Chemical interactions between plants in Mediterranean vegetation: the influence of selected plant extracts on Aegilops geniculata metabolome

Scognamiglio, M.; Fiumano, V.; D'Abrosca, B.; Esposito, A.; Choi, Y.H.; Verpoorte, R.; Fiorentino, A.

Phytochemistry 106 (2014) 69–85

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Chemical interactions between plants in Mediterranean vegetation: The influence of selected plant extracts on *Aegilops geniculata* metabolome

Monica Scognamiglio [a,*], Vittorio Fiumano [a], Brigida D'Abrosca [a], Assunta Esposito [a], Young Hae Choi [b], Robert Verpoorte [b], Antonio Fiorentino [a]

[a] Department of Environmental Biological and Pharmaceutical Sciences and Technologies, Second University of Naples, via Vivaldi 43, I-81100 Caserta, Italy
[b] Natural Products Laboratory, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 25 February 2014
Received in revised form 7 May 2014
Available online 26 July 2014

Keywords:
Allelochemicals
Flavonoids
Hydroxycinnamate derivatives
NMR-based metabolomics
Oxidative stress

ABSTRACT

Allelopathy is the chemical mediated communication among plants. While on one hand there is growing interest in the field, on the other hand it is still debated as doubts exist at different levels. A number of compounds have been reported for their ability to influence plant growth, but the existence of this phenomenon in the field has rarely been demonstrated. Furthermore, only few studies have reported the uptake and the effects at molecular level of the allelochemicals.

Allelopathy has been reported on some plants of Mediterranean vegetation and could contribute to structuring this ecosystem. Sixteen plants of Mediterranean vegetation have been selected and studied by an NMR-based metabolomics approach. The extracts of these donor plants have been characterized in terms of chemical composition and the effects on a selected receiving plant, *Aegilops geniculata*, have been studied both at the morphological and at the metabolic level. Most of the plant extracts employed in this study were found to have an activity, which could be correlated with the presence of flavonoids and hydroxycinnamate derivatives. These plant extracts affected the receiving plant in different ways, with different rates of growth inhibition at morphological level.

The results of metabolomic analysis of treated plants suggested the induction of oxidative stress in all the receiving plants treated with active donor plant extracts, although differences were observed among the responses. Finally, the uptake and transport into receiving plant leaves of different metabolites present in the extracts added to the culture medium were observed.

1. Introduction

Chemical interaction among plants is the basic concept of allelopathy, defined as the beneficial or harmful influence of chemical substances mainly produced from the secondary metabolism of plants, microorganisms and fungi that affect the growth and development of nearby plants and microorganisms, and interfere with the development and maintenance of agricultural and natural ecosystems (De Albuquerque et al., 2011). After the glorious birth of this concept, it has been and still is highly debated, and is receiving renewed attention in recent years (Cipollini et al., 2008; Weir et al., 2004).

The understanding of this complex phenomenon can shed light over several important questions: from the ecological points of view, to understand how plants can interact by chemicals to determine the structure of plant communities (Bais et al., 2003; Chou, 1999) and to change the successional processes of vegetation dynamics (Fernandez et al., 2006); or how alien plants can invade existing ecosystems (Callaway and Ridenour, 2004; Callaway and Walker,1997). Furthermore, the identification of allelochemicals can suggest roles for many secondary metabolites which have no clear assigned role up to now, while also furnishing the basis for the development of environmentally friendly herbicides (Macías et al., 2007). Last, but not least, the study of the molecular mechanisms involved in the biosynthesis and in the effects of allelochemicals can open new horizons in plant molecular biology.

An allelopathic interaction implies a donor and a receiving plant. The donor is plant which produces and releases the allelochemical while the receiving one is the plant sensitive to it.

Allelopathic interactions can be considered elucidated when the following evidence is available: the existence of chemicals (or their mixtures) able to influence other plant performance; the observation that metabolites are actually released into the soil and can somehow reach the receiving plant; the modes of action and the possible molecular mechanisms involved have been established.

* Corresponding author. Tel.: +39 0823 274579; fax: +39 0823 274571.
E-mail address: monica.scognamiglio@unina2.it (M. Scognamiglio).

http://dx.doi.org/10.1016/j.phytochem.2014.07.006
0031-9422/

The isolation and characterization of putative allelochemicals is well documented as the analytical procedures do not differ from that used for other phytochemicals that are identified by bioactivity guided fractionation (Macías et al., 1999; Scognamiglio et al., 2012). However, the molecular mechanisms are rarely known, although the availability of new techniques and approaches offers useful tools to learn more about the interactions (Golisz et al., 2008; Inderjit et al., 2009). On the other hand, the actual activity in field conditions is not easy to determine and will require a further advancement in the available approaches.

