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RESEARCH ARTICLE

Different phytotoxic effect of *Lolium multiflorum* Lam. leaves against *Echinochloa oryzoides* (Ard.) Fritsch and *Oriza sativa* L.

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Abstract

Rice cultivation, particularly prone to weed issues, requires practices able to effectively control them, however reducing the use of herbicides, responsible for damage to human health and ecosystem sustainability. Alternative strategies for weed management can be based on plant-plant interaction phenomena. In this context, a group of organic farmers has developed a pragmatic approach for weed containment using *Lolium multiflorum* Lam. as a cover crop before rice. The present study aimed to confirm the farmer field observations reporting a preferential inhibitory effect of *L. multiflorum* on *Echinochloa oryzoides* (Ard.) Fritsch, one of the most yield-damaging rice weed, compared with *Oryza sativa* L. The study showed that *L. multiflorum* was able to significantly reduce the seed germination of *E. oryzoides*. It was found to be more susceptible than *O. sativa* both to the effect of the aqueous extract and powder of *L. multiflorum* leaves (23–79% vs. 3–57% and 26–100% vs. 23–31%, respectively). In addition, the leaf extract was able to affect *E. oryzoides* growth starting from 20% concentration both in relation to the root and shoot length while *O. sativa* exhibited differences compared with the control only under the influence of extract 50%. The *L. multiflorum* leaf characterization by NMR and UPLC-HR-MS analyses led to the identification of 35 compounds including several polyphenols, glycosyl flavonoids and glycosyl terpenoids, as well as different amino acids and organic acids. Some of them (e.g. protocatechuic and gallic acids) are already known as allelochemicals confirming that *L. multiflorum* is a source of plant growth inhibitors.

Keywords Allelopathy · Cover crop · Early watergrass · Italian ryegrass · Organic rice · Weed biocontrol

Introduction

Weed control strategies play a key role in the success of agricultural production. This is even more true for rice, a crop particularly prone to weed issues. The incidence of weeds is the main constraint for rice production and the main cause of the yield gap between conventional and organic farming (Delmotte et al. 2001; Shennan et al. 2017; Hazra et al. 2018). On the other hand, the intensive use of herbicides in high-input cropping systems is becoming increasingly problematic in terms of environmental pollution. Their low biodegradability and high persistence pose considerable hazards for soil and water quality, and, to a certain extent, for human health (Kim et al. 2017). Several European countries report levels for one or more pesticides in groundwater that exceed quality standards. In some areas of northern Italy, between Piedmont and Lombardy regions, where 94% of national rice production is concentrated, the highest degradation of groundwater and surface water quality due to pesticide and herbicide

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contamination was found (Ispra 2018). For all these reasons, the European authorities have prohibited the use of active substances potentially harmful to human health or the environment (e.g. Directive 79/117/EE; Directive 91/414/CEE).

In addition to the restrictions on the use of certain chemicals belonging to traditional phytosanitary plans, there are also problems related to the progressive ineffectiveness of some products in the control of weed populations. The wide and continuous use of herbicides with a limited diversification of the action mechanisms in mono-cropping systems has caused resistance phenomena worldwide. Among rice weeds, some *Echinochloa* species such as *E. colona* (L.) Link, *E. crus-galli* L., *E. oryzoides* (Ard.) Fritsch have evolved herbicide resistance in many countries by reducing farm productivity (Fischer et al. 2000; Talbert and Burgos 2007; Malik et al. 2010; Wright et al. 2018). In particular, *E. oryzoides* was found resistant to ALS-inhibitors, ACCase-inhibitors and lipid inhibitors (Fischer et al. 2000; Altop et al. 2014). *E. oryzoides*, known as early watergrass, is also particularly fearsome for the rice production because it emerges before the other *Echinochloa* species, thus reaching greater competitiveness. This results in a lower rice plant density and severe yield losses.

Against this background, new weed management strategies, able to delay or reduce their germination and growth, are necessary (Gibson et al. 2002; Awan et al. 2015). The development of eco-friendly herbicides or agroecological practices effective in reducing the *Echinochloa* species incidence is a key aspect to enhance the sustainability of the rice cultivation. Plant-plant interference, intended as a negative effect of one plant on another, is a widely investigated aspect to find a useful alternative tool (Kadioglu and Yanar 2004; Jose et al. 2016; Lim et al. 2017). In the agroecosystem integrated management, the introduction into crop rotations of allelopathic crops affecting the weeds' growth is a promising agroecological practice (Wezel et al. 2014). In this regard, some interesting results emerged from the on-farm research carried out by a group of researchers and some organic rice farmers in North Italy (Orlando et al. 2020). The participatory research identified a promising management strategy for weed control within the organic rice cropping system. After the cultivation of a winter herbage as a cover crop, the rice no-till sowing is carried out directly on the standing cover crop. It is not incorporated into the soil as green manure but shredded and used as green mulching, briefly irrigated before the rice paddy flooding, postponed for about a month. The farmer experience has highlighted that when *Lolium multiflorum* Lam. is chosen as a cover crop, a considerable reduction of *E. oryzoides* incidence is detected. These observations suggested the existence of a species-specific mechanism of interaction between the two species, beyond the well-known effect of competitiveness for light, nutrients and space (Teasdale 1996; Bastiaans et al. 2008). Based on their empirical knowledge, farmers have speculated that the presence of the *L. multiflorum* green mulching affects both rice and weed growth. Nevertheless, the weed is more susceptible, and this difference can be useful to obtain a competitive advantage in favour of rice. To validate this hypothesis, the possibility of successfully managing a plot- or field-scale experiment was evaluated untenable. The organic rice field is, in fact, a complex system where many variability sources interact in the short and long term, contributing to determine the weeds dynamics, with resulting high inter- and intra-field and inter- and intra-season variability (Stoop et al. 2009; Orlando et al. 2020).

