **Mulching.** It is accomplished by partial or total coverage of the soil with the use of opaque material. Thus, germination or development of weeds are limited.
- **Flooding.** This technic is based on the important action of weed control provided by water. The presence of water on soils prepared in advance for the false seeding encourage the contemporary emergence of weeds and makes their control easier (Ferrero and Casini, 2001c).

- **Heat transfer.** They represent a direct method of weed management. These strategies are based on the use of flaming by gas, hot water and water steam actions (Virbickaite *et al.*, 2006). Weed control is done through exposure to an intense heat wave (Bond and Grundy, 2001). Among heat-based systems, solarization represents a technique of soil disinfection and disinfestation through the use of solar energy and the soil application of transparent polyethylene film. In favourable climatic environments, this technique has demonstrated excellent efficacy in the control of weeds, fungal pathogens and nematodes.

3.1.4. Biological means

Biological control is defined as the use of a biotic agent with the aim to restrain or diminish a population of a pest (Boyetchko, 1997). In the case of weed control, natural enemies or plant pathogens are used in order to decrease the density of a target plant species below its economic threshold of damage (Scheepens *et al.*, 2001).

In crops, four different techniques of weed control can be found: inoculative or classical approach, inundative or microbial herbicide approach (Müller-Schärer *et al.*, 2000), conservative approach and biological broad spectrum control (Ferrero and Casini, 2001d).

- **Inoculative or classical approach.** It is intended to contain naturalized exotic weed species by introducing one or numerous exotic organisms that came from origin area of the weed. The introduction of such organisms proceeds until they have been successfully established. In general, insects and pathogenic fungi of plants represent the biotic agents employed for this weed control method (Scheepens *et al.*, 2001).
- **Inundative or microbial herbicide approach.** In this case weed control is based on the release, over the whole weed population to be controlled, of a high amount of the control agent. In order this method to be effective, it is necessary to repeat the application of the agent during every growing season or multiple times for each crop cycle (Scheepens *et al.*, 2001).
- **Conservative approach.** It involves the management of indigenous weeds by conservation and enhancement of their control agents (predators, parasites and pathogens). In order to achieve this aim, it is necessary try to limit the presence of organisms that, in nature, regulate the population of the biological agent (Ferrero and Casini, 2001d).
- **Biological broad-spectrum control.** This is a non-selective control method. It has ancient origins, dating back to when sheep, cattle and horses were used to manage vegetation at the margins of cultivate fields or to burn stubble after harvesting cereals.

To date, more interesting examples are the application of this approach to the control of aquatic vegetation in some particular situations, such as artificial basins or irrigation drains (Ferrero and Casini, 2001d).

3.1.5. Ecological means

All improvements and adaptation of the management methods of a crop or of a crop system that allow to regulate the presence of weed species and to limit their negative effect on crop performance can be considered as ecological control means (Bastiaans *et al.*, 2008).

An important ecological tool of weed control is represented by the use of cover crops (Kruidhof, 2008). They can be inserted in rotation between a crop and the next or can be present concurrently during a part or all of a crop cycle (Upadhyaya and Blackshaw, 2007). The function of cover crops is to suppress weed emergence and growth mainly as consequence of soil shading and inter-specific competition (Ferrero and Casini, 2001e).

3.2. Chemical control means: herbicide applications

Herbicides represent the principal method for weed control. Introduced at the end of 1940, they have been responsible of a profound change in weed management replacing the manual labour, the use of animals and the mechanical interventions (Heap, 2014). They were characterized by high success because of their efficacy (Shaner, 2014), their significant contribution in increasing crop yield (Heap, 2014) and for allowing a cheap and easy weed management (Shaner, 2014).

The first synthetic herbicides to be broadly applied were the phenoxyacetic acids; this category of herbicides was detected at the time of the World War II and were developed commercially after the war.

New classes of herbicides were found and adopted for weed management across the 1950s and early 1960s. These classes included the substituted ureas and uracils, the chlorobenzoic acids and phenylacetic acids derivatives, the s-triazines, triazoles, and other heterocyclic derivatives (Shaner, 2014).

As happens for all technologies, herbicides have been confronted with some challenges: safety, environmental problems and the appearance of weeds resistant to them are the most important ones (Heap, 2014).

4. Herbicide weed resistance

Resistance to herbicides can be considered as a common and foreseeable consequence of natural selection (Heap, 2014).

Resistance is defined as “*the inherited ability of a plant and at least a part of its descent to survive following exposure to an herbicide able to effectively control, when applied correctly, the weed population*”. The transmissibility of the character “resistance” to the next generation is, therefore, the main aspect of resistance phenomena (Lucchin *et al.*, 2001).

It may happen that, within a weed population, there are individuals characterized by the presence of rare mutations that confers resistance to an herbicide before it has been applied (Heap, 2014). The resistance development is the consequence of a selection process driven by the repeated adoption of the same herbicides or the same mechanisms of action (Shaner, 2014).

Being the most economical form of weeding, herbicides found quickly use where labour costs were high. However, the method for herbicide application varied according to the context considered. In Europe and North America, where the herbicide costs (compared with total input) were low and the crops often subsidized, high herbicide doses were usually applied. On the contrary, where subsidies to crops were not provided (as in the case of Australia), the herbicide doses used by farmers were the minimum required and frequently a low presence of weeds was accepted. The herbicide application rate was closely correlated to the selection pressure. Europe and North America were the first areas to resort to the use of herbicides as well as to adopt new mechanisms of action. It has been hypothesized that first cases of herbicide resistance occurred in these regions as a consequence of the chronology and the high selection pressure (Gressel, 2009).

The first documented case of herbicide weed resistance dates back to 1957; it has occurred in wild carrot in Ontario, Canada (Shaner, 2014). Nevertheless, other events of resistance have not been reported until 1965.

The first severe case of herbicide weed resistance appeared in 1968 when a population of *Senecio vulgaris* showed resistance to simazine or atrazine (Shaner, 2014).

A remarkable worsening of herbicide resistance phenomenon was observed in the 1980-1995 period (Figure 2), when the number of cases increased from 41 in 1980 to 191 in 1995. This increase was mainly due to a sudden and fast selection of populations resistant to ALS and ACCase inhibitor herbicides (Shaner, 2014).

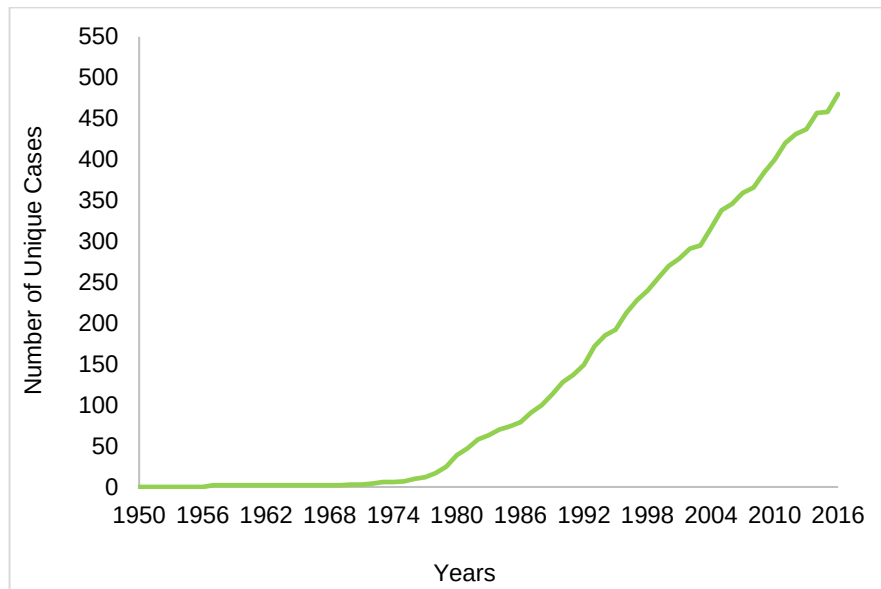


Figure 2. Trend of the increase in cases number of herbicide resistant weeds worldwide. (Modified from Heap, 2017a).

4.1. Mechanisms of resistance to herbicides in weeds

The mechanisms of herbicide resistance developed by plants may appear as a result of alterations occurring at the genetic level or as a consequence of physiological adaptations.

Resistance phenomena often result from alterations in the target site structures on which the herbicide acts. Such modifications are the consequence of mutations that may cause a reduction in the linkage efficacy between herbicide and target site or an obstacle to linkage formation.

In other cases, the appearance of resistance involves plant metabolism with possible alteration of the herbicide absorption or translocation in weed.

The resistance mechanisms that can be selected by plants are different, as well as the resulting resistance behaviour. Main resistance behaviour are (Follis *et al.*, 2011):

- simple resistance: resistance to an herbicide or to herbicides of the same chemical class;
- cross resistance: resistance to herbicides belonging to different chemical classes;
- multiple resistance: as a result of the presence of multiple mechanisms able to cause resistance to one or more herbicides.

4.1.1. Target site resistance

The development of target site herbicide resistance can be the consequence of an alteration in the target site or of a gene amplification/overexpression.

- **Alteration in the target-site.** This mechanism of resistance is characterized by the presence of structural changes; these changes are the consequence of point mutations in herbicide-binding proteins (Matzrafi *et al.*, 2014). Resistance phenomena to the herbicides ALS-inhibitors, ACCase-inhibitors, dinitroanilines and triazines are frequently the consequence of target-site resistance development (Heap, 2014). Some of the herbicide-binding proteins in which the point mutations can occur are: D1 protein in the photosystem II (PSII) complex (resistance to triazines), acetolactate synthase (ALS; resistance to ALS-inhibitors), acetyl-CoA-carboxylase (ACCase; resistance to ACCase-inhibitors), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; resistance to glyphosate).

Target-site resistance can occasionally be the consequence of an increased number of copies of target gene, such as in the case of EPSPS (Matzrafi *et al.*, 2014).

The development of this type of resistance is the consequence of the repeated application of a single herbicide (or group of connected herbicides) and/or to the exclusion of alternative techniques of weed control. It was showed that the evolution of weed resistance is also influenced by the dose of herbicide: a high dose tends to improve the progress of target-site resistance (Yuan *et al.*, 2007).

- **Gene amplification/overexpression.** In this mechanism of resistance an increase in the synthesis of target enzyme occurs. As a consequence of this, it is necessary that a greater concentration of herbicide reaches the target site so that the enzyme is inhibited and the plant death is caused (Heap, 2014).

4.1.2. Non-target site resistance

Non-target site resistance occurs as a consequence of mutations that involves parts different from the target site (Yuan *et al.*, 2007). On the contrary to what occurs for the target-site, non-target-site resistance can result in the appearance of resistance to herbicides characterized by different mechanisms of action (Petit *et al.*, 2009, 2010). Moreover, compared to target-site resistance, the non-target-site seems to occur as a more generic reaction to herbicides. The mechanisms governing the non-target-site resistance can be considered a subset of reactions that plants implement against abiotic stresses (Yuan *et al.*, 2007; Délye, 2013). This type of resistance may be constitutive, stress-induced, or in part probably both (Cummins *et al.*, 2009). It has been demonstrated that the non-target-site resistance type constitutive is related to greater

secondary metabolism to plant level (Cummins *et al.*, 2009). This hypothesis is supported by the fact that the herbicide usage is a stress; this stress primes reaction pathways in all weed individuals regardless of whether they are sensitive to the herbicide (Délye, 2013).

The mechanisms that induce non-target-site resistance are supposed to be different and variable within and between species. This is in line with the evidences supporting that resistance is controlled by a multigenic complex (Petit *et al.*, 2010); however, cases of monogenic control can be found (Mithila *et al.*, 2012).

Furthermore, the mechanisms of non-target-site resistance may also change on the basis of herbicide (Powles and Yu, 2010; Beckie and Tardif, 2012) (Délye *et al.*, 2013). This type of resistance holds a key role in the selection of resistance to ACCase-inhibitor herbicides; moreover, non-target-site is most probably the main mechanism of resistance to ALS-inhibitors in grass weeds (non-target-site cases to ALS-inhibitors found in broadleaf weeds were rarely found) (Délye, 2013).

The main mechanisms of non-target-site resistance showed by weeds can be classified in: enhanced metabolism, decrease in absorption and/or translocation, sequestration of the herbicide.

- **Enhanced metabolism.** This is a type of resistance based on the increased capacity of a plant to metabolize the herbicide before it causes its death (Heap, 2014). It is a process based on multiple steps involving various types of enzymes. This process can be essentially separated into three phases (Kreuz *et al.*, 1996; Délye, 2013). The first is represented by the transformation of the herbicide molecule into a metabolite more hydrophilic. The second phase consists in the conjugation of the metabolite obtained in the first phase with a plant acceptor molecule. In the third phase, after additional conjugations, cleaving and/or oxidation steps, the metabolite is ejected from the vacuole and/or the cell walls; outside of them, additional degradations may happen (Délye, 2013).

Enhanced metabolism seems to be the main mechanism underlying resistance to herbicides included in groups ACCase and ALS-inhibitors (Powles and Yu, 2010; Beckie and Tardif, 2012).

- **Decrease in absorption and/or translocation.** In this case herbicide translocation to the site of action is hampered for some reason. This allows the plant to survive the herbicide application (Heap, 2014).

Plants resistant to all the principal herbicide mechanisms of action (EPSPS inhibitors (Michitte *et al.*, 2007; Vila-Aiub *et al.*, 2012), ALS-inhibitors (White *et al.*, 2002) and ACCase inhibitors (De Prado *et al.*, 2005)) have developed the ability to reduce the herbicide absorption. In several cases, resistant plants have differences in the physical and chemical characteristics of the cuticle, that may cause a reduced

retention of the herbicide solution on the leaves and/or a reduced herbicide absorption through the cuticle.

The reduced translocation results in a limitation of the herbicide flow inside the plants and/or in the its compartmentation (Délye, 2013).

- **Sequestration of the herbicide.** Resistance is due to the herbicide sequestration into cell wall or into vacuoles. This behaviour causes a reduction of the concentration of herbicide at the level of the site of action (Heap, 2014).

4.1.3. Cross-resistance

Cross-resistance occurs when a single mechanism among those listed above causes resistance to more than one herbicide (Heap, 2014).

The mechanism of cross-resistance can be target site type or non-target site type (Beckie and Tardif, 2012).

- **Target site cross-resistance.** Target-site cross resistance occurs when an alteration in the enzyme causes resistance to several herbicides which act on the same target site (Heap, 2014). This type of resistance does not necessarily involve all classes of herbicides characterized by similar mechanism of action or all herbicides that are included in a given class (Powles and Preston, 1995).
- **Non-target-site cross resistance.** Non-target site cross-resistance is considered as cross resistance to different classes of herbicides. This type of resistance is due to a mechanism (s) different from resistant enzyme target sites (Powles and Preston, 1995).

A particular case of cross-resistance is **negative cross-resistance**, which occurs when a weed resistant to a certain herbicide exhibits an enhanced sensitivity to other herbicides (Lucchin *et al.*, 2001).

4.1.4. Multiple resistance

Multiple resistance occurs when an individual developed different mechanisms of resistance to one or more herbicides that belong to the same or to diverse chemical families (De Prado and Franco, 2004). It represents one of the most serious current and future problems of management of herbicide resistance (Powles and Preston, 1995).

This type of resistance is commonly the results of a sequential selection of resistance mechanisms occurring when herbicides that acts on different sites of action are used for prolonged time or as a consequence of accumulation of resistance genes by pollen movement (Heap, 2014). Cases of multiple resistance selection mediated by pollen flow happen in outcrossing species, such as *Lolium rigidum*, *Alopecurus myosuroides*, *Kochia scoparia* and various *Amaranthus* spp. (Beckie and Tardif, 2012).

The commonest cases of multiple resistance occur in the case in which a singular plant or population is characterized by the presence of two or more resistance mechanisms that cause resistance to a single herbicide or to a class of herbicides. The most complex cases occur when, in a sequential manner or simultaneously, herbicides belonging to different chemical classes select two or more distinct mechanisms of resistance. Management of herbicide-resistant weeds is particularly difficult when within the same individual different resistance mechanisms, both target-site and non-target site are present (Powles and Preston, 1995). Currently, the most frequent cases of multiple resistance are represented by weed species resistant to two mechanisms of action (Figure 3).

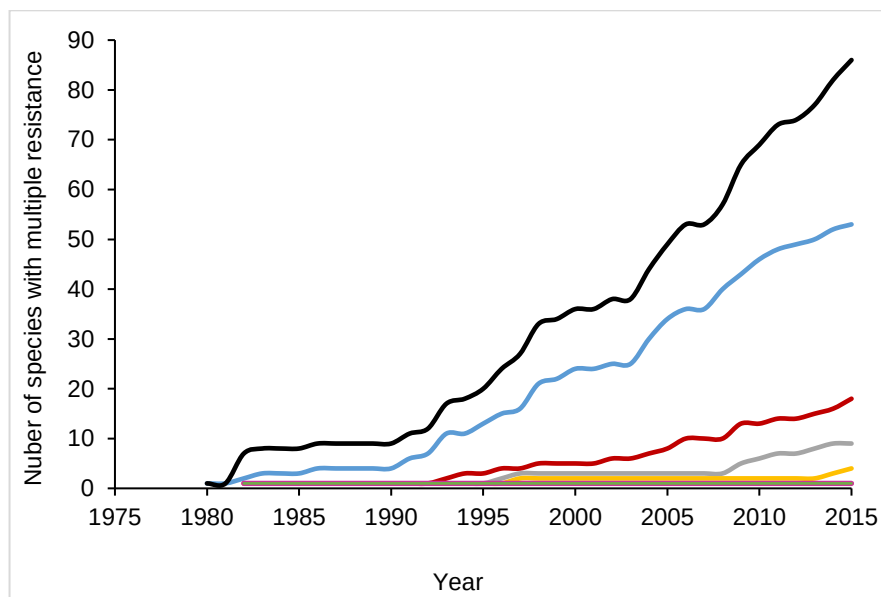


Figure 3. Increase in the number of weed populations affected by resistance to multiple mechanisms of action (MoAs). Blue line: 2 MoAs; red line: 3 MoAs; grey line: 4 MoAs; orange line: 5 MoAs; violet line: 6 MoAs; green line: 7 MoAs; black line: cumulative. (Modified from Heap, 2017a).

4.2. Factors of induction to the development of herbicide resistance

The propensity for the development of herbicide resistance depends, principally, on three elements: weed, herbicide and cultural practices (Vaughn and Fuerst, 1985).

4.2.1. Weed

Several patterns affect the levels of selection of weeds resistant to herbicides with different mechanisms of action. The ability to select resistance is mainly due to the characters of the weed species sprayed, in particular: fecundity, breeding system, generation time, fitness in absence of the herbicide, seed longevity, gene movement through pollen and seeds, seed dormancy. Moreover, the frequency, number and fitness of genes responsible for resistance to every mechanism of action and the amount of individuals sprayed during the years contribute to the development of resistance. The last of the above aspect is of fundamental importance: when an herbicide is applied over high extension and controls a large number of species, it is likely that several weeds develop resistance (Heap, 2014).

4.2.2. Herbicide

Herbicide features involved in the development of resistance are represented by: site of action, mechanism of action, persistence and absence of herbicide rotation.

- **Site of action.** An herbicide with a single site of action tends to select resistant weeds faster than an herbicide characterized by various mechanisms of action (Bhatti *et al.*, 2013).
- **Mechanism of action.** Herbicides against which weeds have developed an increased metabolism are more exposed to the selection of resistant cases compared to herbicides with resistance caused by mutation in the site of action (Dyer *et al.*, 1993).
- **Persistence.** The application of herbicides characterized by a strong residual activity involves the constant elimination of the susceptible biotypes. This allows the resistant species to be selected (Koch *et al.*, 2004).
- **Absence of herbicide rotation.** Herbicide rotation, as well as herbicide mixture, is considered to be of fundamental importance in the management of resistance (Beckie and Reboud, 2009). The use of the same herbicides or the same mechanisms of action may result in the appearance of populations no longer controlled by them (Heap, 2014).

4.2.3. Cultural practices

Among some of the cultural practices applied by farms and potentially responsible for the appearance of weed populations resistant to herbicides there are: monoculture, minimum tillage or no tillage, application of the same herbicide or combination of herbicides with the same mechanism of action, use of inappropriate herbicide doses (in

particular the application of lower or higher doses compared to the recommended field rate) (Bhatti *et al.*, 2013).

4.3. Herbicide involved in resistant selection

Among the groups of herbicides that selected the most resistant weeds are counted: ALS inhibitors, ACCase inhibitors, PSII inhibitors, synthetic auxins, bipyridilliums and glycines (Heap, 2014).

ALS-inhibitors, in particular, are the herbicides that have selected the greatest number of resistance cases over time (Figure 4).

ALS inhibitors. They represent the group of herbicides that has been applied for more than 30 years in all the main crops and for the control of most of the weed species. The site of action of ALS inhibitors is the enzyme acetolactate synthase. This enzyme is particularly prone to show gene point mutations in consequence of which the appearance of resistance phenomena to these herbicides occurs.

The first recorded case of resistance developed against ALS inhibitors was a metabolic resistance; it occurred in 1986 in Australia, when a population of *Lolium rigidum* showed resistance to chlorsulfuron (Heap, 2014).

The first case of target site resistance occurred in 1987 in USA in *Lactuca serriola* (Mallory-Smith *et al.*, 1990).

Currently, the major cases of resistance are found in weeds of rice and wheat (Heap, 2014).

ACCase inhibitors. Since 1974, the year of their introduction, the ACCase inhibitors were applied in various crops.

Currently, over 25 million hectares of cereals are affected by the presence of weeds resistant to these class of herbicides (Heap, 2014).

Eleven are the known target-site amino acid substitutions in the acetyl CoA carboxylase gene; these substitutions result in a modified and insensitive form of the ACCase enzyme (Brown *et al.*, 2002; Delye, 2005; Liu *et al.*, 2007; Hochberg *et al.*, 2009).

Metabolic resistance occurs through the cytochrome P450; this type of resistance is common in *Lolium rigidum* and *Alopecurus myosuroides* (Heap, 2014).

PSII inhibitors. They are a group of herbicides characterized by predisposition to the selection of resistance cases; this occurs because the initial frequency of the individuals not controlled is relatively great and because these herbicides were applied on a large scale for a long time (Heap, 2014).

Synthetic auxins. Introduced for the first time in the 1940s, synthetic auxins are among the most applied herbicides still nowadays (Heap, 2014).

Currently, 34 are the weed species that developed resistance to this class of herbicides (Figure 4).

Bipyridilliums. The first cases of weed resistance to bipyridilliums was reported in 1980s.

The principal mechanisms of resistance developed by weeds are represented by reduced translocation and sequestration (Heap, 2014).

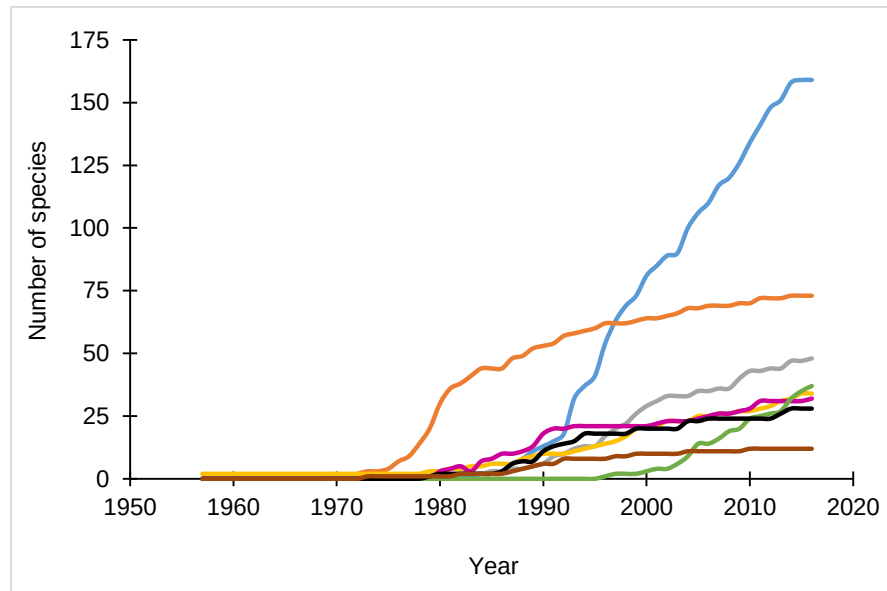


Figure 4. Increase in the number of weed species resistant to different mechanisms of action worldwide. Blue line: ALS-inhibitors; red line: triazines; grey line: ACCase inhibitors; orange line: synthetic auxins; violet line: bipyridilliums; green line: glycines; black line: ureas, amides; brown line: dinitroanilines (Modified from Heap, 2017a).

4.4. Weeds resistant to herbicides and difficult to control

Among the herbicide resistant weeds having a high relevance ad global level, the genera *Lolium*, *Amaranthus*, *Conyza* and *Echinochloa* includes populations that pose serious threats to weed management in several cropping systems (Figure 5).

These genera are characterised by a high genetic variability and the ability to develop resistance to a broad number of herbicide sites of action.

Lolium rigidum has evolved resistance to 12 herbicide mechanisms of action. *L. rigidum* is characterized by a great genetic variability and quickly develops resistance phenomena to almost any herbicide applied for its

control. The frequent development of target site and non-target site cross resistance as well as the rapid appearance of multiple resistance to a broad range of herbicides through outcrossing makes this weed particularly problematic. Multiple resistance phenomena are also found in *L. multiflorum*.

The second most troublesome weed in the world for the selection of resistance cases is *Avena fatua*. This weed infests everywhere cereal crops and had developed resistance to the herbicides used. Compared to *L. rigidum*, *A. fatua* is characterized by self-crossing (about only 5% of out-crossing) and by the production of a smaller number of seeds per plant; for these reasons, the weed is not as fast as *L. rigidum* in developing multiple resistance phenomena. Nevertheless, worldwide and in cereals, *A. fatua* is more prevalent than *L. rigidum* and its ability to evolve herbicide resistance can be considered an aspect of high economic relevance.

Other weeds that have developed resistance phenomena to herbicides causing serious economic injury are: *Echinochloa crus-galli*, *Conyza canadensis*, *Echinochloa colona*, *Alopecurus myosuroides*, *Eleusine indica*, *Chenopodium album*, *Kochia scoparia*, *Amaranthus retroflexus*, *Amaranthus tuberculatus*, *Amaranthus palmeri* (Heap, 2014).

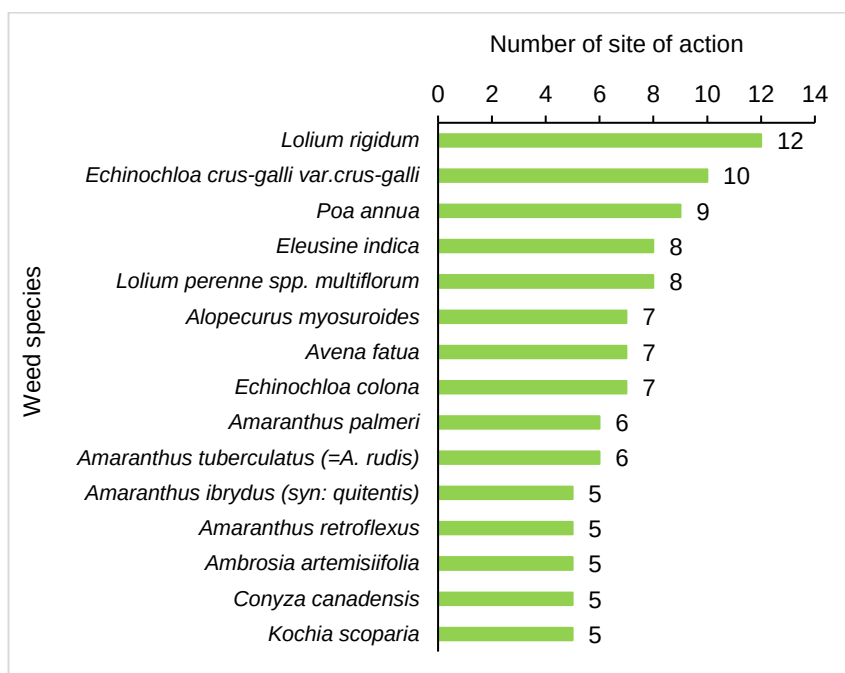


Figure 5. Weed species that have developed resistance to herbicides with different sites of action. (Modified from Heap, 2017a).

4.5. Situation of herbicide weed resistance worldwide

Nowadays, 480 unique cases (weed x site of action) of herbicide resistance phenomena are found overall and concern the large majority of known sites of action of herbicides (23 out of 26) and 163 different herbicides. Resistance cases are reported in 251 species (146 dicots and 105 monocots) and affect 91 crops in 69 countries.

Globally, the higher number of resistant weeds is reported in the USA with 160 cases, followed by Australia (88 unique cases), and Canada (65 unique cases) (Heap, 2017b). The crops affected by the greater number of resistance cases are wheat (74 resistant weed species) and corn (61 resistant weed species), followed by rice (51 resistant weed species) and soybean (47 resistant weed species) (Figure 6).

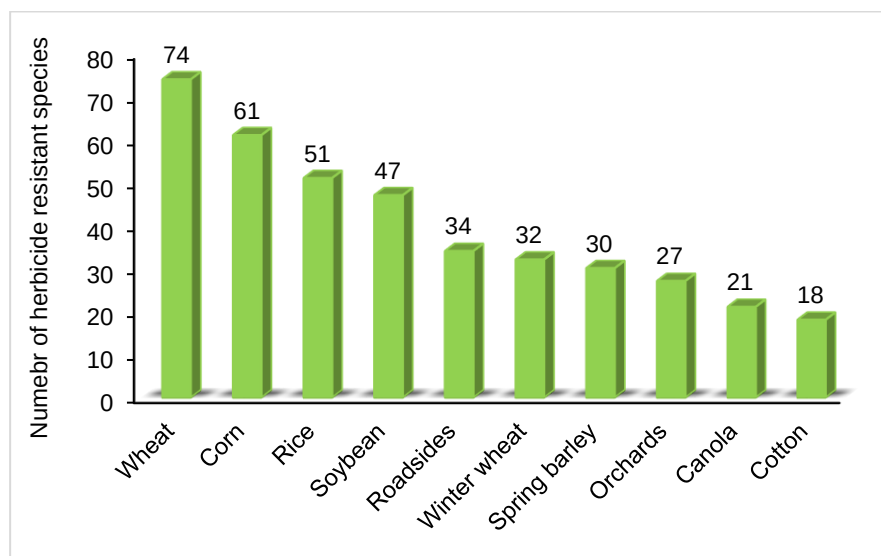


Figure 6. Top ten crops affected by herbicide resistant weed species. (Modified from Heap, 2017a).

4.6. Situation of herbicide weed resistance in Europe

4.6.1. France

In Europe, France has the major number of resistance cases (48) of weeds resistant to herbicides. The first case of herbicide resistance was detected in 1978 when a population of *Chenopodium album* and a population of *Poa annua* resulted no longer controlled by atrazine. The first of these two cases occurred in corn, the second one in orchards.

From 1978 until 1989 several weed species developed resistance to atrazine.

The first case of weed resistance to ACCase inhibitor herbicides was found in 1993 and affected a population of *Alopecurus myosuroides* while the first appearances of resistance to ALS-inhibitors were found in 2006 in populations of *A. myosuroides*, *L. rigidum*, *Apera spica-venti*, *Avena sterilis* and *A. fatua* (Heap, 2017b).

4.6.2. Spain

In Spain, 37 are the cases of resistance to herbicides reported up to now. The first occurred in 1981 in a population of *Panicum dichotomiflorum* no longer controlled by atrazine.

The first appearances of resistance to ACCase and ALS-inhibitor herbicides were found, respectively, in 1992 in a population of *L. rigidum* and in 2000 in a population of *Alisma plantago-aquatica* and *Cyperus difformis*.

The first case of multiple resistance was detected in 1992 in a population of *L. rigidum*, that showed resistance towards ACCase inhibitors and the PSII inhibitors. Another case of multiple resistance to two sites of action occurred in 1993 in a population of *Papaver rhoeas* and interested the ALS-inhibitors and the synthetic auxins.

Multiple resistance to three sites of action was found, for the first time, in 2015. This resistance affected ACCase inhibitors, ALS-inhibitors and PSII inhibitors and was developed by a population of *Alopecurus myosuroides* (Heap, 2017b).

4.6.3. Germany

In Germany, resistance to herbicides counts 32 cases. The first one was observed in 1978, when a population of *Stellaria media* resulted no longer controlled by atrazine.

Five cases of multiple resistance to two sites of action were found in 1980, 1983, 2008, 2009. The weed populations interested were, respectively, *A. retroflexus* (multiple resistance to Photosystem II inhibitors and PSII inhibitors), *A. myosuroides* (multiple resistance to ACCase inhibitors and PSII inhibitors), *L. perenne* (multiple resistance to ACCase inhibitors and ALS-inhibitors), *A. myosuroides* (multiple resistance to ACCase inhibitors and ALS inhibitors) and *A. fatua* (multiple resistance to ACCase inhibitors and ALS-inhibitors).

A case of multiple resistance to three sites of action (ACCase inhibitors, ALS-inhibitors and PSII inhibitors) occurred, for the first time, in 2009 in a population of *A. spica-venti* while the first appearance of multiple resistance of four sites of action was detected in 2007 in a population of *A. myosuroides* (Heap, 2017b).

4.6.4. United Kingdom

In United Kingdom, herbicide resistance phenomena are found in 27 cases. The first was reported in 1977 in a population of *Senecio vulgaris* that developed resistance to metribuzin and simazine.

The first appearance of resistance to ACCase inhibitors was detected in 1982 while the first case of resistance to ALS-inhibitors was recorded in 1984; both these two first appearances of resistance were found in *A. myosuroides*.

Multiple resistance to three sites of action (ACCase inhibitors, ALS- inhibitors and microtubule mitotic disrupters) was reported for the first time in 1993 in a population of *A. sterilis* and the next year in a population of *A. fatua* (Heap, 2017b).

4.6.5. Italy

In Italy, a total of 30 cases of herbicide resistance phenomena have been reported up to now. The first case of resistance occurred in 1978 when a population of *Solanum nigrum* resulted no longer controlled by atrazine. The first case of resistance to ACCase inhibitors was discovered in 1992 and affected a population of *A. sterilis*, while the first appearance of resistance to ALS-inhibitors was found in 1994 in a population of *Alisma plantago-aquatica*.

The first case of multiple resistance to two sites of action was detected in 1998 in a population of *Papaver rhoeas* and interested ALS- inhibitors and synthetic auxins. Other phenomena of multiple resistance to two sites of action occurred in 2002 in *L. perenne* ssp. *multiflorum* (multiple resistance between ACCase inhibitors and ALS inhibitors), in 2004 in *E. erecta* (multiple resistance towards PSII inhibitors and cellulose inhibitors) and in *A. sterilis* (multiple resistance towards ACCase inhibitors and ALS-inhibitors), in 2008 in *L. perenne* ssp. *multiflorum* (multiple resistance towards ACCase inhibitors and EPSP synthase inhibitors), in 2009 in *E. crus-galli* var. *crus-galli* (multiple resistance towards ACCase inhibitors and ALS-inhibitors) and in 2012 in *L. perenne* ssp. *multiflorum* (multiple resistance towards ALS inhibitors and EPSP synthase inhibitors) (Heap, 2017b).

Currently, sixteen are the weed species that have developed resistance to various mechanisms of action in indifferent crops. Among these we count *Avena sterilis*, *Echinochloa crus-galli*, *Lolium* spp., *Oryza sativa* (GIRE®, 2017)

The main cases of resistance are found in rice fields. The high risk of herbicide resistance selection is due to several factors: the direct sowing, the monoculture, the introduction of chemical control through the application of selective herbicides and the limited number of mechanisms of action available (Follis *et al.*, 2011). It is estimated that weed control is

carried out through the application of ALS-inhibitors in about 90% of the Italian rice fields.

A contribution in the reduction of the number of mechanisms of action used was caused by the introduction of the Clearfield technology®, a technology that is based on the cultivation of rice varieties tolerant to imidazolinone herbicides (Panozzo *et al.*, 2013) and that represents an important mean for the control of *Oryza sativa* (weedy rice). The repeated application of imidazolinones exerts a selection pressure such as to increase the possibility of resistance development in weeds that infest rice fields, particularly *Echinochloa* spp. (Scarabel *et al.*, 2012).

Among the main species that can be found in rice fields and have developed herbicide resistance phenomena are counted: *Alisma plantago-aquatica*, *Cyperus difformis*, *E. crus-galli.*, *Oryza sativa*, *Schoenoplectus mucronatus* (GIRE®, 2017). *Alisma plantago-aquatica*, as well as *Cyperus difformis* and *Schoenoplectus mucronatus*, evolved resistance to ALS inhibitors starting from 1994 (Panozzo *et al.*, 2013).

In various locations, *Echinochloa* spp. can be considered one of the major rice weeds; in Italy, they represent the main obstacle in rice cultivation (Vidotto *et al.*, 2007).

Echinochloa spp. are characterized by a C₄ photosynthetic pathway and by a high competitive benefit in the case of growth together with C₃ crops (Tabacchi *et al.*, 2006).

The genus *Echinochloa* is characterised by a high variability in morphological, biological and physiological traits. The panicle length can vary from 2 cm to 20 cm, its form can be open pyramidal or closed and columnar, while the panicle bearing can be erect or prostrate. Furthermore, *Echinochloa* spp. show awnless or awned spikelets, with red or green awns (Tabacchi *et al.*, 2006).

Echinochloa spp. are widely distributed around the world and can infest several crops (Panozzo *et al.*, 2013). Some of them (especially *Echinochloa crus-galli*) are highly adaptable to various environment conditions and are able to grow both in dry and in flooded soils. Some studies demonstrate that the competition established by *Echinochloa* spp. can be partly affected by delay in germination and is negligible when rice plots are retained free of weeds for 30 days or more after seeding (Vidotto *et al.*, 2007).

Echinochloa spp. can be discerned in “red” or “white” on the basis of the presence or absence of pigmentation at the basal share of the plant and it has been shown that this morphological trait (red or white pigmentation) could correspond to a different herbicide sensitivity (Tabacchi *et al.*, 2006).

Another important weed infesting rice worldwide is *Oryza sativa* (weedy rice). In Italy, it is found in at least 70% of the total rice cultivation area.

Because of the presence of morphological and physiological common traits, weedy rice has been classified within the same genus and species of the cultivated rice (Fogliatto *et al.*, 2011).

In Italian rice fields, weedy rice was reported, for the first time, at the beginning of the 19th century. However, its management has become an issue from 1920 as a consequence of the adoption of direct seeding and rice monoculture (Fogliatto *et al.*, 2011). Since 1990, the spread in rice fields of this weed increased notably, presumably because of the presence of a wide number of weedy rice seeds in the commercial seed lots and of the cultivation of low-competitive rice varieties.

Weedy rice is characterized by the presence of broad variability in anatomical, biological and physiological traits.

The distinction between weedy populations and rice cultivated can be easily performed only after tillering. Starting from this stage, the weed is commonly characterized by the presence of some distinct traits that permit to distinguish it from the crop; these traits are represented by: numerous and slender tillers, hispid, light green leaves, tall plants and shattering capacity of the grains. Most weedy rice grains present a red pigmentation because of the anthocyanin content; however, phenotypes characterized by white pericarp have been reported (Ferrero *et al.*, 1999).

This weed species is able to adapt fast to various environments and settings because of the polymorphism that characterized it (Fogliatto *et al.*, 2011).

The yield losses caused by weedy rice infestations can vary largely, as response to the weed density and competitive ability of the varieties (Ferrero *et al.*, 1999). In particular, it has been observed that the crop yield reduction ranged from 1% to 13% in the case of weedy rice plants density equal to 1-5%, up to 30% in the case of weedy rice plants density equal to 12-20% (Busconi *et al.*, 2012).

5. Effect of abiotic stress on weed biology

Plant growth may be affected by various abiotic and biotic stresses (Ahuja *et al.*, 2010). To cite some but a few, high and low temperature, drought, alkalinity, salinity, (Parvaiz *et al.*, 2008), high CO₂ (Ahuja *et al.*, 2010), UV stress and pathogen disease are among the most important (Parvaiz *et al.*, 2008).

Abiotic stress occurs when environmental conditions determine growth reduction, and eventually yield losses. The mechanisms by which the plants react to the abiotic stresses are dynamical and complex and can be both elastic (reversible) and plastic (irreversible) (Cramer *et al.*, 2011). In their adaptation to these stresses some of the processes involved are the activation of cascade of molecular networks included in the perception of the stress, the transmission of the signal and the expression of particular genes, and metabolites connected to stress (Vinocur and Altman, 2005). Moreover, the plant reactions to stress may change on the basis of the tissue or organ exposed (Cramer *et al.*, 2011). More in general, plants implement different biochemical and physiological reactions aimed to react and survive to stress (Seki *et al.*, 2003). At the root level, for example, reactions to stress are tissue or cell specific and are very dissimilar based on the type of stress associated. Furthermore, the complexity of the reaction depends on the grade and duration of the stress (it can be acute or chronic) (Cramer *et al.*, 2011).

The impact of environmental stresses on plant growth has become a topic of high relevance in relation to effect of climate changes on plant resources, biodiversity and food security overall (Ahuja *et al.*, 2010). The environmental stresses exerted by climate changes may affect every aspect of plant structure and represent a severe challenge in a perspective of sustainable development of agriculture in a period characterized by a significant increase in the world population (Ahuja *et al.*, 2010).

5.1. Salt stress

Under saline stress conditions, the absorption and over-accumulation of Na⁺ into the cells involves the appearance, in plants, of ionic and osmotic stress (Zhang *et al.*, 2008). The high presence of salt can cause a variable degree of impacts at whole-plant level, including crop growth reduction, yield losses, and plant death.

Salt stress affects all key plant processes, including photosynthesis, protein synthesis, energy and lipid metabolism. The very first detectable effects of salt stress are a decrease in leaf area development, which is eventually stopped if the stress persists and become stronger (Hasegawa *et al.*, 2000; Zhu, 2002).

Furthermore, an increase in the salt concentration during plant growth induces water stress due to the increment of osmotic potential, the increase (at soil level) of ions or other plant toxic components, the increment of ions at plant tissues level (Howard and Mendelssohn, 1999). Moreover, different mutual occurrences are induced by saline stress, such as the inhibition of the enzyme activities in metabolic pathway, the reduction in the efficiency of carbon use, the decomposition of protein and membrane structures (Tsugane *et al.*, 1999).

The formation of active oxygen species, comprising superoxide, hydroxyl radicals, hydrogen peroxide and singlet oxygen, generated during the water stress induced by the saline conditions is thought to play a key role in the inhibition of plant growth (Lee *et al.*, 2001).

5.2. Salt tolerance mechanisms in plants

In order to manage the saline stress, plants implement a wide range of biochemical and molecular mechanisms. Biochemical strategies involved in the formation of products and in the implementation of actions aimed to increase tolerance to salinity are probably acting in an additive and presumably synergistic way (Parida and Das, 2005).

Biochemical pathways involved in salt tolerance are selective storage or exclusion of ions, check of ion interception through roots and translocation in leaves, compartmentalization at the cellular and whole plant levels, production of compatible solutes, alteration in the photosynthetic function, change in membrane conformation, induction of antioxidative species and plant hormones.

The mechanisms that induce salt tolerance are characterized by a different degree of complexity (Bohnert *et al.*, 1995), involving numerous biochemical pathways and aiming at the preservation of the principal processes including photosynthesis, respiration, effective use of water and processes that help to maintain such significant traits as cytoskeleton, cell wall, or plasma membrane-cell interactions (Botella *et al.*, 1994), chromosome and chromatin structure changes (DNA methylation, polyploidization, amplification of specific sequences, DNA elimination) (Walbot and Cullis, 1985).

5.2.1. Ion regulation and compartmentalization

The absorption and compartmentalization of ions are of fundamental importance not only in the case of regular development but also in the case of growth in saline environment, when ion homeostasis is disrupted by the stress (Adams *et al.*, 1992).

High concentration of Na⁺ at the cytoplasm level cannot be tolerated by plants. In consequence of this, under salt conditions, the plant tries to compartmentalize Na⁺ into the vacuole or to compartmentalization of ions

in various tissues in order to facilitate their metabolic processes (Zhu, 2003).

5.2.2. Induction of biosynthesis of solutes

Cytoplasm accumulates compounds characterized by a low molecular mass in order to compensate the ionic balance in the vacuoles. Since such compounds do not hinder the ordinary metabolic processes, they are defined as compatible solutes ; moreover, they substitute water in biochemical process (Hasegawa *et al.*, 2000). Proline (Khatkar and Kuhad, 2000; Singh *et al.*, 2000), glycine betaine (GB) (Wang and Nii, 2000; Khan *et al.*, 2000), sugars (Bohnert *et al.*, 1995; Kerepesi and Galiba, 2000), and polyols (Orthen *et al.*, 1994; Bohnert *et al.*, 1995) are some of the main compatible solutes.

Polyols contain a significant portion of CO₂ adsorbed and provide to various activities in the form of compatible solutes, chaperones characterized by low molecular mass, eliminators of oxygen radicals induced by the stress (Smirnoff and Cumbes, 1989; Bohnert *et al.*, 1995). Polyols work in two manners difficult to distinct in mechanistic way: osmotic adaptation and osmo-protection. In the case of osmotic adaptation, polyols work as osmolytes in order to promote the water retention in the cytoplasmic level and allow the sodium sequestration to the vacuole or apoplast. Cellular structures are preserved through the interaction of these osmolytes with membranes, protein complexes or enzymes. Such compounds present hydrogen-bonding traits by which they are able to protect macromolecules against negative consequence resulting from increased ionic strength in the adjacent media (Crowe *et al.*, 1992). They balance the loss of water that occurs in phase of stress through close combination with protein and membrane constituents (Yancey *et al.*, 1982).