Recently, a new approach based on the advances in the omics technologies, has been developed for allelochemical studies (D'Abrosca et al., 2013; Scognamiglio et al., 2014). In this approach, NMR-based metabolomics is applied to analyse donor plants, the extracts of the donor plants are screened for their activity on a test species, and the receiving plant's morphological and metabolic changes are measured. By using multivariate data analysis, plant extract components that correlate with growth inhibition activity can be identified and also synergistic and additive effects can be found. From the full analysis of the receiving plant, clues about the modes of action can be further explored and the fate of the allelochemicals in the receiving plant can be observed. This systems biology approach omits the problems of a reductionist approach based on searching single pure compounds and determining their mode of allelopathic action on other plants, as synergy between compounds may play an important role.

Mediterranean ecosystems offer a good background for allelopathy studies, as chemical interference has been shown to be one of the main important mechanisms regulating plant diversity (Chou, 1999), affecting intra and interspecific plant competition and thus dynamical vegetation processes (Fernandez et al., 2006). Moreover, climatic stress has been shown to increase the production of allelochemicals (Rice, 1984) amplifying the importance of allelopathic interactions under Mediterranean-climate conditions.

In order to study chemical interference occurring among Mediterranean plants, a plant community has been selected and a screening has been carried out with the NMR-based metabolomics approach. On the basis of previous observations, high plant diversity and low presence of alien species have been shown for the study area (Esposito et al., 1999, 2006), thus suggesting a potential role of allelopathy as a mechanism regulating its structural and functional features (Bonanomi et al., 2012; Esposito et al., 2008). Hence, sixteen species belonging to this plant community have been tested for their activity on a coexisting plant, *Aegilops geniculata*. Receiving plants performance in terms of growth has been registered and the metabolome of *A. geniculata* has been analysed in order to detect possible changes after the treatments with plant extracts.

2. Results

2.1. Experimental design

To determine possible effects of plant extracts (Table 1) on a selected receiving plant both donor and receiving plants were subjected to metabolomics analysis (Fig. 1).

For each donor plant three biological replicates were analyzed by a metabolomic based approach in order to define the composition of the plant extract. The same samples were extracted in order to obtain samples useful for the bioassay.

These extracts, obtained in a way analogous as for the extracts analyzed by NMR metabolomics, were used to treat *A. geniculata* (hence three replicates were carried out for each donor plant) as described in the experimental section. Finally, receiving plant samples were analyzed by NMR based metabolomics and multivariate and quantitative data analysis was carried out.

2.2. Donor plant extract composition

Plant extract composition has been defined by comparing NMR data with an in-house library, with databases (Cui et al., 2008) and with some literature data (Lubbe et al., 2013; Verpoorte et al., 2007; Wolfender et al., 2013). For some metabolites, the support of 2D-NMR techniques was needed. ^{1}H-NMR data and extract composition are given in Table 2.

2.3. Morphological analysis of treated plants

Morphological measurements were carried out on leaves and on roots of *A. geniculata* plants treated with extracts. These data were expressed as % variation from control value after normalization. The results (Fig. 2) show that leaves were less affected than roots. However, roots of the receiving plants showed higher variability than leaves. *Petrorhagia saxifraga* and *Lobularia maritima*, caused an inhibition of root length of more than 50% from control. Plants treated with *Carex distachya*, *Pistacia lentiscus*, *Rosmarinus officinalis* and *Cistus* spp. extracts showed even an inhibition of root length of around 100%: from the moment the extracts were added to the nutrient solution, no further root growth was observed.

The data were subjected to HCA. Both variables, root and shoot length, were taken into consideration. From this analysis, four main groups were observed: group I one was made up by controls and plants treated with *P. saxifraga*. The treatment with this plant extract did not significantly affect plant performance. Group II was made up by plants treated with *Phillyrea angustifolia* and *R. officinalis* of which the extracts caused the highest inhibition of both root and leaf growth. Group IV was made up by the treatments with *P. lentiscus*, *C. distachya*, *L. maritima* and *Teucrium chamaedrys*. This group was characterized by an inhibition of root growth of 50% or more, while the inhibition of the leaf growth was less than 50%. The remaining extracts constituted group III which was further split into two subgroups: *Arbutus unedo*, *Bellis sylvestris*, *Myrtus communis* and *Melilotus neapolitana* which inhibited both root and leaf in a similar manner (and always higher than 50%), while for the other plants (*Cistus* spp., *Medicago* spp. and *Teucrium polium*) an inhibition of about 50% was observed for leaves and a more marked inhibition for roots.