Accordingly, the present study was aimed to verify in vitro the phytotoxic activity of *L. multiflorum* leaves against *E. oryzoides* integrating our previous results (Vitalini et al. 2020). Likewise, the possible effects on *O. sativa* were also evaluated and different approaches simulating the release of phytotoxic compounds from the producing plant towards the target plant were considered.

Materials and methods

Plant material

Seeds of *E. oryzoides* were collected during 2018 from an organic rice field of the 'Terre di Lomellina" farm, located in the province of Pavia (North-West Italy). The same farm provided the seeds of *O. sativa* L. (cv. Rosa Marchetti) and *L. multiflorum*. All seeds were stored at 4 °C. Before use, their surface was sterilized with 1% sodium hypochlorite by shaking for 10 min, then repeatedly rinsed with distilled water.

Furthermore, also *L. multiflorum* leaves were harvested in the rice fields of the "Terre di Lomellina" farm, air-dried at shade and room temperature (25 °C), then kept in paper bags until extraction. A voucher specimen (No. LMTL210) was deposited at the Department of Agricultural and Environmental Sciences, Milan State University (Milan, Italy), after its identification according to Flora d'Italia (Pignatti 1982).

Aqueous extract bioassay

To reproduce the field conditions, *L. multiflorum* aqueous extract was prepared as previously described (Vitalini et al. 2020). Powdered leaves were mixed with distilled water (1:10, w/v), then shaken at room temperature. After 24 h, the mixture was filtered through gauzes to remove residues and centrifuged at 2300 g for 30 min. The obtained extract was used as such (100%) as well as diluted with distilled water to give final concentrations of 1%, 10%, 20% and 50%. The seeds of *E. oryzoides* and *O. sativa* were sown in Petri dishes (9 cm) on filter paper. Ten sterilized seeds of each species

were placed on two filter papers and soaked with 5 mL of each dilution. The same volume of distilled water was used as control (0% concentration). All Petri dishes were prepared in a vertical laminar flow hood by using sterile materials and sealed with parafilm before incubation in a growth chamber at 25 °C/16 h light and 18 °C/8 h dark cycle for 7 days. Five petri dishes were realized for each combination of "species × *L. multiflorum* treatment" by setting up the experimental design as follows: *E. oryzoides* or *O. sativa* seeds × 6 concentration levels of *L. multiflorum* extract (including distilled water as control) × 5 replicates.

Leaf powder bioassay

The leaf powder bioassay was carried out according to Vitalini et al. (2020) with some modifications. Different quantities (0.25 g, 0.36 g, 0.5 g) of *L. multiflorum* powdered leaves were spread on two filter papers in Petri dishes (9 cm). Then, ten sterilized seeds of *E. oryzoides* or *O. sativa*, respectively, were placed and 5 ml of distilled water was added. The same volume of distilled water was used in the control samples (0 g of leaf powder).

All Petri dishes were prepared in a vertical laminar flow hood by using sterile materials and sealed with parafilm before incubation in a growth chamber at 25 °C/16 h light and 18 °C/8 h dark cycle for 7 days. Five petri dishes were realized for each combination of "species × *L. multiflorum* treatment" by setting up the experimental design as follows: *E. oryzoides* or *O. sativa* seeds × 6 concentration levels of *L. multiflorum* extract (including distilled water as control) × 5 replicates.

Seed germination measurements

The seed germination was recorded daily. On the seventh day, root and shoot length (mm) of each germinated seed was measured on graph paper under a stereomicroscope and the average value per Petri dish was calculated. The obtained data were used to compute the following germination indices:

$$\text{Germination percentage} = \frac{Germinated\ seed\ number}{Seed\ total\ number} \times 100 \quad (1)$$

$$\text{SVI} = (Mean\ Root\ length + Mean\ Shoot\ length) \times Germination\ percentage \quad (2)$$

$$\text{MGT} = \frac{\sum D \times Germinated\ seed\ number}{\sum Germinated\ seed\ number} \quad (3)$$

where SVI is the Seedling Vigour Index (Eq. 2; Abdul-Baki and Anderson 1973) and MGT is the mean germination time (Eq. 3; Ellis and Roberts 1981) calculated taking into account D that is the number of days from the beginning of germination, plus the number of seeds germinated on day D.