5.2.3. Induction of species with antioxidative action

The active oxygen species need to be removed in order to preserve the ordinary plant development. Yet, active oxygen species operate both as toxic compounds at the cellular level and as mediator agents in stress tolerance stimulation. In order to mitigate the toxicity of the active oxygen species, the plant cells are equipped with a highly effective antioxidant protection system.

Antioxidants can be separated in two categories: the first one is represented by nonenzymic constituents enclosing lipid-soluble and tocopherols connected to the membrane, water-soluble reductants, ascorbic acid and glutathione; the second one is represented by enzymatic constituents enclosing superoxide dismutase (SOD), catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), ascorbate peroxidase (APX) (EC 1.11.1.11) and glutathione reductase (GR) (EC 1.6.4.2) (Lee *et al.*, 2001).

SODs represent the first constituents involved in the detoxification of active oxygen species in plants; they are a set of metalloenzymes responsible to convert superoxide in hydrogen peroxide and singlet oxygen and provide protection to cells against the superoxide produced as a result of oxidative stress (Asada and Kiso, 1973; Fridovich, 1986). At the level of the chloroplast and the cytosol, the activation of APX and GR causes the oxidation and the re-reduction of ascorbate and glutathione (Lee *et al.*, 2001).

The removal of the toxic product of SOD is catalysed by APX through the oxidation of ascorbate to monodehydroascorbate while the regeneration of ascorbic acid is catalysed by GR (Smirnoff, 1993). Moreover, hydrogen peroxide can be reduced to water by the catalase activity, even though the affinity of this enzyme for H₂O₂ is very low (Graham and Patterson, 1982).

Various trials conducted on different plant systems showed that environmental stresses caused by drought, chilling and salt alter the number and the actions of the enzymes involved in the detoxification of active oxygen species (Lee *et al.*, 2001).

5.2.4. Stimulation of plant hormones

In presence of high saline quantities, plants are induced to enhance the synthesis of hormones such as abscisic acid (ABA) and cytokinins (Thomas *et al.*, 1992; Vaidyanathan *et al.*, 1999). It has been noted that ABA attenuates the effect of inhibition of photosynthesis, development and translocation of assimilates that is caused by salt (Popova *et al.*, 1995). Under stress conditions, ABA acts by stimulating the closing of the stomata through rapid change of ionic fluxes in guard cells. Furthermore, ABA plays a role in alterations of gene expression (Leung and Giraudat, 1998).

Under saline stress, the increase of ABA causes a rise in Ca²⁺ absorption allowing to maintain the integrity of membranes; this condition means that, in presence of deep level of external saline concentration, plants can regulate the absorption and translocation in a long term (Chen *et al.*, 2001).

5.2.5. Alteration in photosynthetic process

The photosynthetic pathway is obstructed by salt stress through reduction of water potential. As a consequence, the increase of effectiveness in water use represents the principal goal of salinity tolerance (Parida and Das, 2005).

5.2.6. Molecular mechanism of salinity tolerance

The tolerance to saline conditions is a multigenic character and various genes are involved in the codification of salt-stress proteins. These genes are responsible of codification of the following functional groups: photosynthetic enzymes, enzymes for the synthesis of compatible solutes, vacuolar-sequestering enzymes, radical-scavenging enzymes. The majority of the genes included in the functional groups listed above have been recognized as salt inducible when exposed to stress situations (Wu *et al.*, 1996).

Introduction to the conducted studies

The phenomenon of weed resistance to herbicides is an issue of global relevance. The main cause of the occurrence of this phenomenon is represented by the repeated use of the same herbicides or the same mechanisms of action (Matzrafi *et al.*, 2014). The selection of weed populations resistant to herbicides leads to a less efficient weed management, which in turn results in a stronger competition between weeds and crops in the exploitation of resources and, eventually, in a significant decrease in yields.

In addition to the increased competition from weeds due to herbicide resistance, and to the impact of pest and diseases, crop yields are affected by several other abiotic stresses. Among these, a crucial role is played by salt stress; one of the causes of the occurrence of this stress on plants is represented by the occurrence of saltwater intrusion phenomena.

The studies described below were then realised by considering two main aims. The first one was to analyse the consequence resulting from the repeated use of the same herbicide mechanisms of action on different weed species; the second one was to analyse the influence of salt stress on the biology of two different weed species and of one crop; moreover, the studies were aimed to assess the potential relationship between salt stress and response to herbicide applications.

The first study proposed below refers to the first objective, while the following three studies refer to the second.

The first study was carried out at the Research Centre Agroscope (Nyon - Switzerland) with the aim to verify the appearance of herbicide resistance in various *L. multiflorum* and *A. spica-venti* populations escaped to herbicide field treatments in different Switzerland locations.

The trials were performed in greenhouse; seeds of each weed population were sown into biodegradable alveolar trays filled with soil previously sterilized.

The herbicide mechanisms of action tested for resistance development were ALS and ACCase inhibitors in the case of *L. multiflorum* populations, ALS, ACCase and PSII inhibitors in the case of *A. spica-venti*.

In order to confirm the development of resistance, the populations of both weed species not controlled by the herbicide treatments were subjected to genetic analysis.

The results underlined the appearance of herbicide resistance (target-site, non-target-site and multiple) in some of the weed populations tested during the trials.

The second and the third study were organized with the aim of evaluating the effect of saline conditions on germination of two weed species and a crop; the potential relationship between herbicide resistance and response to salt stress was also analysed.

The second study was conducted on six *Echinochloa* spp. populations, three sensitive and three resistant to ALS-inhibitor herbicides while the third study was carried out on five weedy rice populations, four sensitive and one resistant to the ALS-inhibitor herbicides, and on two rice varieties (Baldo and CL80).

The two studies were carried out in growing chamber under controlled conditions; seeds of weed populations and crop were placed in Petri dishes and subjected to a range of salinity comprised between 0 mM and 400 mM.

During the tests, the percentage of germination, the time required for germination and the growth of roots and shoots were evaluated.

The results showed that weed populations and rice varieties were affected by salinity.

The fourth study was aimed at evaluating the effect of salinity on germination and seedling growth of two weed species (barnyard grass and weedy rice) and a crop (rice), in combination with their response to herbicide treatments.

Trials were conducted in greenhouse; weed populations and crop variety were grown in alveolar trays filled with sand and placed in trays containing 2 l of solution. The salinity range to which weed populations and crop were exposed was comprised between 0 mM NaCl and 250 mM NaCl.

These trials were divided in two subsets: one was set up to assess the effect of growing the plants in different saline conditions only, while in the other the effect of combining salt stress and herbicide treatment was studied.

The physiological parameters analysed were: seed germination, speed of germination, plant height, shoot and root fresh weight, SPAD, content of chlorophyll *a*, chlorophyll *b* and carotenoids.

First study

**Monitoring of the development of herbicide
resistance in Switzerland in 2016**

Winter wheat, together with potatoes, represents one of the main crops of Switzerland (Mouron *et al.*, 2016). In 2014, the area covered by wheat was 88,350 hectares with a production of 550,826 tons (FAOSTAT, 2017). Weeds are considered the most important obstacle for wheat production, as they can be potentially responsible of yield losses greater than those due to pests and diseases (Jabran *et al.*, 2017). It was evaluated that the actual production loss resulting from the presence of weeds is equal to 7.7% while the potential decrease of yield could be equal to 23% (Oerke, 2006).

Weeds compete with crop in the use of resources (light, nutrients and water) and impede the growth through the synthesis of substances with allelopathic effect. Such competition weeds-crop may cause, for wheat, a decrease in leaf area, number of seeds, weight of 1000 seeds and grain yield (Jabran *et al.*, 2017).

The most efficient and cost-effective methods for weed control is represented by herbicides (Matzrafi *et al.*, 2014). Globally, the herbicide classes most utilized for weed management in wheat are represented by a limited number of mode of actions, and in particular by synthetic auxins, ALS-inhibitors and ACCase inhibitors (Jabran *et al.*, 2017). Thus, the repeated use of the same herbicide or the same mechanism of action may result in weed populations no longer controlled by them (Shaner, 2014). Wheat is characterized by a number of herbicide resistant weed species greater than that of the other crops. Worldwide, the development of resistance phenomena to different sites of action has been reported on 298 populations belonging to 74 weed species (Jabran *et al.*, 2017).

The main weeds that have selected herbicide resistance in wheat are, globally, represented by *Lolium* spp., *Avena* spp., *Kochia* spp., *Alopecurus* spp., *Phalaris* spp., *S. media* and *Raphanus* spp. (Jabran *et al.*, 2017).

In Switzerland, the first case of herbicide resistance was reported in 1977, when a population of *Chenopodium album* (Tschuy and Wirth, 2015) resulted resistant to atrazine (Heap, 2017b). Over the next nine years, other broadleaf weeds developed resistance to triazines; among these weeds, we count *Amarantus retroflexus*, *C. polyspermum*, *Conyza canadensis*, *Senecio vulgaris* and *Solanum nigrum* (Heap, 2017b).

The resistance issues assumed importance from 1996 because of the appearance of the first case of resistance to isoproturon in *Apera spica-venti* (Tschuy and Wirth, 2015).

The first case of resistance to ALS-inhibitor herbicides was reported in 2006 in *A. spica-venti* (Tschuy and Wirth, 2015) while the first appearance of resistance to ACCase inhibitor herbicides was detected in 2010 in *Alopecurus myosuroides* (Tschuy and Wirth, 2015).

Currently, the development of herbicide resistance phenomena concerns five different mechanisms of action. The weed species involved (Tschuy and Wirth, 2015) are three monocotyledonous (*A. spica-venti*, *Alopecurus myosuroides* and *Lolium multiflorum*) and a dicotyledonous (*C. album*).

From the point of view of the spatial distribution, the cases of resistance result to be concentrated in some cantons (Agroscope, 2017) (Figure 1).

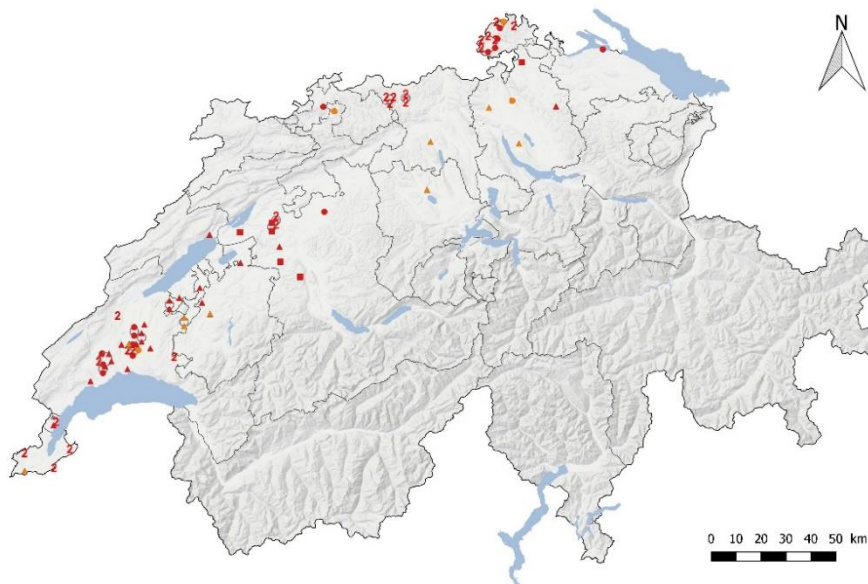


Figure 1. Situation of herbicide weed resistance in Switzerland. Different colours indicate different survival range (orange: 70-85%; red: 85-100%). Symbols indicate the classes of herbicides that developed resistance (Circle: ACCase inhibitors; triangle: ALS inhibitors; square: PSII inhibitors; rhombus: PSII inhibitors (Ureas and amides); star: other). Number 2 indicate double resistance (Modified from Agroscope, 2016).

L. multiflorum

L. multiflorum is an annual weed (Gundel *et al.*, 2008) diffused all around the world (Mahmood *et al.*, 2016). It creates various obstacles in weed control in various cropping systems, including winter cereals in Europe (Mahmood *et al.*, 2016), and it is considered a weed species with a high tendency to develop herbicide resistance phenomena (Mahmood *et al.*, 2016). The first case of resistance in Europe was found when a population of this weed resulted resistant to the ACCase inhibitor diclofop-methyl (Heap, 2017b).

In Switzerland, the first cases of herbicide resistance in *L. multiflorum* were found in 2013 and 2014, with populations no longer controlled by glyphosate in vineyards where this herbicide was repeatedly applied for more than fifteen years. Moreover, in 2014, resistance phenomena to fluazifop were observed in cereals (Tschuy and Wirth, 2015).

Currently, the cases of *L. multiflorum* herbicide resistance result to be concentrated in the Vaud Canton (Figure 2).

A. spica-venti

A. spica-venti results to be one of the most common annual winter weeds in Central and Eastern Europe. The presence of this weed is prevalent in soils with light texture. *A. spica-venti* infests not only winter cereals but also oilseed rape and spring cereals characterized by early sowing (Gerhards and Massa, 2011).

In Switzerland, *A. spica-venti* is widely diffused in cereal crops cultivated in the plateau areas. Moreover, the spread of this weed is growing because of the high presence in crop rotations with winter cereals and the increase in the adoption of minimum tillage.

The first case of resistance was found in 2006, when an *A. spica-venti* population resulted resistance to sulfonylureas (Tschuy and Wirth, 2015). In 2012 and 2013 the first cases of double resistance were observed (Tschuy and Wirth, 2015). Nowadays, *A. spica-venti* populations that developed herbicide resistance are present in different Cantons (Figure 2).

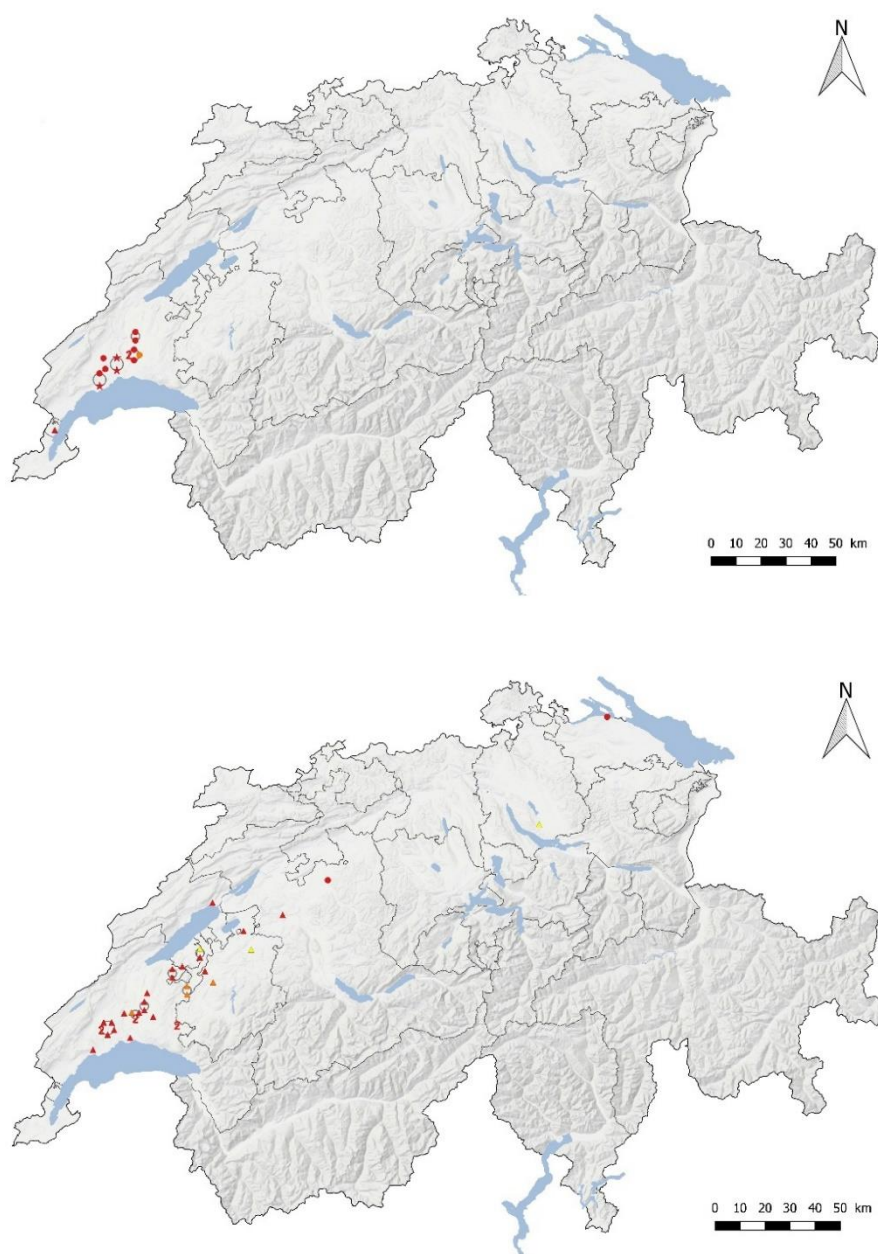


Figure 2. Situation of *L. multiflorum* and *A. spica venti* resistance to herbicides in Switzerland (from top to bottom). Different colors indicate different survival range (yellow: 55-70%; orange: 70-85%; red: 85-100%). Symbols indicate the classes of herbicides that developed resistance (Circle: ACCase inhibitors; triangle: ALS inhibitors; square: PSII inhibitors; rhombus: PSII inhibitors (Ureas and amides); star: other). Number 2 indicate double resistance (Modified from Agroscope, 2016).

The monitoring of the appearance of new cases of herbicide resistance is of crucial importance, especially in areas in which the selection of new resistant weed populations is stressed by the repeated use of few or the same mechanisms of action.

The study was carried out with the aim of evaluating the appearance of resistance phenomena to different herbicide mode of action by *L. multiflorum* and *A. spica-venti* populations. Furthermore, an additional objective was to identify, by genetic analysis, the type of resistance (target site or non-target site) developed by the weed populations no longer controlled by the tested herbicides.

Materials and methods

Plant material

The trials were conducted on nineteen *L. multiflorum* populations and on twelve *A. spica venti* populations. Seeds came from different Switzerland locations and were collected in fields in which the weed populations escaped to the herbicide treatments applied. In particular, in the case of *L. multiflorum*, seeds were collected in the Vaud, Freiburg, Lucerne, Thurgau, Geneva, Aargau and Graubünden cantons. In the case of *A. spica-venti*, seeds came from the Berne, Vaud, Freiburg, Neuchâtel and Graubünden cantons (Table 1 and 2).

Table 1. Tested *L. multiflorum* populations.

Population	Population code
Bournens	Bour.
Boussens	Bou.
Bussigny	Bus.
Cheseaux-sur-Lausanne	Che.
Daillens	Dai.
Essertines-sur-Rolle	Ess.
Goumoens	Gou.
Goumoëns-le-Jux	Goum.
Marbach	Mar.
Montagny Commugny	Mon.
Ottoberg	Ott.
P. Satigny	P. Sa.
Remetschwil	Rem.
Senarclens	Sen.
Verdeaux Pampigny	Ver.
Villigen	Vil.
Vuillerens	Vui.
Yens	Yen.
Zizers	Ziz.

Table 2. Tested *A. spica-venti* populations.

Population	Population code
Aefligen	Aef.
Boussens	Bou.
Demoret	Dem.
Frasses	Fras.
Les Esserts	Les. E.
Les Troncs	Les. T.
Marchissy	Mar.
Montherod	Mon.
Payerne	Pay.
Selfkant	Sel.
St. Blaise	St. B.
Zizers	Ziz.

Germination screening

In order to evaluate the germination capacity of the tested weed populations, a germination screening was carried out. Twenty seeds for each population of *L. multiflorum* and *A. spica-venti* were placed into Petri dishes lined with one filter paper imbibed with 5 mL of tap water. Petri dishes were sealed with Parafilm in order to prevent drying and contamination. Three replicates for each population were organized. Thereafter, Petri were incubated in a growth chamber at a constant temperature of 25°C and 12-h daylight.

Seed germination was recorded once a week for three weeks.

Greenhouse trials

The trials were conducted in greenhouse under controlled conditions (20°C average daily temperature and 12-h daylight). All *L. multiflorum* and *A. spica-venti* populations were tested in the autumn 2016 at the Research Station Agroscope-Changins (Nyon, Switzerland).

Seeds of the two weed species were sown in biodegradable alveolar trays composed by six holes (8.0 X 8.0 cm) and filled with sandy-loam soil previously sterilized.

L. multiflorum populations were sprayed at 3-4 leaf stage while *A. spica-venti* populations at 2-3 leaf stage. Each species was sprayed at different rates of commercial formulation of four herbicides. In particular *L. multiflorum* populations were sprayed with: fluazifop-p-butyl (ACCase inhibitor), pinoxaden (ACCase inhibitor), cycloxydim (ACCase inhibitor) and mefenpyr-diethyl+ iodosulfuron-methyl-sodium (ALS inhibitor) (Table 3). *A. spica-venti* populations were treated with: mefenpyr-diethyl+

iodosulfuron-methyl-sodium (ALS inhibitor), sulfosulfuron (ALS inhibitor), fluazifop-p-butyl (ACCase inhibitor) and isoproturon (PSII inhibitor) (Table 4).

All treatments were arranged in a complete randomised design with three replicates.

Herbicide treatments were conducted using a cabinet track sprayer equipped with a single flat fan nozzle calibrated to deliver a spray volume of 300 l ha⁻¹ at a pressure of 2.29 bar and at a travel speed of 3.00 km h⁻¹. All herbicides were applied at four rates: zero (untreated check), recommended field rate (N), two times the recommended field rate (2N) and four times the recommended field rate (4N).

A population known to be sensitive to the tested herbicides was used as control, both in the case of *L. multiflorum* and in the case of *A. spica-venti*. The control level of tested herbicides was determined 21 days after their application by counting the number of plants survived to the treatments. The herbicide efficacy was expressed as a percentage of resistance (0% absence of resistance: all individuals dead, 100% total resistance: all individuals survived).

Table 3. Herbicides applied in the case of *L. multiflorum* populations.

Active ingredient	Concentration	Field rate* (N)
Fluazifop-p-butyl	13.4%	2 l ha ⁻¹
Pinoxaden	9.71%	0.45 l ha ⁻¹
Cicloxydim	10.8%	3 l ha ⁻¹
Mefenpyr-diethyl	26.6%	200 g ha ⁻¹
Iodosulfuron-methyl-sodium	8.84%	

*: rates are given as formulated products

Table 4. Herbicides applied in the case of *A. spica-venti* populations.

Active ingredient	Concentration	Field rate* (N)
Mefenpyr-diethyl	26.6%	200 g ha ⁻¹
Iodosulfuron-methyl-sodium	8.84%	
Sulfosulfuron	80%	25 g ha ⁻¹
Fluazifop-p-butyl	13.4%	2 l ha ⁻¹
Isoproturon	45.9%	3 l ha ⁻¹

*: rates are given as formulated products

Data analysis

According to the results obtained by the visual control of herbicide efficacy, a preliminary descriptive analysis was performed by including each weed population in a class of resistance / sensitivity, according to the following criteria (modified from Paterson, 2016; Table 5): class 0-high

sensitivity (SS), class 1-sensitivity (S), class 2-medium sensitivity (MS), class 3-medium resistance (MR), class 4-resistance (R), class 5-high resistance (RR). The attribution of each population to a certain class was performed for each herbicide at each rate (field rate, two times the field rate and four times the field rate).

Table 5. Classes of resistance / sensitivity applied to the response of *L. multiflorum* and *A. spica-venti* populations to the tested herbicides. The percentages indicate the plants that survived the herbicide treatment.

Classes of resistance / sensitivity					
0	1	2	3	4	5
SS	S	MS	MR	R	RR
< 25%	25-40%	40-55%	55-70%	70-85%	>85%

Statistical analysis

The number of plants survived to the herbicide treatments was expressed as percentage of survival. The analysis of variance was performed on this percentage in order to verify the presence of significant differences between the populations of each weed species in terms of response to every tested herbicide. The analysis was conducted on data transformed according to the *arcsin* function; the homogeneity of variances was tested with the application of Levene test.

Means were separated using Tukey's post-hoc test.

ANOVA was carried out using the Statistical Software SPSS 23.

Genetic analyses

Genetic analyses were carried out with the aim to identify the type of resistance (target site or non-target-site) developed by populations of *L. multiflorum* and *A. spica-venti* that escaped the herbicide treatments applied during the trials.

The genetic analyses included DNA-Extraction, PCR and DNA-Sequencing and were carried out by PlantaLyt GmbH (Hannover, Germany) and by the company GATC (Konstanz, Germany).

Results and discussion

L. multiflorum populations

The trials allowed to confirm the presence of resistance in some of the populations that escaped field herbicide treatments. Eight out of the nineteen tested populations resulted resistant or highly resistant to at least two of the herbicides applied (three ACCase inhibitors and one ALS-inhibitors). Seven of the resistant populations came from the Vaud canton while one population came from the canton of Freiburg. The concentrations of the resistance cases in the Vaud canton could be considered a consequence of the type of crops present. This Canton ranks first in the production of various crops including wheat (Statistique Vaud, 2016) and *L. multiflorum* is considered a most important weed for this cereal (GIRE®, 2017). Probably this led to a standardization of the management of this weed, with the use of the same herbicides and the consequent appearance of resistance phenomena.

In the case of ACCase inhibitors, all the eight populations were resistant or highly resistant to one or all of these herbicides. These results are similar to those observed by Petersen *et al.* (2016), in trials carried out on populations of *Lolium* spp. from Germany and Switzerland sprayed with ACCase inhibitors (including pinoxaden and cycloxydim, that were used also in our study) and ALS-inhibitors. The development of resistance phenomena to ACCase inhibitors in *L. multiflorum* was reported also by White *et al.*, (2005) and similar results were also found by De Padro *et al.* (2000), in studies conducted on *L. multiflorum* populations sensitive and resistant to ACCase inhibitor herbicides and treated with various ACCase inhibitors at different doses. At the end of the tests, the findings obtained by the authors confirmed the presence of resistance cases in the populations already no longer controlled in field and showed the presence of resistance in one of the sensitive populations (70% of plants survived to the ACCase inhibitor PP-604 applied at the dose of 75 g ha⁻¹).

As regards the ALS-inhibitor herbicide applied during our trials, one population resulted highly resistant while another showed to be moderately resistant. Both these two populations resulted also resistant to one of the tested ACCase inhibitors. The development of resistance phenomena to ALS-inhibitors was also found by Petersen *et al.* (2016), during the trials that they carried out on *Lolium* spp. populations. Similar findings were reported by Llewellyn *et al.* (2001): in trials carried out on *L. rigidum*, the authors found populations that resulted no longer controlled by the applied ALS inhibitors.

Moreover, at the end of our study, two populations showed multiple resistance to ACCase and ALS-inhibitors. In effect, *L. multiflorum* is considered one of the few species that frequently selected resistance phenomena to various herbicides (Délye *et al.*, 2013). The appearance of

multiple resistance in the populations tested in our trials may be related to the allogamy and to the cross-pollination phenomena that characterize *Lolium* spp.. Multiple resistance can indeed be considered the result of the accumulation of resistance alleles in progeny due to pollen movement in out-crossing species including *L. rigidum*, *A. myosuroides*, *Kochia scoparia* and various *Amaranthus* spp. (Beckie and Tardif, 2012). The development of multiple resistance was also reported by Llewellyn *et al.* (2001), in the case of *L. rigidum* populations: in their study, the authors found that various populations were resistant both to ACCase inhibitors and ALS-inhibitors.

Analysis of applied herbicides

Fluazifop-P-butyl

At the end of the trials, the results showed that the majority of the tested populations resulted highly sensitive to the fluazifop-P-butyl at the rates N, 2N and 4N.

A decrease in the percentage of highly resistant populations (RR) was observed when the rate was increased: the percentage was 26.32% at the rates N and 2N, and 17.65% at the rate 4N.

Moreover, a decreasing in the number of populations resistant (R) was observed: the percentage of populations belonging to this class was 10.53%, 5.26%, and to 0.00% at the rates N, 2N and 4N, respectively (Figure 3).

The data analysis performed showed that the majority of populations can be divided in highly resistant (RR) and highly sensitive (SS).

It is possible to note that, at rate 2N, the populations resulted to be RR at the recommended field rate (N) maintained almost unchanged their level of resistance to fluazifop-p-butyl.

At rate 4N, the percentage of survived plants decreased also for many of the populations that resulted RR at the rates N and 2N. Three of the tested populations (Gou., Goum. and Yen.) represented an exception to this behavior maintaining a level of resistant plants greater than 90%.

In the case of populations Bour. and Bou. the survival rate resulted equal to 100% at 2N and close or above to 50% at 4N (Figure 4).

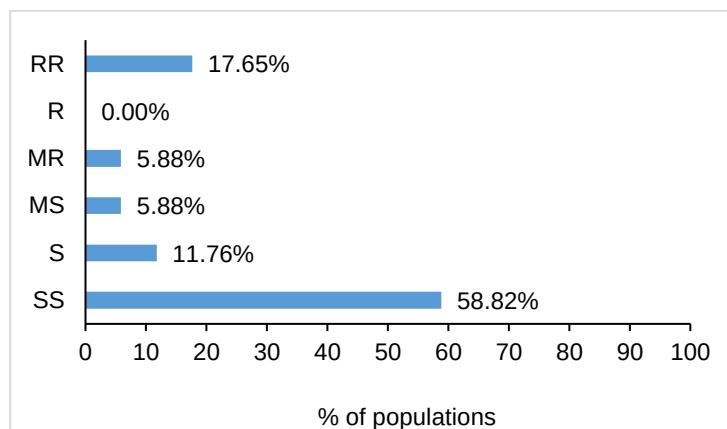
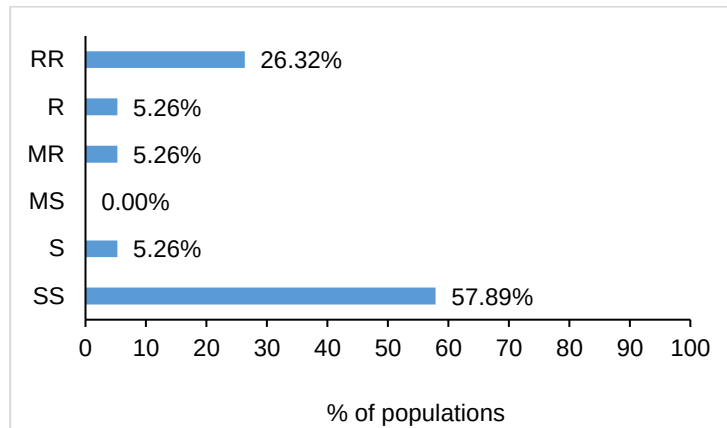
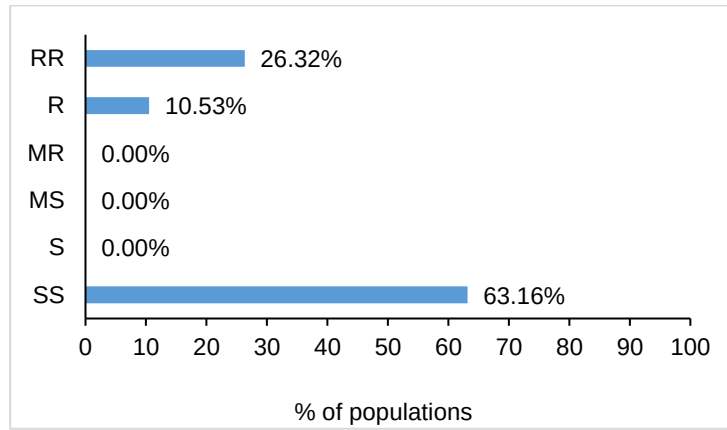


Figure 3. Division into classes of sensitivity / resistance for fluazifop-p-butyl herbicide applied at rates N, 2N, 4N (from top to bottom).

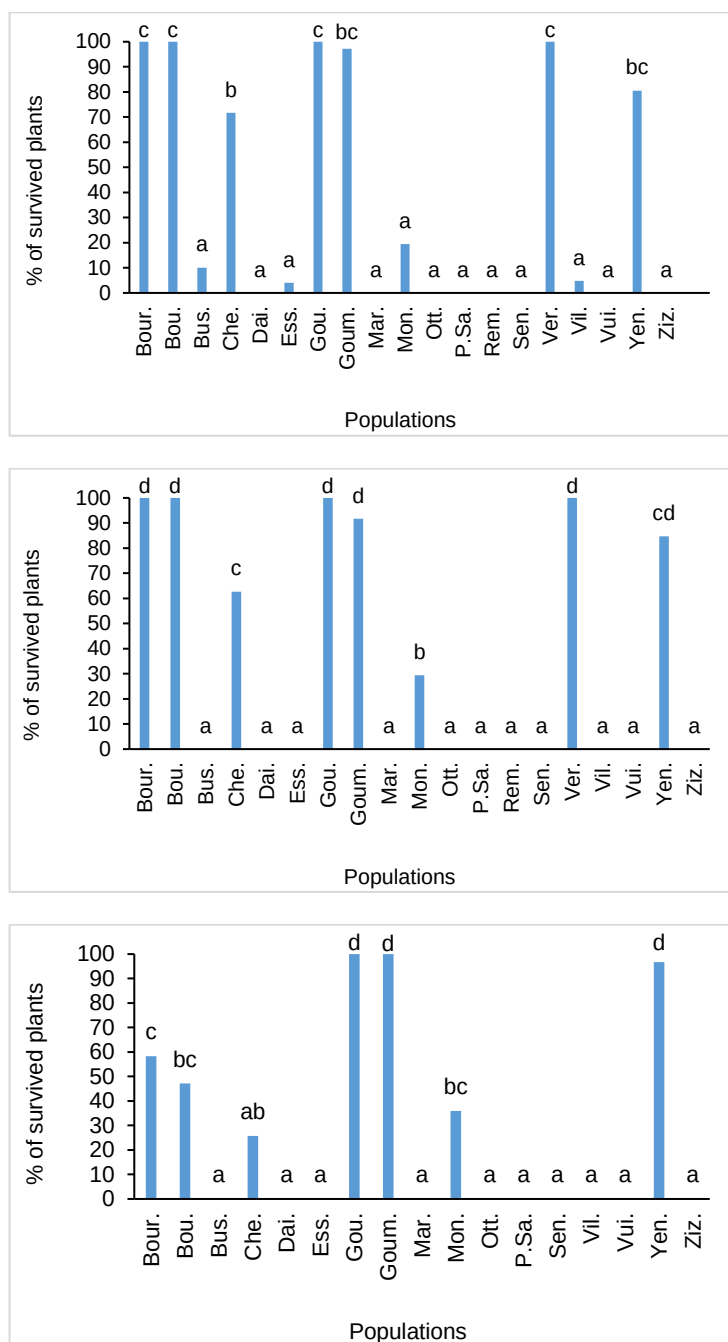


Figure 4. Plants of each population (expressed as percentage) survived to fluazifop- p-butyl application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Pinoxaden

The results showed that the tested herbicide presented a limited capacity to control the development of the weed.

At the field rate (N), pinoxaden was the herbicide characterized by the greatest number of highly resistant populations compared the other herbicides tested: in effect, the percentage of populations belonging to class RR was equal to 36.84%. The number of populations included in this class was lower at higher herbicide rates although remaining on quite high percentage (Figure 5), underlining the development of marked tolerance to pinoxaden. This result could be the consequence of the evolution, in these populations, of target-site resistance mechanism.

The results obtained at the end of the trials showed that, at the field rate (N), eight of the tested populations developed high resistance to pinoxaden, with a percentage of survived plants greater than 80% (in three case this percentage was equal to 100%).

It is interesting to note that the resistance level showed by populations at rate N was equal or slightly lower also at the two times (2N) and four times (4N) the field rate. In particular, populations Bou. and Goum. were characterized by a percentage of survival to pinoxaden equal to 100%. In the case of population Mont., the level of survival plants resulted greater than 50% while in the case of the populations Yen. it was greater than 60% at rate 2N and approximately equal to 40% at rate 4N (Figure 6).

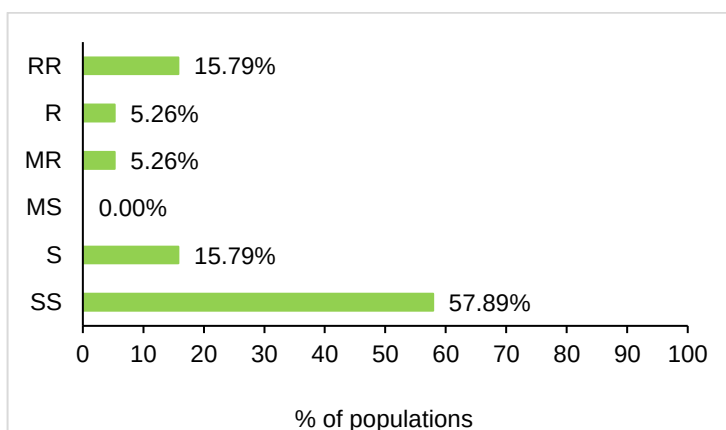
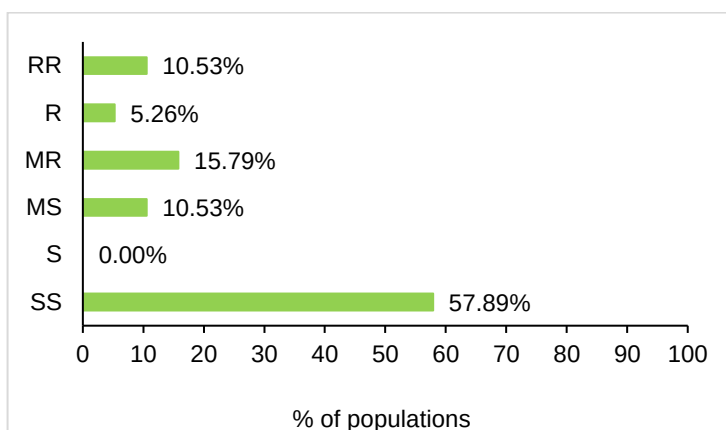
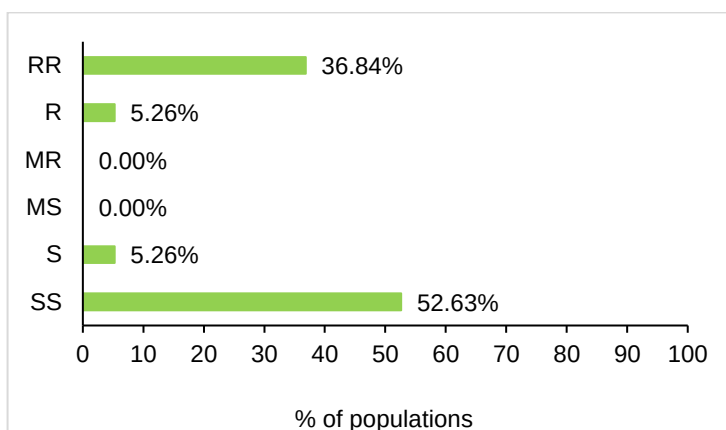


Figure 5. Division into classes of sensitivity / resistance for pinoxaden herbicide applied at rates N, 2N, 4N (from top to bottom).

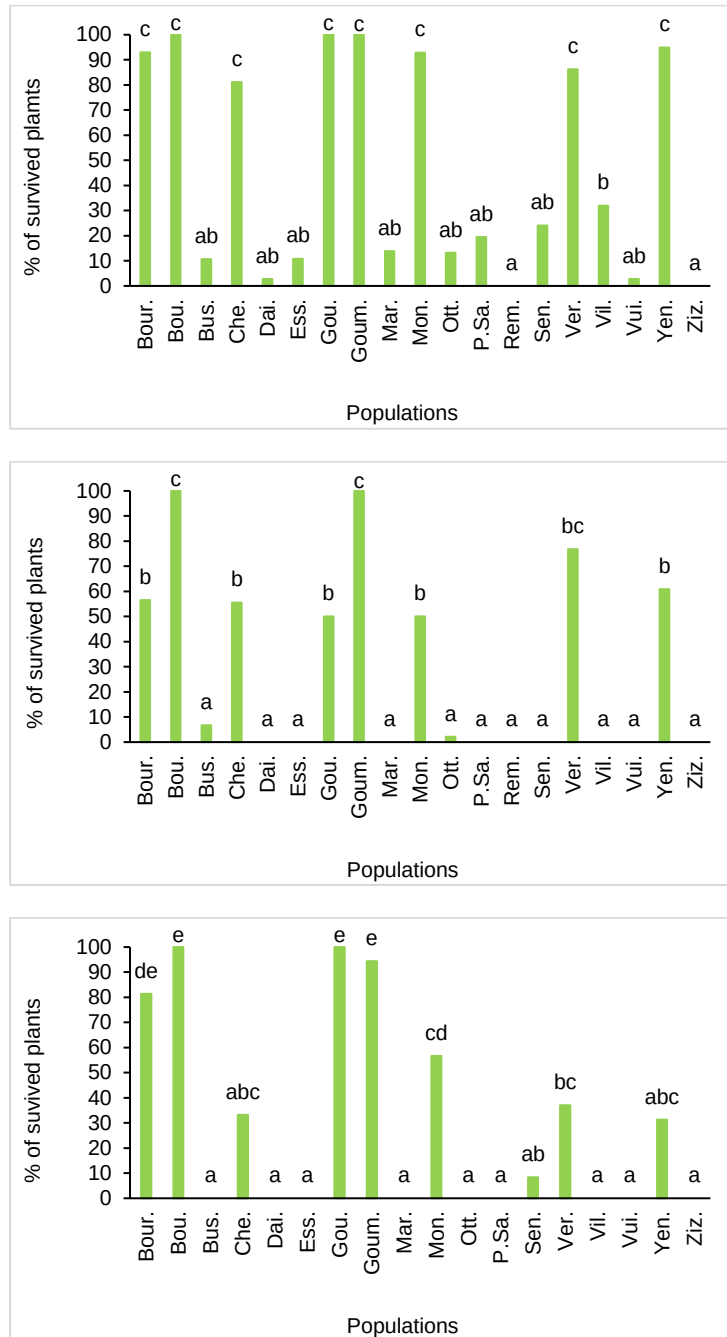


Figure 6. Plants of each population (expressed as percentage) survived to pinoxadem application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Cicloxydim

The results obtained at the end of the experimentation underlined that, to date, cicloxydim is characterized by a good ability to control *L. multiflorum*: the percentage of populations included in the class SS resulted greater than 70% already at the field rate (N). However, despite this great number of populations characterized by high sensitivity, it is important to consider that the percentage of populations belonging to class RR at the rates 2N and 4N remained high compared to that at rate N (Figure 7). This finding suggests that the populations no longer controlled developed a marked level of tolerance to the herbicide (neither a rate equal to 4 times that of field is able to contain their growth). Such tolerance is likely to result from the fact that these populations developed a resistance of type target site.

The results obtained from the analysis of data obtained at the end of the study suggested that the populations characterized by resistance at rate N maintained a high level of survival plants also at two times (2N) and four times (4N) the field rate.

In the case of populations Gou. and Goum. the percentage of survived plants was equal to 100% at all the three herbicide rates; in the case of the population Bou. the level of survival was interested by a slight decrease (from 100% to about 90%) at rate 4N.

A greater decrease of the resistance level between the rate N and the rate 4N was noted in the case of the populations Bour. and Che. (Figure 8).

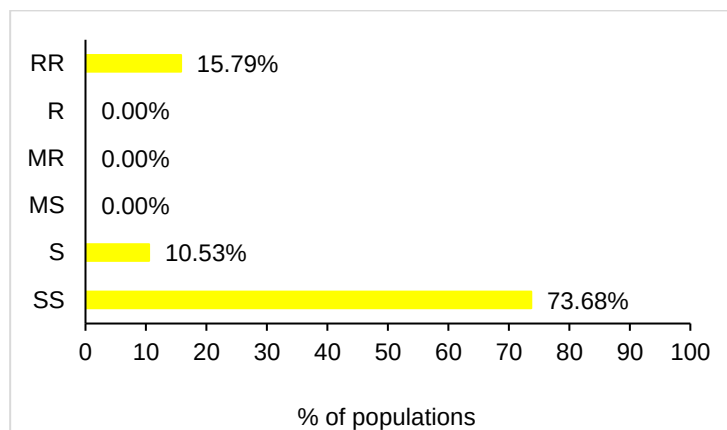
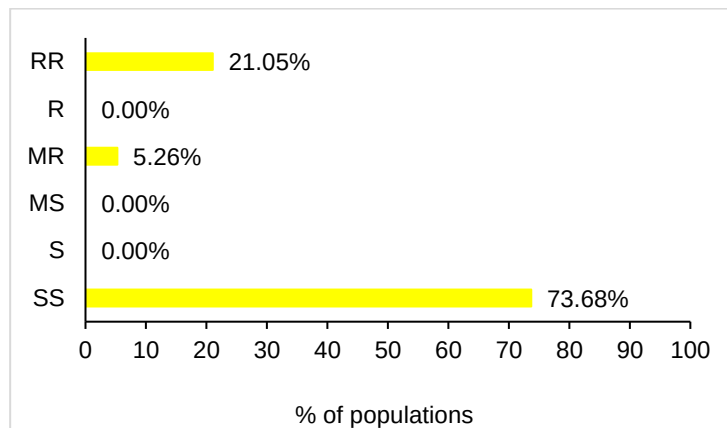
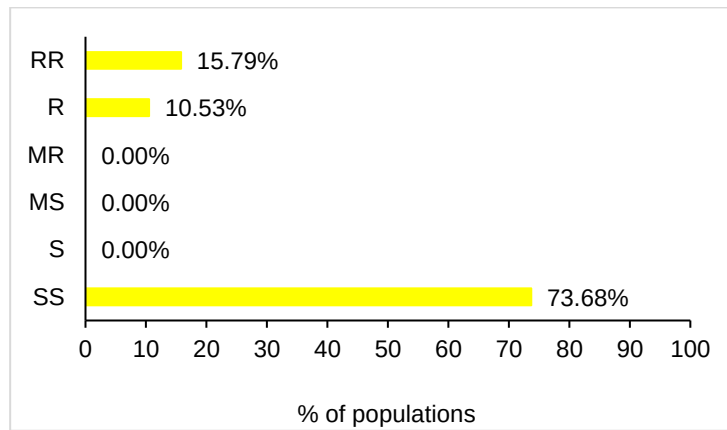


Figure 7. Division into classes of sensitivity / resistance for cicloxydim herbicide applied at rates N, 2N, 4N (from top to bottom).