Ultra-performance liquid chromatography/electrospray ionization-high resolution mass spectrometry (UPLC/ESI-HR-MS)

The UPLC/ESI-HR-MS analysis was carried by coupling an Acquity UPLC separation module (Waters, Milford, MA, USA) with in-line photodiode array (PDA) eλ detector (Waters) to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer and an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions were as follows: spray voltage + 3.5/− 3.5 kV, sheath gas flow 35, auxiliary gas flow 15, temperature 300 °C, and capillary temperature 350 °C. Positive mass calibration was performed with Pierce LTQ ESI Positive Ion Calibration Solution (Thermo Scientific Pierce, Rockford, IL, USA), containing caffeine, the tetrapeptide MRFA and Ultramark 1621. Negative mass calibration was performed with Pierce ESI Negative Ion Calibration Solution (Thermo Scientific Pierce), containing sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621. Four μL of sample (20% diluted in water from crude extracts) were separated using a Waters Acquity BEH C18 column (150 × 2.1 mm, 1.7 μm, 130 Å) (Waters, Milford, MA, USA) kept at 40 °C, and using 0.1 mL 100 mL-1 of formic acid in H2O MilliQ-treated water (solvent A) and 0.1 mL 100 mL-1 formic acid in acetonitrile (solvent B). For the UPLC separation, a linear elution gradient was applied (isocratic 5% B for 5 min then 5% to 50% of solvent B in 20 min) at a flow rate of 0.2 mL min-1. The LC eluate was analysed by Full MS and data-dependent tandem MS analysis (dd-MS^2) of five of the most intense ions (Top 5). The resolution was set at 70,000 and 17,500 and the AGC targets were 1×10^6 and 1×10^5 for Full MS and dd-MS^2 scan types, respectively. The maximum ion injection times were 50 ms. The MS data were processed using Xcalibur software (Thermo Scientific) and Mnova MS plug-in (MestreNova 14.0.1, Mestrelab). Metabolites were determined according to their calculated exact mass and absorption spectra. Their structures were confirmed by high-resolution tandem MS (HR-MS/MS) by comparison with reported assignments in literature or databases.

NMR spectroscopy

Freeze-dried samples were suspended in D_2O at a final concentration of 10 mg/mL, sonicated (37 kHz, 20 min, Elmasonic P 30H, Elma Schmidbauer GmbH, Singen, Germany) and centrifuged (22,000 g, 5 min, 20 °C, ScanSpeed 1730R Labogene, Lynge, Sweden). 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS, final concentration 0.5 mM) was added to the supernatant as internal

reference for concentrations and chemical shift. The pH of each sample was verified with a microelectrode (Mettler Toledo, Columbus, OH, USA) and adjusted to 7.4 with NaOD or DCl. All pH values were corrected for the isotope effect. The acquisition temperature was 25 °C. All spectra were acquired on an Avance III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (^{1}H, ^{13}C, ^{15}N/^{31}P and ^{2}H) cryogenic probe. ^{1}H NMR spectra were recorded with *cpmgpr1d*, *noesygppr1d*, *ledbpgppr2s1d* pulse sequences (Bruker library) and 256 scans, a spectral width of 20 ppm, and a relaxation delay of 5 s. They were processed with 0.3-Hz line broadening, automatically phased and baseline corrected. Chemical shifts were internally calibrated to the DSS peak at 0.0 ppm. The ^{1}H,^{1}H-TOCSY (total correlation spectroscopy) spectra were acquired with 48 scans and 512 increments, a mixing time of 80 ms and relaxation delay of 2 s. ^{1}H,^{13}C-HSQC (heteronuclear single quantum coherence) spectra were acquired with 64 scans and 512 increments, relaxation delay 2 s. The NMR data were processed using MestreNova 14.1.0 software (Mestrelab Research, Santiago de Compostela, Spain). Compounds identification and assignment were done with the support of 2D NMR experiments, and comparison with reported assignments. For metabolite quantification, the simple mixture analysis tool (Cobas et al. 2011) integrated in MestreNova software package was exploited to set a semi-automatic protocol for the identification and quantification of metabolites, by creating specific metabolite libraries for the different analysed matrices. In this protocol, the global spectrum deconvolution algorithm was employed to deconvolute the overlapping regions and to perform the absolute quantification of metabolites with resonances in crowded spectral areas, too. When possible, the concentration was calculated looking at the mean value of the different signals assigned to the same metabolite.

Statistical analysis

The simple factorial experiment design with five replicates was followed. It contained the two target plant species (*E. oryzoides* and *O. sativa*) and the treatments performed with *L. multiflorum* extract (different concentration levels) or powder (different quantities) as fixed factors considered nominal and ordinal variables, respectively. In addition, the germination indices (germination percentage, SVI, MGT, root length and shoot length) were dependent variables. Analysis of variance was carried out separately for the aqueous extract bioassay and leaf powder bioassay. One-way ANOVA and the Tukey-B post hoc test evaluated the effects of *L. multiflorum* on *E. oryzoides* and *O. sativa* highlighting the most significant impacts. Two-way ANOVA analysed the interactions between the target species and the *L. multiflorum* extract or powder highlighting possible differences in the species response to the different treatments. The significance of F values was tested at p value ≤ 0.05. All statistical analyses were performed using the IBM SPSS.25.

Results

Leaf extract bioassay

Results of the aqueous extract bioassay are shown in Table 1. The interaction "species x *L. multiflorum* treatment" significantly affected the germination percentage, root length, shoot length and SVI (p values < 0.05) with differences between *E. oryzoides* and *O. sativa* in their responses to the increasing concentrations.

As for intra-species analysis, reductions in *E. oryzoides* germination were found at all five leaf extract concentrations (1% to 100%) compared with the distilled water used as a negative control (0%). In detail, 1%, 10% and 20% concentrations showed a similar impact, decreasing the seed germination by 26%, 23% and 30%, respectively. The 50% extract further reduced the number of germinated seed up to 42%. A strong effect was detected for 100% extract able to stop seed germination at 21% (i.e. seed germination decreased by 79%). Otherwise, the germination percentage of *O. sativa* remained unchanged compared with the control at all tested concentrations except at 100% leaf extract. Under its effect, the rice seeds showed a drastic reduction in germination by 57% less than the control.