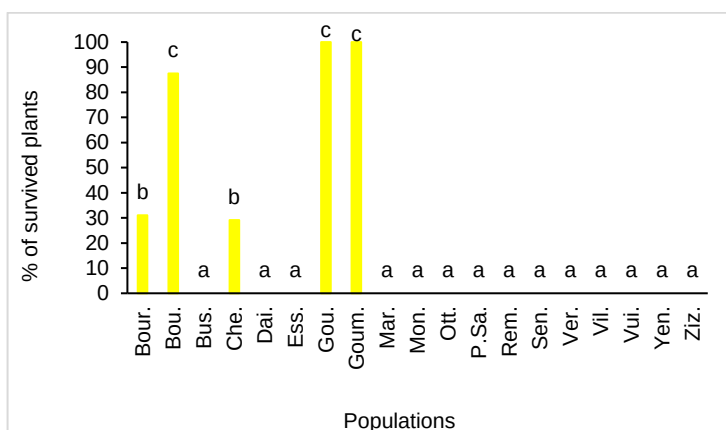
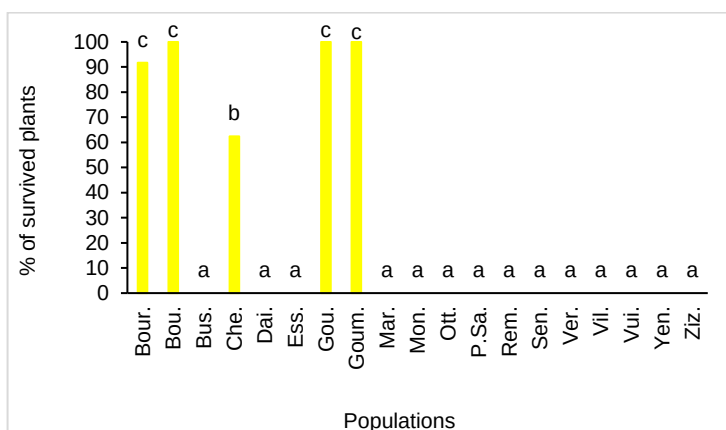
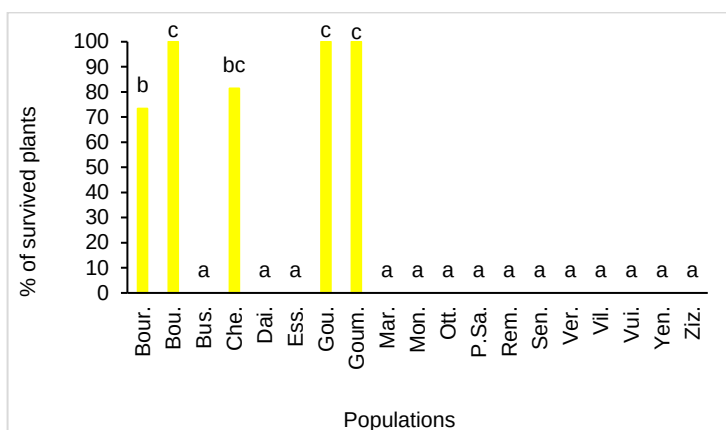


Figure 8. Plants of each population (expressed as percentage) survived to cicloxydim application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Mefenpyr-diethyl+ Iodosulfuron-methyl-sodium

The results indicate a high ability of the tested herbicide in controlling *L. multiflorum* populations. Compared with the other herbicides, the application of Mefenpyr-diethyl+ iodosulfuron-methyl-sodium resulted in the lowest number of populations classified as R or RR (Figure 9).

The percentage of populations included in class SS was 78.95%, 89.47% and 94.74% at the rates N, 2N and 4N, respectively.

The amount of populations included in the class RR resulted limited: at the rates N and 2N only the 5.26% of populations fell into this class while at the rate 4N no population was included in class RR.

The analysis of results underlined that, in general, there was a decrease of resistance cases with the increase of herbicide rate.

It is possible to observe that, for various populations, the reduction of resistance was marked, with a survival rate equal to 0% at the rates 2N and 4N. In the case of other populations, the response to herbicide was more variable: at the rate 2N, the percentage of plants escaped to herbicide treatment was equal to 0% while at the rate 4N it increased slightly.

Population Mon. represented an exception, with a level of resistance that remained high at all the three herbicide rates. In particular, the percentage of survived plants resulted greater than 90% at the rates N and 2N and still greater than 70% at the rate 4N (Figure 10).

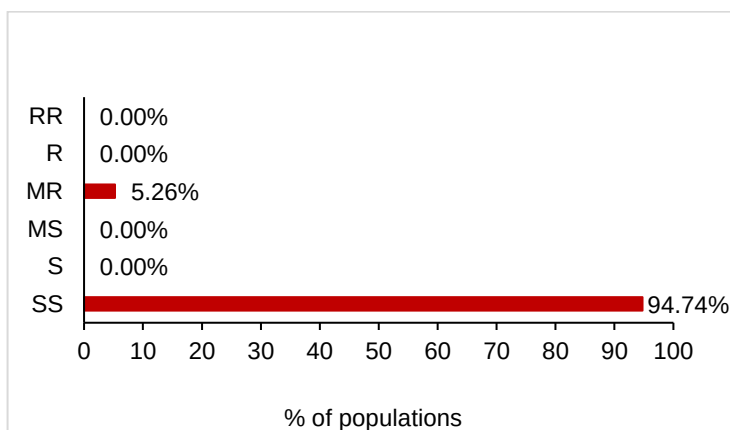
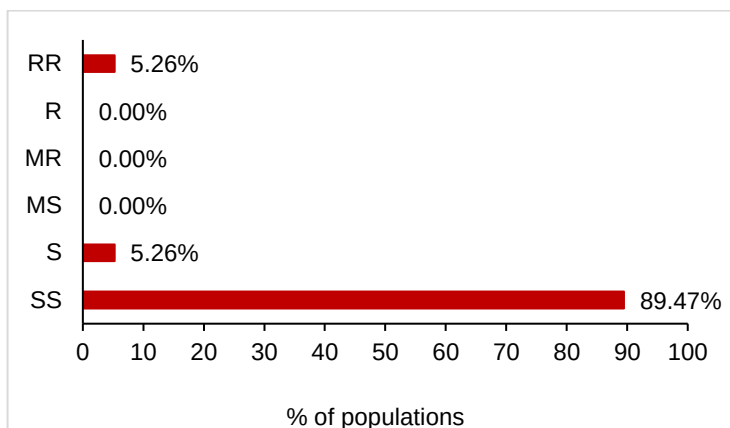
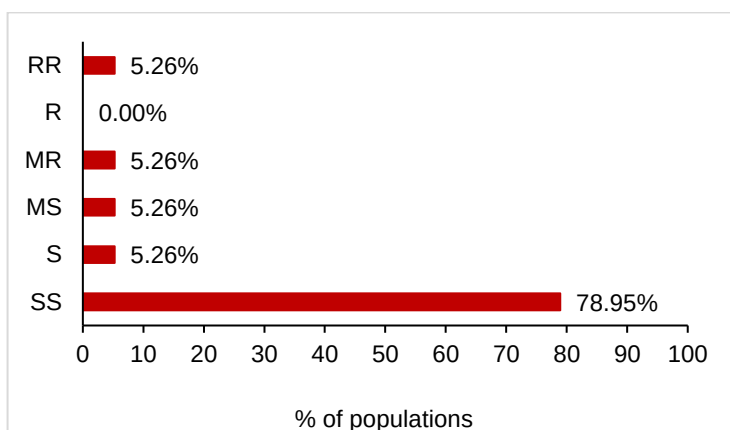


Figure 9. Division into classes of sensitivity / resistance for Mefenpyr-diethyl+iodosulfuron-methyl-sodium herbicide applied at rates N, 2N, 4N (from top to bottom).

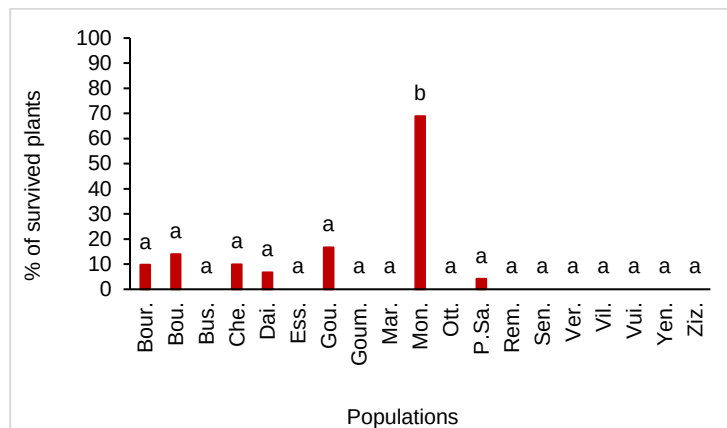
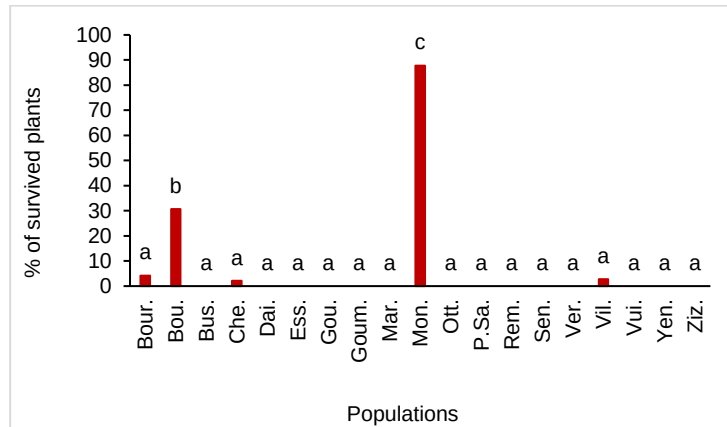
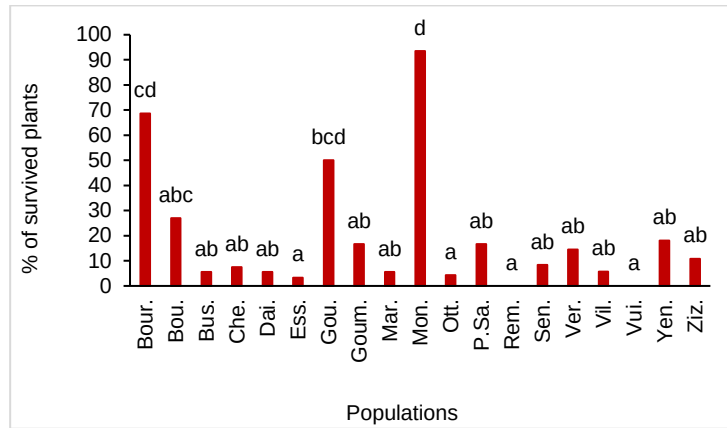


Figure 10. Plants of each population (expressed as percentage) survived to Mefenpyr-diethyl+ iodosulfuron-methyl-sodium application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

A. spica-venti

The results obtained showed that eight of the twelve *A. spica-venti* populations were resistant or highly resistant to one, two or three of the four tested herbicides. Six of the resistant populations came from the Vaud canton while the other resistant populations came from Freiburg, Neuchâtel and Graubünden cantons.

In the case of ACCase inhibitor herbicide, only two of the eight resistant populations were characterized by resistance to it.

In the case of ALS-inhibitor herbicides, seven populations resulted resistant or highly resistant to one or both the active ingredients used during our trials. Similar results were found by Massa *et al.* (2011), in the trials conducted on 72 populations of *A. spica-venti* whose seeds were collected in fields where herbicide treatments showed unsatisfactory results. The populations were tested in greenhouse by applying different ALS inhibitors, including sulfosulfuron. At the end of the trials, the authors found all populations resulted resistant to the ALS-inhibitors applied; in particular, the efficacy of sulfosulfuron ranged from 0 to 20% at the recommended field rate and from 0 to 30% at four times the field rate.

In the case of the PSII inhibitor herbicide, two of the tested populations showed resistance to it.

Furthermore, cases of multiple resistance were found during the study. In particular, one population developed multiple resistance to the ACCase and the PSII inhibitors, one to ACCase and ALS-inhibitors and another one to ALS and PSII inhibitors.

The development of this type of resistance could be due to the application of one of the herbicide tested for controlling many weed generations. Since the non-target-site resistance may result in multiple resistance, the use of a same herbicide on many generations may have led to the selection of non-target-site resistance mechanisms to one herbicide, which can in turn result in the development of non-target-site resistance to another herbicide (Délye *et al.*, 2013).

Analysis of applied herbicides

Mefenpyr-diethyl+ Iodosulfuron-methyl-sodium

The results showed that there was a reduction in the number of populations highly resistant to the herbicide with the increase of the rate: the percentage of populations belonging to the class RR ranged from 41.67% at the rate N to 33.33% at the rate 4N. A different response from what was expected was observed in the case of class R: in effect, there was an increase in the number of populations included in this class with the increase of the herbicide rate (Figure 11). This result was probably due to the low germination capacity found in some of the populations tested (data not shown) and, as a consequence, to the low number of plants sprayed.

The data analysis performed at the end of the trails showed that there were numerous cases of resistance between the tested populations.

It is possible to note that, in various populations, the level of resistance remained high at all the three herbicide rates. In particular, in the case of the populations Dem. and Pay. the percentage of survived plants resulted equal to 100%.

As regards the population Les E. the level of resistance decreased from 100% at the rates N and 2N to little more than 70% at the rate 4N.

In the case of the other tested populations, instead, there was a decrease of resistance percentage with the increase of herbicide rate.

A particular behavior was found for the population Les T.: it was characterized by an increase in resistance level with the increase of herbicide rate (from about 10% at the rate N to 100% and about 90%, respectively, at the rates 2N and 4N) (Figure 12).

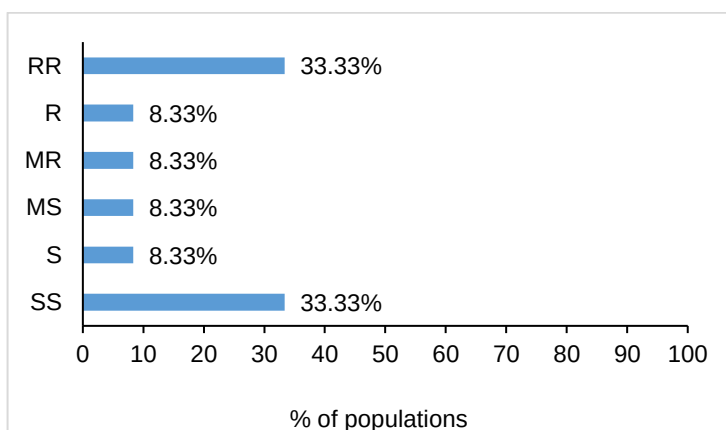
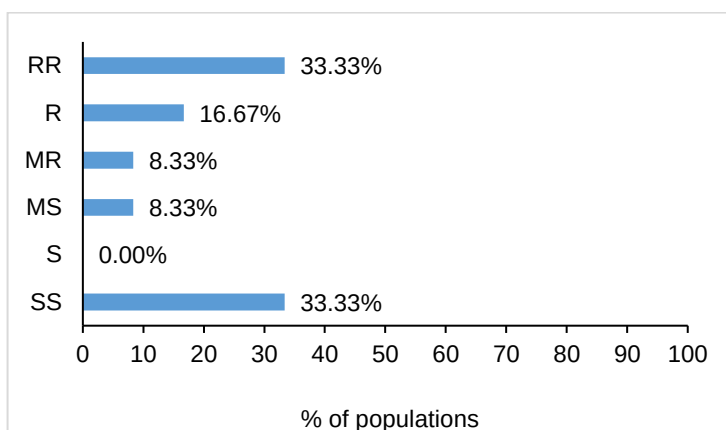
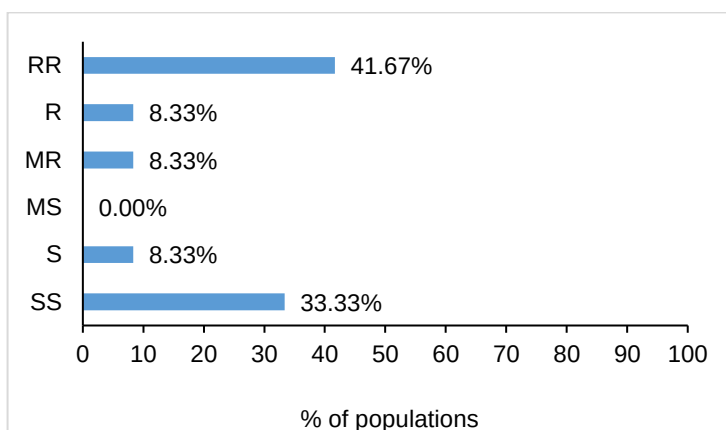


Figure 11. Division into classes of sensitivity / resistance for mefenpyr-diethyl+ iodosulfuron-methyl-sodium applied at rates N, 2N, 4N (from top to bottom).

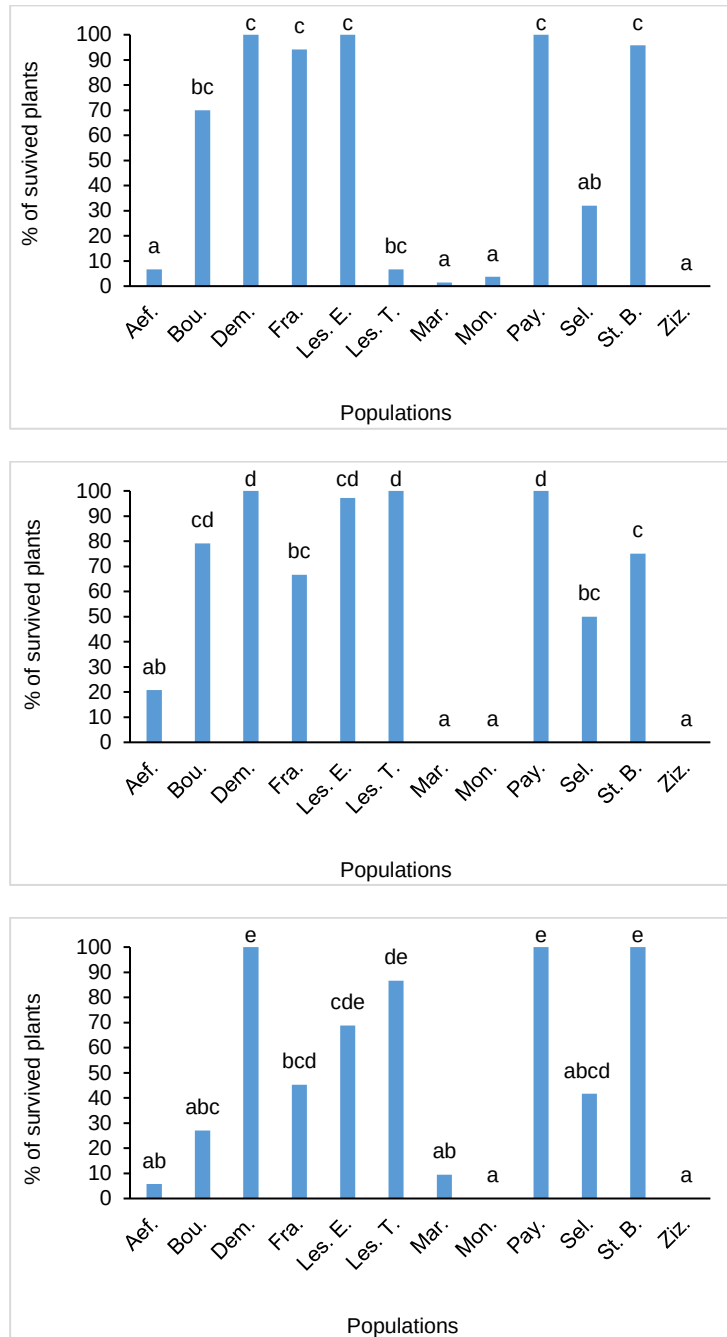


Figure 12. Plants of each population (expressed as percentage) survived to mefenpyr-diethyl+ iodosulfuron-methyl-sodium application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Sulfosulfuron

The results showed that a very high number of resistance cases among the tested populations at all the three rates to which this herbicide was applied.

At the rates N and 2N, the percentage of populations included in class SS resulted less than 50% while, at the rate 4N, only half the populations was included in this class.

Moreover, at the rate 4N, the percentage of populations in class RR was still very high and equal to 25% (Figure 13).

The results obtained showed that the majority of the tested populations developed high levels of resistance to sulfosulfuron both at the field rate (N) and at two times the field rate (2N). In particular, the populations Dem., Fra., Les E., Pay. and St. B. were characterized by percentage of survived plants equal to 100% or slightly lower in the case of both the herbicide rates.

Moreover, it is interesting to note that the level of resistance in population Dem. and St. B. remained very high (between 90% and 100%) also at the rate 4N.

A marked decrease in percentage of surviving was, instead, observed for population Pay. at the rate 4N (from 100% at the rates N and 2N to about 10% at the rate 4N).

Population Les T. resulted characterized by an increase of resistance with the increase of herbicide rate (Figure 14).

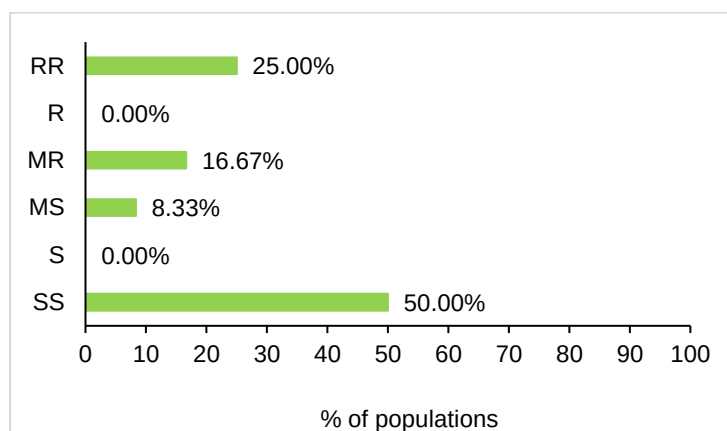
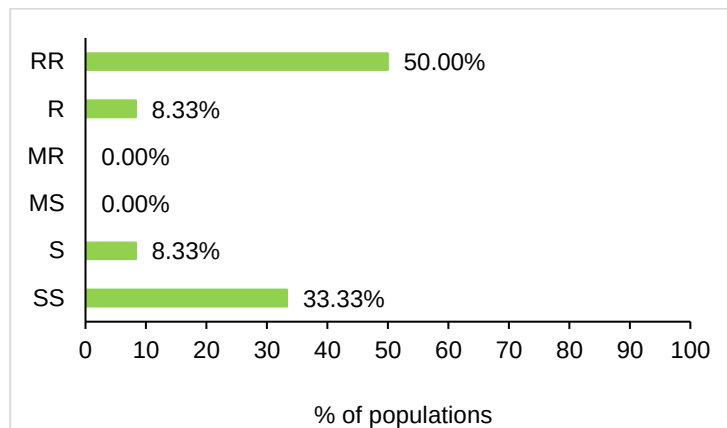
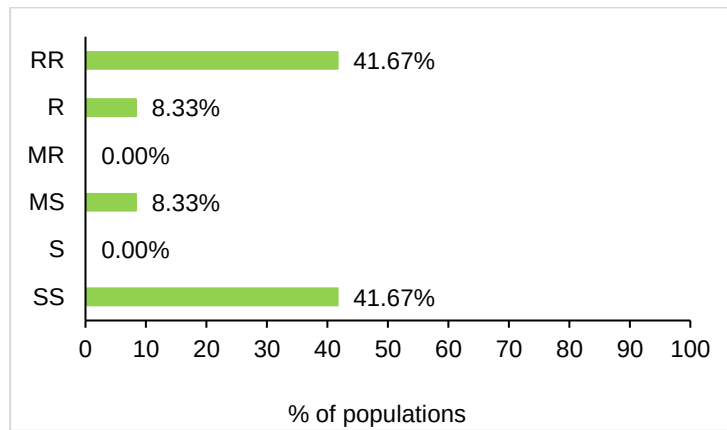


Figure 13. Division into classes of sensitivity / resistance for sulfosulfuron applied at rates N, 2N, 4N (from top to bottom).

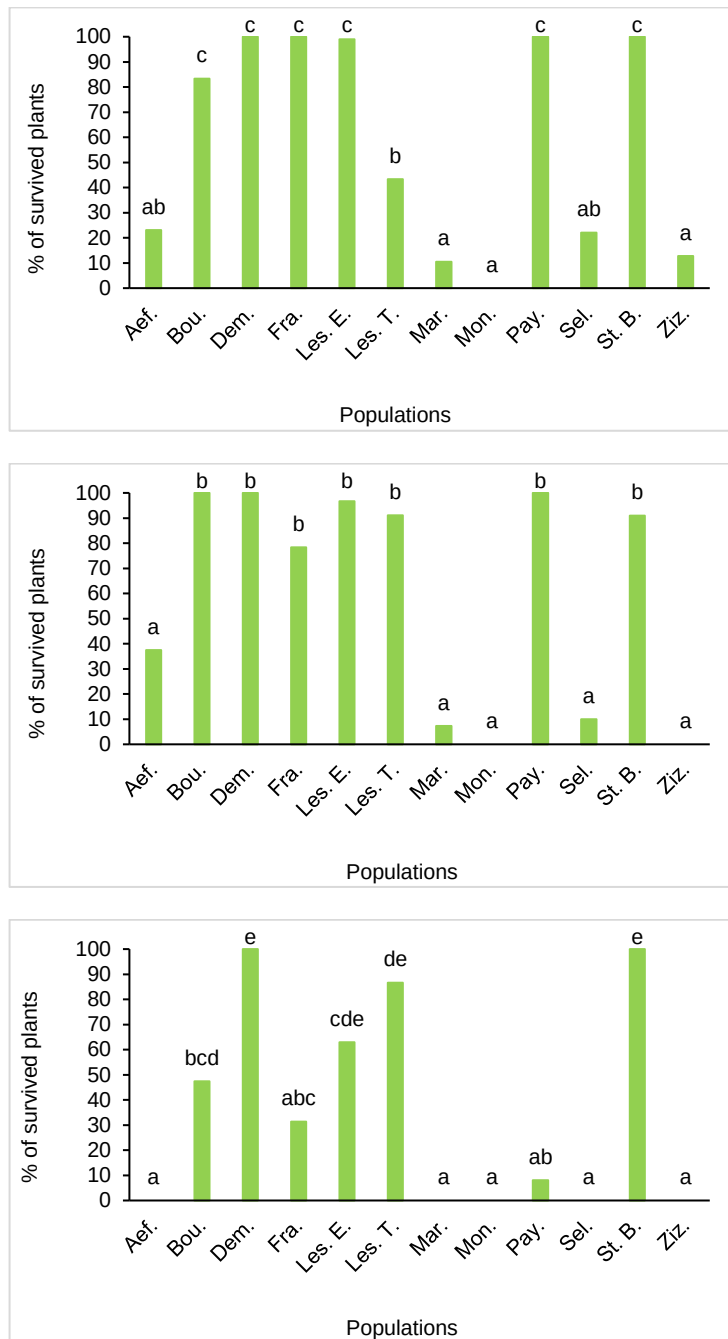


Figure 14. Plants of each population (expressed as percentage) survived to sulfosulfuron application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Fluazifop-P-butyl

At the end of the trials, the results showed that this tested herbicide presented a discrete ability of controlling the tested weed.

At the field rate (N), the 50% of tested populations fell into class SS while about the 17% resulted highly resistant to the herbicide (RR).

At the rates 2N and 4N the percentage of populations highly sensitive was greater than 90% while no population was in the class RR (Figure 15).

The analysis of results obtained at the end of the study underlined that, in general, the tested herbicide selected cases of resistance only in the case of the field rate. Moreover, at this rate, only the population Sel. showed a percentage of survival close to 100%.

At the rates 2N and 4N, it was possible to observe that all the tested populations were controlled to the herbicide application. The population Sel. was an exception: it showed a level of resistance that remained high both at the rate 2N (70%) and at the rate 4N (about 40%) (Figure 16).

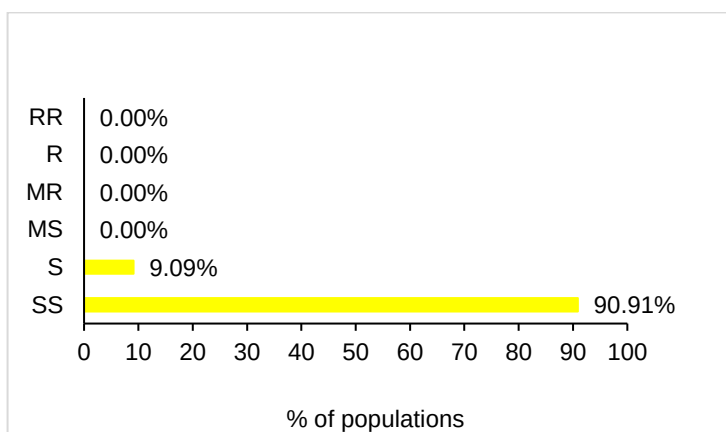
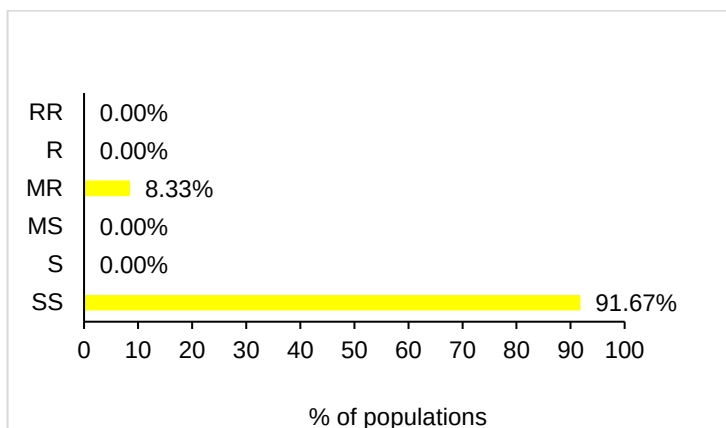
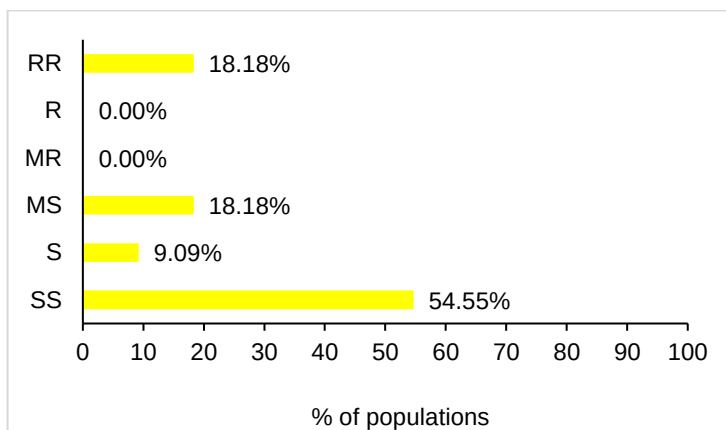


Figure 15. Division into classes of sensitivity / resistance for fluazifop-p-butyl applied at rates N, 2N, 4N (from top to bottom).

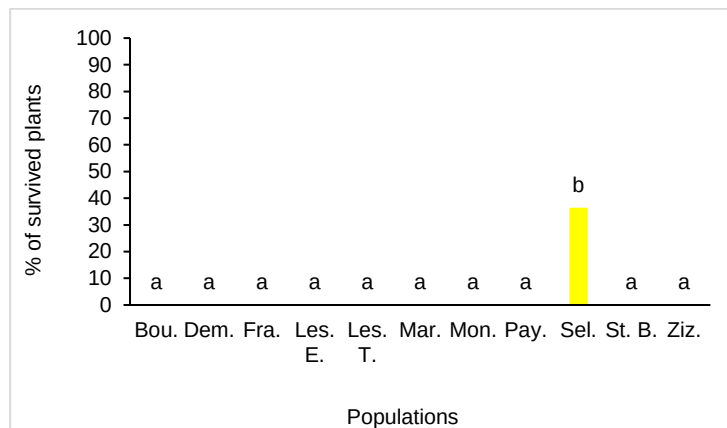
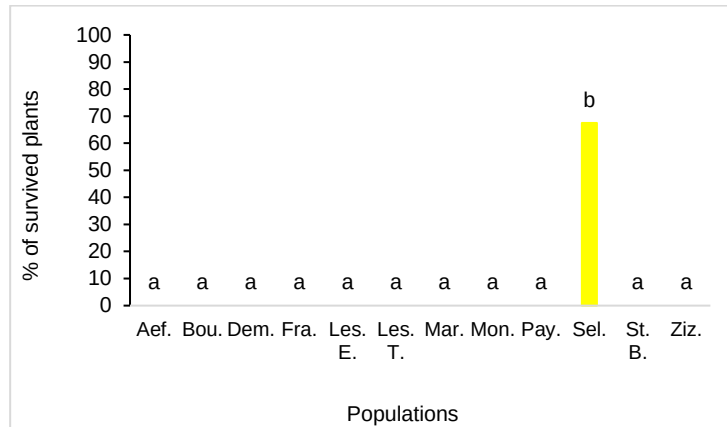
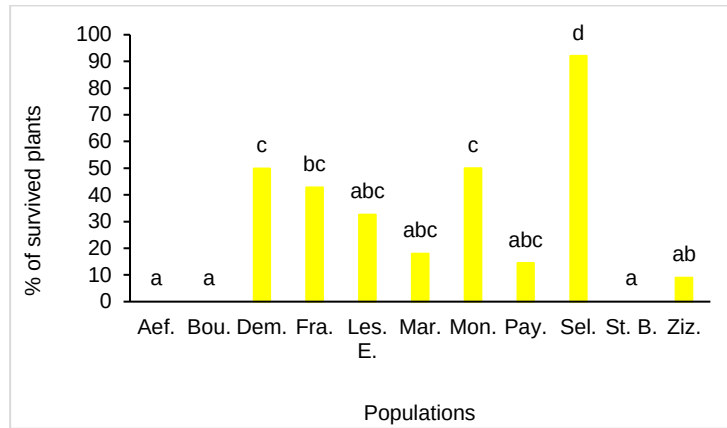


Figure 16. Plants of each population (expressed as percentage) survived to fluazifop-p-butyl application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Isoproturon

As observed for fluazifop-P-butyl, the results showed that isoproturon was less susceptible to evolve resistance phenomena compared to mefenpyr-diethyl+ iodosulfuron-methyl-sodium and sulfosulfuron. The percentage of populations included in the class SS was greater than 80% already at the rate N. The number of populations belonging to the class RR remained constant in all the three herbicide rates. The percentage of populations grouped in the R class was 8.33%, 0.00% and 0.00% at the rates N, 2N, and 4N, respectively (Figure 17).

The high efficacy of isoproturon was also found by Soukup *et al.* (2006): during their trials, they observed that this herbicide was able to provide a 100% control of *A. spica-venti* populations tested already at 0.5 of the recommended field rate.

The findings obtained at the end of the trials suggested the limited ability of the tested herbicide to developed resistance phenomena at all the three rates.

At the rate N, only one population (Sel.) showed a percentage of survived plants equal to 100% while for all the other populations this percentage resulted lower than 20%.

It is interesting to note that population Sel. was characterized by a percentage of plants escaped to herbicide application equal to 100% also at the rates 2N and 4N (Figure 18).

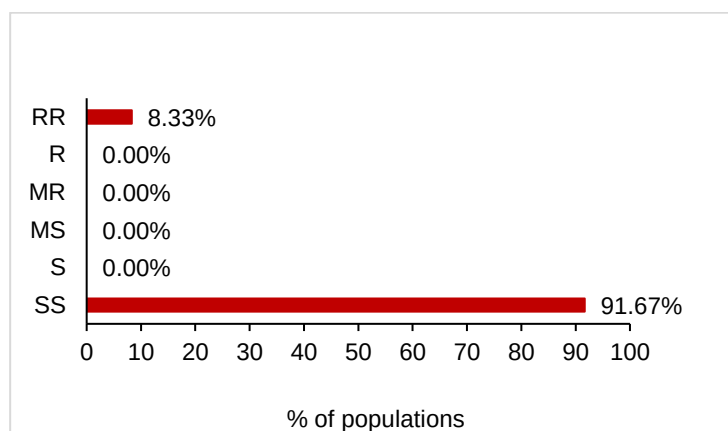
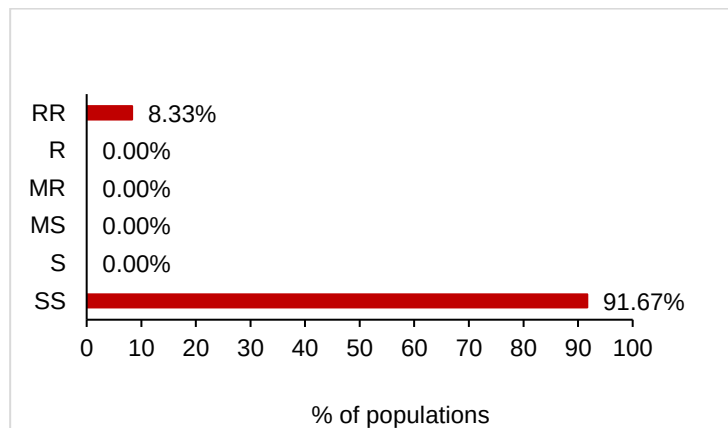
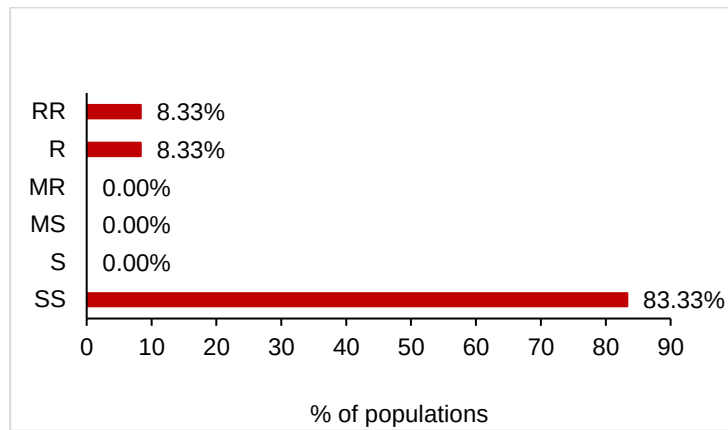


Figure 17. Division into classes of sensitivity / resistance for isoproturon applied at rates N, 2N, 4N (from top to bottom).

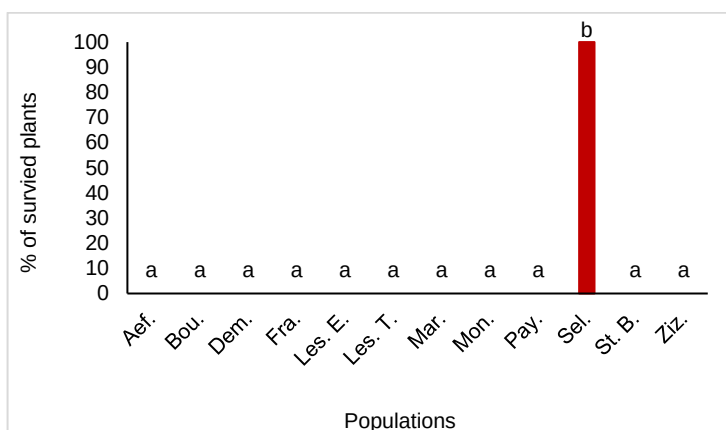
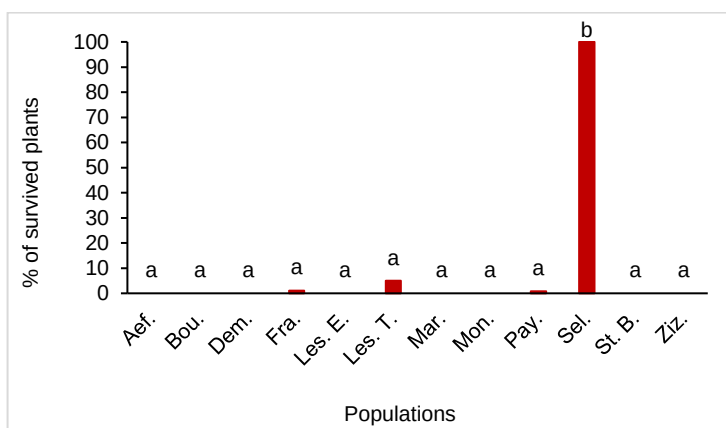
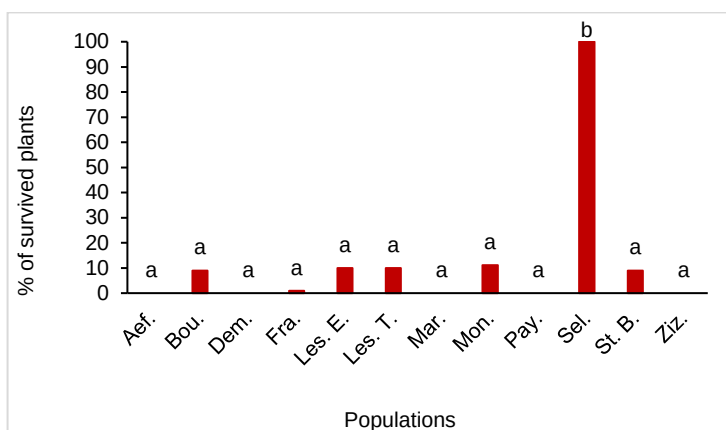


Figure 18. Plants of each population (expressed as percentage) survived to isoproturon application at rates N, 2N, 4N (from top to bottom).

Different letters above the bars indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Genetic analyses

L. multiflorum

The genetic analysis carried out on the populations not controlled by treatments with the tested herbicides (Table 6 and Table 7) confirmed the development of resistance phenomena, both target-site and non-target-site.

Six out of the eight populations resistant to ACCase-inhibitors showed a mutation in the gene encoding for ACCase, while the other two populations are then supposed to be resistant because of a non-target-site resistance mechanism. In the case of target-site resistance, the most frequent mutation was Ile-1781-Leu; this mutation was found in five populations, while the other resistant population showed a mutation type Ile-2041-Asn (Table 4). The mutation in Ile-1781-Leu and Ile-2041-Asn were also found by Scarabel *et al.* (2011), in *Lolium* spp. populations, by Yu *et al.* (2007) in resistant *Lolium* populations and by Martins *et al.* (2014) in the case of Ile-2041-Asn in *L. multiflorum* resistant populations.

In the case of the ALS-inhibitor, four populations surviving the application of this herbicide developed a resistance non-target-site (Table 5). This mechanism results to be the prevalent in ALS-inhibitor resistant grasses (Délye *et al.*, 2013). In the case of *L. rigidum*, the development of non-target-site resistance phenomena that affect up to 16 herbicides with 9 different mechanisms of action was reported in various studies (Délye *et al.*, 2013).

Table 6. Type of resistance (TSR = target site; NTSR = non-target site) developed for tested ACCase inhibitor herbicides in *L. multiflorum* populations.

Population	Resistance type	Point gene mutation in TSR
Bour.	TSR	Ile-1781-Leu
Bou.	TSR	Ile-1781-Leu
Che.	TSR	Ile-1781-Leu
Gou.	TSR	Ile-1781-Leu
Goum.	TSR	Ile-1781-Leu
Mon.	NTSR	-
Vil.	NTSR	-
Yen.	TSR	Ile-2041-Asn

Table 7. Type of resistance (TSR = target site; NTSR = non-target site) developed for tested ALS-inhibitor herbicide in *L. multiflorum* populations.

Population	Resistance type
Bour.	NTSR
Bou.	NTSR
Gou.	NTSR
Mon.	NTRS

A. spica-venti

As observed for *L. multiflorum*, also in the case of *A. spica-venti* the genetic analyses conducted on the populations not controlled by the herbicide applications confirmed the presence of resistance. In the case of ALS inhibitors, the analyses showed that five populations developed target-site resistance while three populations resulted non-target-site resistant (Table 8). The genetic mutation responsible for the appearance of target-site resistance is type Pro-197-His in all the target site resistant populations. Cases of target site resistance due to a genetic alteration was also found by Massa *et al.* (2011): during their trials, they observed that a Thr mutation at Pro-197 affected 67 *A. spica-venti* populations. Moreover, Adamczewski *et al.* (2012), underlined the presence of a gene point mutation at position Pro-197 in two of the three populations resulted cross-resistant to sulfonylurea herbicides. Mutations in the gene encoding for the ALS inhibitors were also observed by Hamouzová *et al.* (2014). Among the populations that, during our trials, were affected by target-site mutation, two were found to have multiple resistance to the ALS inhibitors and the ACCase inhibitor. Similar results were observed also by Adamczewski *et al.* (2012), which found that two biotypes characterized by multiple resistance to these two classes of herbicides showed mutation at level of Pro-197.