Root length, shoot length and SVI (p values = 0.000) of *E. oryzoides* were significantly reduced by 20%, 50% and 100% extract concentrations. In particular, the root length decreased from 22% to 98%, shoot length from 30% to 85% and SVI from 55% to 97%, respectively. In the case of *O. sativa*, only 50% and 100% concentrations drastically impacted its roots (− 62% and − 90%, respectively) and the resulting SVI (− 59% and − 91%). Differently, all concentrations were unable to affect the shoot development (p value = 0.07). As for the MGT, both species showed a remarkable increase only under 100% extract effect (27% for *E. oryzoides* and 13% for *O. sativa*) without significant interaction (p value = 0.1).

Leaf powder bioassay

Concerning the plant powder bioassay, the obtained results are shown in Table 2. The interaction "species × *L. multiflorum* treatment" significantly affected the germination percentage (p value = 0.000) highlighting differences between *E. oryzoides* and *O. sativa* in their responses at 0.50 g of leaves, while the two species showed a similar trend with respect to the other germination indices (p value > 0.05).

The intra-species analysis showed that 0.50 g of *L. multiflorum* leaves completely suppressed *E. oryzoides* germination (0%), preventing the determination of the other

Table 1 Germination indices measured on filter paper for *E. oryzoides* and *O. sativa* under the effect of different concentrations of *L. multiflorum* leaf extract

Species	Leaf extract concentration (%)	Germination (%)	MGT	Root length (mm)	Shoot length (mm)	SVI
E. oryzoides	0	97.0 ± 4.5 a	5.5 ± 0.4 a	50.0 ± 11.9 b	25.2 ± 3.6 ab	7215 ± 1394 a
	1	71.7 ± 25.2 ab	5.6 ± 0.4 a	73.0 ± 4.5 a	28.6 ± 4.7 a	9070 ± 608 a
	10	75.0 ± 19.9 ab	5.5 ± 0.5 a	76.0 ± 5.9 a	33.2 ± 1.8 a	9783 ± 1174 a
	20	67.5 ± 5.0 ab	5.7 ± 0.5 a	39.1 ± 3.9 b	17.6 ± 2.9 b	3258 ± 1190 b
	50	56.7 ± 8.6 b	5.7 ± 0.2 a	10.3 ± 12.3 c	17.5 ± 3.7 b	1905 ± 1026 bc
	100	20.0 ± 23.1 c	7.0 ± 0.0 b	1.2 ± 1.5 c	3.7 ± 4.5 c	200 ± 240 c
	F	15.6	5.4	31.8	24.6	41.2
	p value	0.000*	0.002*	0.000*	0.000*	0.000*
O. sativa	0	98.1 ± 3.8 a	5.0 ± 0.2 a	39.4 ± 6.7 a	16.5 ± 3.8 a	5458 ± 999 a
	1	93.3 ± 5.4 a	4.8 ± 0.2 a	35.6 ± 16.0 a	12.4 ± 11.4 a	4384 ± 2693 ab
	10	93.3 ± 5.4 a	4.9 ± 0.3 a	38.8 ± 5.2 a	15.7 ± 4.0 a	4922 ± 1086 ab
	20	95.0 ± 6.4 a	5.0 ± 0.3 a	44.6 ± 7.9 a	18.9 ± 11.4 a	5666 ± 1442 a
	50	90.0 ± 0.0 a	5.2 ± 0.1 a	15.1 ± 11.7 b	9.8 ± 0.3 a	2242 ± 1050 bc
	100	42.5 ± 9.6 b	5.6 ± 0.1 a	3.8 ± 1.9 b	7.8 ± 0.5 a	500 ± 172 c
F		64.4	7.6	14.0	2.6	12.5
p value		0.000*	0.000*	0.000*	0.07	0.000*
Interaction species × treatment						
F		3.5	1.9	6.0	5.4	7.7
p value		0.009*	0.1	0.001*	0.001*	0.000*

Values are mean ± standard deviation, asterisk and different letters indicate statistically significant differences at p value ≤ 0.05 among treatments in each species

indices. Even 0.25-g and 0.36-g treatments involved significant germination decreases, by 26% and 46%, respectively. The germination percentage of *O. sativa* was affected by all three treatments with similar decrease values compared with the control (23% to 31%).

Root and shoot length of *E. oryzoides* were influenced by 0.36-g treatment with a reduction of 70% and 42%, respectively, while SVI was inhibited also by 0.25 g of leaves (53% and 69%).

The root growth and SVI in *O. sativa* showed an increasing reduction shifting from 0.25 g to 0.50 g (24% to 82% and 49% to 71%, respectively) while shoot elongation was not significantly affected (p value = 0.09).

All treatments were not able to affect the MGT in both species (p values > 0.05).