As concerns the ACCase inhibitor and the PSII inhibitor all the populations resulted resistant during the greenhouse trials developed a non-target-site resistance (Table 9 and 10). Moreover, one population was characterized by multiple resistance to the all the tested herbicide mechanisms of action while three populations resulted affected by multiple resistance to the ALS inhibitors and the PSII inhibitor.

Non-target-site resistance is considered to be the prevalent form of resistance to ACCase and ALS-inhibitors in grasses. Furthermore, the development of non-target-site resistant against ACCase and ALS-inhibitors could be the result of an enhanced metabolism, that seems to be the main mechanism of resistance for these two classes of herbicides (Délye *et al.*, 2013).

Cases of non-target-site resistance in *A. spica-venti* were also evidenced by Adamczewski *et al.* (2012), in a study carried out on different populations of this weed: the authors reported that the resistance of two populations to the sulfonylureas tested was not found to be target-site and it was likely due to an alteration of the metabolism.

Table 8. Type of resistance (TSR = target site; NTSR = non-target site) developed for tested ALS-inhibitor herbicides in *A. spica-venti* populations.

Population	Resistance type	Point gene mutation in TSR
Aef.	NTSR	-
Bou.	TSR	Pro197-His
Dem.	TSR	Pro197-His
Fras.	TSR	Pro197-His
Mon.	TSR	Pro197-His
Pay.	TSR	Pro197-His
Sel.	NTSR	-
St. B.	NTSR	-

Table 9. Type of resistance (TSR = target site; NTSR = non-target site) developed for tested ACCase inhibitor herbicide in *A. spica-venti* populations.

Population	Resistance type
Fras.	NTSR
Pay.	NTSR
Sel.	NTSR

Table 10. Type of resistance (TSR = target site; NTSR = non-target site) developed for tested PSII inhibitor herbicide in *A. spica-venti* populations.

Population	Resistance type
Bou.	NTSR
Mon.	NTSR
Sel.	NTSR
St. B.	NTSR

Conclusions

The findings obtained in this study showed that some of the tested *L. multiflorum* and *A. spica-venti* populations exhibited herbicide resistance. In the case of *L. multiflorum*, eight populations evolved resistance phenomena; moreover, among these resistant populations, four were characterized by multiple resistance to ACCase and ALS inhibitors.

Furthermore, the study showed that the resistance phenomena were concentrated in the Canton of Vaud (7 cases out of 8).

The evolution of herbicide resistance in the *L. multiflorum* populations were confirmed by the genetic analyses, which showed that the resistance to the ACCase inhibitors was due to a mutation in the gene encoding these herbicides (target site resistance) while resistance to the ALS inhibitor was due to the appearance of a non-target site mechanism of resistance.

As far as the target site resistance to the ACCase inhibitors is concerned, the most frequent mutation in the populations tested during the trials was mutation type Ile-1781-Leu. It is reported that the alteration in the ACCase is considered the cause of the appearance of high levels of resistance in this weed (Hassan *et al.*, 2002).

Regarding *A. spica-venti*, eight populations resulted to be characterized by the development of resistance to at least 2 of the tested herbicides. Moreover, among these resistant populations, one evolved multiple resistance to the ALS and the ACCase inhibitors, one to ACCase and PSII inhibitors and another one to ALS and the PSII inhibitors.

As occurred in the case of *L. multiflorum*, also in the case of *A. spica-venti* populations the appearance of resistance cases was verified by the genetic analyses. The populations resulted characterized by herbicide resistance both target-site and non-target-site. In grasses, the form non-target-site is believed to be the most common in the case of ACCase and ALS inhibitor herbicides (Délye *et al.*, 2013).

The results obtained at the end of the study suggest that herbicide resistance is a significant issue even in Switzerland. The increasing importance of this phenomenon is stressed not only by the numerous cases of resistance to one of the herbicides tested in this study but also by the appearance of multiple resistance phenomena in some of the tested weed populations.

These results underline the need for a correct application of herbicides and for a use of a proper combination of chemical and agronomical means in the weed management.

The use in the same growing season or during more growing season of mixtures or rotation of herbicides with different mechanism of action result

to be condition of primary importance in the prevention of appearance of resistance phenomena.

The adoption of herbicides with different modes of action allows to reduce the selection pressure of resistance to any one herbicide. In preventing this phenomenon, it is also essential to take into account the different types of resistance that may appear. In the case of multiple target-site-resistance, the application of herbicide mixtures has greater efficacy in delaying its development compared to herbicide rotation (Lagator *et al.*, 2013).

However, the herbicide mixtures significantly reduce the resistance only in the case in which they are composed by herbicides characterized by similar levels of effectiveness against target weeds, otherwise one of the herbicides will exert the strongest pressure, and eventually increase the chances to select populations resistant to it. (Beckie, 2006).

Furthermore, the application in mixture of an herbicide which has already developed resistance is definitely not an efficient strategy for management of herbicide resistance (Jacquemin *et al.*, 2009).

Management of non-target-site resistance, since it can be responsible for the onset of multiple resistance, should be based, when possible, on the use of herbicides characterized, when known, by different metabolic pathways in their degradation, in addition to herbicides with different mechanisms of action (Délye *et al.*, 2013).

Second study

Effect of salinity on *Echinochloa crus-galli* germination as affected by herbicide resistance

Paper submitted to *Italian Journal of Agronomy* (now in editing status)

Abstract

Salinity is one of the major abiotic stresses that may affect yield and quality of crops. Salinization, in combination with the presence of aggressive weeds, such as barnyard grass (*Echinochloa* spp.), can be considered one of the factors responsible for reducing yield in rice fields. The aims of the study were to evaluate the salt effect on germination and first seedling growth of six different Italian common barnyard grass (*E. crus-galli*) populations (three sensitive and three resistant to ALS-inhibitor herbicides) and to verify the presence of differences in salt response between populations sensitive and resistant to the ALS-inhibitor herbicides. Germination tests were conducted under nine different NaCl concentrations (from 0 mM to 400 mM). Significant differences in germination capacity were found between sensitive and resistant populations from 0 mM to 250 mM NaCl; in particular, germination capacity of the sensitive populations was higher (up to 90%) than that of the resistant ones (about 70%). The increase in salinity over 250 mM reduced progressively the germination capacity: from 300 mM onwards, no significant differences were found between sensitive and resistant populations and the germination resulted inhibited for two of them (one sensitive and one resistant). Speed of germination and root and shoot length of seedlings were also inversely related to salt concentration. Time required for achieving 50% of final germination capacity was extended from about three days at 0 mM NaCl up to about 10-12 days at 400 mM NaCl. Root length and shoot length ranged from 9.88 cm and 6.16 cm, at 0 mM NaCl, to 0.36 cm and 0.41 cm, at 400 mM NaCl. According to the results, there is no a clear evidence that response to saline conditions was related to resistance towards ALS-inhibitor herbicides, as in some cases significant differences were found between populations showing a similar herbicide sensitivity. Responses of barnyard grass to salinity are may play a role in the importance of this weed in future scenarios of salt intrusion: for example, a lower speed of germination at increasing salt levels could suggest a delayed emergence of this weed during crop establishment and first growth. To evaluate the real consequences in terms of competitions towards the crop, future studies are needed for assessing the response to salinity of the main rice varieties cultivated in the environment in which the *E. crus-galli* populations tested in this study were collected.

keywords

salinity, weeds, rice, barnyard grass, herbicide resistance

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Introduction

Salinity represents one of the major limitations for yield and quality of a number of different crops (Maggio *et al.*, 2011). According to the estimates of the Food and Agriculture Organization of the United Nations, about 20% of irrigated land worldwide is affected by the increase of the salinity level (Rozema and Flowers, 2008). This phenomenon is accentuated by the competition for fresh water between agricultural and civil uses, which is worsened by climate changes, growing population (Maggio *et al.*, 2011), socio-economic development and water contamination (Balía and Viezzoli, 2015). Salinity conditions are relevant not only to arid and semiarid environments and to the southern regions of the world, but also to the Mediterranean coastal areas. In Europe, 26 countries (Maggio *et al.*, 2011), in particular Spain, Portugal, Italy, Greece (Ghiglieri *et al.*, 2012) and France (Puard *et al.*, 1999) are interested by salinization phenomena (Maggio *et al.*, 2011). In the coastal areas, salinization of aquifers is usually caused by saltwater intrusion (Mongelli *et al.*, 2013) as a result of groundwater overexploitation (Balía and Viezzoli, 2015).

In Italy, this phenomenon is found in various regions, such as Sardinia (Capaccioni *et al.*, 2005), the Catania Plain (Capaccioni *et al.*, 2005), Tuscany (Barrocu, 2003), the Tiber Delta (De Luca *et al.*, 2005), Campania, Calabria (Barrocu, 2003) and the Adriatic coast (Ghiglieri *et al.*, 2012), especially the Po Plain (Antonellini *et al.*, 2008).

Some of these areas, in particular the Po Plain and the Oristano province (Sardinia), are used for rice cultivation. In these areas, the process of salinization may contribute to reduce crop yield, together with the presence of highly problematic weeds. In fact, salinity represents one of the environmental conditions that affect seed germination and plant growth (Sadeghloo *et al.*, 2013b), both in weeds and in crops. The knowledge of the ability of seeds to germinate in different environmental conditions, including salinity, is considered of fundamental importance not only for crop establishment, but also for estimating weed development in agricultural ecosystems (Koger *et al.*, 2004; Benvenuti, 2011).

Among the main weed species that infest rice fields, barnyard grasses (several species of the genus *Echinochloa* P. Beauv.), weedy rice (*Oryza sativa* L.), and sedges (mainly the genus *Cyperus* L.) are some of the most troublesome, able to cause significant yield losses (Panozzo *et al.*, 2013).

In particular, *Echinochloa* spp. have been key weeds in almost all rice systems worldwide, including European and Italian rice fields. These weeds are characterized by C4 photosynthetic pathway and some species are able to grow both in dry and flooded conditions (Vidotto *et al.*, 2007). *Echinochloa* spp. exhibit great competition effects towards rice, especially during early stages of cultivation. Gibson *et al.* (2002), for example, found that the competition established by *Echinochloa* spp. is

significantly lower if a rice field is maintained free from these weeds during the first 30 days after seeding.

Echinochloa spp. can be distinguished in “red” or “white” biotypes on the basis of the different pigmentation at the basal sheaths of the plant. It has been established that this different plant pigmentation could reflect differences in herbicide sensitivity of the weed (Tabacchi *et al.*, 2006). Problems associated with *Echinochloa* spp. management have worsened in the last decade, due to the selection of populations resistant to different herbicides applied in rice field, in particular ALS and ACC-ase inhibitors (Panozzo *et al.*, 2013). In the search of alternative and effective techniques for controlling these weeds, several methods have been tested, including biocontrol agents (Hershenhorn *et al.*, 2016) and the use of rice varieties tolerant to herbicides (Kraehmer *et al.*, 2017).

Even though the area of rice cultivation affected by salinization is increasing at global level, the response of rice weeds to salt conditions and the potential influences on weed ecology and competition effects have been poorly investigated so far. The stress due to the presence of salt causes physiological and biochemical alterations and tolerance to salinity in plants is related to the synthesis (induced by the stress) of several compounds, including abscisic acid (ABA), glycinebetaine, organic acids, proline, polyamine and polyols (Cowan *et al.*, 1992). The functions of these compounds in response to salt stress seem to be those of chemical signals, osmotic adjustment, free radical scavenging, preservation of proteins and membrane integrity. As concerns the grass weeds, it was found that the tolerance to saline conditions is due to their capacity in uptake and translocation of Na⁺, maintaining K⁺, and osmotic regulation through the accumulation of proline and glycinebetaine, even though salt tolerance in *E. crus-galli* seems to be not related to Na⁺ adsorption changes (Yamamoto *et al.*, 2003).

In the case of *Echinochloa* spp., in particular, it is not yet entirely clear what is the effect of salt stress on their behaviour and physiological pathways. In these terms, a potential delay in germination or a negative influence on seedling establishment and first growth due to salinity could potentially alter the role of these weeds in rice production systems. In addition, the knowledge of the interactions between response to salinity and other agronomical relevant traits, such as herbicide resistance, is essential to estimate potential future impacts of salinity on *Echinochloa* spp. management in rice. In particular, it is not clear if there is interaction between herbicide resistance and salt stress and what could be the biochemical mechanisms involved.

This study aimed to evaluate the effect of salinity on germination of different common barnyard grass (*E. crus-galli* (L.) P. Beauv.) populations collected in the Italian rice area and to verify the presence of differences in terms of salt sensitivity between populations that are susceptible or resistant to ALS-inhibitor herbicides.

Materials and methods

Seed Collection

Seeds of six common barnyard grass populations (*E. crus-galli*) were used during the trials. The seeds were collected between 2010 and 2012 in Italian rice fields which underwent repeated applications of penoxsulam since many years. Penoxsulam is a broad-spectrum ALS-inhibitor herbicide used in rice to control common barnyard grass, water-plantain (*Alisma plantago-aquatica* L.), red stem (*Ammannia coccinea* Rottb.) and other weeds. After collection, the seeds were let dry at room temperature for about one week and then stored at +5°C. At different timings, seedlings from an aliquot of the collected seed lots were tested for resistance to ALS-inhibitor herbicides via a greenhouse bioassay and a molecular study that confirmed the presence of target site resistance in three populations (labelled *r1*, *r2*, and *r3*) due to Trp-574-Leuc point mutation in the ALS gene (unpublished data). Thus, the other three populations (*s1*, *s2*, and *s3*) were considered sensitive to penoxsulam. For the present study, seeds of the six populations were taken for the same collected seed lots.

Effect of water salinity on germination

In order to evaluate the effect of water salinity on the germination, 20 seeds for each population were placed in Petri dishes (9 cm diameter) lined with one filter paper imbibed with 5 mL of deionized water or saline solution.

Nine different salt concentrations were applied. Salt solutions were prepared by dissolving NaCl in deionized water at the following concentrations: 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, and 400 mM. These nine salt concentrations were selected among those applied in previous studies (Chauhan and Johnson, 2009a; Sadeghloo *et al.*, 2013b; Opeña *et al.*, 2014) and according to the water salinity levels found in some European rice cultivation areas (Isla *et al.*, 2003b; Gay *et al.*, 2010b; Moret-Fernández and Herrero, 2015) in order to be able to compare the results of our trial with those found in other studies carried out in analogous conditions and in order to use saline concentrations that can be found in the area from which the six common barnyard grass populations tested came from. The number of doses was selected following the guidelines provided by Holland-Letz and Kopp-Schneider (2015).

Three replicates for each salt treatment were used. Petri dishes were sealed with Parafilm to avoid drying and contamination. Afterward, they were incubated in a growth chamber at a constant temperature of 25 °C and arranged in a randomized complete block design.

Seed germination was recorded every day for 15 days. Moreover, at the end of the incubation period (15th day), length of roots and shoots of a sample of 10 seeds for each Petri dish was determined.

Two runs of the entire experiment were carried out.

Statistical analyses

ANOVA was carried out on data of germination capacity (total of number of seeds germinated by the end of germination test), speed of germination and length of roots and shoots in order to test the effects of population, NaCl concentration and experiment run. The test was conducted using the open source programme and environment *R* (R Core Team, 2016)

A regression analysis was performed on data of germination capacity. The fitted model was the following 3-parameter log-logistic regression model (Streibig *et al.*, 1993; Knezevic *et al.*, 2007; Vidotto *et al.*, 2013):

$$Y = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \quad (1)$$

here Y is the germination capacity, x is the NaCl concentration in mM, d is the upper limit, and b is the relative slope at the point of inflection e .

Model fitting was performed using the function *drm* of the add-on package *drc* of the programme *R* (Ritz and Streibig, 2012; Ritz *et al.*, 2015).

The same model was also used to describe the effect on speed of germination, as the relationship between duration of germination test and cumulated number of germinated seeds. The analysis was conducted separately for each salt concentration with the time (days) as the parameter x in Eq. (1) and cumulated germination as the dependent variable Y . Furthermore, the same analysis was applied to model the relationship between NaCl concentrations (independent variable) and either the length of roots or length of shoots (dependent variables).

The effective concentrations required to reduce by 50% either germination capacity or shoot or root length in comparison to the values obtained at 0 mM salt concentration (EC_{50}) were estimated from the fitted models using the function *ED* of the package *drc*. In the case of cumulated germination, the number of days required to obtain 50% of final total cumulated germination was calculated. The values of EC_{50} were used to perform pair-wise comparisons between populations, or between averages among resistant or susceptible populations, by calculating a Sensitivity Index (SI):

$$SI = \frac{EC_{50}(A)}{EC_{50}(B)} \quad (2)$$

where A and B refer to two generic populations under comparison. SI was also calculated by considering $EC_{50}(A)$ and $EC_{50}(B)$ in Eq. 2 as estimated by pooling data of resistant or susceptible populations, respectively.

The significance of SI of each comparison was calculated by using the function *EDcomp* of the package *drc* of the *R* programme.

Results and discussion

Germination capacity

The results showed that the germination of the tested weed biotypes was affected by the salt treatments (Figure 1).

The analysis of variance showed significant differences in terms of germination capacity between resistant and sensitive populations from 0 mM NaCl to 250 mM NaCl (Table 1). At these saline doses, the germination capacity of the sensitive populations ranged from about 84% to 94% and resulted greater than that of the resistant ones, which ranged from about 60% to 71%.

Table 1. Seed germination capacity (expressed as percentage of germinated seeds) at 15 days of resistant (R) and sensitive (S) populations from 0 mM to 400 mM NaCl concentrations. R and S are averages from ALS-herbicide resistant (*r1*, *r2*, and *r3*) and sensitive (*s1*, *s2* and *s3*) populations, respectively.

Population	0 (mM)	50 (mM)	100 (mM)	150 (mM)	200 (mM)	250 (mM)	300 (mM)	350 (mM)	400 (mM)
R	69.44	71.11	69.44	67.50	60.55	63.33	50.55	36.11	13.89
S	91.11	93.89	90.55	92.78	87.78	83.89	69.44	34.44	12.78
t-test significance	*	*	*	*	*	*	ns	ns	ns

R: populations resistant to ALS-inhibitor herbicides (*r1*, *r2*, and *r3*); S: populations susceptible to ALS-inhibitor herbicides (*s1*, *s2*, and *s3*); *: $P \leq 0.05$; ns: $P > 0.05$.

Seed germination remained quite stable up to a saline concentration of 250 mM, with values slightly above 60% and 80% for resistant and sensitive populations, respectively. These germination values were similar to those recorded in previous studies on *E. glabrescens* Munro ex Hook in which at a salt concentration of 200 mM the germination was more than 60% and averaged about 73% at 250 mM NaCl (Opeña *et al.*, 2014); similar germination level (68%) was also found on *E. crus-galli* at a salt concentration of 225 mM (Sadeghloo *et al.*, 2013b). Conversely, in trials carried out by Chauhan and Johnson (2009a), germination of *E. colona* (L.) Link was totally inhibited at the salt concentration of 200 mM. In our study, increasing salt concentration above 250 mM reduced seed germination, until about 13% germination at 400 mM NaCl, with no differences between resistant and sensitive populations. At 400 mM NaCl germination was inhibited for the populations *s2* and *r2*, while it ranged from 5% to 33% for the populations *s1* and *s3*, respectively (Figure 1). At the same NaCl concentration Sadeghloo *et al.* (2013b) found that germination was completely inhibited. Previous studies on *E. colona* and

E. glabrescens also found a linear decrease of germination with the increase of salt concentration (Chauhan and Johnson, 2009a; Opeña *et al.*, 2014).

The EC_{50} for germination capacity was the lowest for the resistant population *r2* (EC_{50} 274.47 mM), while the highest for the sensitive population *s3* (EC_{50} 380.73 mM) (Table 2). The pairwise comparison between populations showed significant differences between population *s2* and population *r3* and between population *r2* and *s3* (Table 3). Furthermore, significant differences were also found between the resistant populations *r2* and *r3*. According to these results, there is no a clear evidence that response to saline conditions was influenced by sensitivity towards ALS-inhibitor herbicides, as in some cases significant differences were found between populations showing a similar herbicide sensitivity (such in the case of *s2* vs *s3* and *r2* vs *r3*).

Table 2. NaCl concentration required to reduce by 50% seed germination capacity (EC_{50}) of the tested populations. Vales in brackets are the standard error.

Population code	EC_{50}
<i>r1</i>	377.46 (58.18)
<i>r2</i>	274.47 (45.63)
<i>r3</i>	379.91 (28.25)
<i>s1</i>	339.61 (19.02)
<i>s2</i>	286.21 (27.66)
<i>s3</i>	380.73 (35.94)

Table 3. *SI* values of germination capacity (ratio between EC_{50} of two populations) of the pairwise comparisons between tested populations. *SI* values are tested against the hypothesis that they are not dissimilar to 1 and the p-values of this test are reported.

Compared populations	<i>SI</i>	P value
<i>r1/s1</i>	1.11	ns
<i>r1/s2</i>	1.32	ns
<i>r1/r2</i>	1.37	ns
<i>r1/s3</i>	0.99	ns
<i>r1/r3</i>	0.99	ns
<i>s1/s2</i>	1.19	ns
<i>s1/r2</i>	1.24	ns
<i>s1/s3</i>	0.89	ns
<i>s1/r3</i>	0.89	ns
<i>s2/r2</i>	1.04	ns
<i>s2/s3</i>	0.75	*
<i>s2/r3</i>	0.75	*
<i>r2/s3</i>	0.72	*
<i>r2/r3</i>	0.72	*
<i>s3/r3</i>	1.00	ns

*: $P \leq 0.05$.

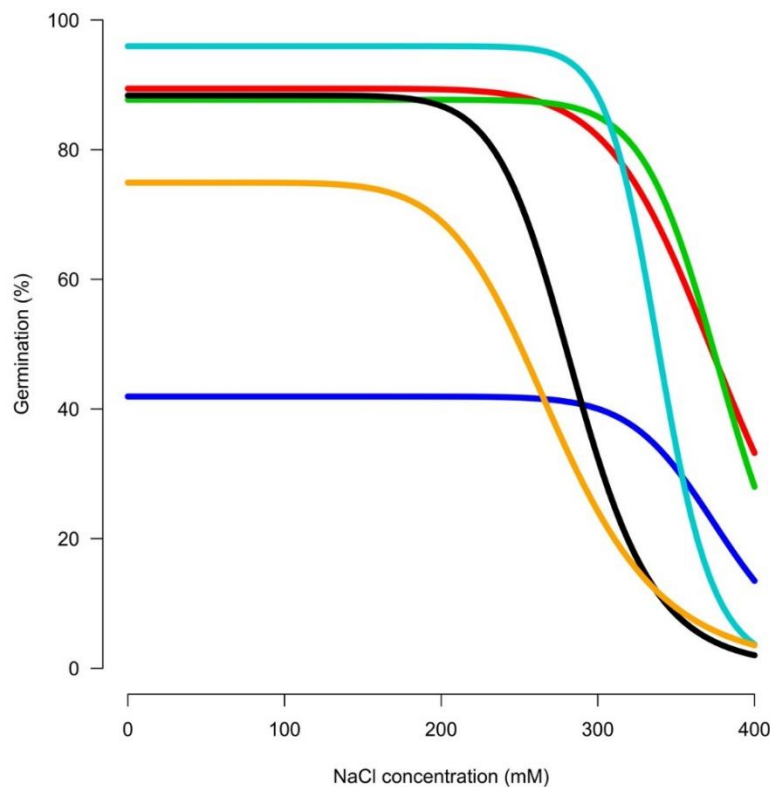


Figure 1. Seed germination (%) of the tested barnyard grasses populations as function of NaCl concentration, as fitted by the following log-logistic model $Y = d/[1+\exp\{b(\log(x) - \log(e))\}]$. Blue line: population *r1*; orange line: *r2*; green line: *r3*; cyan line: *s1*; black line: *s2*; red line: *s3*.

Speed of germination

The results obtained showed that the appearance of the first germinated seeds delayed with the increase of NaCl concentration (Figure 2). An analogous trend was found by Hakim *et al.* (2011) during a germination test conducted on different weeds, including *E. crus-galli* and *E. colona*. At 0 mM NaCl concentration the seeds began to germinate between the second and the third day from the start of germination test and reached their maximum germination between the fourth and the ninth day. Furthermore, at 0 mM NaCl only populations *s1* and *r2* showed significant differences in the time required for achieving 50% of their final germination capacity: in particular, population *s1* required 2.48 days while population *r2* needed 3.84 days (Table 4; Figure 2). For the resistant populations, the time required to reach 50% of germination capacity ranged, on average, from a minimum of 2.85 days

at 50 mM NaCl to a maximum of 12.42 days at 400 mM NaCl; averaging among sensitive populations, EC_{50} ranged from a minimum of 3.42 days to a maximum of 10.26 days, respectively, at 50 mM and 400 mM salt concentration (Figure 2). At the higher saline concentrations, the barnyard grass populations tested in our trials showed a speed of germination greater than that found by Hakim *et al.* (2011): in fact, in their study, the authors observed that *E. crus-galli* and *E. colona* began to germinate after 12-15 days.

Pairwise comparisons showed the presence of significant differences between resistant and sensitive populations at 50 mM, 200 mM, 250 mM and 300 mM NaCl concentrations (Table 4). Furthermore, significant differences were found within resistant and within sensitive populations at salt concentrations of 50 mM (s_1 vs s_2 and r_2 vs r_3), 200 mM (r_2 vs r_3), 250 mM (r_1 vs r_2) and 300 mM (s_1 vs s_2). As observed for germination capacity, also in the case of speed of germination there is no a clear evidence that sensitive and resistant populations react differently to increasing NaCl concentrations.

Table 4. SI values of speed of germination (ratio between EC_{50} of two populations) of the pairwise comparisons between tested populations. SI values are tested against the hypothesis that they are not dissimilar to 1 and the p-values of this test are reported. Only comparisons giving P values ≤ 0.05 are included.

NaCl concentrations (mM)	Compared populations	SI	P value
0	s_1/r_2	0.64	*
50	s_1/s_2	0.78	*
	s_1/r_2	0.78	*
	s_1/r_3	1.47	*
	s_2/r_3	1.89	*
	r_2/r_3	1.89	*
	s_2/r_3	1.73	*
200	r_2/r_3	1.61	*
250	r_1/s_2	0.64	*
	r_1/r_2	0.60	*
	s_1/r_3	1.43	*
	s_2/r_3	1.79	*
300	r_1/s_2	0.55	*
	s_1/s_2	0.69	*
	s_2/r_3	1.94	*

*: $P \leq 0.05$.

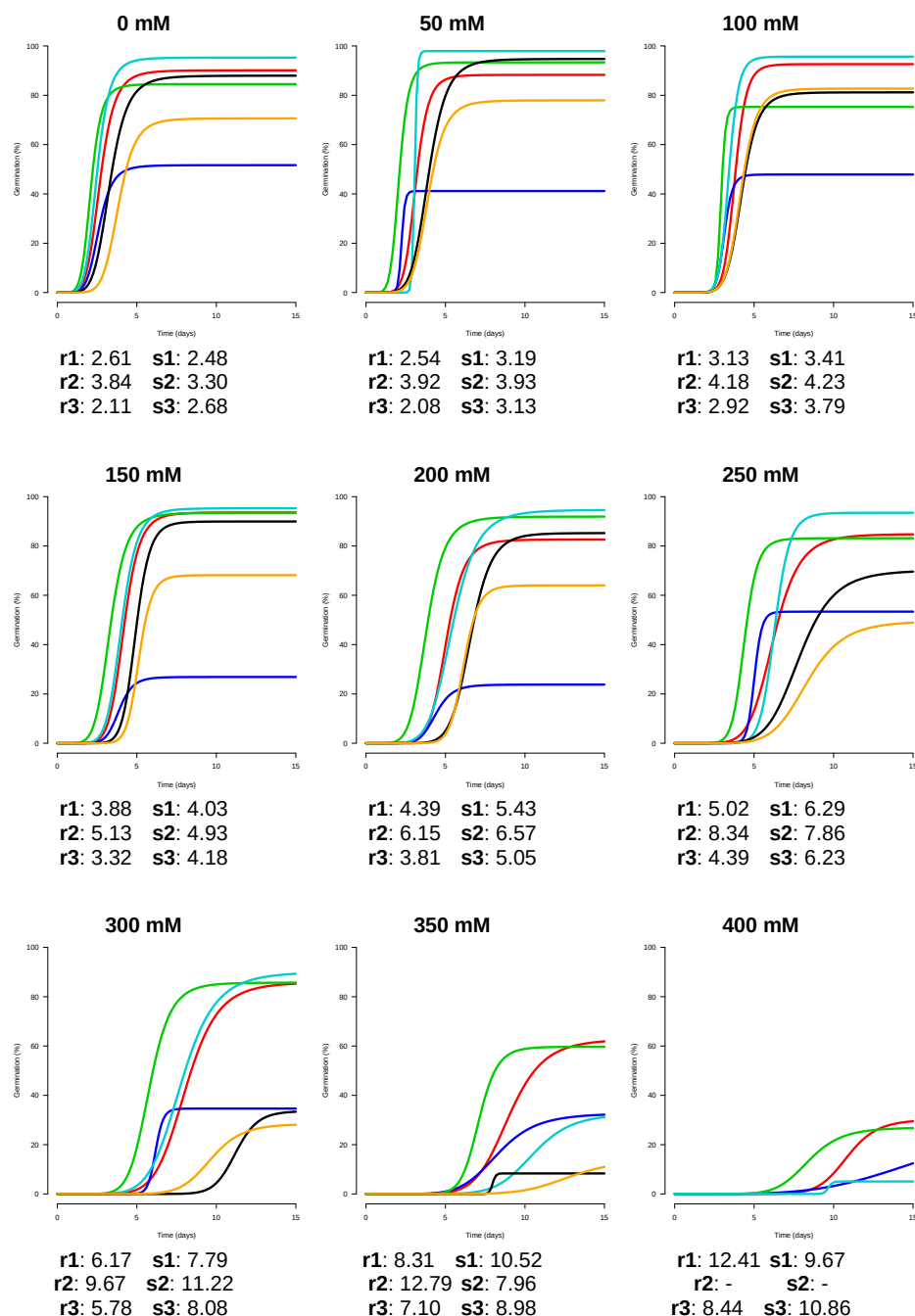


Figure 2. Cumulated percent of germinated seeds as function of duration of germination test, as fitted by the following log-logistic model $Y = d / \{1 + \exp[b(\log(x) - \log(e))]\}$ (graphs) and EC_{50} values (days) of the different populations at the tested NaCl concentrations. Blue line: population r_1 ; orange line: r_2 ; green line: r_3 ; cyan line: s_1 ; black line: s_2 ; red line: s_3 .

Length of roots and shoots

The results showed that, in all the tested populations, the length of roots decreased with increasing NaCl concentrations (Figure 3). Root length ranged, on average, from 9.88 cm at 0 mM NaCl to 0.36 cm at 400 mM NaCl. A similar trend was found by Hakim *et al.* (2011) on *E. crus-galli* and *E. colona*, in which a progressive reduction of root length with the increase of saline concentration was observed.

The value of EC_{50} for root length in resistant populations averaged 162.60 mM, with a minimum of 96.36 mM for the population *r2* and a maximum of 230.98 mM for the population *r3*. In the case of the sensitive populations, EC_{50} for root length averaged 159.76 mM, with a minimum of 114.81 mM for the population *s2* and a maximum of 210.10 mM for the population *s3* (Table 5).

The pairwise comparisons between populations underlined the presence of significant differences in root length among some of the resistant and sensitive populations (Table 6).

Regarding the length of shoots (Figure 4), a similar behaviour was observed, with shoot length decreasing with the increase of salt concentration. Shoot length ranged, on average, from 6.16 cm at 0 mM NaCl to 0.41 cm at 400 mM NaCl. Hakim *et al.* (2011) obtained analogous results on different species, including *E. crus-galli* and *E. colona*. In general, the values of EC_{50} for shoot length were higher than those recorded for root length, indicating that shoot growth was apparently less affected by salinity than root growth. In resistant populations, EC_{50} for shoot length was on average 246.10 mM, with a minimum of 226.47 mM for the population *r2* and a maximum of 261.67 mM for the population *r1* (Table 5). In the case of sensitive populations, EC_{50} for shoot length was 217.00 mM, with a minimum of 199.23 mM for the population *s2* and a maximum of 249.67 mM for the population *s3*. The pairwise comparisons between populations underlined the presence of significant differences in shoot length among some of the resistant and sensitive populations (Table 6). As observed for germination capacity and speed of germinations, there are no clear trends of different tolerance to salinity conditions in the tested populations that could be attributable to sensitivity to ALS-herbicides. Nevertheless, a similar behaviour was observed in terms of response of shoot length to salinity within resistant populations.

Table 5. NaCl concentration required to reduce of 50% root and shoot length (EC_{50}) of populations *r1*, *r2*, *r3*, *s1*, *s2* and *s3*, average EC_{50} for resistant and sensitive populations and their comparison. Vales in brackets are the standard error.

Population code	EC_{50} root length	EC_{50} shoot length
<i>r1</i>	160.47 (13.47)	261.67 (12.57)
<i>r2</i>	96.36 (12.24)	226.47 (11.61)
<i>r3</i>	230.98 (7.59)	250.15 (8.49)
<i>s1</i>	154.38 (9.29)	202.09 (11.56)
<i>s2</i>	114.81 (9.06)	199.23 (6.77)
<i>s3</i>	210.10 (10.24)	249.67 (6.38)

Table 6. Comparison between *r1*, *r2*, *r3*, *s1*, *s2* and *s3* populations for NaCl concentration required to reduce by 50% (*SI*) root length and shoot length.

Compared populations	Root length		Shoot length	
	<i>SI</i>	P value	<i>SI</i>	P value
<i>r1/s1</i>	1.04	ns	1.29	*
<i>r1/s2</i>	1.40	*	1.31	*
<i>r1/r2</i>	1.67	*	1.15	ns
<i>r1/s3</i>	0.76	*	1.05	ns
<i>r1/r3</i>	0.69	*	1.05	ns
<i>s1/s2</i>	1.34	*	1.01	ns
<i>s1/r2</i>	1.60	*	0.89	ns
<i>s1/s3</i>	0.73	*	0.81	*
<i>s1/r3</i>	0.67	*	0.81	*
<i>s2/r2</i>	1.19	ns	0.88	*
<i>s2/s3</i>	0.55	*	0.80	*
<i>s2/r3</i>	0.50	*	0.80	*
<i>r2/s3</i>	0.46	*	0.91	ns
<i>r2/r3</i>	0.42	*	0.90	ns
<i>s3/r3</i>	0.91	ns	1.00	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

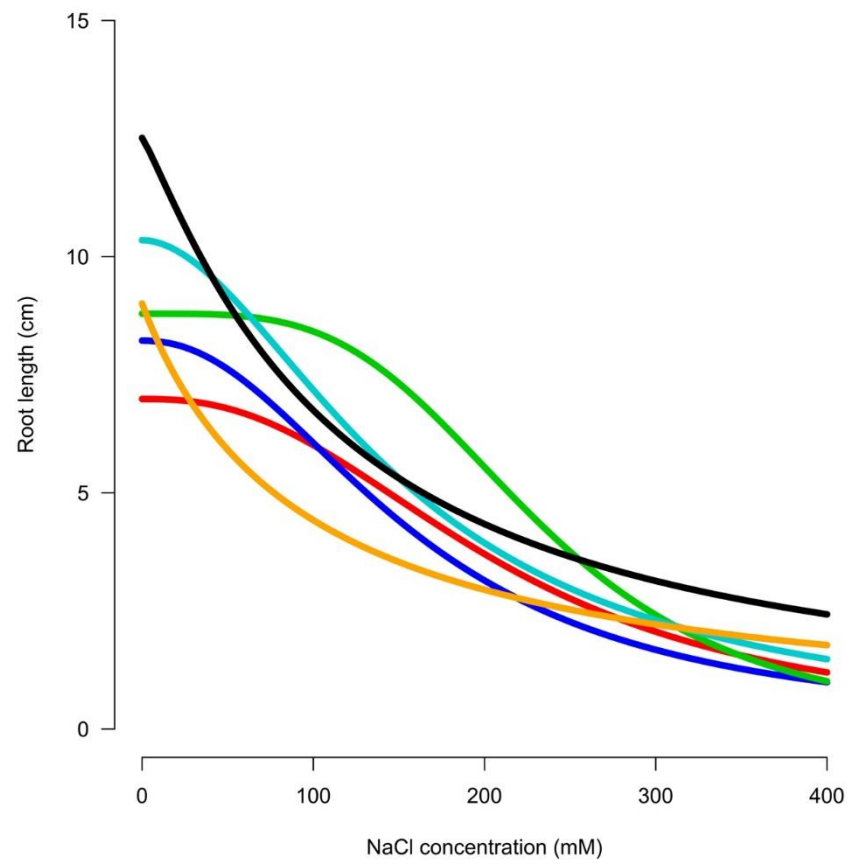


Figure 3. Root length of the different populations at the tested NaCl concentrations, as fitted by the following log-logistic model $Y = d / \{1 + \exp[b(\log(x) - \log(e))]\}$. Blue line: population *r1*; orange line: *r2*; green line: *r3*; cyan line: *s1*; black line: *s2*; red line: *s3*.

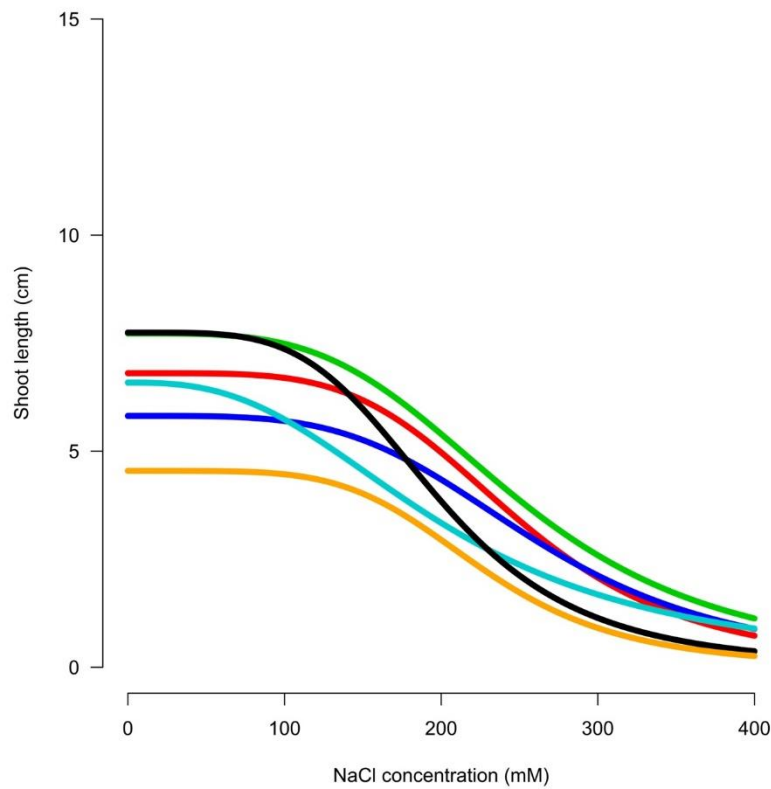


Figure 4. Shoot length of the different populations at the tested NaCl concentrations, as fitted by the following log-logistic model $Y = d / \{1 + \exp[b(\log(x) - \log(e))]\}$. Blue line: population *r1*; orange line: *r2*; green line: *r3*; cyan line: *s1*; black line: *s2*; red line: *s3*.

Conclusions

The results obtained in this study suggest that in the tested *E. crus-galli* populations, seed germination capacity, speed of germination, root and shoot growth were affected by saline conditions.

A remarkable barnyard grass tolerance to moderate salinity levels was observed: from 0 mM to 250 mM NaCl the seed germination capacity was up to 90% in the sensitive populations while it was about 70% in the resistant ones. Moreover, one of the populations (s3) showed a good tolerance to salinity, with a percentage of germination equal to 33%, even at 400 mM NaCl. Although the resistant tested populations exhibited an intrinsically lower germination capacity, as shown by the lower values recorder in non-saline conditions (0 mM NaCl), the response to salinity is similar to that observed in sensitive populations.

These results are interesting both from an ecological and agronomic perspective. For example, the reduction of speed of germination at increasing salt levels could suggest a reduced competitive activity of barnyard grass. Moreover, these results could be potentially exploited for predicting weed emergence dynamics through modelling, also in crops different from rice (Masin *et al.*, 2010, 2012).

The real consequences in terms of competitions towards the crop should be evaluated also taking into consideration the negative impact that salinity could have also on germination and first growth of the crop itself. Future studies are then needed for assessing the response to salinity of the main rice varieties cultivated in the environment in which the *E. crus-galli* populations tested in this study were collected.

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Third study

Effect of saline conditions on weedy rice and rice germination

One of the main obstacle for crop production is represented by weeds: they be considered one of the principal causes of reduction in quality and crop yield (Shaner, 2014).

In Italian rice field, the weed species able to cause serious issues to the crop are various; among these weedy rice (*Oryza sativa* L.), barnyard grasses (*Echinochloa* spp. P. Beauv) and sedges (*Cyperus* spp. L.) are counted (Panozzo *et al.*, 2013). Weedy rice, in particular, is present in the large majority of rice cultivation area (Fogliatto *et al.*, 2011). In Italy, this weed was firstly found more than a century ago, nevertheless its control has become highly difficult since 1920 because of the diffusion of direct seeding and rice monoculture (Fogliatto *et al.*, 2011). The level of infestation of the weed was affected by a sudden increase since 1990. The reasons of the rise in spread of weedy rice are still not clear, but some have proposed among the causes were the high number of weedy rice seeds presented in the commercial seed lots and the large cultivation of semi-dwarf indica-type rice varieties, which are characterized by lower growth and competitiveness than the majority of the varieties cultivated up to that period (Scarabel *et al.*, 2012).

This weed is able to adapt speedily to various environments and settings because of the polymorphism that characterized it (Fogliatto *et al.*, 2011). The crop yield reduction caused by the high competitive ability of this weed (Vidotto *et al.*, 2001) ranged from 1% to 13% in the case of weedy rice plant density equal to 1-5%, up to 30% in the case of weedy rice plant density equal to 10-20% (Busconi *et al.*, 2012).

The main mean of controlling this weed is represented by the herbicide applications (Panozzo *et al.*, 2013). However, since weedy rice belong to the same species of rice, its control resulted to be more problematic than other weeds (Gealy *et al.*, 2003), as the herbicides applied to control the weed could cause serious damage also to rice (Sudianto *et al.*, 2013). In order to overcome this limitation, in 2002 Clearfield® rice was introduced by the Louisiana State University Agricultural Centre. The Clearfield® technology was built around the selection of rice varieties characterized by tolerance to the imidazolinone herbicides (Sudianto *et al.*, 2013), which are otherwise not selective towards rice. In Italy, imazamox represents the herbicide applied in association with these varieties (Scarabel *et al.*, 2012).

The introduction of Clearfield® rice varieties and the repeated use of the herbicide imazamox involved also the introduction of the potential risk of herbicide resistance selection. This risk materialized in 2010, when a weedy rice biotype was found to be resistant to imazamox (Heap, 2017b). In Italy, in particular, several weedy rice populations resistant to imazamox have been identified by Busconi *et al.* (2012), and Scarabel *et al.* (2012), only few years after these varieties were first cultivated in Italy. Nowadays, Clearfield® technology is still very effective for management of weedy rice and several other weeds, but the selection of weedy rice

populations resistant to imidazolinones poses serious issues on the sustainability of this technology in the future.

In addition to herbicide resistance issues, which can make rice much prone to yield losses caused by weeds, rice cultivation is becoming endangered also by abiotic stresses due to consequences of climate changes. Among them, saltwater intrusion problems are more and more frequent in the last years in different rice cultivation areas.

Based on the estimates of the United Nations Food and Agriculture Organization, about 20% of irrigated lands is interested by the increase of the salt level (Rozema and Flowers, 2008). This phenomenon is stressed by the competition for fresh water between agriculture and civil uses, as result of climate changes, growing population (Maggio *et al.*, 2011), socio-economic development and contamination (Balía and Viezzoli, 2015). In Europe, 26 countries are affected by cases of salinization (Maggio *et al.*, 2011); among these, we count Spain, Portugal, Italy, Greece (Ghiglieri *et al.*, 2012), and France (Gay *et al.*, 2010a). In Italy, saltwater intrusion phenomena are found in some areas including Sardinia, the Catania Plain (Capaccioni *et al.*, 2005), Tuscany (Barrocu, 2003), the Tiber Delta (De Luca *et al.*, 2005), Campania, Calabria (Barrocu, 2003), and Adriatic coast (Ghiglieri *et al.*, 2012), especially the Po Plain (Antonellini *et al.*, 2008). Among these, the Po Plain and the Oristano Province are both interested by rice cultivation.

This study was conducted with the aims to evaluate the effect of salinity on germination of Italian weedy rice populations and cultivated rice varieties and to investigate the presence of a potential relationship between response to salinity and sensitivity to the imidazolinone herbicide imazamox.

Materials and Methods

Seed collection

In order to evaluate the effect of salinity, a series of germination trials were carried out.