NMR and UPLC-HR-MS analysis

Aqueous extract of *L. multiflorum* leaves was characterized by mean of a combined analytical approach based on NMR spectroscopy and UPLC separation coupled with high resolution mass (HR-MS) analysis that has been already reported for characterization of plant extracts (Amigoni et al. 2017; Palmioli et al. 2019). In particular, UPLC separation was mainly targeted to the qualitative identification of polyphenols and secondary metabolites, whereas NMR spectroscopy data were complementary used for primary and secondary metabolites identification and quantification, including non-ionizable compounds. The chromatographic trace extracted at 320 nm, the characteristic absorbance of polyphenols, and ^{1}H-NMR profile with signal attribution of aqueous extract of *L. multiflorum* leaves were reported in Fig. 1 a and b, respectively. Detailed spectrometric HR-MS data used for compound identification are reported in Table 3. Overall, data analysis allowed the identification of 35 compounds, including several polyphenols, glycosyl flavonoids and glycosyl terpenoids. Among them, we clearly identified protocatechuic acid, 5-*p*-coumaroylquinic acid, apigenin and naringenin 6,8-di-*C*-glucoside and different glycosides of kaempferol and isorhamnetin. Moreover, we detected glycosyl terpenoids such as blumenol C-9-*O*-(2'-*O*-β-glucuronosyl)-β-glucoside, also known as blumenin, nor-isoprenoid trihydroxymegastigmane-4,7-dien-3-one-9-*O*-β-glucoside, also known as sauroposide, and the ubiquitous monoterpenoid lactone loliolide (Fig. 1a). In addition, ^{1}H NMR profile (Fig. 1b) showed also the presence of several amino acids, choline, γ-aminobutirric acid and different organic acids and polyphenols, including shikimic and gallic acids. After the

Table 2 Germination indices measured on filter paper for *E. oryzoides* and *O. sativa* under the effect of different quantity of *L. multiflorum* powdered leaves

Species	Powdered leaf quantity (g)	Germination (%)	MGT	Root length (mm)	Shoot length (mm)	SVI
O. sativa E. oryzoides	0.00	95.0 ± 5.8 a	5.4 ± 0.2 a	51.7 ± 13.0 a	28.5 ± 8.8 a	7552 ± 1595 a
	0.25	70.0 ± 9.1 b	6.0 ± 2.0 a	50.3 ± 25.5 a	22.3 ± 0.7 ab	3526 ± 937 b
	0.36	50.0 ± 8.2 c	5.8 ± 0.1 a	15.7 ± 8.3 b	16.5 ± 3.7 b	2317 ± 943 b
	0.50	0.0 ± 0.0 d	n.d.	n.d.	n.d.	n.d.
	F	141.8	0.4	5.6	4.7	20.9
	p value	0.000*	0.7	0.03*	0.04*	0.000*
	0.00	97.5 ± 5.0 a	5.0 ± 0.0 a	45.5 ± 10.0 a	18.2 ± 4.6 a	6205 ± 1586 a
	0.25	67.6 ± 20.0 b	5.5 ± 1.6 a	34.6 ± 2.2 b	22.5 ± 3.7 a	3151 ± 1215 b
	0.36	67.5 ± 19.0 b	5.3 ± 0.1 a	17.2 ± 2.7 c	12.7 ± 2.1 a	2037 ± 651 b
	0.50	75.0 ± 13.0 b	5.3 ± 0.4 a	8.2 ± 2.1 c	14.7 ± 8.3 a	1790 ± 893 b
F		5.6	0.2	38.1	2.7	12.6
p value		0.001*	0.8	0.000*	0.09	0.001*
Interaction species × treatment						
F		35.9	0.02	1.0	1.9	0.5
p value		0.000*	0.9	0.4	0.2	0.6

Values are mean ± standard deviation, asterisk and different letters indicate statistically significant differences at p value ≤ 0.05 among treatments in each species

manual identification of compounds, specific library was built using the simple mixture analysis tool implemented in the MestReNova 14.1 software. Simple Mixture Analysis allows for the simultaneous quantification of all metabolites contained in a complex mixture. The library developed with this approach is available as .exp. files (Palmioli and Airoldi 2019). Overall, the most abundant metabolites in crude extract were 2,3-butanediol (24.5 mM), succinate (19.8 mM), proline (12.95 mM), acetate (5.14 mM), alanine (4.91 mM), aminobutirric acid (4.07 mM), shikimic acid (2.95 mM), protocatechuic acid (2.92 mM) and lactate (2.34 mM).

Discussion

Till now, the effects of *L. multiflorum* phytotoxic activity were studied on the main grown crops, especially on rice (Li et al. 2008a, b; Lehoczky et al. 2011; Jang et al. 2018a, b), while the negative impact on weeds was only partially investigated (Vitalini et al. 2020; Jang et al. 2018b).

Our results confirmed the inhibitory action of *L. multiflorum* against rice weeds (Vitalini et al. 2020; Jang et al. 2018b). Furthermore, the obtained data supplemented the previous information on the effectiveness of *L. multiflorum* used as a cover crop. In particular, its leaves were able to significantly decrease the *E. oryzoides* germination, in both performed bioassays, unlike inflorescences, stems and roots (Vitalini et al. 2020). However, similarly to these organs, *L. multiflorum* leaves also significantly reduced the *E. oryzoides* seedling vigour. As it often happens, the roots were the vegetative structures most affected by the treatments (Vitalini et al. 2020; Favaretto et al. 2018).

In general, the phytotoxic effect was found to be dose- and species-dependent with a higher susceptibility of *E. oryzoides* compared with *O. sativa*. These data support the hypothesis based on the real-world farming environment experiences collected from some rice farmers, pioneers in the Italian organic rice sector (Orlando et al. 2020). The differences found between *E. oryzoides* and *O. sativa* in the in vitro assays are at the basis of their agronomic practices (Orlando et al. 2020). *L. multiflorum* has a greater impact on *E. oryzoides* than *O. sativa* in their early growth stages providing some competitive advantages to rice whose lower density, also evidenced by some in vitro treatments, is faced by farmers with a more abundant seed sowing. So, all in all, *L. multiflorum*, used as a cover crop before rice cultivation, can give a valuable contribution to the organic or low-input management of weeds. As a result, it may be expected that in the rice fields, the *L. multiflorum* residues can release compounds with a selective effect and, consequently, a potential role in weed control (Tabaglio et al. 2013). Considering the results reported by Li et al. (2008b), it is also possible to suppose that, among the decomposition products of *L. multiflorum* residues, there are some rice stimulators whose activity, mediated by soil

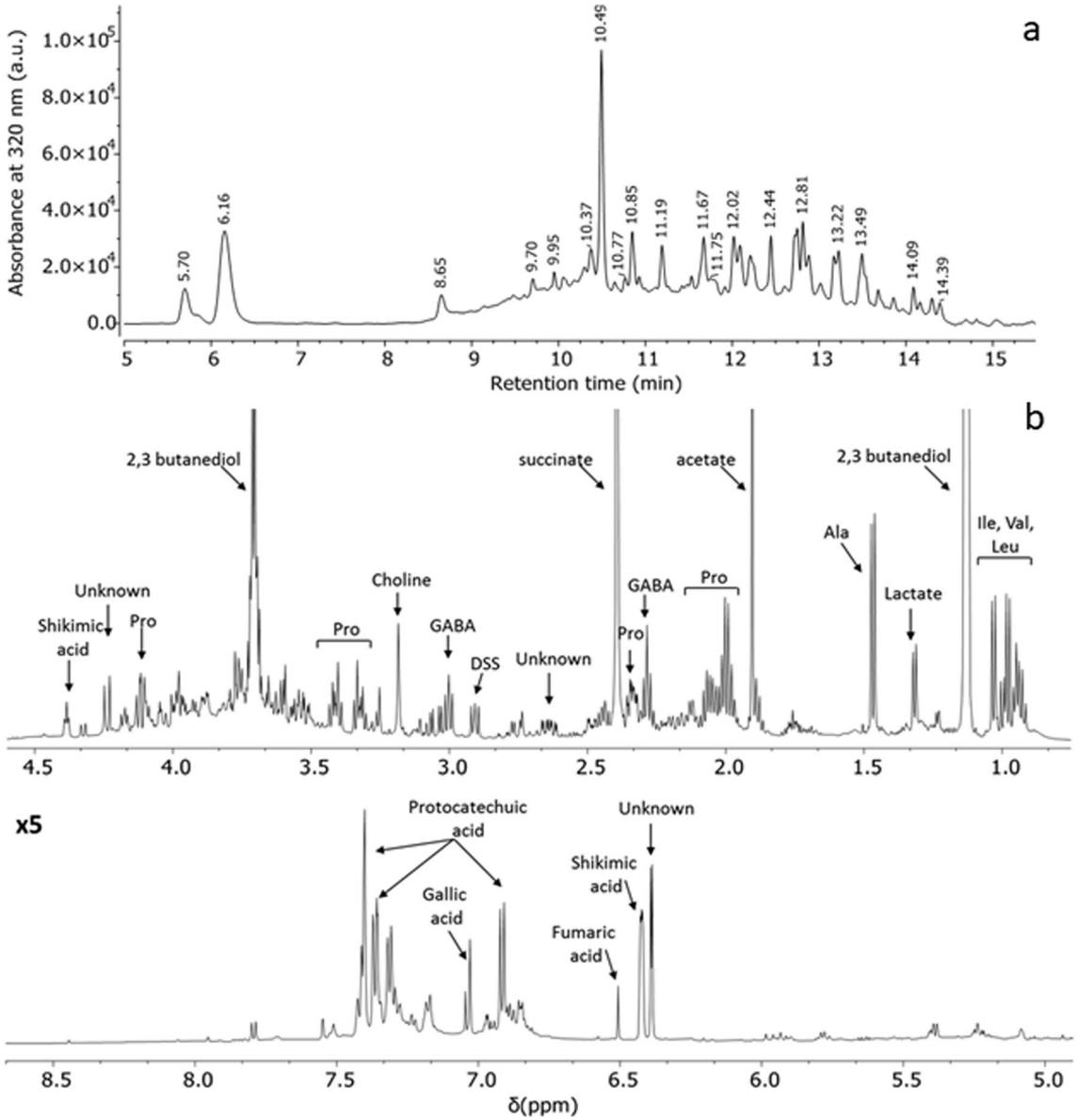

Fig. 1 UPLC separation trace extracted at 320 nm (**a**) and ^{1}H-NMR profile (**b**) of freeze-dried leaf extract from *Lolium multiflorum* dissolved in D_2O at a concentration of 10 mg/mL (DSS 0.5 mM, pH 7.4, 25 °C)

microorganisms, promote the growth of rice seedlings. In addition, it seems that *E. oryzoides* can be more susceptible to the *L. multiflorum* than *O. sativa,* at least in the administered quantities, due to the smaller size of its seeds (Synowiec et al. 2017).