Five weedy rice populations were tested for their tolerance to salinity conditions. The populations were selected among a larger set of about 150 populations originally resulting from a collection campaign carried out during 2008-2009 in a rice field area across Piemonte and Lombardia. Based on the results obtained from previous field and greenhouse tests, four populations (labelled s1, s2, s3 and s4) resulted to be sensitive to the herbicide imazamox, while one (labeled r) escaped field treatment with imazamox and resistance towards this herbicide was observed in greenhouse in a preliminary study.

Moreover, two rice varieties (Baldo, a conventional variety and CL80, a Clearfield® variety) were tested.

Effect of water salinity on germination

Twenty seeds of each population were placed in Petri dishes (9 cm diameter). The Petri dishes were lined with one filter paper imbibed with either 5 mL of deionized water or saline solution and sealed with Parafilm to avoid drying and contamination. Nine different saline concentrations were applied; these were prepared by dissolving NaCl in 5 mL of deionized water in concentrations equal to 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM and 400 mM. The salt concentrations were selected based on previous studies (Chauhan and Johnson, 2009a; Sadeghloo *et al.*, 2013a; Opeña *et al.*, 2014) and on the water salinity levels of some European rice cultivation areas (Badia, 2000; Amezketa, 2006; Gay *et al.*, 2010a).

Petri dishes were incubated in growing chamber at constant temperature of 25°C. The experimental unit was represented by a single Petri dish and all treatments (5 populations x 9 NaCl concentrations) were arranged in a complete randomized design with three replicates.

Germinated seeds were counted every day for fifteen days. Furthermore, at the end of the germination test (15th day) root and shoot length of a sample of ten seeds for each Petri was measured.

The entire experiment was conducted twice.

Statistical analyses

ANOVA was performed on data of germination capacity (percentage of seeds germinated at the 15th day) to test for differences in terms of response to salinity between weedy rice sensitive and resistant populations and between rice varieties. ANOVA was carried out using the open source programme and environment R (R Core Team, 2016).

A 3-parameters log-logistic regression model was fitted to data of seed germination capacity (Streibig *et al.*, 1993; Knezevic *et al.*, 2007; Vidotto *et al.*, 2013):

$$Y = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \quad (1)$$

Where Y is the seed germination capacity, x is the NaCl concentration expressed in mM, d is the upper limit, and b is the relative slope at the point of inflection e .

Model fitting was obtained using the function *drm* of the add-on package *drc* of the programme *R*.

The same type of model was also applied to study the effect of saline conditions on speed of germination. The analysis was performed separately for each single NaCl concentration. In this case, in Eq. (1) the parameter x represented the time (days) while the dependent variable Y the cumulated germination.

Moreover, the same model was used to describe the effect of the different NaCl concentrations (parameter x) on root and shoot length (independent variable Y).

The salt concentrations required to reduce by 50% seed germination capacity, root length and shoot length compared to the values observed at 0 mM NaCl (EC_{50}) were calculated from the fitted models by the use of the function *ED* of the package *drc*. The values of EC_{50} were then utilized to carry out pair-wise comparisons among all populations and varieties, by calculating a *Sensitivity Index* (*SI*):

$$SI = \frac{EC_{50}(A)}{EC_{50}(B)} \quad (2)$$

Where A and B indicate two populations or two varieties or a population and a variety under comparison.

All the *SI* values were tested for their difference from 1 by the use of the function *EDcomp* of the package *drc* of the *R* programme.

Results and discussions

The results obtained at the end of the trials showed that the saline conditions affected germination of both weedy rice populations and rice varieties (Figure 1).

A *t*-test analysis showed the presence of significant difference between the sensitive and resistant populations from 0 mM to 350 mM NaCl. At these salt concentrations, the average germination capacity of sensitive weedy rice populations resulted higher than that of the resistant one and ranged, on average, from 93% to 29% (Table 1).

Germination of sensitive populations remained high up to 300 mM and resulted greater than 60%. The percentage of germination found at 250 mM (about 70%) was in agreement with a study by Sadeghloo *et al.* (2013a), that reported 68% germination of barnyardgrass at 225 mM.

Resistant population seemed to be less tolerant to salinity: the germination capacity was lower than 50% already at a salt concentration of 250 mM.

In our study, the increase of salinity above 350 mM resulted in a decrease of germination percentage with no differences between sensitive and resistant populations.

Table 1. Seed germination capacity (% of germinated seeds) at 15 days of sensitive and resistant weedy rice populations from 0 mM to 400 mM NaCl.

NaCl concentration (mM)	Population		<i>t</i> -test significance
	S	R	
0	93.33	76.67	*
50	97.50	90.00	*
100	94.58	80.00	*
150	92.08	71.67	*
200	83.33	53.33	*
250	70.42	48.33	*
300	61.25	36.67	*
350	28.75	0.00	*
400	8.33	1.67	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

In the case of rice varieties, a *t*-test analysis showed the presence of significant differences, in terms of percentage of germination, between Baldo and CL80 (Table 2).

Seed germination capacity of Baldo ranged from 97% at 0 mM to 42% at 400 mM and resulted higher than that CL80 which ranged from 88% at 0 mM to 3% at 400 mM.

Seed germination of the rice varieties remained above 50% up to the salt concentration of 250 mM (65% and 57% for Baldo and CL80, respectively).

Table 2. Seed germination capacity (% of germinated seeds) at 15 days of Baldo and CL80 rice varieties from 0 mM to 400 mM NaCl.

NaCl concentration (mM)	Variety		t-test significance
	Baldo	CL80	
0	96.67	88.33	*
50	100.00	95.00	*
100	98.33	85.00	*
150	98.33	86.67	*
200	95.00	58.33	*
250	65.00	56.67	*
300	61.67	35.00	*
350	63.33	15.00	*
400	41.67	3.33	*

* $P \leq 0.05$.

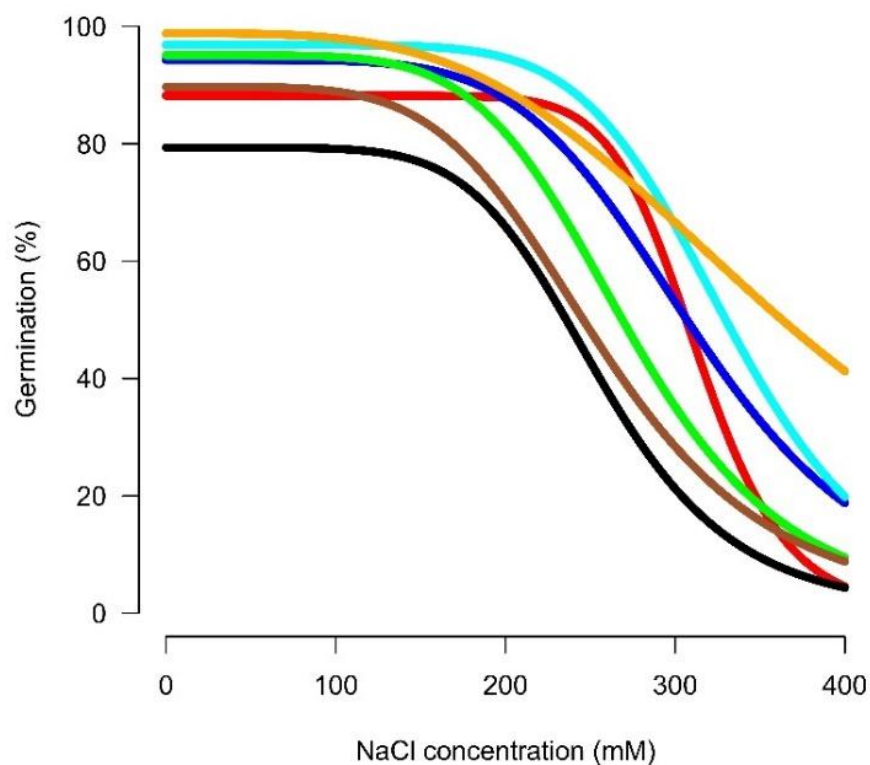


Figure 1. Seed germination capacity (%) of tested weedy rice populations and rice varieties as function of NaCl concentration as fitted by the following *log-logistic model* $Y = d/[1+\exp[b(\log(x) - \log(e))]]$. Red line: population s1, blue line: s2, green line: s3, cyan line: s4, black line: r, orange line: Baldo variety; brown line: CL80 variety.

The salt concentration required to reduce by 50% (EC_{50}) (Table 3) seed germination of the sensitive weedy rice populations was 297.92 mM NaCl, with a maximum of 332.69 mM NaCl for population s4 and a minimum of 273.71 mM NaCl for population s3. In the case of the resistant population r, the EC_{50} was 256.31 mM NaCl.

For the rice varieties, the EC_{50} was 365.35 mM NaCl in the case of Baldo and to 257.41 mM NaCl in the case of CL80.

None of the pairwise comparisons among all weedy rice populations and rice varieties showed significant differences, as the calculated *SI* values (ratio between EC_{50} of two populations/varieties) never differ significantly from one.

Table 3. NaCl concentration required to reduce by 50% seed germination capacity (EC_{50}) of the tested weedy rice populations and rice varieties.

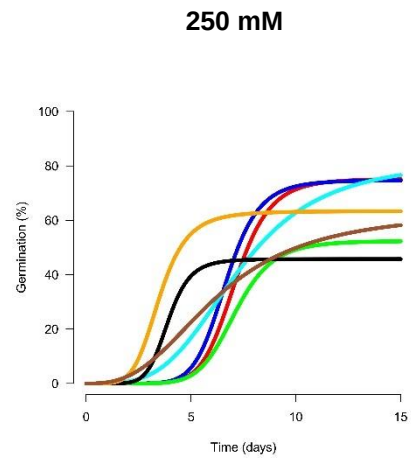
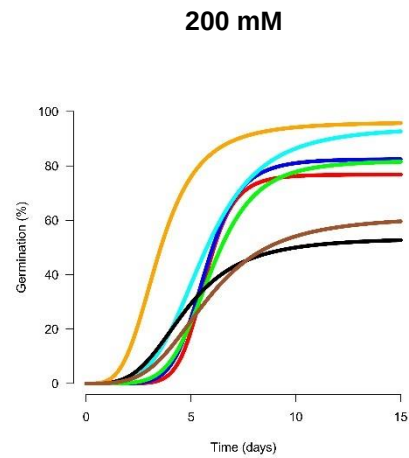
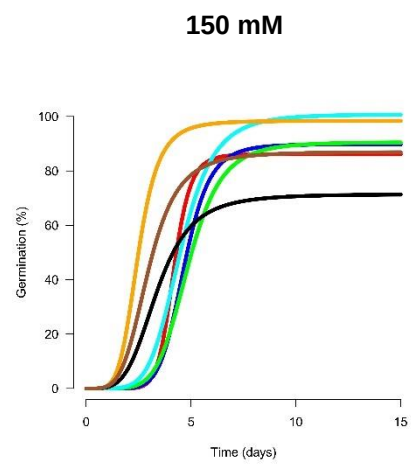
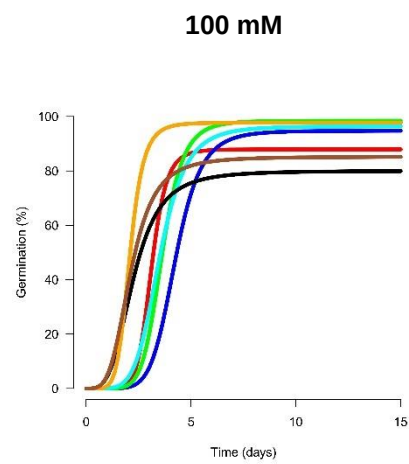
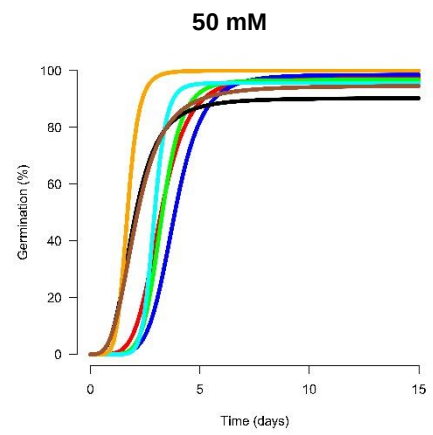
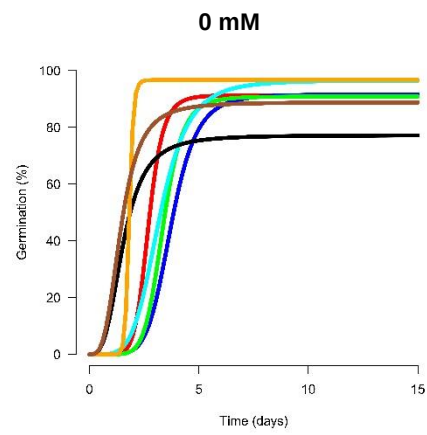
Population	EC_{50}
s1	313.41
s2	313.47
s3	273.71
s4	332.69
r	256.31
Baldo	365.35
CL80	257.41

Speed of germination

The results obtained at the end of the trials showed that salinity influenced speed of germination both in the case of weedy rice populations and rice varieties. In particular, germination times were prolonged as salt concentration increased (Figure 2). These results were in agreement with what found by Hakim *et al.* (2011): the authors found that the mean germination time of the weeds tested (among which weedy rice) increased with increasing of salinity concentration.

At 0 mM NaCl, the sensitive populations began to germinate between the second and the third day from the start of the incubation period, reaching their maximum between the seventh and the ninth day; the resistant started to germinate at the first day and reached its maximum germination capacity at the fifth day.

In the case of rice varieties, seed germination began between the first and the second day from the start of the study (Figure 2) with a maximum of germination at the third day. Moreover, at 0 mM NaCl, no significant differences were found between weedy rice populations and rice varieties in the time required to reach 50% of seed germination.



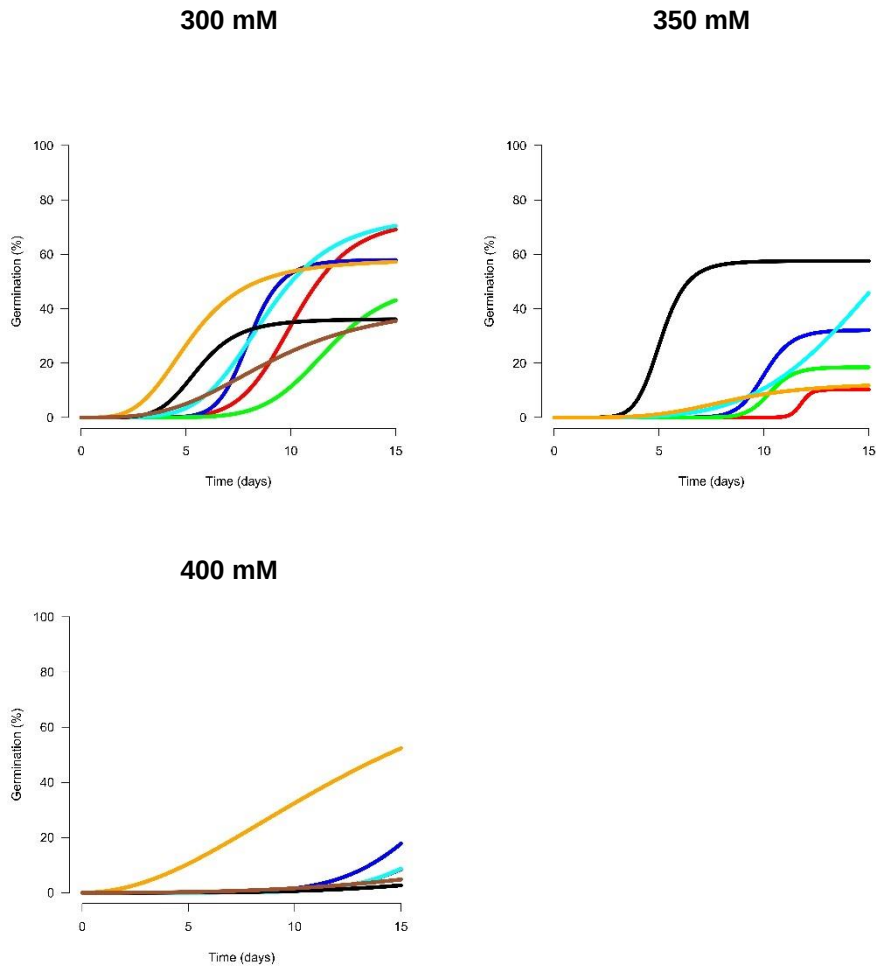


Figure 2. Cumulated seed germination capacity (%) as function of trial period based on the fitting of the log-logistic model of weedy rice populations and rice varieties at the different NaCl concentration as fitted by the following *log-logistic model* $Y = d/[1+\exp[b(\log(x) - \log(e))]]$. Red line: population s1, blue line: s2, green line: s3, cyan line: s4, black line: r, orange line: Baldo variety; brown line: CL80 variety.

The time required to reach 50% of seed germination (Table 4) ranged, on average, from a minimum of 3.30 days at 50 mM to a maximum of 20.47 days at 400 mM in the weedy rice sensitive populations while ranged from a minimum of 1.95 days at 50 mM to a maximum of more than 15 days at 400 mM in the case of the resistant one. Furthermore, at 400 mM, the germination of the sensitive population s3 began on the last day of the germination test.

As concern rice varieties, the EC_{50} ranged from 1.69 days at 50 mM to 6.50 at 400 mM for Baldo and from 2.08 days at 50 mM to 8.00 days at 400 mM for CL80.

On average, the weedy rice populations and the rice varieties tested in our trials resulted faster to reach 50% of seed germination than the weedy rice tested by Hakim *et al.* (2011), which found an average time of germination ranging from 6.22 days at control to 14.88 days at a salt concentration slightly lower than the 350 mM applied in our trials.

The pairwise comparisons (Table 5) showed the presence of significant differences between sensitive weedy rice populations and rice at 50 mM NaCl (s3 vs Baldo), 100 mM NaCl (s2 and s3 vs Baldo), 150 mM NaCl (s2, s3 and s4 vs Baldo) and 350 mM NaCl (s1 vs Baldo).

Table 4. Time (days) required to reach 50% of germination capacity (EC_{50}) of the tested weedy rice populations and rice varieties at the different NaCl concentrations.

NaCl concentration (mM)	Population / variety							
	s1	s2	s3	s4	Mean of S	r	Baldo	CL80
0	2.70	3.74	3.37	3.20	3.25	1.53	0.82	1.44
50	3.20	3.84	3.25	2.91	3.30	1.95	1.69	2.08
100	3.11	4.25	3.62	3.50	3.62	2.24	2.08	2.12
150	4.17	4.67	4.86	4.49	4.55	3.38	2.48	2.97
200	5.67	5.68	5.94	5.59	5.72	4.73	3.48	5.76
250	7.12	6.67	7.11	7.23	7.03	3.91	3.50	6.22
300	7.62	8.02	11.76	8.78	9.04	5.60	5.24	9.18
350	11.78	10.04	10.31	12.00	12.48	-	5.09	8.48
400	12.00	12.50	>15	14.00	12.83	>15	6.50	8.00

Table 5. *SI* values of the time required for the first germination (calculated as the ratio between EC_{50} of two weedy rice populations, two rice varieties or a weedy rice population and a rice variety). Only pairwise comparisons with P value ≤ 0.05 are showed.

NaCl concentration (mM)	Compared populations / varieties	<i>SI</i>	P value
50	s3/Baldo	1.92	*
100	s2/Baldo	2.04	*
	s3/Baldo	1.74	*
150	s2/Baldo	1.88	*
	s3/Baldo	1.96	*
	s4/Baldo	1.81	*
350	s1/Baldo	2.31	*

*: P value ≤ 0.05

Root and shoot length

The results obtained at the end of the measurements showed that root growth was affected by salinity conditions. A decrease of length with increasing salt concentration was observed (Figure 3). These results are in agreement with what found by Hakim *et al.* (2011): in their study, the authors noticed that root and shoot length of various weed species (included weedy rice) decrease with the increase of saline concentration. For the sensitive weedy rice populations root length ranged, on average, from 9.81 cm at 0 mM NaCl to 0.25 cm at 350 mM NaCl; at 400 mM NaCl, no root development was observed. In the case of the *r* population, the length ranged from 7.37 cm at control to 0.72 cm at 300 mM; as found for the sensitive populations, at 400 mM NaCl there was no root growth. In the case of rice varieties, root growth ranged from 7.87 cm at 0 mM NaCl to 0.31 cm at 400 mM NaCl in the case of Baldo and from 4.38 cm at 0 mM NaCl to 0.47 cm at 350 mM NaCl in the case of CL80; moreover, for CL80 no root development was observed at 400 mM. A similar trend of root growth was found by Momayezi *et al.* (2009): in their study on different rice genotypes with the application of NaCl and Na₂SO₄, the authors noted a decrease of root length with the increase of saline concentration. Conversely, differently from what observed in our trials, Hakim *et al.* (2011), found less root growth of the weedy rice tested at salt concentrations similar to some of those applied in our study.

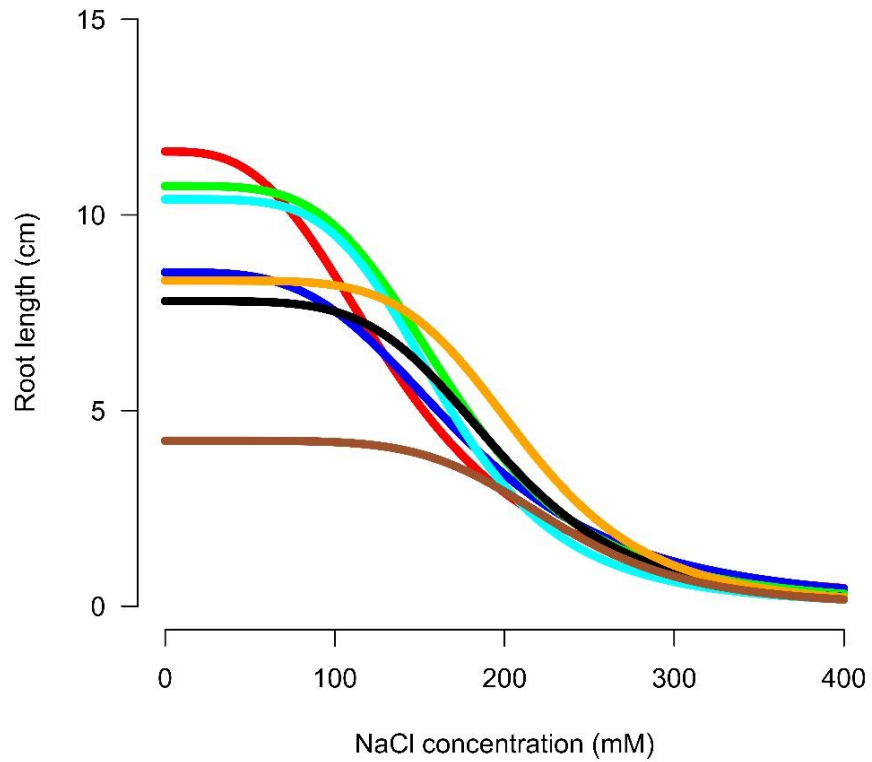


Figure 3. Root length of the weedy rice populations and the rice varieties at the different NaCl concentrations as fitted by the following *log-logistic model* $Y = d/[1 + \exp\{b(\log(x) - \log(e))\}]$. Red line: population s1, blue line: s2, green line: s3, cyan line: s4, black line: r, orange line: Baldo variety; brown line: CL80 variety.

The salt concentration required to reduce by 50% (EC_{50}) root length (Table 6) of the weedy rice sensitive populations was, on average, equal to 163.44 mM NaCl with the maximum in population s2 (177.25 mM NaCl) and the minimum in population s1 (138.68 mM NaCl). In the case of the *r* population, the EC_{50} was 197.68 mM NaCl.

In the rice varieties, EC_{50} was 212.18 mM NaCl in the case of Baldo and 230.35 mM NaCl in the case of CL80.

The pairwise comparisons (Table 7) showed the presence of significant differences between the sensitive and the resistant weedy rice populations and between weedy rice populations and rice varieties. Furthermore, significant differences were found within the sensitive weedy rice populations.

Table 6. NaCl concentration required to reduce by 50% (EC_{50}) root length of the weedy rice populations *s1*, *s2*, *s3*, *s4*, *r* and the varieties Baldo and CL80

Population / variety	EC_{50} root length
<i>s1</i>	138.68
<i>s2</i>	177.25
<i>s3</i>	172.04
<i>s4</i>	165.80
<i>r</i>	197.68
Baldo	212.18
CL80	230.35

Table 7. Comparison between weedy rice populations *s1*, *s2*, *s3*, *s4*, *r* and rice varieties Baldo and CL80 for the NaCl concentration required to reduce by 50% root length (SI_{50}).

Compared populations / varieties	Root length	
	SI	P value
<i>s1/s3</i>	0.81	*
<i>s1/s2</i>	0.78	*
<i>s1/s4</i>	0.84	*
<i>s1/Baldo</i>	0.65	*
<i>s1/CL80</i>	0.60	*
<i>s1/r</i>	0.70	*
<i>s2/s4</i>	1.07	*
<i>s2/Baldo</i>	0.84	*
<i>s2/CL80</i>	0.77	*
<i>s2/r</i>	0.90	*
<i>s3/s2</i>	0.97	ns
<i>s3/s4</i>	10.4	ns
<i>s3/Baldo</i>	0.81	*
<i>s3/cl80</i>	0.75	*
<i>s3/r</i>	0.87	*
<i>s4/Baldo</i>	0.78	*
<i>s4/CL80</i>	0.72	*
<i>s4/r</i>	0.84	*
Baldo/CL80	0.92	ns
Baldo/r	1.07	ns
CL80/r	1.17	*

*: P value ≤ 0.05 ; ns: P value > 0.05 .

Shoot length showed a behaviour similar to that found in the case of roots, with a decrease of shoot development with increase of salt concentration (Figure 4).

Shoot length of the tested weedy rice populations ranged, on average, from 6.70 cm at 0 mM NaCl to 0.18 cm at 400 mM NaCl. In general, sensitive populations were characterized by a shoot development higher than that of the resistant one.

In rice varieties, shoot length ranged, on average, from 6.46 cm at 0 mM NaCl to 0.61 cm at 400 mM NaCl. At all the applied salt concentrations, Baldo produced shoots longer than those of CL80. However, this greater shoot development cannot be correlated with a higher salt tolerance of Baldo, as CL80 produced shorter shoots already in non-saline conditions (3.71 cm in CL80 vs 9.22 cm in Baldo).

As already observed in the case of roots, the development of shoots found in our study was greater than that noted by Hakim *et al.* (2011), in the case of weedy rice exposed to saline concentrations similar to some of those applied during our germination trials.

The salt concentration required to reduce by 50% (EC_{50}) (Table 8) shoot growth of the sensitive weedy rice populations was, on average, 187.25 mM NaCl with the maximum of 202.50 mM NaCl for population s4 and the minimum of 175.72 mM NaCl for population s3. In the case of the resistant population r, the EC_{50} was 186.29 mM NaCl.

In the rice varieties, the EC_{50} was 209.26 mM NaCl in the case of Baldo and 224.89 mM NaCl in the case of CL80.

The pairwise comparisons (Table 9) showed the presence of significant differences between the sensitive weedy rice population s4 and the resistant population r and between weedy rice populations and rice varieties (s1 vs Baldo and CL80, s2 vs Baldo and CL80, s3 vs Baldo and CL80, s4 vs CL80). Furthermore, significant differences were found within the sensitive weedy rice populations (s1 vs s4 and s3 vs s4) and within rice varieties. Based on this last result, salt response, in terms of shoot development, of the tested weedy rice populations seems to be independent of their sensitivity to herbicide.

The differences noted between Baldo and CL80 suggested the presence of variability in crop growth under saline conditions.

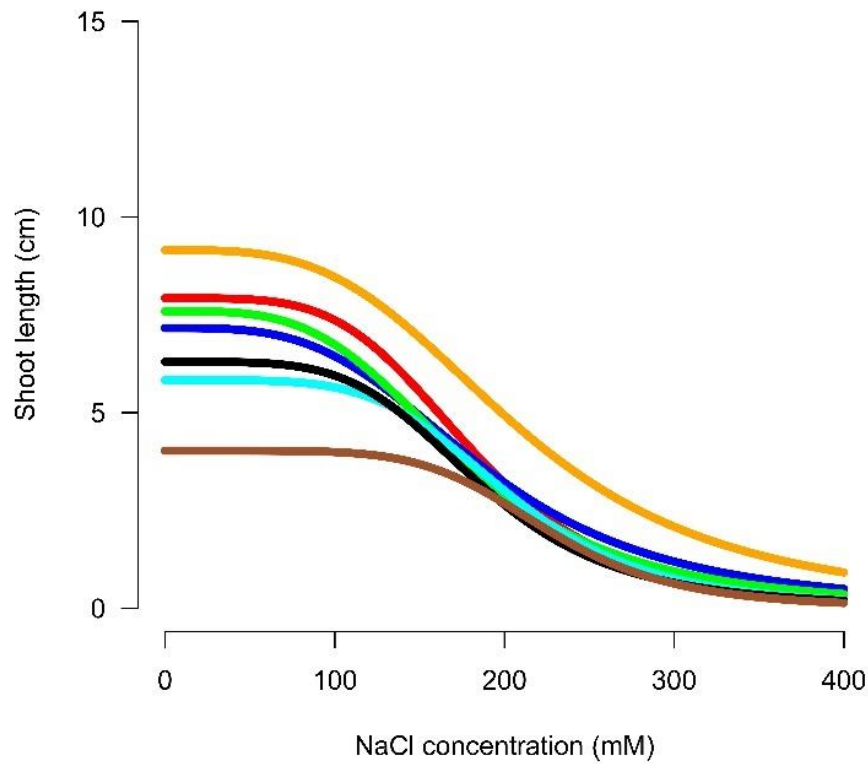


Figure 4. Shoot length of the weedy rice populations and the rice varieties at the different NaCl concentrations as fitted by the following *log-logistic model* $Y = d/[1+\exp\{b(\log(x) - \log(e))\}]$. Red line: population s1, blue line: s2, green line: s3, cyan line: s4, black line: r, orange line: Baldo variety; brown line: CL80 variety.

Table 8. NaCl concentration required to reduce by 50% (EC_{50}) shoot length of the weedy rice populations *s1*, *s2*, *s3*, *s4*, *r* and the varieties Baldo and CL80.

Population / variety	EC_{50} shoot length
<i>s1</i>	182.56
<i>s2</i>	188.22
<i>s3</i>	175.72
<i>s4</i>	202.50
<i>r</i>	186.29
Baldo	209.26
CL80	224.89

Table 9. Comparison between weedy rice populations *s1*, *s2*, *s3*, *s4*, *r* and rice varieties Baldo and CL80 for the NaCl concentration required to reduce by 50% shoot length (SI_{50}).

Compared populations / varieties	Shoot length	
	SI	P value
<i>s1/s3</i>	1.04	ns
<i>s1/s2</i>	0.97	ns
<i>s1/s4</i>	0.90	*
<i>s1/Baldo</i>	0.87	*
<i>s1/CL80</i>	0.81	*
<i>s1/r</i>	0.98	ns
<i>s2/s4</i>	0.93	ns
<i>s2/Baldo</i>	0.90	*
<i>s2/CL80</i>	0.84	*
<i>s2/r</i>	1.01	ns
<i>s3/s2</i>	0.93	ns
<i>s3/s4</i>	0.87	*
<i>s3/Baldo</i>	0.84	*
<i>s3/CL80</i>	0.78	*
<i>s3/r</i>	0.94	ns
<i>s4/Baldo</i>	0.97	ns
<i>s4/CL80</i>	0.90	*
<i>s4/r</i>	1.09	*
Baldo/CL80	0.93	*
Baldo/ <i>r</i>	1.12	*
CL80/ <i>r</i>	1.21	*

*: P value ≤ 0.05 ; ns: P value > 0.05 .

Conclusions

The findings suggest that both weedy rice populations and rice varieties tested in our study were affected by salinity: with the increase of salt concentration, a decrease of seed germination capacity, speed of germination and root and shoot development was observed.

The differences in response to salt stress noted between the tested weedy rice populations cannot be associated with the different sensitivity to the imidazolinone herbicides. The lower germination capacity of the resistant population can be considered as a behaviour characterizing the population itself; this is confirmed by the fact that a lower seed germination was also found in absence of saline stress (0 mM NaCl).

The findings of the study underlined a good tolerance of the tested weedy rice populations to high saline conditions: at 250 mM NaCl seed germination capacity was 70% in the case of sensitive populations and 43% in the case of the resistant one.

A result of great interest is also represented by the high tolerance to salt stress showed by Baldo, which germination capacity was greater than 60% up to a salt concentration of 350 mM. Furthermore, at 400 mM NaCl, the percentage of germinated seeds (42%) resulted higher than that of the CL80 and of all the weedy rice populations tested.

The high tolerance to saline conditions found in the rice variety Baldo represents a result of great importance: this suggest the ability of the crop to growth even under stress conditions. Moreover, the great seed germination capacity increases the competitive ability of the rice compared to the weeds, which is of crucial importance during the first crop growth stages. Weeds interfere with crop development because of the competition they use for the exploitation of light, water and nutrients (Smith, 1968; Dew, 1972; Kropff, 1988). The comparison of the germination time of weeds and crop is a key element in the definition of competition (Dew, 1972; O'Donovan *et al.*, 1985; Kropff and Spitters, 1991): weeds characterized by simultaneous or slightly later germination, compared to that of the crop, are causing greater production losses than weeds that emerge in later stages of growth (Smith, 1968; Dew, 1972; Kropff, 1988; Liu *et al.*, 2009).

Furthermore, because of the current presence of saline conditions in some European cultivation areas and the potential expansion of this phenomenon, the obtained results provide useful information about what might be the behaviour of weed and crop under saline environment.

Fourth study

**Salt effect on germination and growth of
barnyard grass, weedy rice and rice**

The development of plants is influenced by numerous environmental stresses (Ahuja *et al.*, 2010); among these are included: high and low temperature, drought, salinity, alkalinity (Parvaiz *et al.*, 2008), high CO₂ (Ahuja *et al.*, 2010). These stresses are considered environmental status responsible for causing, in plants, a reduction in development and yield under the optimum levels (Cramer *et al.*, 2011).

Salinity represents one of the principal environmental stresses than can affect growth and productivity of plants. The deleterious results caused to the plants by the presence of high salt levels can be highlighted at the whole-plant level; they appear as death of the plant and/or as yield reductions. All the primary metabolic processes that characterize the plants (photosynthesis, protein synthesis, energy and lipid metabolism) are involved in the set of responses to salt stress (Parida and Das, 2005). Currently, the effect produced on plant growth by environmental stresses (Ahuja *et al.*, 2010), among which the salinity is one of the main (Pattanagul and Thitisaksakul, 2008), has become an important issue as consequence of the impact due to climate change on plant resources, biodiversity and food security overall (Ahuja *et al.*, 2010). Climate change, in combination with growing populations (Maggio *et al.*, 2011), socio-economic development and contamination (Balía and Viezzoli, 2015), is responsible for the strong competition for fresh water use between agriculture and other human activities (Maggio *et al.*, 2011). This, in turn, is one of the reasons for the increase in salinity levels in irrigated lands, as stated also by the United Nations Food and Agriculture Organization, which reported an increment in salt concentration on about 20% of the irrigated lands (Rozema and Flowers, 2008). In Europe in particular, 26 countries (Maggio *et al.*, 2011), including Spain, Portugal, Italy, Greece (Ghiglieri *et al.*, 2012) and France (Gay *et al.*, 2010a) are interested by cases of salinization (Maggio *et al.*, 2011). In Italy, problems of salt stress resulting from saltwater intrusion can be detected in Sardinia (Capaccioni *et al.*, 2005), in the Catania Plain (Capaccioni *et al.*, 2005), in Tuscany (Barrocu, 2003), in the Tiber Delta (De Luca *et al.*, 2005), in Campania and Calabria (Barrocu, 2003), in the Adriatic coast (Ghiglieri *et al.*, 2012), especially in the Po Plain (Antonellini *et al.*, 2008).

In some of these areas, the presence of salt stress can be an obstacle to the growth of various crops such as rice in the case of the Po Plain and the Oristano Province (Sardinia).

Rice is considered a crop relatively tolerant to salt stress (Chauhan *et al.*, 2013). Rice plant is able to tolerate the saline stress during the germination phase while it results to be susceptible to this stress during seedling emergence and early growth. During the following vegetative growth, the plant usually tolerates more the saline conditions, but it becomes again more sensitive during reproductive growth stages (Mittal *et al.*, 2016).

The presence of high levels of salinity causes a reduction in yields while very high salinity conditions can cause the total failure of the crop (Chauhan *et al.*, 2013).

The stress due to salinity is worsened by the presence of other limiting factors, and by the competition due to weeds in particular. Moreover, it should be noted that, in general, most of weeds are less susceptible to environmental stresses than crops (Hakim *et al.*, 2011).

Among the major weeds of rice fields, we count *Echinochloa* spp. and weedy rice (Panozzo *et al.*, 2013). The response of these weed species to saline conditions is variable: *E. crus-galli* is considered to be able to tolerate conditions of high salinity (Wilson and Read, 2006), while weedy rice is found to be sensitive to exposure to salt (Hakim *et al.*, 2013).

The study was carried out with the aim to evaluate the saline effect on germination and growth of barnyard grass and weedy rice populations and of a rice variety. Moreover, the relationship between salt stress and the response to herbicide application was also investigated.

Materials and methods

Plant material

The study was conducted on four barnyard grass populations, three weedy rice populations and one rice variety.

Seeds of the four barnyard grass populations were collected in Italian rice fields in which the repeated application of the ALS-inhibitor penoxsulam occurred. Three (*s1*, *s2* and *s3*) of the four barnyard grass populations tested were sensitive to the ALS-inhibitor herbicide penoxsulam (*S* populations) while, based on greenhouse screening and molecular bioassays, the last one (*r*) resulted resistant.

Seed of the three weedy rice populations (labelled *wr1*, *wr2* and *wr3*) were chosen among various populations coming from different Italian rice fields and resulted sensitive to the main ALS and ACCase inhibitor herbicides used in rice crop. Moreover, of these three weedy rice populations, one was awnless, one awned and one mucronate.

The rice variety tested during the study was Baldo, one of the most widespread varieties in the Mediterranean Basin and characterized by high commercial importance.

Saline solution

In order to bring the experimental conditions of the study as close as possible to those that can be found in certain real field conditions, the salt concentrations applied during the trials were equal to 0 mM, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM. These concentrations are similar to

the salinity levels that can be found in some European rice cultivation areas (Badia, 2000; Isla *et al.*, 2003a; Gay *et al.*, 2010a).

Whole-plant bioassay

The trials were carried out in greenhouse under controlled conditions (25°C average daily temperature, 12-h daylength). All the species were tested in spring 2017 at the experimental facilities of the Department of Agriculture, Forestry and Food Science of the University of Turin (Italy).

The test was divided into two subsets: the first, in which only the saline effect on weed populations and rice varieties was analysed (“salt”) and the second, in which the effect of the potential interaction between salinity and herbicide treatment on weeds was tested (“salt+herbicide”). The two subsets were sown the same day and all the assessments were carried out with the same timing in the two subsets.

Weed populations and the rice variety were sown into alveolar trays composed by 5 columns X 4 rows of holes (2.7x2.7x5.8 cm) filled with sand, by placing three seeds in each hole. Each column of the tray hosted one of the four weed populations or the rice variety. In each tray, all the populations and the variety were present (so that a population or the variety occupied only one column in each tray). The column occupied by a certain population/variety was attributed randomly for each tray.

The alveolar trays were placed into rectangular plastic trays filled with 2 l of solution characterized by different saline concentrations.

Three replicates for each salt concentration were considered. Each set of alveolus and tray was considered as a replicate.

Plants were maintained in the same trays for the whole duration of the test.

The solution was prepared using tap water ($EC = 1718.75 \mu S\ cm^{-1}$) and NaCl in different proportion in order to obtain desired salt concentrations. The nutrient solution Hoagland's No. 2 Basal Salt Mixture was added at the dose of 3.2 g to the 2 l of solution.

After seven days from the beginning of the test, three times a week 250 ml of tap water were added to the solution present in the trays in order to compensate the solution losses due to evaporation and/or adsorption and to correct the EC by bringing it to the pre-set values.

The pH was measured every day for the duration of the study. At the beginning of the test, the pH value was equal to 6.60; during the trial, the value increased and stabilized at 7.75. In order to operate in conditions similar to what could happen in field, the pH value was not corrected.

The herbicides applied to subset “salt+herbicide” were represented by the ALS-inhibitor penoxsulam in the case of barnyardgrass populations and by the ALS-inhibitor imazamox in the case of the weedy rice populations. Both the herbicides were applied at the field rate (penoxsulam, active ingredient = $40\ g\ ha^{-1}$; imazamox, active ingredient = $35\ g\ ha^{-1}$) using a cabinet track sprayer equipped with a single flat fan nozzle (Teejet

DG8002-VS) calibrated to deliver a spray volume of 300 l ha⁻¹ at a pressure of 203 kPa. Barnyard grass populations and weedy rice populations were sprayed at 2-3 leaf stage.

Since the rice variety Baldo is a conventional variety, herbicide treatment was not applied and it was tested for saline response only. Consequently, the results related to the rice variety are present only in the subset “salt”.

Seed germination of barnyard grass and weedy rice populations and of rice variety was recorded every day in each salt concentration, in both the “salt” and “salt + herbicide” subsets. The counting of germinated seeds was conducted once a day for fifteen days.

The number of seeds germinated at the fifteenth day were then expressed as a percentage of germination.

Plant height was determined immediately before herbicide treatment and at 7, 14 and 21 days after treatment; in the case of subset “salt” the height was detected with the same timing of the subset “salt+herbicide”. The height was measured from the ground level to the apex of the longest leaf. For each population / variety, the measurements were carried out on four plants for each of the three replicates. The measured plants were randomly selected.

Aboveground fresh weight of each weed population and of the rice variety for both the subsets was detected 21 days after herbicide treatment. Moreover, root fresh weight of the weed populations and the rice variety was determined. The fresh weight of shoots and roots was determined by measuring the total weight of all the plants grown in all replicates of the different treatments compared.

SPAD measurement

After 21 days from the herbicide treatment in the subset “salt+herbicide”, the SPAD value was measured for each weed population and rice variety using a SPAD 502 Konica Minolta - Chlorophyll Meter.

SPAD values were detected in the middle portion of the first true leaf.

For each population / variety, the measurements were carried out on four plants for each of the three replicates. The plants for which SPAD was measured were randomly selected

Chlorophyll and carotenoids extraction

Chlorophyll and carotenoids content of each weed populations and rice variety for each salt treatment and in both the subsets was measured at 21 days after herbicide application in subset “salt+herbicide”. An amount of 0.2 g of leaves collected from each replicate was added with 10 mL of 80% acetone and finely ground by the use of a dispersing instrument for a minute. The samples obtained were stored in darkness for 24 h (Guo *et al.*, 2017) at 4°C; after the storage period, each sample was centrifuged. Spectrophotometric measurement of chlorophyll *a*, *b* and carotenoids was then performed on the supernatant samples obtained after centrifugation.

The wavelengths used were 662, 645 and 470 nm for the chlorophyll *a*, chlorophyll *b* and carotenoids, respectively. The amount of these three pigments was determined using the Lichtenthaler and Wellburn formulas and expressed as milligrams per gram of fresh weight (mg g⁻¹ FW) (Zhan *et al.*, 2009).

Statistical analyses

ANOVA was conducted to test the effect of population, salt dose and presence / absence of herbicide application for the different parameters considered (seed germination, plant height, plant fresh weight, root fresh weight, SPAD, chlorophyll and carotenoids content). The analysis was conducted by using the open source programme and environment *R* (R Core Team, 2016).

A 3-parameters log-logistic regression model was performed on data of seed germination capacity (total of number of seeds germinated at the fifteenth day). The fitted model applied was the following (Streibig *et al.*, 1993; Knezevic *et al.*, 2007; Vidotto *et al.*, 2013):

$$Y = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \quad (1)$$

Where *Y* is the seed germination capacity, *x* is the NaCl concentration expressed in mM, *d* is the upper limit, and *b* is the relative slope at the point of inflection *e*.

Model fitting was executed by the application of the function *drm* of the add-on package *drc* of the programme *R*.

The same *f* model was also applied to study the effect of saline conditions on speed of germination. The analysis was performed separately for each single NaCl concentration; in this case, the parameter *x* in Eq. (1) represented the time (days), while the dependent variable *Y* was the cumulated germination.

The salt concentrations required to reduce by 50% seed germination capacity, root length and shoot length compared to the values observed at 0 mM NaCl (*EC*₅₀) were calculated from the fitted model by the use of the function *ED* of the package *drc*.

The values of *EC*₅₀ thus obtained were utilized to carry out pair-wise comparisons between populations and varieties, by determination of a Sensitivity Index (SI):

$$SI = \frac{EC_{50}(A)}{EC_{50}(B)} \quad (2)$$

Where *A* and *B* indicate two populations or a population and the variety under comparison.

The significance of *SI* of each comparison was obtained by the use of the function *EDcomp* of the package *drc* of the *R* programme.

Since the initial experiment conditions were the same in the two subsets, the germination data were first analysed to check if they could be represented by a single model by pooling the data of the “salt” and “salt+herbicide” subsets. The data was fitted first as a pooled dataset and then as two separate datasets, one for each subset. ANOVA was conducted to verify the presence of significant differences between the two analyses. Given that no differences were found, the germination data of the two subsets were analysed a single dataset.