In any case, the observed phytotoxic effect of *L. multiflorum* aqueous extract suggested that its leaves contained inhibitor compounds. Some of them have been reported as allelochemicals. For example, 2,3-butanediol identified in the *Caragana intermedia* Kuang and H.C.Fu root aqueous extract was considered as one of the allelochemicals for *Medicago sativa* Linn. through a concentration-dependent effect (Chen et al. 2017). Protocatechuic and gallic acids were among the main allelopathic compounds isolated from the aqueous extract of *Delonix regia* (Hook.) Raf. able to reduce the germination of the *Lactuca sativa* L. seeds more than 30% compared with the control (Li et al. 2010). Both compounds from the ethyl acetate fraction of the aqueous extract of *Merostachys riedeliana* Rupr. leaves showed also inhibitory effects on *Leucaena leucocephala* (Lam.) de Wit (Jose et al. 2016). Gallic acid identified in leachates of bark, fresh leaves and leaf litter of different *Eucalyptus* species and *Picea schrenkiana* Fisch. et Mey. significantly decreased the seedling growth of *Phaseolus mungo* L. and of the same *P. schrenkiana*, respectively (Li et al. 2010). In the rhizosphere soil of *Ageratum conyzoide* L., gallic acid helped to inhibit the *O. sativa* growth in terms of root length, shoot length and seedling weight (Li et al. 2010). The shikimic acid

Table 3 UPLC/HR-MS data for the major extract components identified in the aqueous extract obtained from *Lolium multiflorum* leaves

#	RT (min)	ID	Name	Molecular formula	Monoisotopic mass	HRMS (+) $[M+H]^+$	Abs. Error (ppm)	HRMS (−) $[M-H]^-$	Abs. Error (ppm)	λ_{Abs} (nm)	MS^2 (+) (rel. int.)	MS^2 (−) (rel. int.)
1	5.70	PCA	Protocatechuic acid	$C_7H_6O_4$	154.0261	155.0330	3.24	153.0190	2.02	260, 294	111 (100), 93 (85), 137 (27), 65 (20)	109 (100)
2	6.16	UNKN	Unknown			322.0538		320.0408		254, 320	322 (100), 169 (74), 276 (48), 258 (36), 286 (33)	153 (100), 166 (88), 320 (62), 122 (44), 109 (23)
3	6.71	Maltol	Maltol	$C_6H_6O_3$	126.0311	127.0384	4.53	–	–	273	–	–
4	8.27	Trp	Tryptophan	$C_{11}H_{12}N_2O_2$	204.0891	205.0964	3.70	203.0827	0.33	272, 279, 288	188 (100), 146 (39)	116 (100), 159 (35), 72 (35), 74 (32), 142 (28)
5	8.64	UNKN	Unknown			306.0583		304.0460		255, 296, 336		
6	10.40		Naringenin 6,8-di-*C*-glucoside	$C_{27}H_{32}O_{15}$	596.1736	597.1793	3.44	595.1676	1.22	251, 322	–	355 (100), 385 (92), 415 (29), 96 (14), 313 (12)
7	10.49	UNKN	Unknown			322.0538		320.0407		254, 305, 314	322 (100), 169 (95), 258 (47), 286 (44), 304 (41), 276 (38), 294 (33)	153 (100), 166 (86), 320 (57), 122 (43), 109 (23)
8	10.85	Vicenin 2	Apigenin 6,8-di-*C*-glucoside	$C_{27}H_{30}O_{15}$	594.1590	595.1625	4.77	593.1508	0.95	270, 325	325 (100), 379 (99), 409 (70)	353 (100), 473 (74), 383 (60), 593 (42)
9	11.18	UNKN	Unknown			306.0586		304.0460		259, 322		
10	11.67		Kaempferol glucoside-glucoronide aglycone	$C_{27}H_{28}O_{17}$ $C_{15}H_{10}O_6$	624.1332 286.0472	625.1374 287.0541	4.07 3.17	623.1245 285.0408	1.33 1.19	267, 338	287 (100)	285 (100)
11	12.00	5-pCoQA	5-pCoumaroyl quinic acid	$C_{16}H_{18}O_8$	338.1007	339.0996	3.88	337.0925	1.21	271, 305	–	191 (100)
12	12.20	UNKN	Unknown	$C_{15}H_{17}O_{13}$	405.0675	406.0732	2.24	404.0613	3.88	255, 335	304 (100), 286 (37), 388 (33)	–
13	12.27	Rutin	Quercetin-3-*O* rutinoside aglycone	$C_{27}H_{30}O_{16}$ $C_{15}H_{10}O_7$	610.1539 302.0421	611.1585 303.0493	3.55 1.93	609.1466 301.0279	0.38 0.73	265, 315–350	303 (100)	300 (100)
14	12.44		Apigenin glucoside-glucoronide aglycone	$C_{27}H_{28}O_{16}$ $C_{15}H_{10}O_5$	608.1383 270.0534	609.1425 271.0592	4.05 3.32	607.1287 269.0457	2.79 0.51	265, 309–350	271 (100)	269 (100)
15	12.70		Diosmin glucoside-glucoronide aglycone	$C_{28}H_{30}O_{17}$ $C_{16}H_{12}O_6$	638.1483 300.0634	639.1531 301.0695	3.82 3.92	637.1410 299.0562	0.36 0.14	265, 290, 320–365	331 (100)	329 (100)
16	12.76		Dillenetin 5-glucoside-7-glucuronide aglycone	$C_{29}H_{32}O_{18}$ $C_{17}H_{14}O_7$	668.1594	669.1632 331.0802	4.43 3.21	667.1505 329.0670	1.70 1.12	265, 290, 320–365	331 (100)	329 (100)
17	12.81		Kaempferol 3-neohesperidoside aglycone	$C_{27}H_{30}O_{15}$ $C_{15}H_{10}O_6$	594.1579 286.0472	595.1635 287.0543	3.79 2.64	593.1483 285.0404	4.32 0.20	265, 290, 320–340	287 (100)	284 (100), 285 (60)