The same procedure was performed also in the case of speed of germination and because no differences were found, data of speed of germination of the two subsets were analysed a single dataset.

Results and discussion

At the end of the trials, the results showed that weed populations and rice variety were affected by saline conditions to which they were exposed. In particular, seed germination, speed of germination, plant growth and physiological parameters have been conditioned by the saline treatments.

Barnyardgrass

Seed germination

The results of the study underlined that seed germination capacity of the tested barnyard grass populations was affected by salinity.

The analysis of variance (Table 1) performed between sensitive populations and the resistant one showed the presence of significant differences only at 0 mM NaCl and 250 mM NaCl. On average, at these two salt concentrations, the germination capacity of sensitive populations resulted greater than that of the resistant one. The fact that significant differences between sensitive and resistant were found even in non-saline conditions (0 mM NaCl) suggested that the different percentage of germinated seeds observed at 250 mM NaCl could not be attributed to the level of sensitivity to ALS-inhibitors but it could likely be related to the natural variability that can be found among different barnyard grass populations.

Table 1. Seed germination capacity (% of germination) at 15 days of sensitive (S) and resistant (R) barnyard grass populations from 0 mM to 250 mM NaCl. S values are averages among sensitive populations s1, s2 and s3; R values refer to the population r.

Population	NaCl concentration (mM)					
	0	50	100	150	200	250
S	67.13	68.06	54.17	31.02	29.61	30.56
R	56.94	75.00	61.11	59.72	15.28	2.78
t-test significance	*	ns	ns	ns	ns	*

*: $P \leq 0.05$; ns: $P > 0.05$.

During our tests, we observed that, as general trend, seed germination decreased with the increase of salt concentration (Figure 1). The average germination ranged from 67% at control to 31% at 250 mM in the case of sensitive populations while ranged from 57% to 31% in the resistant population.

At 50 mM NaCl the average germination resulted greater than 60% both for sensitive and resistant populations while at a salt concentration of 100

mM germination resulted equal to 54% and 47% in the case of sensitive and resistant populations, respectively. About 32% and 30% of seed germination occurred at 150 mM and 200 mM for sensitive populations, while, at the same salt rates, 36% and 24% of germination was observed in the case of the resistant population.

Between the sensitive populations, higher tolerance to salinity was observed for the population s2, which showed a germination capacity greater than 60% up to a salt concentration of 100 mM (Figure 1).

The resistant population r was found to be less sensitive to salinity up to 150 mM NaCl, with a seed germination greater than 50%.

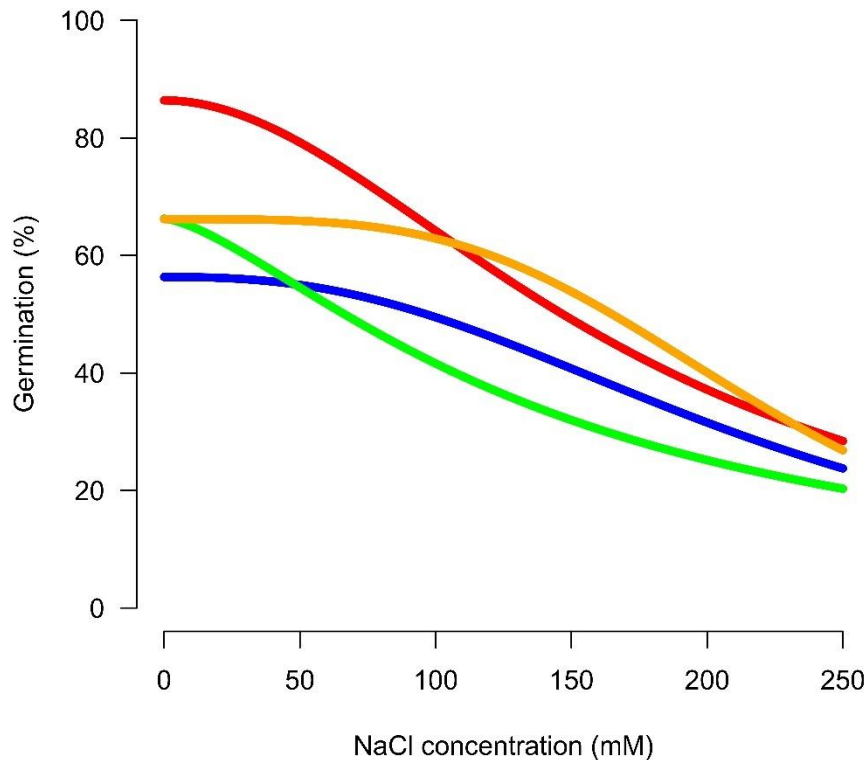


Figure 1. Seed germination capacity (%) of tested barnyard grass populations as function of NaCl concentration as fitted by the following *log-logistic model* $Y = d/[1+\exp[b(\log(x) - \log(e))]]$. Blue line: population s1; red line: s2; green line: s3; orange line: r.

The NaCl concentration required to reduce by 50% (EC_{50}) the seed germination (Table 2), averaged 178.766 mM among sensitive populations, while it was 225.03 mM in the resistant one; however, the analysis of variance showed that there were no significant differences between the EC_{50} of the sensitive and the resistant populations tested.

Table 2. NaCl concentration required to reduce by 50% (EC_{50}) seed germination of tested barnyard grass populations.

Population	EC_{50}
s1	220.33
s2	172.95
s3	143.02
<i>r</i>	225.03

The pairwise comparisons (Table 3) showed that no differences, in terms of germination percentage, were found among all tested populations. These results suggested that a different level of sensitivity to ALS-inhibitors and seed germination capacity under saline conditions is unlikely to occur.

Table 3. SI of germination capacity of the pairwise comparisons between tested populations.

Compared populations	SI	P value
s2/s3	1.21	ns
s2/s1	0.79	ns
s2/r	0.77	ns
s3/s1	0.65	ns
s3/r	0.63	ns
s1/r	0.98	ns

ns: $P > 0.05$.

Speed of germination

The results observed during our trials showed that speed of germination was affected by salinity, as an increase of the number of days required for the germination was observed at increasing salt concentrations (Figure 2).

The controls began to germinate between the seventh and eighth day after seeding and reached their maximum germination between the twelfth and the thirteenth day from the beginning of the trial (Table 4).

In the case of the sensitive populations, the time required to reach 50% of seed germination (Table 5) ranged, on average, from a minimum of 8.33 days at 50 mM NaCl to a maximum of 12.44 days at 200 mM NaCl.

In the case of the resistant population *r*, the EC_{50} ranged from a minimum of 7.15 days to a maximum of 12.00 days, respectively, at 50 mM NaCl and 200 mM NaCl.

At 250 mM NaCl, seed germination of the sensitive populations *s1* and *s2* and of the resistant population *r* began at the fourteenth day from starting of the trial while seed germination of sensitive population *s3* began at the fifteenth day (Table 4). Since, at this salt concentration, seed germination began only a day before the end of the observation period, regression analysis could not be performed and EC_{50} and *SI* were not calculated.

Table 4. Germination start day of the tested barnyard grass populations.

NaCl concentration (mM)	Population			
	<i>s1</i>	<i>s2</i>	<i>s3</i>	<i>r</i>
0	7	7	8	7
50	7	7	7	7
100	8	7	8	7
150	10	8	10	8
200	11	11	12	11
250	14	14	15	14

Table 5. Time (days) required to reach 50% of germination capacity (EC_{50}) of the tested barnyard grass populations.

NaCl concentration (mM)	Population				
	<i>s1</i>	<i>s2</i>	<i>s3</i>	Mean of <i>s</i>	<i>r</i>
0	8.61	8.54	9.32	8.82	7.79
50	8.39	7.72	8.87	8.33	7.15
100	9.92	10.10	10.49	10.17	8.41
150	11.70	12.02	11.94	11.89	10.59
200	12.57	12.37	12.37	12.44	12.00

The pairwise comparisons showed that, at the tested salt concentrations, there were no significant differences between the sensitive populations and the resistant one. Furthermore, no differences were found within the sensitive populations (Table 6).

This finding suggested that, as previously observed in the case of seed germination capacity, the different sensitivity to ALS-inhibitors did not seem to affect the response to saline conditions.

Table 6. *SI* values of the speed of germination (calculated as the ratio between EC_{50} of the tested barnyard grass populations).

Compared populations	NaCl (mM)				
	0	50	100	150	200
<i>s2/s3</i>	ns	ns	ns	ns	ns
<i>s2/s1</i>	ns	ns	ns	ns	ns
<i>s2/r</i>	ns	ns	ns	ns	ns
<i>s3/s1</i>	ns	ns	ns	ns	ns
<i>s3/r</i>	ns	ns	ns	ns	ns
<i>s1/r</i>	ns	ns	ns	ns	ns

ns: $P > 0.05$.

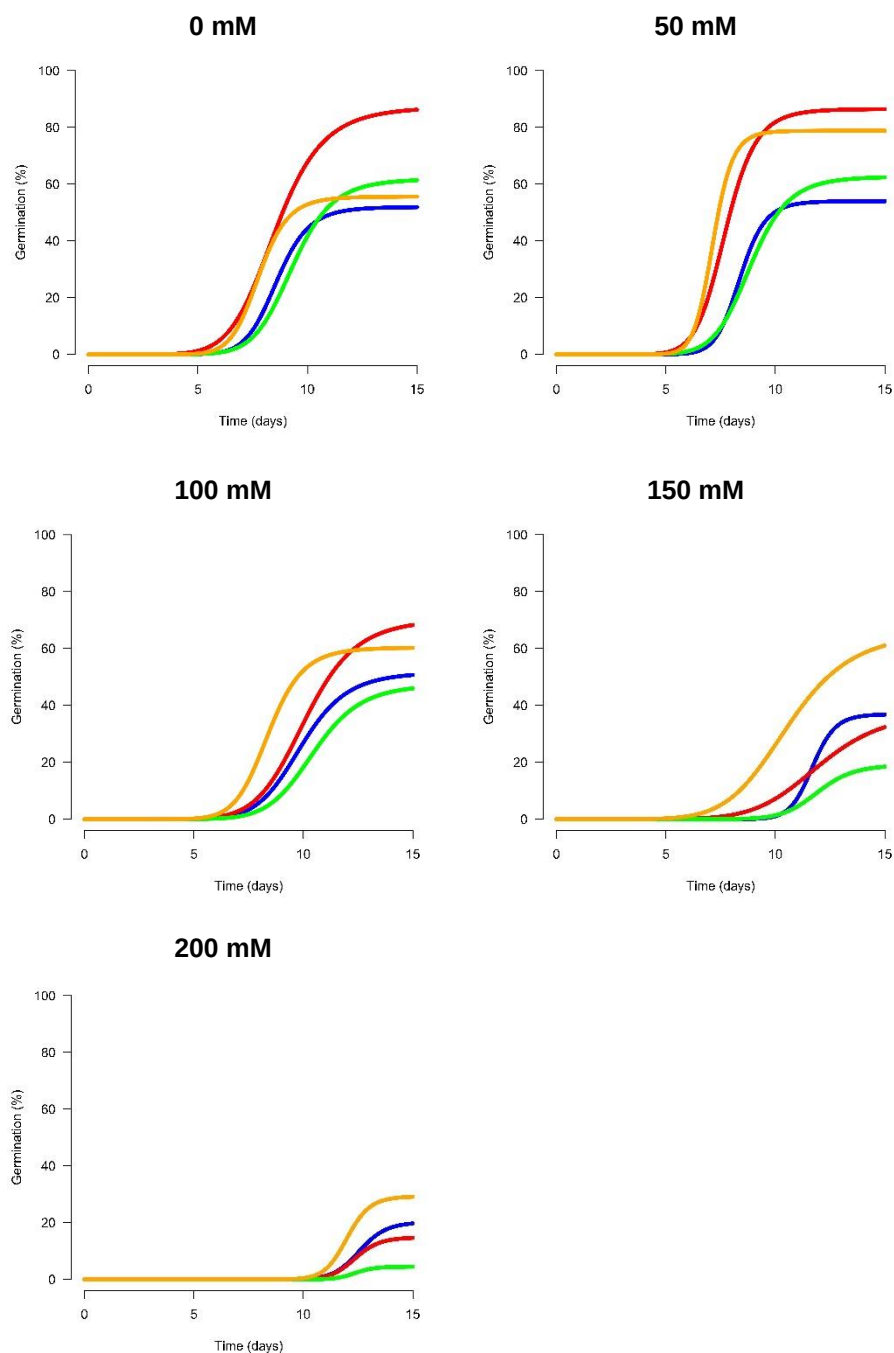


Figure 2. Cumulated seed germination capacity (%) as function of duration of germination period as fitted by the following *log-logistic model* $Y = d/[1+\exp\{b(\log(x)-\log(e))\}]$ of the tested barnyard grass populations at the different NaCl concentrations. Blue line: population s1; red line: s2; green line: s3; orange line: r.

Seedling development

During the study, it was observed that seedling development was affected by salinity conditions only at the highest NaCl concentrations. At the salt doses of 50 mM NaCl and 100 mM NaCl, a development comparable or greater than that observed in non-saline conditions was recorded in all the tested populations.

A reduction in seedling development capacity was noted at 150 mM NaCl. In the case of the salt doses 200 mM NaCl and 250 mM NaCl, seedling emergence occurred but it was followed by a total desiccation of the plant.

Plant height

The results obtained during the trials showed that plant height was affected by saline conditions both in the subset “salt” and in the subset “salt + herbicide”.

The results underlined that plant growth continued progressively throughout observation period in the case of the subset “salt” while, in some cases, it stopped after the herbicide treatment in the subset “salt + herbicide” (data not shown).

Taking into account the measurement carried out 21 days after the herbicide application, in the case of the subset “salt” the tallest plants (44.92 cm) were found in the population *r* at 0 mM NaCl while the plants with the lowest growth (19.25 cm) was recorded in the populations *s1* at 150 mM NaCl.

In the subset “salt + herbicide”, the tallest plants (31.63 cm) were observed in population *r* at the salt concentration of 50 mM while the shortest ones (7.12 cm) were found in population *s1* at 100 mM NaCl.

The salinity effect on plant height was also detected by Hakim *et al.* (2013) during a study conducted on different weed species, including *E. crus-galli* and *E. colona*. The authors noticed that plant height diminished with the increase of salt concentration and that *E. crus-galli* was one of the least affected species. However, the plant growth observed by the authors resulted higher than those of our tests. This may be due to the fact that in our trials saline conditions were set at the beginning of the study while in the study of Hakim *et al.* (2013) salt treatment occurred after the plants were transplanted and were fully established.

ANOVA carried out on the growth data obtained 21 days after the herbicide application showed the presence of significant effects attributable to the factors “population”, “salt dose”, and “herbicide application”. Moreover, the interaction “population” x “salt dose” x “herbicide application” was significant (Table 7).

Table 7. Plant growth (expressed as height in cm) of tested barnyard grass populations from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Plant height of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population	Height (cm)
s1	21.41
s2	26.90
s3	27.44
r	28.61
ANOVA	
Population	*
Salt dose	*
Herbicide application	*
Population x salt doses	ns
Population x herbicide application	ns
Salt doses x herbicide application	ns
Population x salt doses x herbicide application	*

*: $P \leq 0.05$; ns: $P > 0.05$

In order to examine the salinity effect on growth of the populations treated and untreated with the herbicide, the subsets “salt” and “salt + herbicide” were analysed separately.

Plant height: subset “salt”

The analysis of variance showed a significant effect of “population” and “salt dose” (data not shown). The interaction between these two factors was not significant, even though the significance of F was 0.06, close to the 0.05 threshold.

Considering the factor “population”, significant differences in terms of plant growth at different salt concentrations were found in populations s2, s3 and r (Figure 4).

The resistant population r showed a decrease of plant height with the increase of salt concentration while, in the case of the sensitive populations s1, s2 and s3 there was a slight increase of plant height at 50 mM NaCl followed by a decrease at 100 and 150 mM.

In the case of factor “salt”, significant differences among populations were observed at 0 mM and 50 mM NaCl, while no differences were found at the saline concentrations of 100 mM and 150 mM.

The presence of significant differences also at 0 mM NaCl was due to population s1, which grew significantly less than the other populations already in the case of non-saline conditions. Based on the post-hoc comparisons, at 50 mM NaCl, significant differences were found only between populations s1, which grew the least, and s3, which grew the most.

Plant height: subset “salt + herbicide”

ANOVA underlined the presence of significant differences attributable to the factors “population” and “salt dose”, as well as to the interaction “population” x “salt dose”. This indicated that, under saline conditions, the tested barnyard grass populations reacted differently to the herbicide application. In Figure 3 it is possible to observe that the resistant population *r* was characterized by an average height greater than that of the sensitive ones at 0 mM, 50 mM and 100 mM NaCl. At 50 mM NaCl, it is also possible to note a peak of plant height which in the case of sensitive populations was not found or, if present, was less marked (population *s3*).

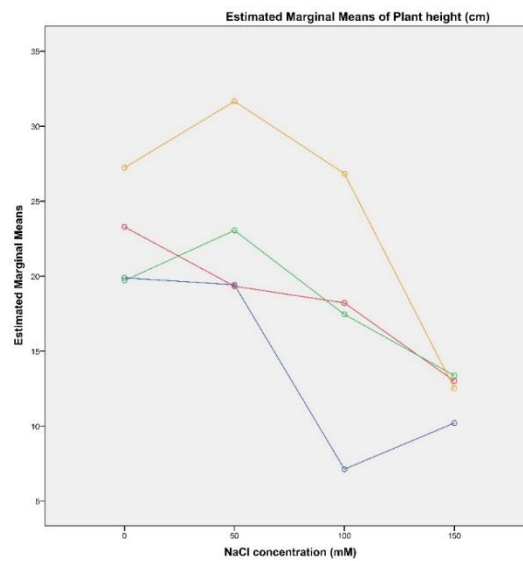


Figure 3. Plant height trend of tested barnyard grass populations under saline conditions in subset "salt + herbicide". Blue line: population *s1*; red line: *s2*; green line: *s3*; orange line: *r*.

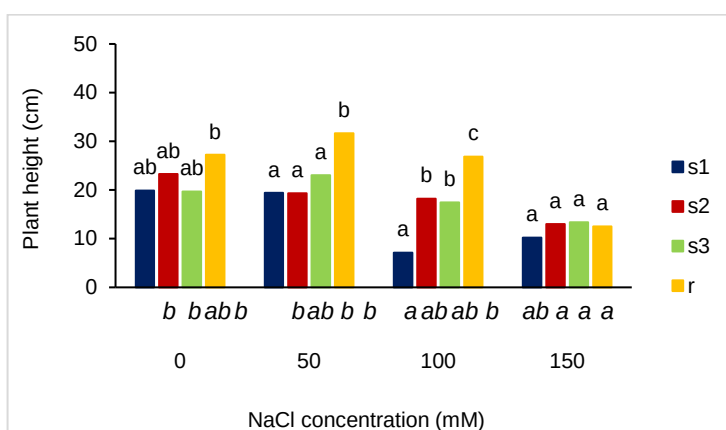
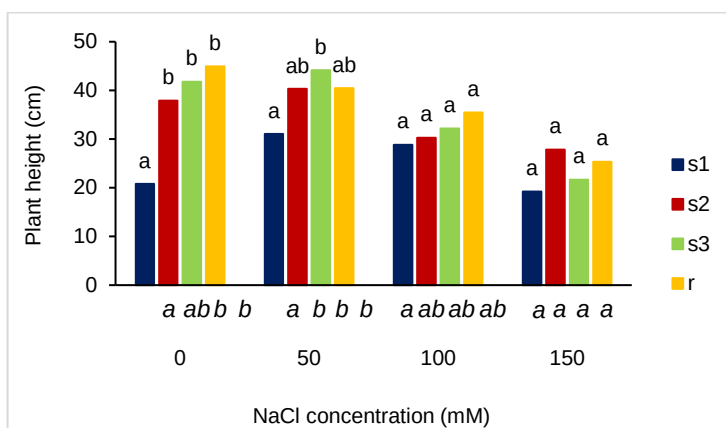


Figure 4. Plant growth (expressed as height) in the subsets “salt” (top) and “salt + herbicide” (bottom) from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Letters above the bars indicate significant differences between populations within the same salt concentration. Letters under the bars indicate significant differences between salt concentration for each population. Different letters indicate significant differences ($P \leq 0.05$) based on Tukey’s post-hoc test.

Shoot fresh weight

The results observed at the end of the study underlined that shoot fresh weight of tested barnyard grass populations was affected by saline conditions both in subset “salt” and in subset “salt + herbicide”. This result was in agreement with what found by Chen *et al.* (2014): in a study on wheat, the authors observed that fresh weight decreased progressively under saline conditions; it should be noted that in this study 0 mM and 120 mM NaCl were tested and that the saline conditions were created only from eight days after after transplanting.

In some cases, at 50 mM NaCl, there was an increase of shoot fresh weight both in the subset “salt” and in the subset “salt + herbicide”, in particular in population *s1* in the subset “salt”.

In the subset “salt”, the highest shoot fresh weight (12.74 g) was found for the population *r* at 50 mM NaCl while the lowest one (0.53 g) was measured for the population *s1* at 150 mM NaCl.

However, shoot fresh weight of this latter population resulted low (1.80 g) already at 0 mM NaCl. Since the average plant height, at the different salt concentrations, ranged between 19.25 cm and 31.04 cm, it is possible that the low shoot fresh weight may be attributed to the presence of elongated plants.

In the case of subset “salt + herbicide”, the highest fresh weight (8.90 g) was observed for population *r* at 50 mM NaCl, while the lowest one (0.28 g) was found for the population *s2* at 150 mM NaCl.

The ANOVA showed a significant effect of factors “population”, “salt dose” and “herbicide application”. Furthermore, also interaction “population” x “herbicide application” and “salt dose” x “herbicide application” were significant (Table 8).

As occurred for the plant height, also in this case the significant effect of the factor “herbicide application” and of the interaction “population” x “herbicide application” can be considered an expected result, as the herbicide treatment influences indeed the development of shoots; moreover, the presence of interaction showed that every single population reacted in a different way to the herbicide treatment.

Furthermore, the significance of the interaction “salt dose” x “herbicide application” indicated that these two factors interacted in influencing the response of the populations considered. In particular, when herbicide application was performed, the barnyard grass populations reacted differently to the different tested salt concentrations.

In order to evaluate the influence of the saline conditions on shoot fresh weight of the untreated and treated populations, the subsets “salt” and “salt + herbicide” were statistically explored in a separate way.

Table 8. Shoot fresh weight (g) of tested barnyard grass populations from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Shoot fresh weight of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population	Shoot fresh weight (g)
s1	1.28
s2	3.92
s3	3.56
r	6.67
ANOVA	
Population	*
Salt dose	*
Herbicide application	*
Population x salt dose	ns
Population x herbicide application	*
Salt doses x herbicide application	*
Population x salt doses x herbicide application	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

Shoot fresh weight: subset “salt”

The analysis of variance showed the presence of a significant effect of the factors “population” and “salt dose” (Figure 6).

Differences between the tested barnyard grass populations in response to saline conditions was found at 50 mM NaCl in the case of the populations s1 and r and at 150 mM NaCl for the populations s1 and s2.

Significant differences between populations was also found at control: in particular, the population s1 showed a shoot fresh weight significantly lower than that of the other populations.

As regards the response of each single population to the different tested salt concentrations, the presence of significant differences was observed for the population s1 between 0 mM NaCl and 50 mM NaCl, for the populations s2 and s3 between 0 mM and 100 mM and between 0 mM and 150 mM NaCl. Moreover, significant differences were found in the case of population r between 0 mM and 150 mM and between 50 mM and 150 mM NaCl.

Shoot fresh weight: subset “salt + herbicide”

The analysis of variance underlined that there was a significant effect of the factors “population” and “salt dose” and of the interaction “population” x “salt dose”. The significance of the interaction suggested that, in presence of herbicide application, the tested populations responded differently to the different salt concentrations. This finding was confirmed by the fact that in the subset “salt” the interaction between the factors “population” and “salt dose” was not significant.

In figure 5 it is possible to note that the population *r* was characterized by a different response trend to saline doses compared to that of the other ones, with a shoot fresh weight higher than that of the sensitive populations.

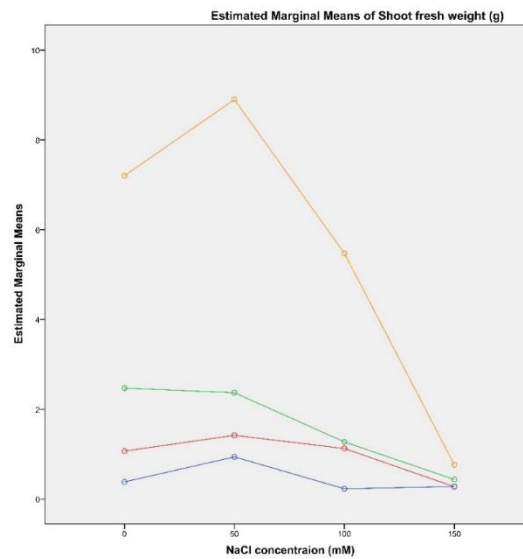


Figure 5. Shoot fresh weight trend of tested barnyard grass populations under saline conditions in subset "salt + herbicide". Blue line: population *s1*; red line: *s2*; green line: *s3*; orange line: *r*.

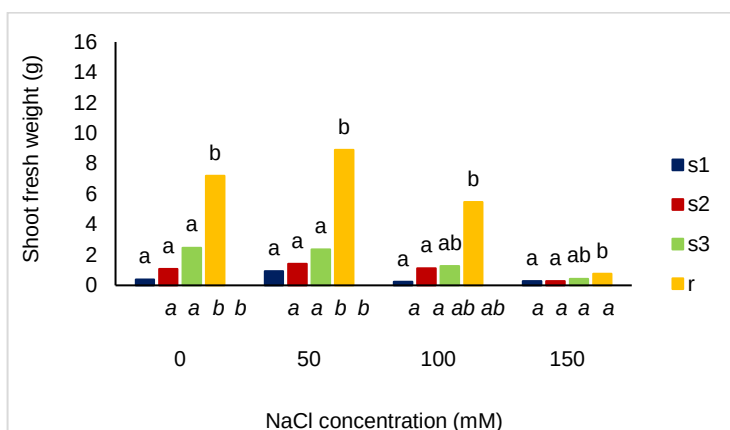
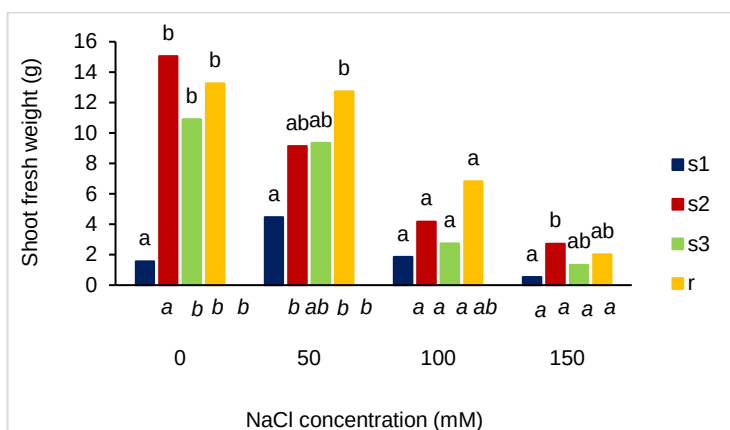


Figure 6. Average shoot fresh weight (g) of the tested barnyard grass populations in the subsets “salt” (top) and “salt + herbicide” (bottom) from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Letters above the bars indicate significant differences between populations within the same salt concentration. Letters under the bars indicate significant differences between salt concentration for each population. Different letters indicate significant differences ($P \leq 0.05$) based on Tukey’s post-hoc test.

Root fresh weight

The results obtained at the end of the study showed that, as occurred for shoot fresh weight, also root fresh weight was affected by saline conditions. According to the analysis of variance, the increase of root weight (compared to that at 0 mM NaCl) observed in some cases at 50 mM NaCl and 100 mM NaCl was, generally, not significant. Population *s1* in the subset “salt” was an exception, as at 50 mM NaCl the population was characterized by a root weight significantly higher than that detected at 0 mM NaCl.

In the subset “salt”, the highest root fresh weight (16.81 g) was found for the population *r* at 50 mM NaCl, while the lowest one (0.68 g) was measured for population *s1* at 150 mM NaCl. As also observed in the case of shoot fresh weight, the average root fresh weight of the population *s1* was low (0.92 g) already at 0 mM NaCl. This behaviour could be attribute to the fact that the plants were thin and elongated.

In the case of the subtest “salt + herbicide” the highest weight (13.26 g) was observed for the population *r* at 50 mM NaCl while the lowest weight (0.06 g) for population *s1* at 150 mM NaCl.

The analysis of variance performed on root fresh weight data measured 21 days after the herbicide treatment on the two subsets showed that the factors “population”, “salt dose”, and “herbicide application” had a significant effect. Furthermore, the interactions “population x salt dose”, “population x herbicide application” and “salt dose x herbicide application” resulted significant (Table 9).

As was done in the case of shoot fresh weight, the subtests “salt” and “salt + herbicide” were statistically explored in a separate way in order to verify the influence of the salt conditions on root fresh weight of untreated and treated populations.

Table 9. Root fresh weight (g) of tested barnyard grass populations from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Shoot fresh weight of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population	Root fresh weight (g)
<i>s1</i>	1.08
<i>s2</i>	4.31
<i>s3</i>	3.58
<i>r</i>	9.38
ANOVA	
Population	*
Salt dose	*
Herbicide application	*
Population x salt dose	*
Population x herbicide application	*
Salt doses x herbicide application	*
Population x salt doses x herbicide application	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

Root fresh weight: subset “salt”

The factors “population” and “salt dose” had a significant effect on root fresh weight, while their interaction resulted not significant.

Significant differences in response to the tested salt concentrations were found between population *s1* and populations *s2*, *s3* and *r* at 0 mM NaCl, between population *s1* and population *r* at 50 mM NaCl. Furthermore, significant differences were observed between population *s1* and population *s2* at 150 mM NaCl.

As regard the effect of the applied saline conditions on each single population, significant differences were found for population *s1* between 0 mM and 50 mM NaCl, for populations *s2* and *s3* between 0 mM and 100 mM NaCl and between 0 mM and 150 mM NaCl (Figure 8).

Root fresh weight: subset “salt + herbicide”

In this case, both “population” and “salt dose” and their interaction shown a significant effect on root fresh weight. This was due to the different response to the tested salt concentration of each barnyard grass population. By observing Figure 7, it is possible to note that the populations *s1*, *s2* and *s3* were characterized by an analogous behaviour in response to salt. A dissimilar response trend can be observed, instead, in the case of the population *r*: it was characterized by a peak at 50 mM NaCl and by an average root fresh weight still high at 100 mM NaCl.

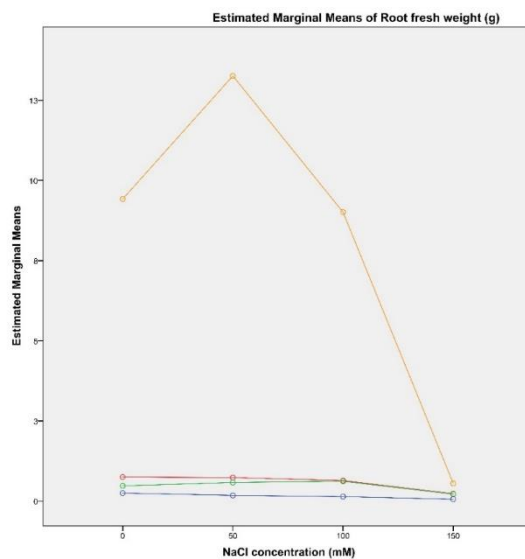


Figure 7. Root fresh weight trend of tested barnyard grass populations under saline conditions in subset "salt + herbicide". Blue line: population *s1*; red line: *s2*; green line: *s3*; orange line: *r*.

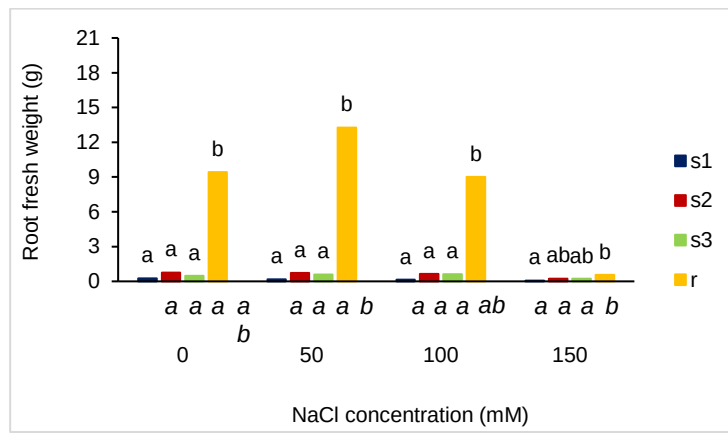
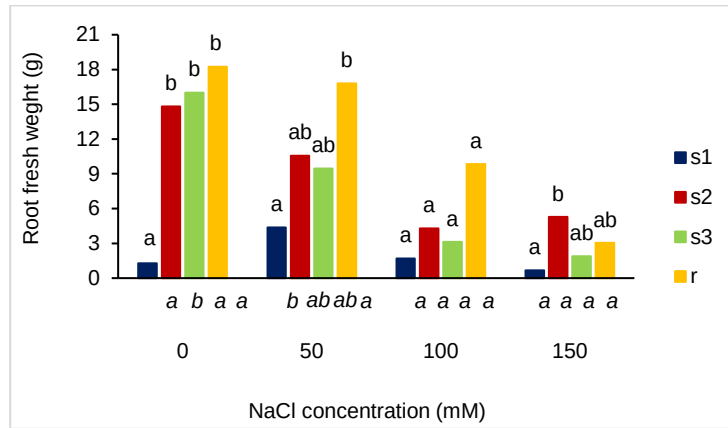


Figure 8. Average root fresh weight (g) of the tested barnyard grass populations in the subsets “salt” (top) and “salt + herbicide” (bottom) from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Letters above the bars indicate significant differences between populations within the same salt concentration. Letters under the bars indicate significant differences between salt concentration for each population. Different letters indicate significant differences ($P \leq 0.05$) based on Tukey’s post-hoc test.

SPAD

ANOVA performed on SPAD values measured 21 days after the herbicide application highlighted the presence of significant differences attributable to the factors “population”, “salt dose” and “herbicide application”. Moreover, the interactions “population” x “herbicide application”, “salt dose” x “herbicide application” and “population” x “salt dose” x “herbicide application” resulted significant (Table 10).

In order to evaluate the effect of herbicide application on the chlorophyll content the two subsets “salt” and “salt + herbicide” were analysed separately.

Table 10. SPAD values of tested barnyard grass populations from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. SPAD values of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population	SPAD value
s1	19.85
s2	20.46
s3	22.96
r	28.12
ANOVA	
Population	*
Salt dose	*
Herbicide application	*
Population x salt dose	ns
Population x herbicide application	*
Salt doses x herbicide application	*
Population x salt doses x herbicide application	*

*: $P \leq 0.05$; ns: $P > 0.05$.

SPAD: subset “salt”

Differences between populations were found at 100 mM NaCl: in particular, the post-hoc comparison showed that population *r* was characterized by a chlorophyll content significantly different compared to that of the other tested populations (Figure 10).

SPAD: subset “salt + herbicide”

The analysis of variance underlined the presence of significant differences due to factors “population” and “salt dose” and to the interaction “population” x “salt dose”. These results suggested that the response to herbicide treatment, in terms of chlorophyll content, of each population changed in relation to the salt concentration applied. This can be observed in Figure 9: all the barnyard grass populations present a different tendency

in response to the tested salt concentrations. Only in the case of the population *s1*, there is a decrease of SPAD values with the increase of salinity; the population *s3* is characterized by a marked increase of SPAD value at 50 mM NaCl while the population *r* shows a steep decrease at 150 mM NaCl.

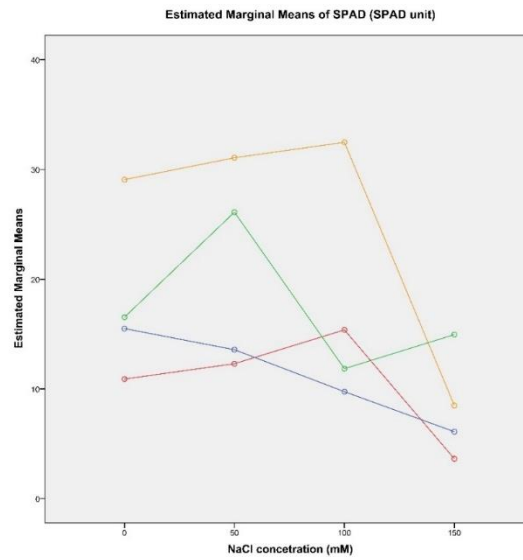


Figure 9. SPAD trend of tested barnyard grass populations under saline conditions n subset “salt + herbicide”. Blue line: population *s1*; red line: *s2*; green line: *s3*; orange line: *r*.

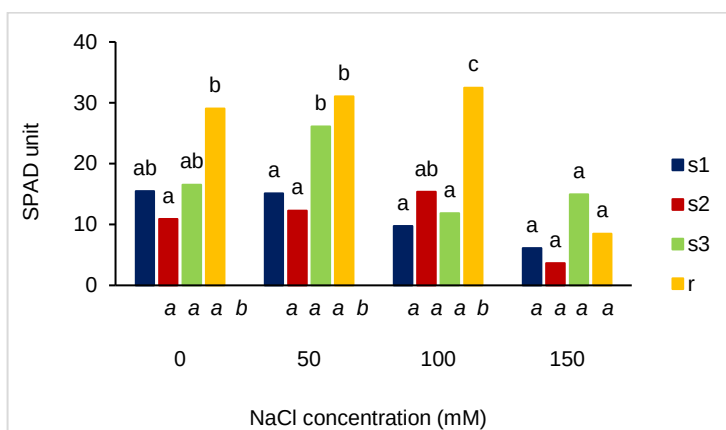
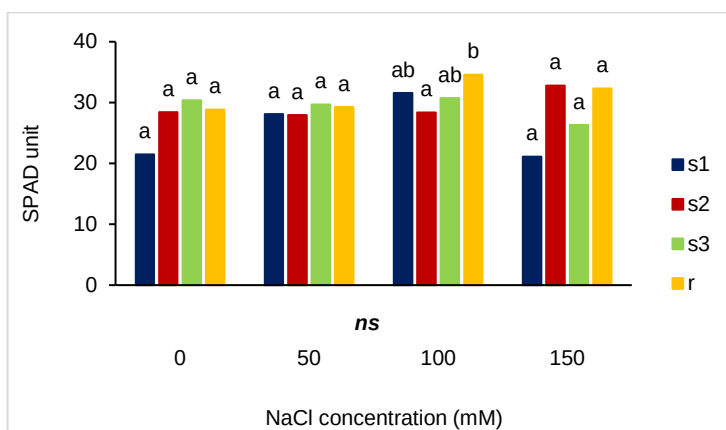


Figure 10. SPAD values in the subset “salt” (top) and “salt + herbicide” (bottom) from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Letters above the bars indicate significant differences between populations within the same salt concentration. Letters under the bars indicate significant differences between salt concentration for each population. **ns** under the bars indicate significant differences salt concentration effect for each population. Different letters indicate significant differences ($P \leq 0.05$) based on Tukey's post-hoc test.

Chlorophyll and carotenoids content

The determination of chlorophyll (*Chl a* and *Chl b*) and carotenoids performed at the end of the trial showed that leaf content of these pigments was affected by salinity conditions.

The analysis of variance carried out on *Chl a* and *Chl b* measured at 21 days after the herbicide application and related to the two considered subsets showed the presence of significant differences attributable to all considered factors and for their interaction in the case of *Chl a* and for all factors and interactions “population” x “salt dose”, “population” x “herbicide application” and “population” x “salt dose” x “herbicide application” in the case of *Chl b* (Table 11 and 12).

In the case of carotenoids, ANOVA underlined the presence of a significant effect for the factors “population”, “salt dose” and “herbicide application” and for all their interactions (Table 13).

In order to evaluate the effect of the different salt doses on the concentration of *Chl a*, *Chl b* and carotenoids and to verify the potential relationship between salinity and herbicide treatment, the two subsets were individually explored.

Table 11. Chlorophyll a content (mg g⁻¹ FW) of tested barnyard grass populations from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. *Chl a* content of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population	<i>Chl a</i> (mg g ⁻¹ FW)
s1	0.87
s2	0.98
s3	1.10
<i>r</i>	1.19
ANOVA	
Population	*
Salt dose	*
Herbicide application	*
Population x salt dose	*
Population x herbicide application	*
Salt doses x herbicide application	*
Population x salt doses x herbicide application	*

*: P≤0.05.

Table 12. Chlorophyll *b* content (mg g⁻¹ FW) of tested barnyard grass populations from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. *Chl b* content of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population	<i>Chl b</i> (mg g ⁻¹ FW)
<i>s1</i>	0.43
<i>s2</i>	0.49
<i>s3</i>	0.72
<i>r</i>	0.80
ANOVA	
Population	*
Salt dose	*
Herbicide application	*
Population x salt dose	*
Population x herbicide application	*
Salt doses x herbicide application	ns
Population x salt doses x herbicide application	*

*: $P \leq 0.05$; ns: $P > 0.05$.

Table 13. Carotenoids content (mg g⁻¹ FW) of tested barnyard grass populations from 0 mM NaCl to 150 mM NaCl 21 days after herbicide application. Carotenoids content of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population	Carotenoids (mg g ⁻¹ FW)
<i>s1</i>	0.23
<i>s2</i>	0.26
<i>s3</i>	0.23
<i>r</i>	0.23
ANOVA	
Population	*
Salt dose	*
Herbicide application	*
Population x salt dose	*
Population x herbicide application	*
Salt doses x herbicide application	*
Population x salt doses x herbicide application	*

*: $P \leq 0.05$.

Chlorophyll and carotenoids: subset “salt”

The content of the *Chl a* (Figure 14), in general, increased with the increase of salt dose. The average pigment content was equal to 1.05 mg g⁻¹ FW, 1.32 mg g⁻¹ FW, 1.31 mg g⁻¹ FW and 1.37 mg g⁻¹ FW at 0 mM NaCl, 50 mM NaCl, 100 mM NaCl and 150 mM NaCl, respectively.

In the case of *Chl b* (Figure 14), an increase (compared to 0 mM NaCl) of its content was found at 50 mM and 100 mM NaCl while a decrease was observed at 150 mM NaCl and ranged, on average, from 0.54 mg g⁻¹ FW at 0 mM NaCl to 1.02 mg g⁻¹ FW at 100 mM NaCl, while at 150 mM NaCl it was equal to 0.76 mg g⁻¹ FW. These results were in disagreement with what noted by Hakim *et al.* (2013). In their study, the authors found that the chlorophyll content decreased with the increase of salt concentration. Our findings are also in disagreement with Chen *et al.* (2014): in a study carried out on wheat based on the application of the saline doses of 0 mM and 120 mM on plants already developed, the authors observed a marked decrease of chlorophyll content 21 days after the salt treatment. Conversely, Guo *et al.* (2017), observed a non-significant decrease (compared to 0 mM NaCl) in the chlorophyll content in the case of wheat grown at a salt concentration of 150 mM (unlike our trials and as in the case of the tests conducted by Hakim *et al.* (2013), in this study the saline treatment occurred on plant already developed).

ANOVA performed for *Chl a* and *Chl b* content underlined the presence of a significant effect of the factors “population”, “salt dose” and their interaction. This result indicated that the content of this pigment depended not only on the biological characteristic of each single population but also on the salinity level to which they were exposed and on the combination of these two variables. In particular, as can be seen in Figure 11, population s1 is characterized by a *Chl a* content which increased markedly at the salt concentration of 50 mM compared to the control and which remained high at the salt concentrations of 100 mM and 150 mM.

A similar trend of chlorophyll content was observed by Manousaki *et al.* (2009). In a study carried out on *Atriplex halimus* and concerning the effect of salinity on metal uptake, the authors noted an increase in the content of this pigment in the case of plants subjected to a combined treatment with low salinity and metals (particularly cadmium and lead).

In the case of *Chl b*, population s3 showed a behaviour different to that of the other populations, with a progressive reduction of the pigment concentration with the increase of saline concentration (Figure 12).

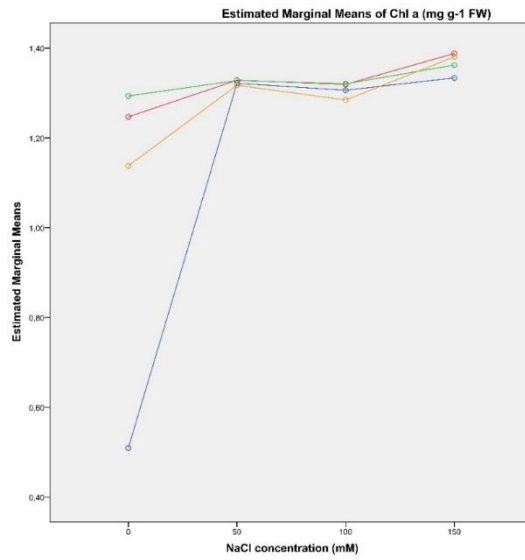


Figure 11. Chlorophyll a content trend of tested barnyard grass populations under saline conditions in subset “salt”. Blue line: population s1; red line: s2; green line: s3; orange line: r.