Table 3 (continued)

#	RT (min)	ID	Name	Molecular formula	Monoisotopic mass	HRMS (+) $[M+H]^+$	Abs. Error (ppm)	HRMS (−) $[M-H]^-$	Abs. Error (ppm)	λ_{Abs} (nm)	MS^2 (+) (rel. int.)	MS^2 (−) (rel. int.)
18	12.99	Sauroposide		$C_{19}H_{30}O_9$	402.1895	–	–	401.1813	0.94	–	–	401 (100), 101 (21), 71 (15), 113 (11), 221 (10)
19	13.10	Blumenin	Blumenol C 9-*O*-(2′-glucuronosyl)glucoside	$C_{25}H_{40}O_{13}$ $C_{13}H_{22}O_2$ aglycone	548.2463 210.1614	549.2522 211.1688	3.63 2.21	547.2369 209.1547	2.86 0.10	–	135 (100), 193 (85), 211 (64), 109 (59), 175 (57), 119 (36), 357 (6)	547 (100), 113 (84), 175 (11), 371 (8)
20	13.17	Calendoflavoside	Isorhamnetin 3-neohesperidoside	$C_{28}H_{32}O_{16}$ $C_{16}H_{12}O_7$ aglycone	624.1696 316.0578	625.1736 317.0647	4.35 2.69	623.1611 315.0514	1.08 1.25	265, 290, 328	317 (100)	314 (100), 315 (100
21	13.23		Kaempferol-glucoside	$C_{21}H_{20}O_{11}$ $C_{15}H_{10}O_6$ aglycone	448.1000 286.0458	449.1059 287.0543	4.34 2.42	447.0916 285.0397	3.79 2.54	265, 290, 326	287 (100)	284 (100), 285 (28)
22	13.48		Kaempferol-glucoside	$C_{21}H_{20}O_{11}$ $C_{15}H_{10}O_6$ aglycone	448.1000 286.0458	449.1060 287.0544	4.14 2.32	447.0915 285.0402	4.05 0.94	265, 290, 326	287 (100)	284 (100), 285 (28)
23	13.55		Isorhamnetin-glucoside	$C_{22}H_{22}O_{12}$ $C_{16}H_{12}O_7$ aglycone	478.1106 316.0578	479.1165 317.0649	3.95 2.02	477.1029 315.0503	1.96 2.37	265, 290, 328	317 (100)	314 (100)
24	13.68		Isorhamnetin-glucoside	$C_{22}H_{22}O_{12}$	478.1106	479.1165	3.95	477.1018	4.22	265, 290, 328	317 (100)	314 (100)
25	13.82		Loliolide	$C_{11}H_{16}O_3$	196.1094	197.1163	4.85	–	–	–		
26	14.08		Kaempferol-acetylglucoside	$C_{23}H_{22}O_{12}$ $C_{15}H_{10}O_6$ aglycone	490.1170 286.0458	– –	– –	489.1034 285.0406	0.97 0.34	265, 290, 326	–	285 (100), 284 (84)
27	14.16		Isorhamnetin-malonyl glucoside	$C_{25}H_{24}O_{15}$ $C_{16}H_{12}O_7$ aglycone	564.1110 316.0578	565.1162 317.0648	4.59 2.40	– –	– –	265, 290, 328	317 (100)	
28	14.39	UNKN	Unknown			254.1006		252.0877		270, 317	208 (100)	208 (100), 164(72), 152 (39), 107 (22)

is the common precursor for the synthesis of different phenolic compounds (e.g. coumarins, terpenoids, phenolic acids) implicated in plant allelopathy observed in both natural and managed ecosystems (Ravazi 2011; Favaretto et al. 2018).

In conclusion, confirmation of the different phytotoxic effects of *L. multiflorum* on *E. oryzoides* and *O. sativa* strengthens the practical knowledge of farmers coming from long-standing direct experiences. Even though the laboratory experiments provide only preliminary results on the potential species-specific relationship and the field conditions can interfere with the dynamics observed in controlled environment, the study data provided evidences to support the use of *L. multiflorum* as a cover crop to reduce the weed incidence and obtain better yields. Its green mulching seems to be a viable and alternative strategy among agroecological practices aimed to improve the sustainability of the crop production.

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Author contributions Stefano Bocchi, Marcello Iriti, Francesca Orlando and Sara Vitalini contributed to the study conception and design. Material preparation, data collection and analysis were performed by Cristina Airoldi, Sumer Alali, Ivano De Noni, Francesca Orlando, Alessandro Palmioli, Valentina Vaglia and Sara Vitalini. The first draft of the manuscript was written by Cristina Airoldi, Francesca Orlando, Alessandro Palmioli and Sara Vitalini. All authors commented on later versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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CORRECTION

Correction to: Different phytotoxic effect of *Lolium multiflorum* Lam. leaves against *Echinochloa oryzoides* (Ard.) Fritsch and *Oryza sativa* L.

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In the title, it should be Oryza instead of Oriza.

Francesca Orlando was co-first author and Stefano Bocchi was co-last author.

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