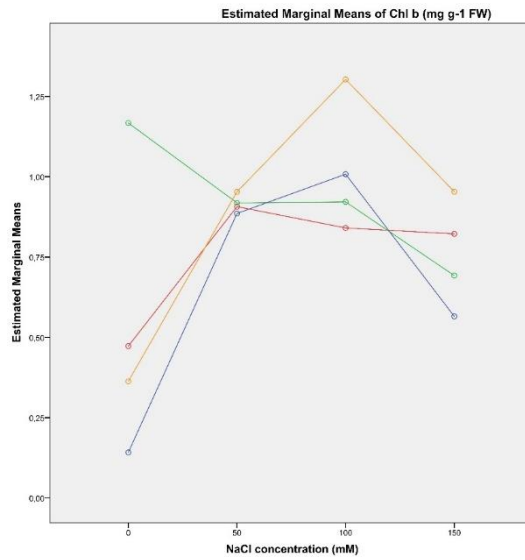


Figure 12. Chlorophyll b content trend of tested barnyard grass populations under saline conditions in subset “salt”. Blue line: population s1; red line: s2; green line: s3; orange line: r.

The content of carotenoids (Figure 14) was characterized by a very variable trend for each of the tested populations at 0 mM, 50 mM and 100 mM NaCl. Conversely, at 150 mM NaCl, a general increase of their concentration was found.

The presence of significant differences in the carotenoids content due to the biological characteristics of the populations, to the salinity conditions and to the combination of these two variables was confirmed by the analysis of variance (Figure 13).

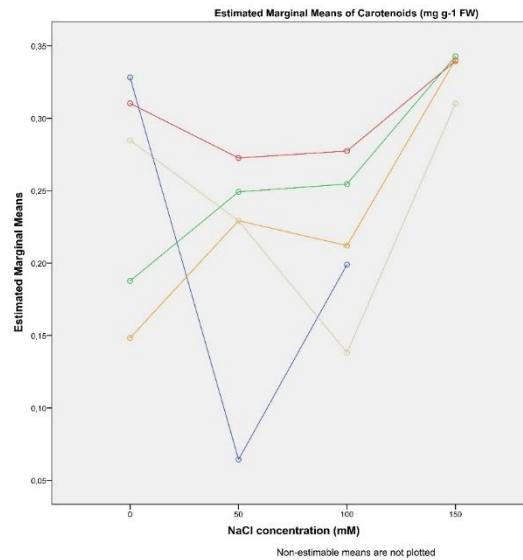


Figure 13. Carotenoids content trend of tested barnyard grass populations under saline conditions in subset "salt". Blue line: population s1; red line: s2; green line: s3; orange line: r.

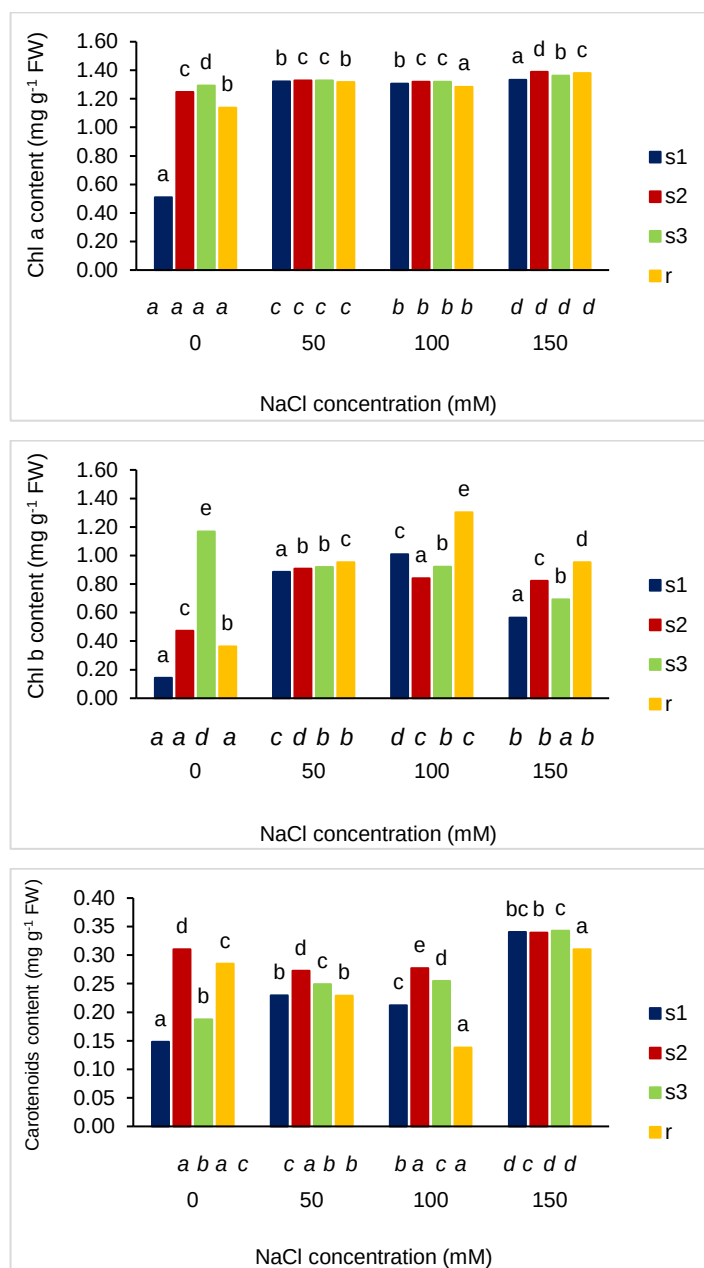


Figure 14. Chlorophyll *a* (top), *b* (middle) and carotenoids (bottom) content (mg g⁻¹ FW) of tested barnyard grass populations in subset “salt” from 0 mM NaCl to 150 mM NaCl. Letters above the bars indicate significant differences between salt concentration for each population within the same salt concentration. Letters under the bars indicate significant differences salt concentration effect for each population. Different letters indicate significant differences ($P \leq 0.05$) based on Tukey’s post-hoc test.

Chlorophyll and carotenoids: subset “salt + herbicide”

As the *Chl a* is concerned, a general increase (compared to that in non-saline conditions) of its leaf content was observed at 50 mM NaCl. At 100 mM NaCl and 150 mM NaCl, a reduction in the pigment concentration was found.

The average values of *Chl a* resulted equal to 0.98 mg g⁻¹ FW, 1.06 mg g⁻¹ FW, 0.94 mg g⁻¹ FW and 0.27 mg g⁻¹ FW, respectively, at 0 mM NaCl, 50 mM NaCl, 100 mM NaCl and 150 mM NaCl.

In the case of *Chl b*, there was a fairly variable trend of its concentration values between the populations from 0 mM NaCl to 100 mM NaCl. For populations *s1* and *s3* *Chl b* concentration at 50 mM NaCl resulted significantly higher than that at 0 mM NaCl; in the case of population *s2*, *Chl b* presented a significant higher content (compared to that at 0 mM NaCl) at 50 mM and 100 mM NaCl concentration while in the case of population *r* the concentration was significantly lower.

At 150 mM NaCl a marked decrease of *Chl b* was found in all the tested populations.

The higher pigment concentration (1.33 mg g⁻¹ FW) was found for populations *r* at the saline dose of 50 mM while the lower concentration (0.08 mg g⁻¹ FW) was observed in the case of population *s2* at 150 mM NaCl (Figure 18).

The analysis of variance conducted for *Chl a* and *Chl b* showed that, in both cases, there were significant differences in terms of chlorophyll content attributable to “population”, “salt dose” and to their interaction (Figure 15 and 16).

As shown by Figure 15, it is possible to note that all populations were characterized by an increase of *Chl a* concentration between 0 mM and 50 mM NaCl. At 100 mM NaCl, instead, each population showed a different trend in the pigment content. In particular, in the case of the population *s1* there was an increase of *Chl a* concentration while a decrease was observed for the populations *s2* and *s3*. In the case of the population *r*, pigment content remains almost the same detected at 50 mM NaCl.

At 150 mM NaCl, the generalized reduction of pigment concentration can be observed.

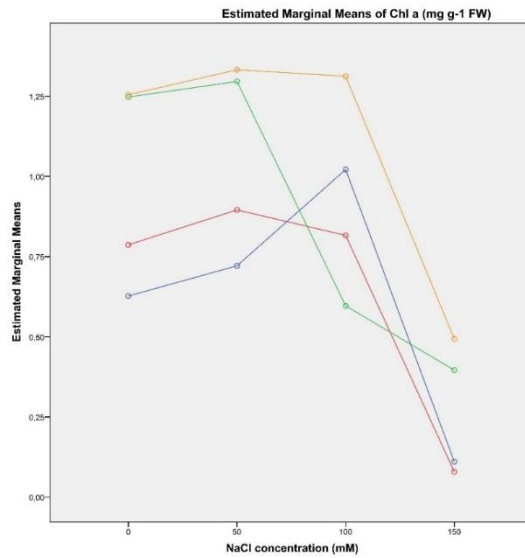


Figure 15. Chlorophyll a content trend of tested barnyard grass populations under saline conditions in subset “salt + herbicide”. Blue line: population s1; red line: s2; green line: s3; orange line: r.

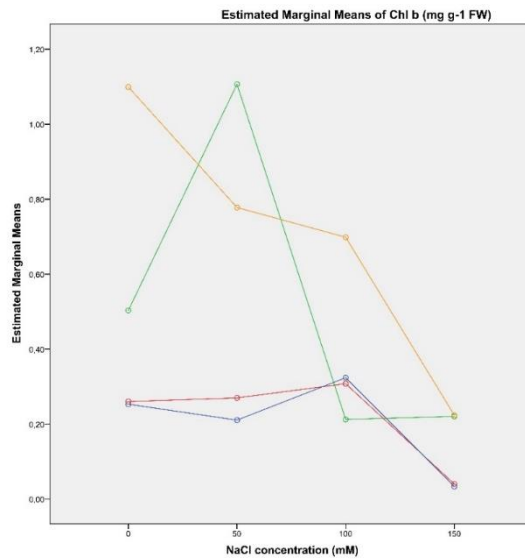


Figure 16. Chlorophyll b content trend of tested barnyard grass populations under saline conditions in subset “salt + herbicide”. Blue line: population s1; red line: s2; green line: s3; orange line: r.

Carotenoid leaf content (Figure 18) showed a variable trend among the populations at 0 mM NaCl, 50 mM NaCl and 100 mM NaCl.

In the case of populations s2 and s3, at 0 mM NaCl, pigment concentration resulted higher than that of the other salt doses.

For population s1, at 50 mM NaCl, carotenoids content was greater than that of 0 mM NaCl while for population r, at 100 mM NaCl, it resulted higher than that at 0 mM NaCl and 50 mM NaCl.

At 150 mM NaCl, carotenoids concentration showed a significant decrease in all tested populations.

The analysis of variance underlined the presence of a significant effect of factors “population” and “salt dose”; furthermore, also the interaction “population” x “salt dose” resulted significant.

As occurred in the case of *Chl a* and *Chl b* these results indicated that the response to herbicide application was the consequence not only of the populations considered and of the salt doses applied but also of the combined action of these two variables.

As shown by Figure 17, a particular behaviour can be underlined for the populations s3 and r. In particular, the first one was characterized by a high decrease of carotenoids content between 0 mM and 50 mM NaCl; this reduction also occurred at 100 mM and 150 mM NaCl. In the case of population r, an increase in carotenoids content can be noted between 0 mM and 100 mM NaCl.

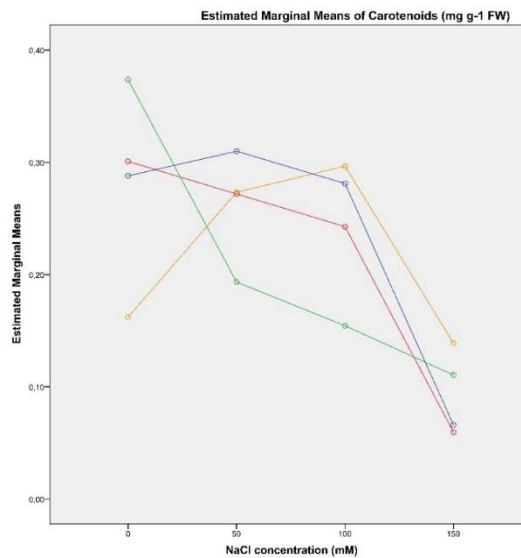


Figure 17. Carotenoids content trend of tested barnyard grass populations under saline conditions in subset “salt + herbicide”. Blue line: population s1; red line: s2; green line: s3; orange line: r.

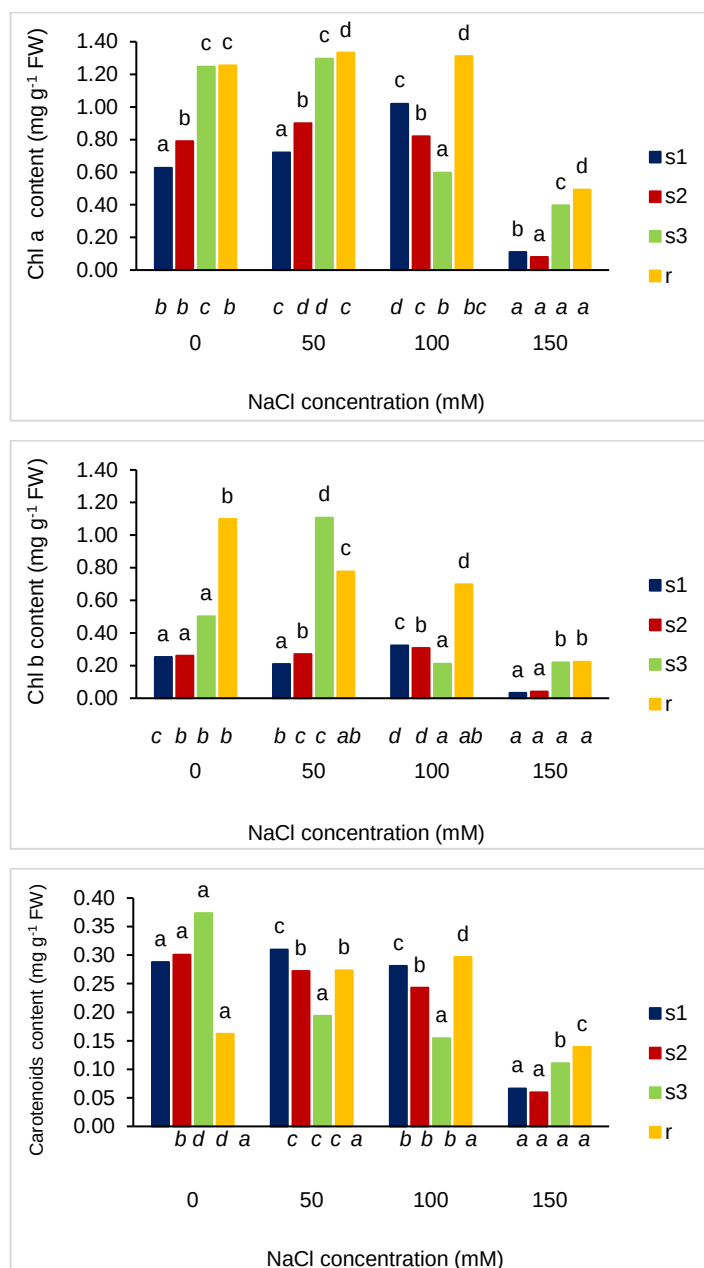


Figure 18. Chlorophyll *a* (top), *b* (middle) and carotenoids (bottom) content (mg g⁻¹ FW) of tested barnyard grass populations in subset “salt + herbicide” from 0 mM NaCl to 150 mM NaCl.

Letters above the bars indicate significant differences between salt concentration for each population within the same salt concentration.

Letters under the bars indicate significant differences salt concentration effect for each population.

Different letters indicate significant differences ($P \leq 0.05$) based on Tukey's post-hoc test.

Comparison between sensitive and resistant populations on plant height, shoot fresh weight, root fresh weight, SPAD, chlorophyll *a* and *b* and carotenoids content

A comparison between S and R populations was attempted by considering all the growth and physiological parameters considered. To this end, data from S populations (s1, s2, and s3) were pooled and compared to data of the population *r* (Table 14 and 15).

In general, greater tolerance to saline conditions was found in the resistant population. In order to understand the extent of this response, the analysis of variance was performed for all physiological parameters by considering the two subsets ("salt" and "salt + herbicide") separately.

As far as the **subset "salt"** is considered, the analysis showed that significant differences between the populations in the response to the different salt doses were present for all the considered parameters.

In the case of plant height, significant differences were detected only in control treatment (0 mM NaCl concentration).

In the case of shoot fresh weight, root weight, SPAD and *Chl a*, significant differences were found for the salt dose of 50 mM and 100 mM or for both.

In particular, in the case of the root weight it was observed that the weight of the resistant population at 50 mM NaCl concentration was more than twice that of the sensitive ones while at 100 mM NaCl concentration it was more than three times.

The content of *Chl b* differed significantly between S and R at the salt doses of 50 mM, 100 mM and 150 mM. Differences in carotenoid content were observed at 100 mM NaCl and 150 mM NaCl.

In the **subset "salt + herbicide"**, in general, the resistant population showed a lower susceptibility to the herbicide application than the sensitive ones even in saline stress conditions. This has been observed in particular for shoot fresh weight, which ranged from 7.20 g to 0.76 g per plant, respectively, at 0 mM and 150 mM NaCl for the resistant population while in the case of the sensitive populations these values ranged from 1.31 g to 0.41 g. This result indicated that salt conditions did not seem to affect the response of the resistant population to the herbicide application. The differences in plant weight between sensitive and resistant populations were confirmed by the analysis of variance. Moreover, significant differences, at some or all the salt doses, were also detected for plant height, root weight, SPAD, *Chl a*, *Chl b* and carotenoids. Even in these cases, in general, the values of the physiological parameters resulted higher in the resistant population than in the sensitive ones.

Table 14. Comparison of growth and physiological parameters between sensitive (S) and resistant (R) barnyard grass populations in the subset “salt”. S values are the averages among populations *s1*, *s2*, and *s3*; R values refer to the population *r* only.

Physiological parameter	NaCl (mM)	Population		P value
		S	R	
Plant height (cm)	0	34.87	44.92	*
	50	38.50	40.45	
	100	30.73	35.45	
	150	23.41	25.36	
Shoot fresh weight (g)	0	7.17	9.44	*
	50	7.65	12.74	
	100	2.92	6.83	
	150	1.53	2.02	
Root fresh weight (g)	0	8.53	13.08	*
	50	8.13	16.81	
	100	3.03	9.84	
	150	2.62	3.04	
SPAD (SPAD unit)	0	27.47	28.86	*
	50	28.56	29.27	
	100	29.81	34.57	
	150	27.86	32.34	
Chlorophyll a (mg g ⁻¹ FW)	0	1.02	1.14	*
	50	1.33	1.32	
	100	1.32	1.28	
	150	1.38	1.38	
Chlorophyll b (mg g ⁻¹ FW)	0	0.59	0.36	*
	50	0.90	0.95	
	100	0.92	1.30	
	150	0.82	0.95	
Carotenoids (mg g ⁻¹ FW)	0	0.22	0.29	*
	50	0.25	0.23	
	100	0.25	0.14	
	150	0.34	0.31	

*: $P \leq 0.05$; ns: $P > 0.05$.

Table 15. Comparison of growth and physiological parameters between sensitive (S) and resistant (R) barnyard grass populations in the subset “salt + herbicide”. S values are the averages among populations s1, s2 and s3; R values refer to the population *r* only.

Physiological parameter	NaCl (mM)	Population		P value
		S	R	
Plant height (cm)	0	21.10	27.23	*
	50	20.67	31.66	*
	100	15.81	26.84	*
	150	12.64	12.52	
Shoot fresh weight (g)	0	1.31	7.20	*
	50	1.58	8.9	*
	100	0.88	5.47	*
	150	0.41	0.76	*
Root fresh weight (g)	0	0.49	9.42	*
	50	0.50	13.26	*
	100	0.47	9.01	*
	150	0.17	0.55	*
SPAD (SPAD unit)	0	14.72	29.06	*
	50	18.46	31.07	*
	100	12.92	32.46	*
	150	8.65	8.49	
Chlorophyll a (mg g ⁻¹ FW)	0	0.89	1.25	
	50	0.97	1.33	*
	100	0.81	1.31	*
	150	0.20	0.49	*
Chlorophyll b (mg g ⁻¹ FW)	0	0.34	1.10	*
	50	0.53	0.78	
	100	0.28	0.70	*
	150	0.10	0.22	*
Carotenoids (mg g ⁻¹ FW)	0	0.32	0.16	*
	50	0.26	0.27	
	100	0.23	0.30	
	150	0.079	0.14	*

*: $P \leq 0.05$; ns: $P > 0.05$.

Weedy rice and rice

Seed germination

The analysis of data collected during the study showed that, as observed in the case of barnyard grass, seed germination of tested weedy rice populations and the rice variety “Baldo” was affected by salinity conditions.

A *t*-test analysis (Table 16) performed between the average response of weedy rice populations and that of the rice variety showed the presence of significant differences, in terms of germination capacity, at 150 mM and 250 mM NaCl. In particular, at 150 mM NaCl the average germination of the weedy rice populations resulted greater than that of the rice while in the case of the salt dose 250 mM Baldo rice was characterized by a germination higher than that of the weedy rice.

Table 16. Seed germination capacity (% of germinated seeds) at 15 days of tested weedy rice populations (WR) and rice variety (R) from 0 mM NaCl to 250 mM NaCl.

Population	NaCl (mM)					
	0	50	100	150	200	250
WR	93.52	93.06	84.72	67.13	27.78	0.93
R	88.89	83.33	77.78	38.89	16.67	8.33
<i>t</i> -test significance	ns	ns	ns	*	ns	*

*: $P \leq 0.05$; ns: $P > 0.05$.

In general, a decrease of seed germination capacity with the increase of salt concentration was observed (Figure 19).

Considering the average among all tested weedy rice populations, the germination ranged from 93.52% at 0 mM NaCl to 0.93% at 250 mM NaCl. Within weedy rice populations, only population *wr1* showed, at 50 mM NaCl, a germination capacity slightly higher than that at 0 mM NaCl. However, the analysis of variance underlined that these differences were not significant.

In the rice variety Baldo, the germination capacity ranged from 88.89% at 0 mM NaCl to 8.33% at 250 mM NaCl.

The average germination of weedy rice populations resulted higher than 50% up to the salt concentration of 150 mM, with the maximum in the population *wr1* (75.00%) and the minimum in population *wr2* (56.94%). At 200 mM NaCl, about 28% of average germination was found with values ranging from 20.83% (population *wr3*) and 43.10% (population *wr1*).

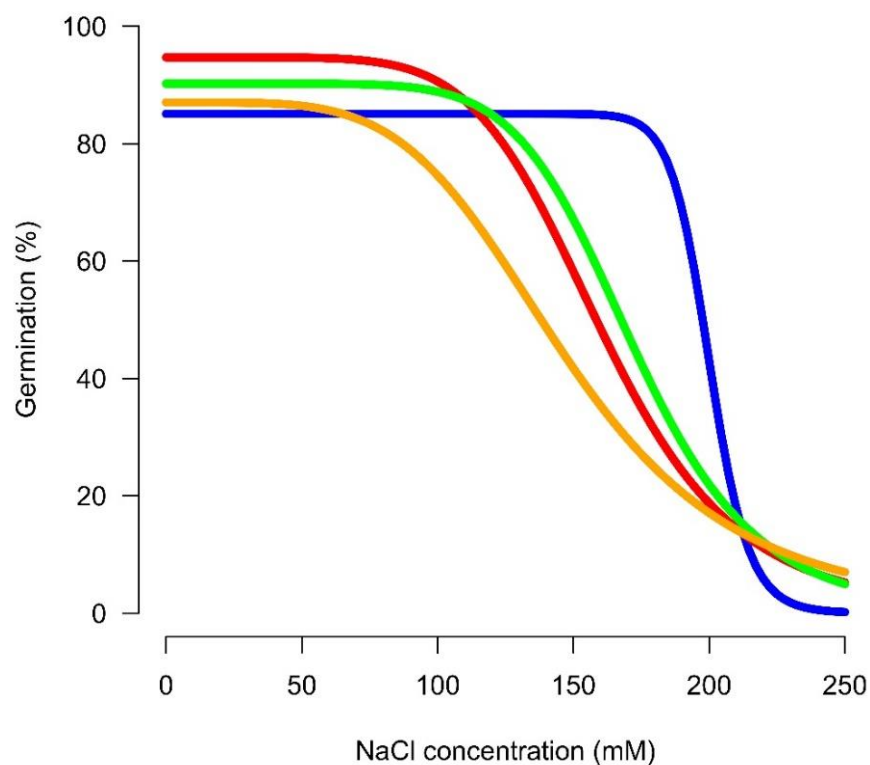


Figure 19. Seed germination capacity (%) of tested weedy rice populations and rice variety as function of NaCl concentration as fitted by the following *log-logistic model* $Y = d/[1+\exp\{b(\log(x) - \log(e))\}]$. Blue line: population wr1; red line: wr2; green line: wr3; orange line: Baldo variety.

The salt concentration required to reduce by 50% seed germination capacity (EC_{50} ; Table 17) in weedy rice was equal, on average, to 178.15 mM NaCl with a minimum of 161.19 mM NaCl for population wr2 and a maximum of 200.85 mM NaCl for populations wr1.

In the case of rice variety, the EC_{50} was 147.39 mM NaCl.

The pairwise comparisons showed significant differences between population wr2 and population wr1 (Table 18); no differences were found between weedy rice populations and rice variety.

Table 17. NaCl concentration required to reduce by 50% (EC_{50}) seed germination capacity of tested weedy rice populations and rice variety.

Population / variety	EC_{50}
<i>wr1</i>	200.85
<i>wr2</i>	161.19
<i>wr3</i>	172.42
Baldo	147.39

Table 18. *SI* value of seed germination capacity of pairwise comparisons between the tested weedy rice populations and the rice variety.

Compared population / variety	<i>SI</i>	P value
<i>wr3</i> /Baldo	1.17	ns
<i>wr3</i> / <i>wr2</i>	1.07	ns
<i>wr3</i> / <i>wr1</i>	0.86	ns
Baldo/ <i>wr2</i>	0.91	ns
Baldo/ <i>wr1</i>	0.74	ns
<i>wr2</i> / <i>wr1</i>	0.81	*

*: $P \leq 0.05$. ns: $P > 0.05$.

Speed of germination

Salinity conditions affected speed of germination (Figure 20). In particular, the time (days) needed for germination to start increased by increasing salt concentration.

In non-saline conditions germination started, on average, 7 days after sowing for weedy rice populations and 4 days after sowing in the case of the rice variety.

In weedy rice populations, a delay in start of germination was observed at the salt doses of 150 mM and 200 mM: in these condition, first germinated seeds were observed, on average, at 8.67 days and 11.33 days after seeding, respectively.

At 250 mM NaCl concentration, the beginning of germination was recorded at the 13th day (in this case, population *wr2* did not show seed germination within 15 days of monitoring) (Table 19).

The time required to reach 50% (EC_{50}) of seed germination ranged, on average, from a minimum of 8.12 days at 50 mM NaCl to a maximum of 12 days at 250 mM NaCl (Table 20).

In the case of the rice variety, in non-saline conditions the first seed germination occurred 4 days after seeding.

At 250 mM NaCl, the seed germination of Baldo began 12 days after seeding (Table 19).

For Baldo, the EC_{50} ranged from a minimum of 5.28 days at 50 mM NaCl to a maximum of 11.69 days at 250 mM NaCl (Table 20).

The pairwise comparisons (Table 21) showed the presence, at 0 mM NaCl, of significant differences between weedy rice populations and rice variety. Moreover, significant differences between the tested weedy rice populations and the rice variety was found at 50 mM NaCl, while no differences were found among weedy rice populations. At the other salt concentrations, there were no differences between tested weedy rice populations and rice variety.

Table 19. Day at which the first germinated seeds were observed on the tested weedy rice populations and rice variety.

NaCl concentration (mM)	Population / variety				
	<i>wr1</i>	<i>wr2</i>	<i>wr3</i>	Mean of <i>wr</i>	Baldo
0	7	7	7	7	4
50	7	7	7	7	4
100	7	7	7	7	4
150	9	8	9	8.67	9
200	12	10	12	11.33	9
250	14	>15	12	13	12

Table 20. Time (days) required reach 50% of cumulated germination (EC_{50}) of the tested weedy rice populations and rice variety.

NaCl concentration (mM)	Population / variety			
	<i>wr1</i>	<i>wr2</i>	<i>wr3</i>	Baldo
0	8.10	8.02	8.20	4.52
50	8.17	7.89	8.31	5.28
100	9.53	8.84	9.49	6.88
150	12.17	11.40	12.39	11.33
200	11.97	13.50	11.85	12.38
250	13.50	-	11.50	11.69

Table 21. *SI* values of the speed of germination (calculated as the ratio between EC_{50}) of the tested weedy rice populations and rice variety. Only comparisons with $P \leq 0.05$ are showed.

NaCl concentration (mM)	Comparison	<i>SI</i>	P value
0	<i>wr3</i> /Baldo	1.81	*
	Baldo/ <i>wr2</i>	0.56	*
	Baldo/ <i>wr1</i>	0.56	*
50	<i>wr3</i> /Baldo	1.57	*
	Baldo/ <i>wr2</i>	0.67	*
	Baldo/ <i>wr1</i>	0.65	*

*: $P \leq 0.05$.

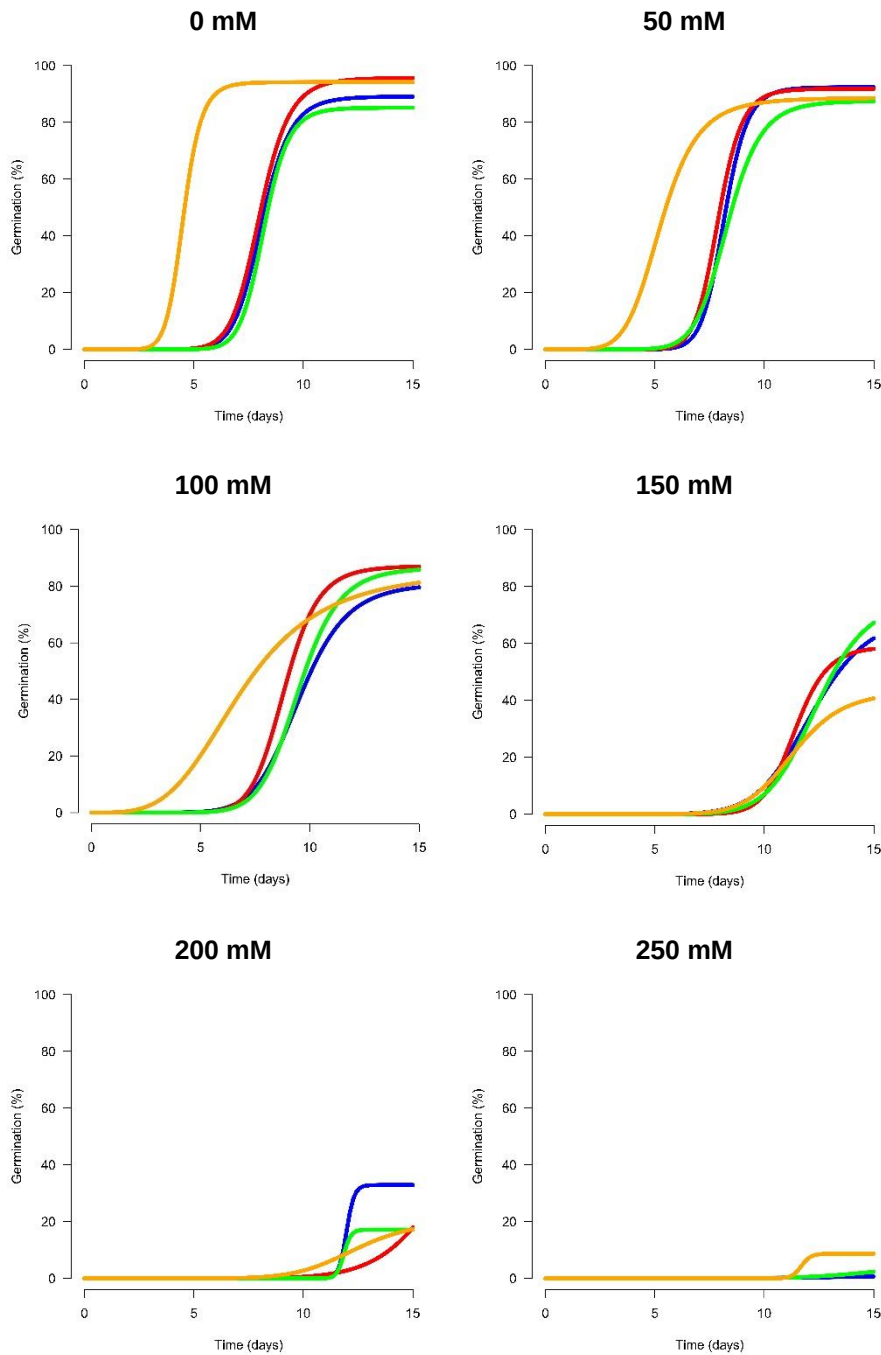


Figure 20. Cumulated seed germination capacity (%) of weedy rice populations and rice variety at the different NaCl concentration as function of trial period, as fitted by the following *log-logistic model* $Y = d/[1+\exp[b(\log(x) - \log(e))]]$. Blue line: weedy rice population wr1; red line: wr2; green line: wr3; orange line: variety Baldo.

Seedling development

The trial was divided in two subsets: “salt” and “salt + herbicide”. In the first one, weedy rice populations and the rice variety Baldo was tested for the response to germination and growth under saline conditions. In the second one, the weedy rice populations were subjected to germination and development under salinity and to an herbicide treatment with imazamox performed at the stage of 2-3 leaves.

Since Baldo was a conventional variety (therefore sensitive to herbicide treatments), it was tested for the response to salinity only. As a consequence, the results were presented only in the case of subset “salt”.

Seedling development was considerably affected by saline conditions starting from 100 mM NaCl, both in weedy rice and rice.

At 50 mM NaCl, seedling development of the weed species and the rice variety appeared to be regular, with seed germination followed by continuous plant growth throughout the trial period.

Noticeable negative effects on plant growth began to appear starting from 100 mM NaCl. At 100 mM NaCl and 150 mM NaCl, after seed germination and first seedling development, plants became completely desiccated within few days. This behaviour was expected for the rice variety, as it is well known that rice is affected by salt stress symptoms starting from EC levels lower than 4 dS/m (Gao *et al.*, 2007), in particular during seedling stage and reproductive period (Hoang *et al.*, 2016). The presence of high content of Na ion (in soil) acts on the biological function of the plant causing numerous negative effects, including disruption of intracellular ion homeostasis, membrane dysfunction and inhibition of metabolic activity. As a consequence, inhibition of development and a decrease of production yields may occur (Gao *et al.*, 2007).

These results are not surprising also in the case of weedy rice, as it belongs to the same species of cultivated rice.

In the case of 200 mM NaCl and 250 mM NaCl, seedlings development stopped at the germination stage.

As a consequence of these findings, the physiological parameters (plant height, shoot and root fresh weight, SPAD value, chlorophyll and carotenoids content) could only be analysed for the salt concentrations of 0 mM and 50 mM.

Plant height

Plant height was affected by saline conditions in both “salt” and “salt + herbicide” subsets.

The analysis of variance carried out on the growth data obtained 21 days after the herbicide application and referred to the two considered subsets showed the presence of a significant effect of “population / variety” and “herbicide application”. (Table 22).

Table 22. Plant growth (expressed as height in cm) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl 21 days after herbicide application. Plant height of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population / variety	Height (cm)
<i>wr1</i>	18.85
<i>wr2</i>	19.19
<i>wr3</i>	21.07
Baldo	21.64
ANOVA	
Population / variety	*
Salt dose	ns
Herbicide application	*
Population/variety x salt dose	ns
Population/variety x herbicide application	ns
Salt dose x herbicide application	ns
Population/variety x salt dose x herbicide application	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

Plant height: subset “salt”

In saline conditions (50 mM NaCl), plant height increased progressively in all tested weedy rice populations and in the tested rice variety. However, the analysis of variance performed on plant height measured 21 days after herbicide application underlined that these increments in plant growth were not significant (Figure 21).

Moreover, no significant differences were found within weedy rice populations and between weedy rice population and rice variety (Figure 21).

The greatest growth was detected for Baldo variety both at 0 mM NaCl (20.04 cm) and 50 mM NaCl (23.23 cm).

wr1 was the population characterized by the lower development at both salt doses; in particular its height was 16.97 cm at 0 mM NaCl and to 17.41 cm at 50 mM NaCl.

The absence of salt effect observed on plant growth is in disagreement to what observed in several studies (for example Jamil *et al.*, 2012; Hakim *et al.*, 2013), which found a reduction in rice development in the presence of salinity.

Plant height: subset “salt + herbicide”

Plant growth of the tested weedy rice populations slightly increased up to the 14th day and then stopped both at 0 mM NaCl and 50 mM NaCl.

Furthermore, plant development recorded at 50 mM NaCl resulted lower than that observed at 0 mM NaCl in all weedy rice populations.

At 0 mM NaCl and 50 mM NaCl, the highest plant height (25 cm and 22 cm, respectively) was found for population *wr3*.

The average height of populations *wr1* and *wr2* was 21 cm at 0 mM NaCl and to 19 cm at 50 mM NaCl.

The analysis of variance carried out on plant height measured 21 days after herbicide application showed that there were no differences between the tested weedy rice populations at the two salt doses; moreover, no significant differences were found for each population at 0 mM NaCl and 50 mM NaCl (Figure 21).

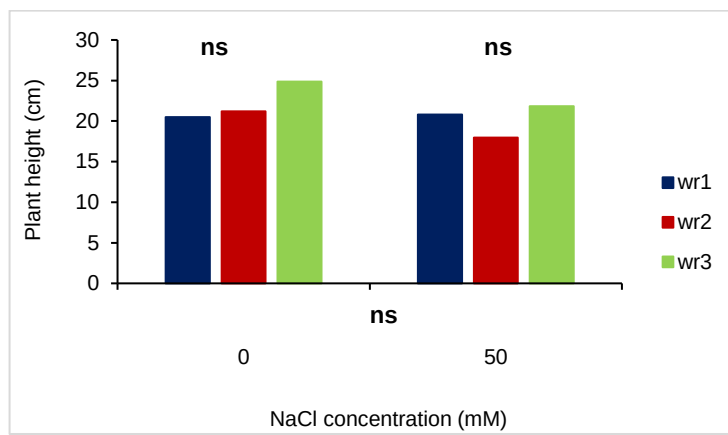
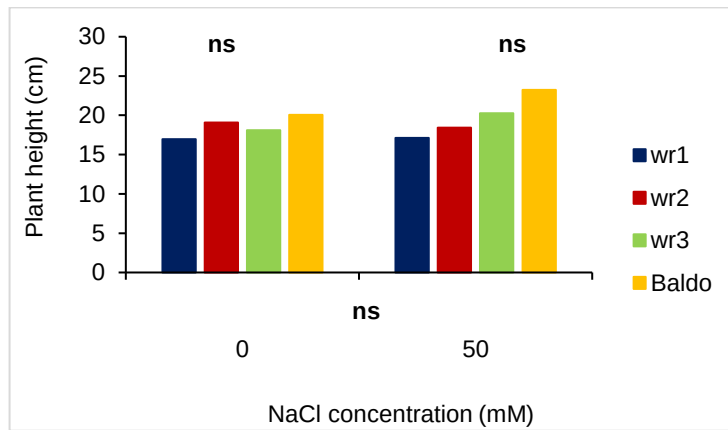


Figure 21. Plant growth (expressed as height in cm) of tested weedy rice populations in the subsets “salt” and “salt + herbicide” at 0 mM NaCl (top) and 50 mM NaCl (bottom) at 21 days after herbicide application.

ns above the bars indicate no significant differences between populations within the same salt concentration.

ns under the bars indicate no significant differences between 0 mM NaCl and 50 mM NaCl for each population.

Different letters indicate significant differences ($P \leq 0.05$) based on Tukey’s post-hoc test.

Shoot fresh weight

The results obtained at the end of the study showed that, as it was observed for the barnyard grass populations, shoot fresh weight of weedy rice populations and rice variety was affected by salinity both in the subset “salt” and in the subset “salt + herbicide”. A decrease in shoot fresh weight between 0 mM NaCl and 50 mM NaCl was recorded. This result is in agreement with what found by Jamil *et al.* (2012): in a study conducted on different rice varieties the authors observed that the shoot fresh weight decreased with the increase of salt concentration. The analysis of variance performed on the fresh weight measurements obtained 21 days after the herbicide application and referred to both subsets underlined the presence of significant differences attributable to the factors “population / variety” and “salt dose” (Table 23).

Shoot fresh weight: subset “salt”

No significant differences were found among tested weedy rice populations / rice variety, neither at 0 mM NaCl nor at 50 mM NaCl (Figure 22).

The average shoot fresh weight of weedy rice populations averaged 1.69 g and 1.29 g per plant at 0 mM NaCl and 50 mM, respectively. In the case of Baldo variety, shoot fresh weight was 2.72 g at 0 mM NaCl and 2.06 g at 50 mM NaCl.

Shoot fresh weight: subset “salt + herbicide”

The analysis of variance showed the presence of a significant effect of “salt dose” (Figure 22). This result was due to the behaviour of population *wr3*, which shoot fresh weight measured at 50 mM NaCl (0.93 g) resulted significantly lower than that found at 0 mM NaCl (1.41 g).

Table 23. Shoot fresh weight (g) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl 21 days after herbicide application. Shoot fresh weight of each population is the overall average of subset “salt” and “salt + herbicide”.

Population / variety	Shoot fresh weight (g)
<i>wr1</i>	1.88
<i>wr2</i>	1.45
<i>wr3</i>	1.18
Baldo	2.39
ANOVA	
Population / variety	*
Salt dose	*
Herbicide application	ns
Population/variety x salt dose	ns
Population/variety x herbicide application	ns
Salt dose x herbicide application	ns
Population/variety x salt dose x herbicide application	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

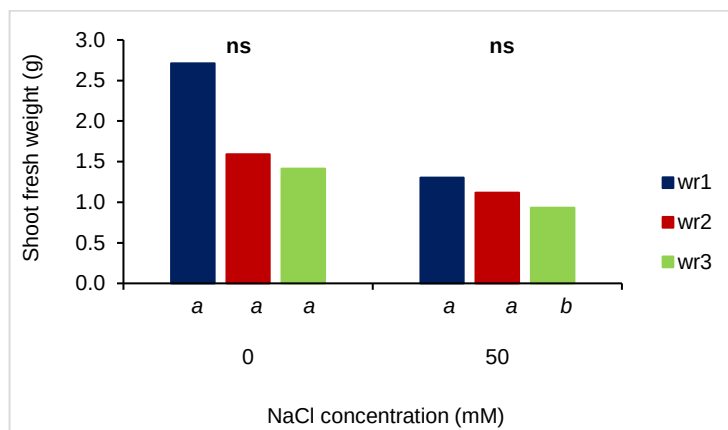
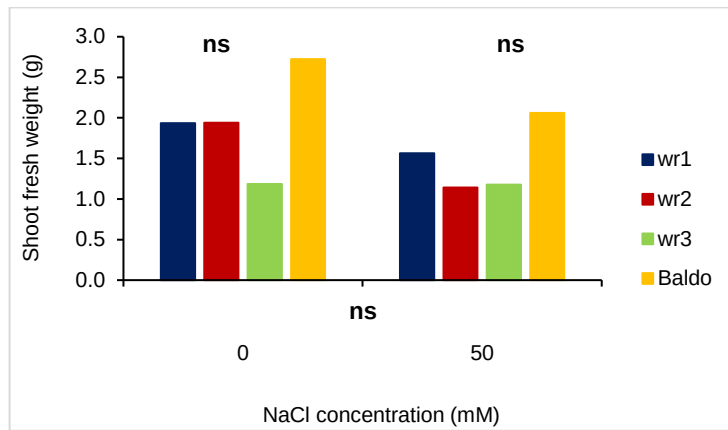


Figure 22. Shoot fresh weight (g) of tested weedy rice populations in the subsets “salt” and “salt + herbicide” at 0 mM NaCl (top) and 50 mM NaCl (bottom) at 21 days after herbicide application.

ns above the bars indicate no significant differences between populations within the same salt concentration.

ns under the bars indicate no significant differences between salt concentrations for each population. Letters under the bars indicate significant differences between 0 mM NaCl and 50 mM NaCl for each population.

Different letters indicate significant differences ($P \leq 0.05$) based on Tukey’s post-hoc test.

Root fresh weight

The results of the measurements carried out at the end of the study showed that root biomass (in terms of fresh weight) was affected by salinity both in the case of weedy rice populations and rice variety. A similar response to salinity was observed by Jamil *et al.* (2012), in the case of three different rice varieties.

The analysis of variance performed on root fresh weight measured 21 days after herbicide application and including the two subsets showed the presence of significant differences due to “population / variety” and “salt dose” (Table 24).

Table 24. Root fresh weight (g) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl 21 days after herbicide application. Root fresh weight of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population / variety	Root fresh weight (g)
<i>wr1</i>	1.71
<i>wr2</i>	1.36
<i>wr3</i>	0.97
Baldo	2.29
ANOVA	
Population / variety	*
Salt dose	*
Herbicide application	ns
Population/variety x salt dose	ns
Population/variety x herbicide application	ns
Salt dose x herbicide application	ns
Population/variety x salt dose x herbicide application	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

Root fresh weight: subset “salt”

Significant differences among weedy rice populations and between weedy rice populations and rice variety were found at 0 mM NaCl. Differences in root development between 0 mM NaCl and 50 mM NaCl were underlined only in the case of Baldo variety.

At 0 mM NaCl and 50 mM NaCl the highest root fresh weight values (2.92 g and 1.67 g, respectively) were found for Baldo variety while the lowest values (0.73 g at 0 mM NaCl and 0.55 g at 50 mM NaCl) were detected for weedy rice population *wr3* (Figure 23).

Root fresh weight: subset “salt + herbicide”

The analysis of variance underlined that there were no significant differences between the factors tested (“population” and “salt dose”).

The highest root weight (2.21 g at 0 mM NaCl and 1.50 g at 50 mM NaCl) was found for the weedy rice population *wr1*; the lowest values at 0 mM NaCl and 50 mM NaCl were recorded for the weedy rice populations *wr2* (1.54 g) and *wr3* (0.78 g), respectively (Figure 23).

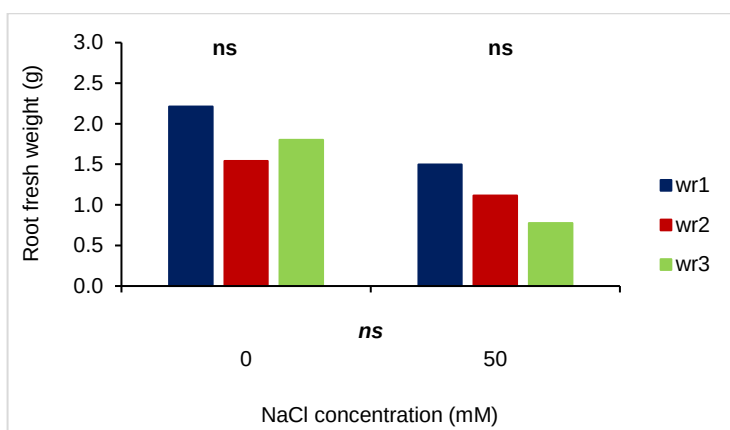
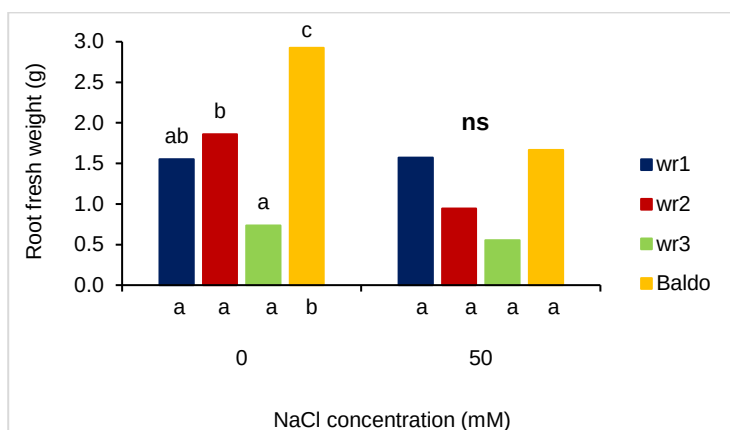


Figure 23. Root fresh weight (g) of tested weedy rice populations in the subsets “salt” and “salt + herbicide” at 0 mM NaCl (top) and 50 mM NaCl (bottom) at 21 days after herbicide application.

Letters above the bars indicate significant differences between populations within the same salt concentration.

Letters under the bars indicate significant differences between 0 mM NaCl and 50 mM NaCl for each population.

ns above the bars indicate no significant differences between populations within the same salt concentration ($P > 0.05$).

ns under the bars indicate no significant differences between 0 mM NaCl and 50 mM NaCl for each population ($P > 0.05$).

Different letters indicate significant differences ($P \leq 0.05$) based on Tukey’s post-hoc test

SPAD

The analysis of variance performed on the SPAD readings and referred to the two subsets showed that both “population / variety” and “salt dose” had a significant effect; furthermore, also the interaction “salt dose” x “herbicide application” was significant (Table 25).

SPAD: subset “salt”

In this subset, saline conditions (50 mM NaCl) resulted in a significant reduction of SPAD values in the weedy rice populations *wr2* and *wr3*, while the population *wr1* and the rice variety Baldo did not show a significant reduction in comparison to control (50 mM NaCl) (Figure 24).

SPAD: subset “salt + herbicide”

In this subset, significant differences were found among populations (Figure 24) at the salt dose of 0 mM. The response of tested weedy rice populations to herbicide application varied only in the absence of salt: at 50 mM NaCl, the three populations responded similarly to the herbicide.

Table 25. SPAD (SPAD unit) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl 21 days after herbicide application. SPAD values of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population / variety	SPAD (SPAD unit)
<i>wr1</i>	8.35
<i>wr2</i>	9.58
<i>wr3</i>	5.70
Baldo	11.69
ANOVA	
Population / variety	*
Salt dose	*
Herbicide application	ns
Population/variety x salt dose	ns
Population/variety x herbicide application	ns
Salt dose x herbicide application	*
Population/variety x salt dose x herbicide application	ns

*: $P \leq 0.05$; ns: $P > 0.05$.

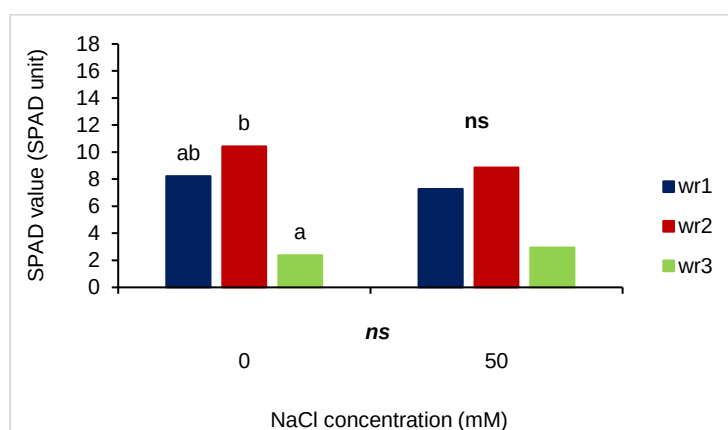
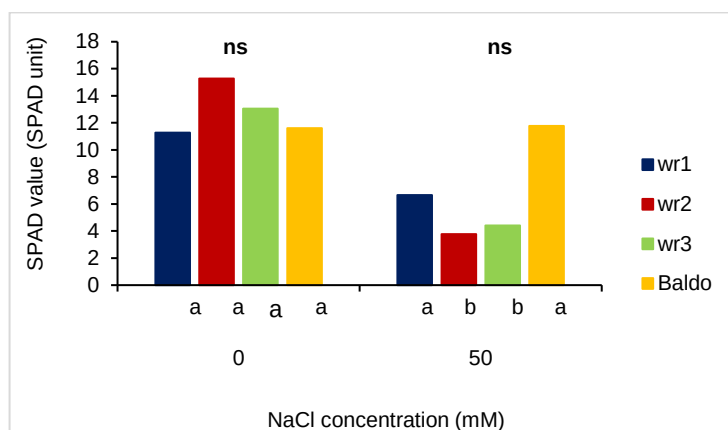


Figure 24. SPAD (SPAD unit) of tested weedy rice populations in the subsets “salt” and “salt + herbicide” at 0 mM NaCl (top) and 50 mM NaCl (bottom) at 21 days after herbicide application.

Letters above the bars indicate significant differences between populations within the same salt concentration.

Letters under the bars indicate significant differences between 0 mM NaCl and 50 mM NaCl for each population.

ns above the bars indicate significant differences between populations within the same salt concentration.

ns under the bars indicate significant differences between 0 mM NaCl and 50 mM NaCl for each population.

Different letters indicate significant differences ($P \leq 0.05$) based on Tukey' post-hoc test.

Chlorophyll *a*, *b* and carotenoids content

The extraction of chlorophyll *a* and *b* (*Chl a* and *Chl b*) and carotenoids carried out at the end of the study for the salt doses of 0 mM and 50 mM underlined that leaf content of these pigments was affected by salinity both in the case of tested weedy rice populations and rice variety. The analysis of variance carried out on concentration of *Chl a*, *Chl b* and carotenoids measured 21 days after the herbicide application and related to the two considered subsets showed the presence of significant differences for all considered factors and for their interaction in all the three pigments (Table 26, 27 and 28).

In order to evaluate the effect of salt on the pigment concentration and to verify the potential relationship between salinity and herbicide application, the two subsets were explored in a separate way.

Table 26. Chlorophyll *a* content (mg g⁻¹ FW) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl 21 days after herbicide application. *Chl a* content of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population / variety	<i>Chl a</i> (mg g ⁻¹ FW)
<i>wr1</i>	0.80
<i>wr2</i>	1.03
<i>wr3</i>	0.80
Baldo	0.72
ANOVA	
Population / variety	*
Salt dose	*
Herbicide application	*
Population/variety x salt dose	*
Population/variety x herbicide application	*
Salt dose x herbicide application	*
Population/variety x salt dose x herbicide application	*

*: $P \leq 0.05$.

Table 27. Chlorophyll *b* content (mg g⁻¹ FW) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl 21 days after herbicide application. *Chl b* content of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population / variety	<i>Chl b</i> (mg g ⁻¹ FW)
<i>wr1</i>	0.19
<i>wr2</i>	0.20
<i>wr3</i>	0.18
Baldo	0.16
ANOVA	
Population / variety	*
Salt dose	*
Herbicide application	*
Population/variety x salt dose	*
Population/variety x herbicide application	*
Salt dose x herbicide application	*
Population/variety x salt dose x herbicide application	*

*: P ≤ 0.05.

Table 28. Carotenoids content (mg g⁻¹ FW) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl 21 days after herbicide application. Carotenoids content of each population is the overall average of subsets “salt” and “salt + herbicide”.

Population / variety	Carotenoids (mg g ⁻¹ FW)
<i>wr1</i>	0.86
<i>wr2</i>	1.11
<i>wr3</i>	0.93
Baldo	0.89
ANOVA	
Population / variety	*
Salt dose	*
Herbicide application	*
Population/variety x salt dose	*
Population/variety x herbicide application	*
Salt dose x herbicide application	*
Population/variety x salt dose x herbicide application	*

*: P ≤ 0.05.

Chlorophyll and carotenoids: subset “salt”

As regard the *Chl a* is concerned the analysis of variance showed a significant effect of both factors “population / variety” and “salt dose”, as well as of their interaction (Figure 25). The significance of the interaction was likely be attributable to the response of the weedy rice population *wr1* to salinity conditions (Figure 25).

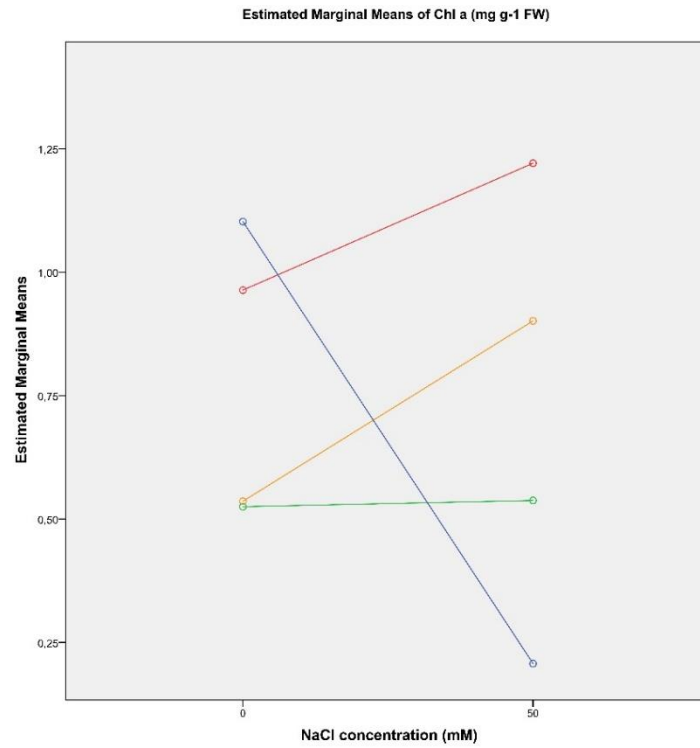


Figure 25. Chlorophyll a content trend of tested weedy rice populations and rice variety under saline and non-saline conditions in subset “salt”. Blue line: population *wr1*; red line: *wr2*; green line: *wr3*; orange line: Baldo variety.

A significant increase in the pigment content between 0 mM NaCl and 50 mM NaCl was found for the weedy rice populations *wr2* and *wr3* and for the rice variety Baldo. Conversely, for the weedy rice population *wr1*, at 50 mM NaCl, the *Chl a* content was lower than that at 0 mM NaCl (0.21 mg g⁻¹ FW vs 1.10 mg g⁻¹ FW) (Figure 28).

Furthermore, as previously observed for the *Chl a*, also in the case of *Chl b*, the analysis of variance underlined the presence of a significant effect of both “population / variety”, “salt dose” and their interaction (Figure 26).

As occurred for *Chl a* content, also for *Chl b* (Figure 28) a significant increase in its content between 0 mM NaCl and 50 mM NaCl was observed, in particular in weedy rice population *wr2* and Baldo variety. A decrease in total chlorophyll content (*Chl a* + *Chl b*) was, instead, detected for weedy rice population *wr1*. This result, which is in agreement with other studies on weedy rice (Hakim *et al.*, 2014) may be due to the increased activity of the chlorophyllase, the enzyme responsible for chlorophyll degradation (Jamil *et al.*, 2012).

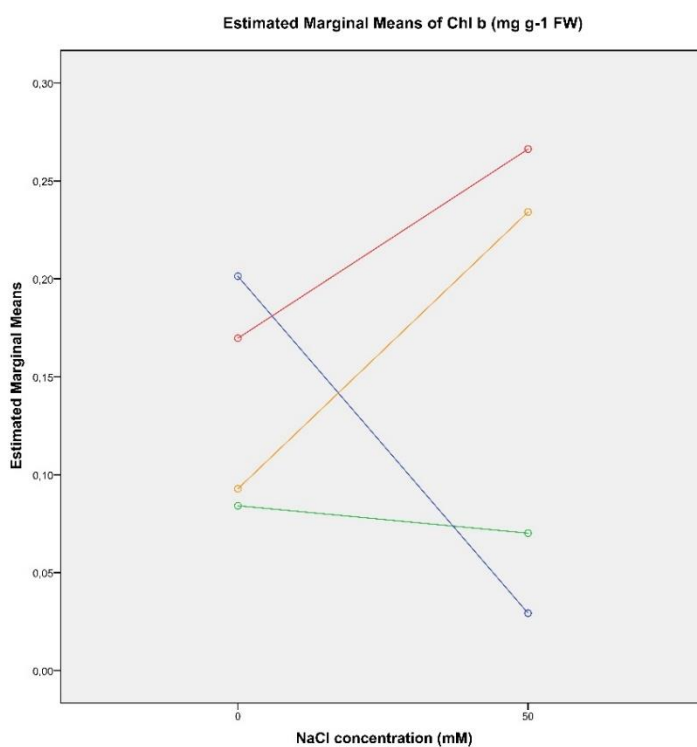


Figure 26. Chlorophyll *b* content trend of tested weedy rice populations and rice variety under saline and non-saline conditions in subset “salt”. Blue line: population *wr1*; red line: *wr2*; green line: *wr3*; orange line: Baldo variety.

Carotenoids content was characterized by an increase between 0 mM NaCl and 50 mM NaCl in the case of weedy rice populations *wr2* and *wr3* and in the case of Baldo variety (Figure 27 and 28).

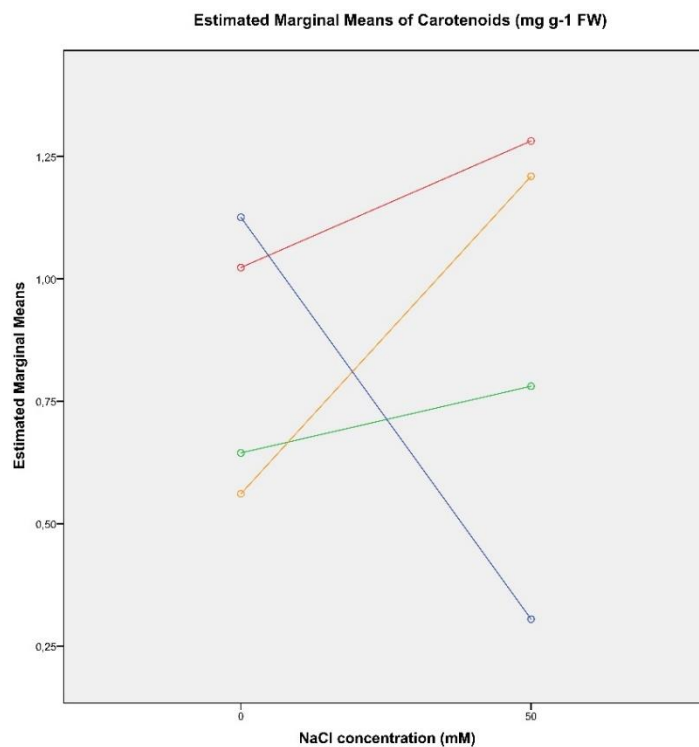


Figure 27. Carotenoids content trend of tested weedy rice populations and rice variety under saline and non-saline conditions in subset “salt”. Blue line: population *wr1*; red line: *wr2*; green line: *wr3*; orange line: Baldo variety.

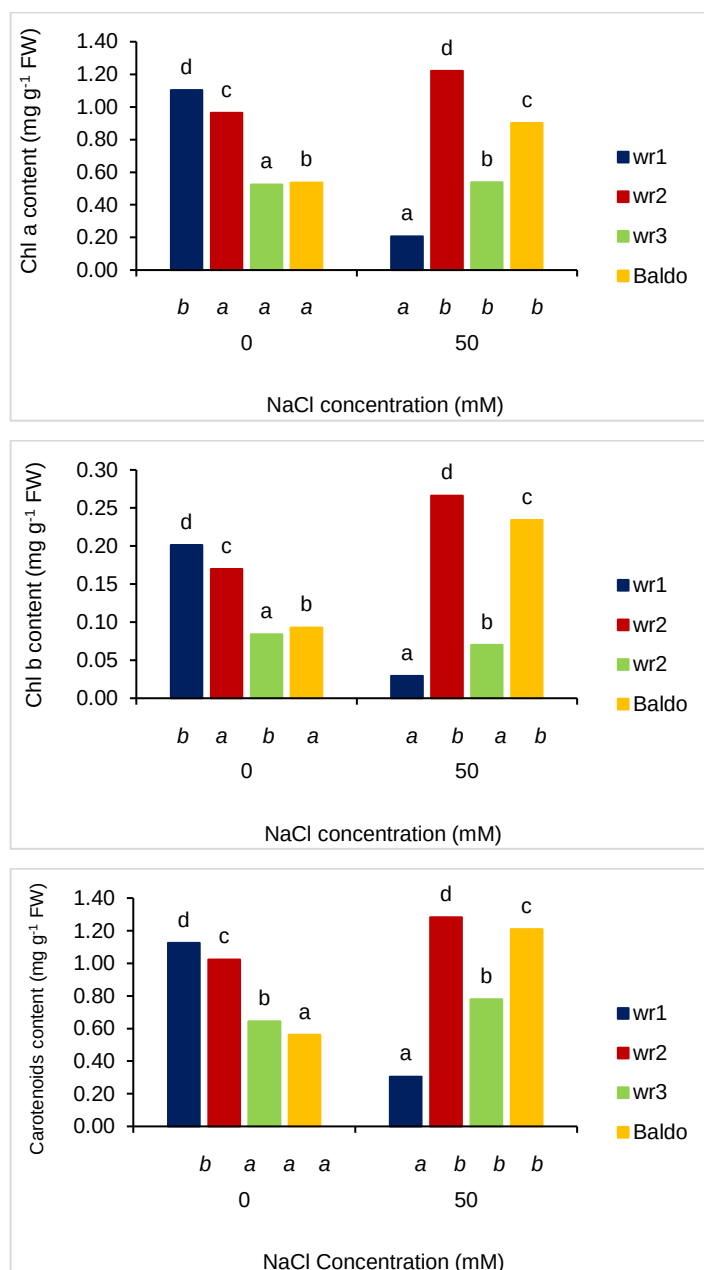


Figure 28. Chlorophyll *a* (top), *b* (middle) and carotenoids (bottom) content (mg g⁻¹ FW) of tested weedy rice populations and rice variety at 0 mM NaCl and 50 mM NaCl in subset “salt” 21 days after herbicide application.

Letters above the bars indicate significant differences between populations within the same salt concentration.

Letters under the bars indicate significant differences between 0 mM NaCl and 50 mM NaCl for each population.

Different letters indicate significant differences ($P \leq 0.05$) based on Tukey's post-hoc test.

Chlorophyll and carotenoids: subset “salt + herbicide”

As occurred for the subset “salt”, also in this case the analysis of variance performed for the concentration of *Chl a*, *Chl b* and carotenoids underlined the presence of significant differences due to “population”, “salt dose” and “population” x “salt dose” interaction (Figure 29, 30 and 31).

With regard to *Chl a*, the content of this pigment decreased significantly from 0 mM NaCl to 50 mM NaCl in the case of populations *wr1* and *wr2* while in the case of population *wr3* there was an increase in its concentration (Figure 32).

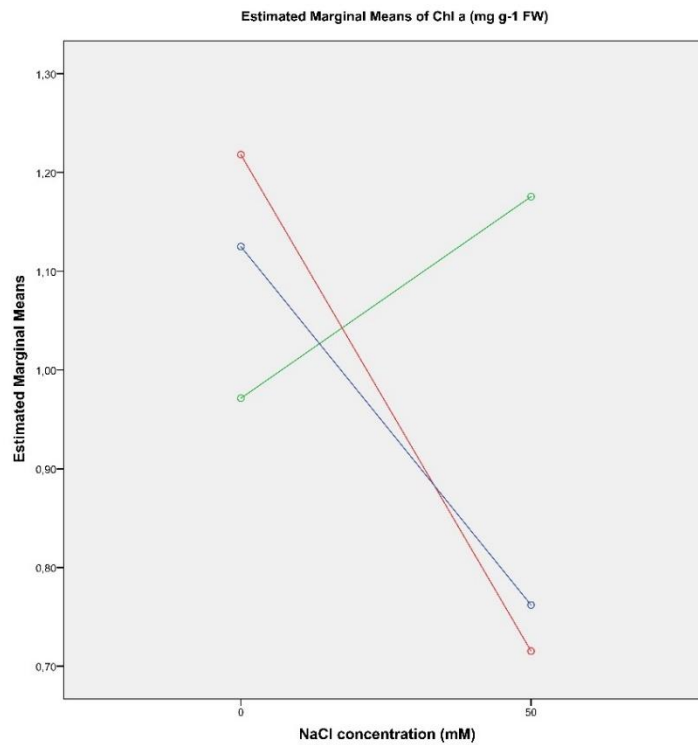


Figure 3. Chlorophyll *a* content of tested weedy rice populations under saline and non-saline conditions in subset “salt + herbicide”. Blue line: population *wr1*; red line: *wr2*; green line: *wr3*.

In the case of *Chl b*, tested weedy rice populations *wr1* and *wr3* showed a significant increase in its content between 0 mM NaCl and 50 mM NaCl (from 0.23 mg g⁻¹ FW to 0.29 mg g⁻¹ FW in the case of *wr1* and from 0.27 mg g⁻¹ FW to 0.29 mg g⁻¹ FW in the case of *wr3*). At 50 mM NaCl, the two populations were characterized by the same pigment content (0.29 mg g⁻¹ FW).

For what concerns the weedy rice population *wr2*, it showed a significant decrease of *Chl b* concentration between saline and non-saline condition; in particular, the pigment content was 0.26 mg g⁻¹ FW at 0 mM NaCl and to 0.11 mg g⁻¹ FW at 50 mM NaCl (Figure 30 and 32).

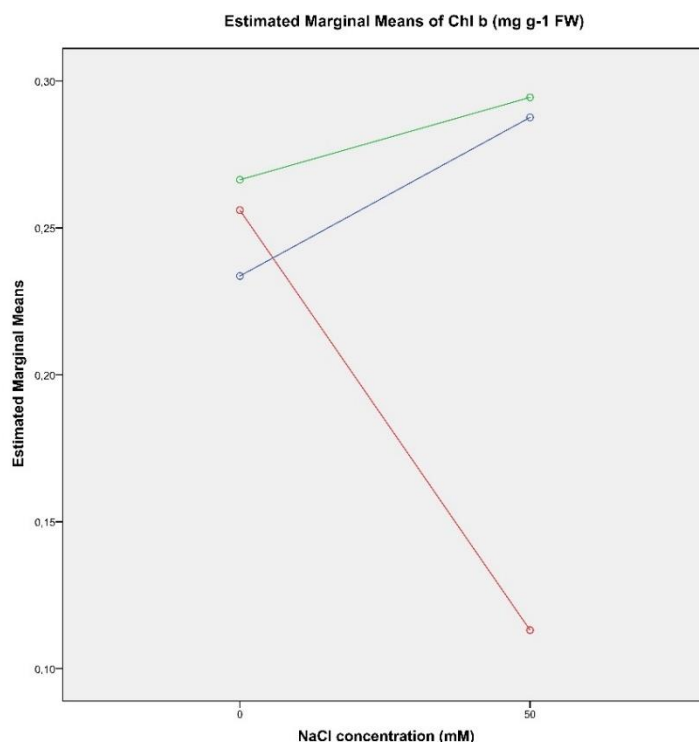


Figure 30. Chlorophyll *b* content of tested weedy rice populations under saline and non-saline conditions in subset “salt + herbicide”. Blue line: population *wr1*; red line: *wr2*; green line: *wr3*.

Leaf content of carotenoids was characterized by a significant decrease from 0 mM NaCl to 50 mM NaCl in the case of populations *wr1* and *wr2*. Furthermore, at 50 mM NaCl, carotenoids concentration of the two populations was not statistically different (0.92 mg g⁻¹ FW and 0.90 mg g⁻¹ FW, respectively, for *wr1* and *wr2* populations).

In the case of population *wr3*, carotenoids concentration presented a significant increase at 50 mM NaCl compared to that observed at 0 mM NaCl (1.27 mg g⁻¹ FW vs 1.03 mg g⁻¹ FW) (Figure 31 and 32).

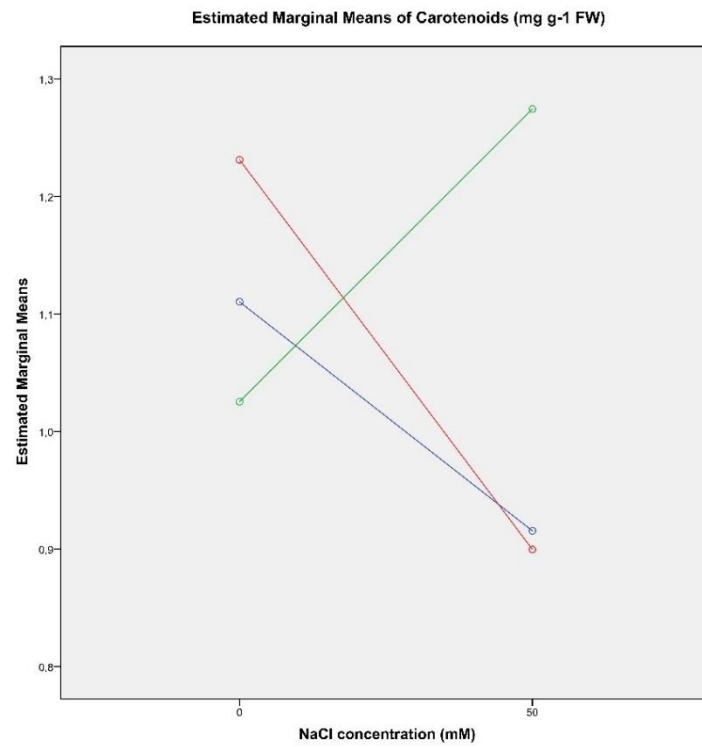


Figure 31 Carotenoids content of tested weedy rice populations under saline and non-saline conditions in subset “salt + herbicide”. Blue line: population *wr1*; red line: *wr2*; green line: *wr3*.

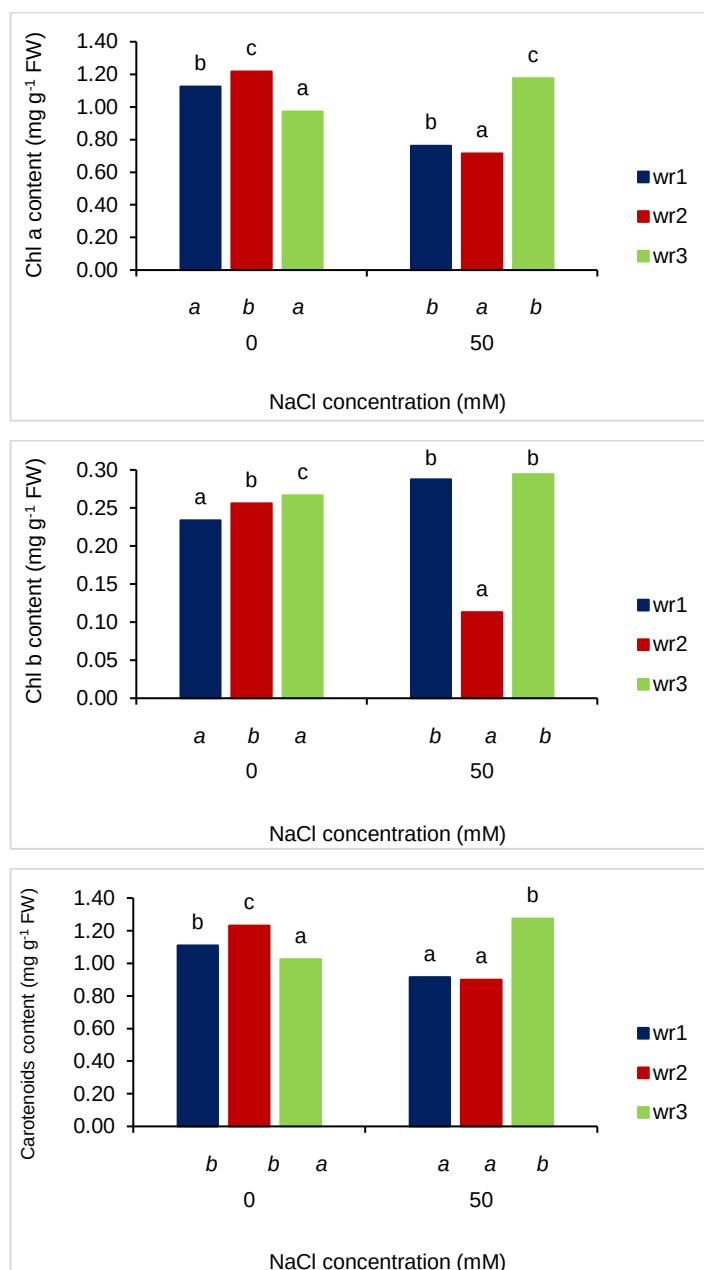


Figure 32. Chlorophyll *a* (top), *b* (middle) and carotenoids (bottom) content (mg g⁻¹ FW) of tested weedy rice populations at 0 mM NaCl and 50 mM NaCl in subset “salt + herbicide” 21 days after herbicide application.

Letters above the bars indicate significant differences between populations within the same salt concentration.

Letters under the bars indicate significant differences between 0mM NaCl and 50 mM NaCl for each population.

Different letters indicate significant differences ($P \leq 0.05$) based on Tukey's post-hoc test.

Comparison between weedy rice populations and rice variety on considered physiological parameters

At the end of the trials, the analysis of the considered physiological parameters by comparing the behavior of weedy rice populations and rice variety was performed (Table 29). Since the rice variety was not subjected to herbicide application, the comparison was only performed in the case of the subset "salt".

In general, the response of tested weedy rice populations and rice variety, in terms of salt tolerance, was found to be variable and dependent on the physiological parameter considered.

A significant higher tolerance of Baldo variety, compared to weedy rice populations, to saline conditions was noted in the case of plant height and SPAD: at 50 mM NaCl, plant growth and SPAD of rice were equal, respectively, to 23.23 cm and 11.78 SPAD value while, in the case of weedy rice, the same variables were 18.61 cm and 4.94 SPAD unit, respectively.

In non-saline conditions, shoot and root fresh weight of Baldo resulted greater (2.72 g and 2.92 g, respectively) than that of weedy rice (1.69 g and 1.38 g, respectively). Conversely, in the same condition, the rice variety was characterized by a carotenoids content (0.56 mg g⁻¹ FW) lower than that of weedy rice populations (0.93 mg g⁻¹ FW).

Table 29. Comparison between physiological parameters of weedy rice populations (WR) and Baldo variety (BV) in the subset "salt".

Physiological parameter	Salt dose (mM)	Population / variety		P value
		WR	BV	
Plant height (cm)	0	18.04	20.04	ns
	50	18.61	23.23	*
Shoot fresh weight (g)	0	1.69	2.72	*
	50	1.29	2.06	ns
Root fresh weight (g)	0	1.38	2.92	*
	50	1.02	1.67	ns
SPAD (SPAD unit)	0	13.20	11.60	ns
	50	4.94	11.78	*
Chl a (mg g⁻¹ FW)	0	0.86	0.54	ns
	50	0.66	0.90	ns
Chl b (mg g⁻¹ FW)	0	0.15	0.09	ns
	50	0.12	0.23	ns
Carotenoids (mg g⁻¹ FW)	0	0.93	0.56	*
	50	0.79	1.21	ns

*: P≤0.05; ns: P>0.05.

Conclusions

The findings obtained at the end of the trials showed that all the examined plant physiological parameters were affected by saline conditions.

A similar pattern of response to salinity between weeds (barnyard grass and weedy rice) and crop (rice) was found in terms of seed germination capacity and speed of germination. The increase of saline dose was accompanied by a reduction in the percentage of germinated seeds and by a delay of the beginning of germination. In the last case, in particular, Baldo variety showed a speed of germination higher than that of barnyard grass and weedy rice at all the salt doses. The trend of this parameter has to be considered a key element in the competition phenomena occurring between crop and weeds. Weeds that are able to germinate before the crop, or at the same time, are those characterized by greater competitiveness and responsible for high yield losses (Swanton *et al.*, 2015).

The findings about speed of germination obtained from the study suggests, therefore, that rice could show a competitive advantage under saline conditions: as seeds germinate faster, crop could suffer less competition from the weeds, at least in the early stage of growth. However, it is necessary to consider that starting from the salt concentration 100 mM, seed germination was followed by the complete plant desiccation.

Barnyard grass populations were affected by salinity starting from the salt dose of 150 mM NaCl, with a reduction in plant growth. This can be considered as adaptation response that allows the plant to survive in the presence of saline stress: this behaviour allows the plants to allocate various resources in order to counteract the stress (Zhu, 2001). At 200 mM NaCl and 250 mM NaCl the seeds were still able to germinate and produce seedlings. Yet, further growth was hampered and the seedlings underwent complete desiccation.

The barnyard grass herbicide resistant population was characterized by greater salt tolerance compared to the herbicide sensitive populations, as grew more, both in terms of plant height, shoot and root fresh weight, SPAD values, chlorophyll and carotenoids content. However, from these results it is not possible to draw the conclusion that there is a relationship between the herbicide resistance and the high salt tolerance found in this population.

In the case of weedy rice, the symptoms on plant growth associated to the presence of salinity were already visible at 100 mM NaCl, when a complete desiccation of the seedlings was observed.

From the comparison between the subsets “salt” and “salt + herbicide”, the herbicide application affected only some of the physiological parameters investigated. A significant effect of the factor “herbicide

application” for the plant height, the *Chl a*, *Chl b* and carotenoids content was found: in particular, a decrease in height, chlorophyll and carotenoids concentration was observed in the subset “salt + herbicide”.

In the case of the rice variety Baldo, as observed for the weedy rice populations, symptoms on plant growth appeared at 100 mM NaCl and were represented by the complete desiccation of the seedlings.

In the case of weedy rice, the populations tested showed a salt tolerance similar or lower compared to that of the rice variety. The similarity between some physiological parameters in response to saline stress can be justified by the fact that crop and weed belong to the same species and therefore may show a similar behaviour.

The good ability to adapt to the salt conditions shown by the barnyard grass populations tested in our study suggests that this weed species would still to be highly problematic in rice field even in a scenario of increased water salinity. Despite the need for germination times longer than those required by rice, this weed does not underwent any growth limitation in the presence of saline concentrations up to 150 mM. Moreover, at a lower salt concentration (50 mM NaCl), growth of shoots and roots was stimulated, leading to values higher than those recorded in the control treatment. An increase in leaves and roots biomass in presence of saline conditions was also found by León-Romero *et al.* (2017), in a study conducted on *Arabidopsis thaliana* to assess the effect of NaCl pre-treatment on plant growth and absorption of toxic metals.

Redondo-Gomez *et al.* (2007), in a study carried out on *Atriplex portulacoides* and based on the application of various saline concentrations (0, 20, 200, 400 and 700 mol m⁻³ NaCl) observed a stimulation of the mean relative growth rate as a result of the exposure to moderate external saline concentrations, with a peak of growth reached at a concentration of 200 mol m⁻³ NaCl. Moreover, Parida *et al.* (2004), also observed an effect of salinity on growth parameters analysed in *Bruguiera parviflora* plants after 45 days of growth in saline conditions: in particular, at a saline concentration of 100 mM, the authors noted an increase in height and root fresh weight significantly higher than that of the control.

This particular behaviour observed in response to some of the tested salt concentrations could be included in the group of stimulation behaviours that are also observed in response to other stresses and that are usually defined with the term “hormesis”. This is a phenomenon of type dose-response based on a stimulation induced by low-dose and on an inhibition induced by high-dose (Calabrese and Baldwin, 2001, 2003; Calabrese, 2008).

The stimulation of the development of some morphological characters (in particular plant height, shoot and root biomass) found in barnyard grass in presence of sub-lethal saline concentrations is an aspect of high criticality: it could result in a greater obstacle of this weed species in relation to crop growth. There is evidence of the presence of high

correlation between competitive capacity and some morphological features of the plant, such as height, tillering, leaf area and leaf angle. Moreover, barnyard grass roots and shoots are considered to be able to limit the rice development in a significant way; a study conducted on water-seeded rice found that the first cause of growth reduction in rice is represented by root competition for resources (Gibson *et al.*, 1999).

The results of our study also suggest that an increase of salinity should not increase the competitive ability of weedy rice, as its response to salinity very close to that of rice.

Moreover, what emerged by the experimentation suggests that it is necessary to take in to account that herbicide application controls the weeds (in particular barnyard grass) also in saline conditions but that the herbicide weed resistant population continues to be such even in presence of salinity.

However, it is necessary to consider that, during the trials, the response to salinity and to the combination of salt and herbicide treatment of a single resistant population was tested. As a consequence, it is not possible to assert with certainty that other herbicide resistant populations can exhibit the same behaviour. If an analogous response to saline conditions is confirmed on other resistant populations, it would be a crucial result. In a future scenario of potential extension of saline areas used for rice cultivation, there would be weeds resistant to herbicides and characterized by high tolerance to environmental stress conditions. This would result in great difficulty in managing weed flora and the competition towards the crop.

A further interesting aspect of the study is that salinity conditions were created from the beginning of the test. So, it was possible to observe a behaviour of weeds and crop in response to the stress to which they were subjected very close to what could be encountered in the field, in the case of cultivation in the presence of saline conditions.

This approach has allowed to monitor what might be the development of barnyard grass and crop in saline environment and to analyse the response to herbicide treatment by plants already potentially stressed by salt.

The high competitive capacity of barnyard grass (compared to weedy rice and rice) under saline conditions and the apparent absence of relationship between salt treatment and herbicide application in an herbicide resistant population appear to be of fundamental importance. The correct management of weeds is therefore an essential condition even in the presence of water salinity. In this condition crop growth is affected not only by the presence of salt but also by the presence of weeds highly tolerant to salinity and, in the most critical cases, also characterized by the development of herbicide resistance phenomena.

Conclusions

The studies presented above focused on two central themes: the development of herbicide resistance by different weed populations and the analysis of weeds and crop response to the appearance of an abiotic stress (in the specific case, salt stress) and the evaluation of the potential interaction between the stress and an herbicide application.

The first theme has been developed through a series of studies carried out on *Lolium multiflorum* and *Apera spica-venti* populations collected in Switzerland. These studies confirmed that the repeated application of the same herbicides or the same mechanisms of action may result in the selection of weed populations no longer controlled by them (Shaner, 2014). The weed populations tested came from crops in which the herbicide rotation was very limited. A finding of further importance is represented by the appearance, in some of these populations, of cross and multiple resistance phenomena (Evans *et al.*, 2016).

The issue of the selection of resistant weed populations is also the consequence of the lacking research, development and commercialization of new herbicides with new mechanisms of actions in almost thirty years (Owen, 2016).

These considerations stress the fundamental importance of a proper management of resistance issue through the recourse of all available strategies.

The application of herbicide with different mechanisms of action is the basis of current strains to avoid the appearance and expansion of herbicide resistance phenomena. Herbicide rotation and sequences allow to reduce the selection pressure of a single target site by using herbicides with different mechanism of action in subsequent growing seasons or during the same season, respectively (Evans *et al.*, 2016). It is important to note, however, that even though herbicide rotation represents the first action in defining a global resistance management strategy, it is not able to delay the selection of all the resistance cases that may appear. Non-target site resistance, for example, can continue to be selected despite the use of this strategy. A weed control strategy which has been reported to perform better than rotation of herbicide mechanisms of action may be the use of herbicide mixtures (Heap, 2014), which exploits the synergistic action of more herbicides applied together (Beckie, 2006).

Nevertheless, the weed control strategies that are considered to be the most effective and reliable on the long run are those based on disturbing and destabilizing weed populations in order to prevent their transformation in severe issues (Heap, 2014). These strategies are applied in a weed integrated management approach for the purpose to limit and delay resistance development by the combined use of chemical and non-chemical means.

Mechanical tools represent one of the major alternative methods of weed control; among these, a crucial role is played by tillage, a system considered to be of interest in regulating and delaying the appearance of resistance phenomena (Beckie, 2006). Mechanical methods interfere with

the evolution of weed communities, and in the dynamics of seedbank in particular (Owen, 2016).

Other weed control means alternative or combined with herbicides are represented by several agronomic practices, including crop rotations, correct choice of sowing time, cover crops, sowing density. With particular reference to the cover crops, their ability to control weeds is greater if they are used in combination with tillage rather than alone (Owen, 2016).

The studies reported here indicated that both weeds and crop are affected by water salinity.

The germination tests showed that the seed germination capacity under saline condition was low to moderate in the case of weeds (barnyard grass and weedy rice) and good in the case of rice, in particular for the Baldo variety, that at the salt dose of 400 mM showed a germination ability still equal to 42%. Moreover, rice was characterized by a shorter time required for the first germination compared to the two weeds.

The positive rice adaptation to saline environment during the germination stage was confirmed by the trials carried out in the greenhouse study. Also in this case the germination percentage was high and the time required for the first germination was shorter than that of the two weeds at all the salt doses applied.

On the contrary, in the growth phases following germination rice and weedy rice showed a reduced salinity tolerance with growth inhibition at the saline concentrations greater than 50 mM. A greater and better adaptation to salinity was found, instead, in the case of the barnyard grass populations. This weed was characterized by a shoot and root growth greater than the control up to a concentration of 150 mM of NaCl. This aspect is of fundamental importance in the definition of the competition relationship between weed and crop, as rice growth is significantly limited by shoot and root competition from barnyard grass (Gibson *et al.*, 1999). In particular, root competition resulting from the presence of weeds may cause a reduction in rice yield of up to 55% (Dass *et al.*, 2017).

Furthermore, the study highlighted that the herbicide resistant barnyard grass population was still tolerant to herbicide application, even in saline conditions. However, it is important to consider that, during the study, the response to salinity and to the combination of salinity and herbicide treatment was analysed for one resistant population only. As a consequence, it is not possible to assert with certainty that other herbicide resistant populations can exhibit the same behaviour.

The findings therefore suggest that despite rice requires less time to germinate compared to the weeds, it is highly disadvantaged by the salt presence during the seedling stage. This, coupled with an apparent absence of lack of damage to the herbicide resistant weed due to salinity, stresses the need to pay close attention to weed control also in presence of an abiotic stress.

These findings obtained at the end of the trials are of great interest in the perspective of potential future extension of crop cultivation to areas affected by the appearance of abiotic stress, such as saltwater intrusion. The effect produced on plant growth by environmental stresses (Ahuja *et al.*, 2010), among which the salinity is one of the main (Pattanagul and Thitisaksakul, 2008), has become an important problematic as a result of the impact due to climate change on plant resources, biodiversity and food security overall (Ahuja *et al.*, 2010).

Climate change, in combination with growing populations (Maggio *et al.*, 2011), socio-economic development and contamination (Balía and Viezzoli, 2015), is responsible for the strong competition for fresh water between agricultural and civil uses (Maggio *et al.*, 2011). Because of that, based on the estimation of the Food and Agriculture Organization of the United Nations an increase of salinity levels in about 20% of the irrigated lands can be globally observed (Rozema and Flowers, 2008).

The stress due to salinity can be associated with the presence of weeds that exhibit high competitiveness compared to the crop and that, in some cases, have developed herbicide resistance phenomena. Furthermore, it is important to consider that, in general, most of weeds result less susceptible to environmental stresses than crops (Hakim *et al.*, 2011).

In the light of this, the results obtained during the trials can give useful indications on the response of weeds and crop to the growth in saline conditions.

The findings can also provide a framework of competitive relationships, between weeds and crop, that can be created in case of appearance of saline stress.

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