2.4. Metabolomic analysis: multivariate data analysis of receiving plants treated with donor plant extracts

The receiving plant metabolome has been previously analyzed in detail (D'Abrosca et al., 2013).

Principal component analysis (PCA) (Fig. 3a) allows the identification of mainly two groups among the treated plants. Most of the plants treated with the extracts are separated from controls along PC1. Controls are clustered in the high-right quadrant together with the plants treated with *P. saxifraga* extracts. The *B. sylvestris* replicates are largely spread through the PCA score plot and it is not possible to attribute this plant to any group. *L. maritima*, *C. distachya*, *Medicago minima* and *T. polium* are separated from controls by PC2 constituting the group of less active extracts, even though one replicate of *M. minima* and *T. polium* is separated from controls also along PC1.

All of the other treatments are separated from controls both along PC1 and PC2.

From the analysis of the loading plots, it was possible to determine which metabolites are responsible for the distribution of plants on the score plot. Along PC1 (Fig. 3b) a clear change of all of the amino acids, and in particular glutamic acid and glutamine,

Table 1
Studied plants.

Species	Family	Herbarium number	Abbreviation
Arbutus unedo L.	Ericaceae	CE000216	AR
Bellis sylvestris Cyr.	Asteraceae	CE000225	BE
Carex distachya Desf.	Cyperaceae	CE000226	CA
Cistus incanus L.	Cistaceae	CE000217	CI
Cistus salviifolius L.	Cistaceae	CE000218	CS
Lobularia maritima Desv.	Brassicaceae	CE000227	LO
Medicago littoralis Rohde	Fabaceae	CE000228	ML
Medicago minima L.	Fabaceae	CE000229	MM
Melilotus neapolitana Ten.	Fabaceae	CE000230	ME
Myrtus communis L.	Myrtaceae	CE000219	MI
Petrorhagia saxifraga L.	Caryophyllaceae	CE000231	PE
Phillyrea angustifolia L.	Oleaceae	CE000220	FI
Pistacia lentiscus L.	Anacardiaceae	CE000221	LE
Rosmarinus officinalis L.	Lamiaceae	CE000222	RO
Teucrium chamaedrys L.	Lamiaceae	CE000223	CH
Teucrium polium L.	Lamiaceae	CE000224	PO

and of organic acids is observed. These metabolites are negatively correlated with PC1, while oblongaroside, *cis*-aconitic acid, betaine and sucrose are positively correlated with this component.

The metabolites varying along PC2 (Fig. 3c) are once again *cis*-aconitic acid and oblongaroside, together with GABA, citric acid, betaine and choline (direct correlation). Along PC2 also a decrease in sugars and glutamic acid and glutamine is observed.

2.5. Metabolomic analysis: HCA of receiving plants treated with donor plant extracts

In order to minimize the effects of natural variation of the extracts obtained by biological replicates, and in order to better define groups among active plants for further analysis, the bucketed NMR data were used for HCA. Instead of the single replicates, mean values for each bucket ($n = 3$) were used for this analysis.

The HCA analysis shows a single group of all the active extracts (group H) (Fig. 4).

P. saxifraga extract is not differentiated from controls, while the effects of *C. distachya* and *L. maritima* extracts (group L) are only slightly different (in accordance with PCA analysis). *T. polium* and *M. minima* extracts are clustered with the most active plants, with these plants further divided into group Ha, comprising the extracts of these two plants, along with *T. chamaedrys*, *M. neapolitana, R. officinalis* and *B. sylvestris,* and group Hb, constituted by *P. angustifolia*, *A. unedo*, *P. lentiscus*, *Cistus* spp., *M. communis* and *Medicago littoralis* extracts.

2.6. Quantitative analysis of A. geniculata metabolites

Quantitative analysis was carried out on *A. geniculata* extracts (Table 3) derived from the treatments with the most active donor plants. The metabolites to be quantified were chosen based on the results of PCA.

Some metabolites varied in the same way (even though to a different extent) in all the treated plants. Aspartic acid and asparagine were quantified together and they were always increased, while *cis*-aconitic acid was always decreased. Analogously, aromatic compounds increased after almost all the treatments.

The analysis showed an increase of all the amino acids (excluding alanine) in *A. unedo* treated plants. Concerning organic acids, citric and malic acids were increased, while *cis*-aconitic acid was decreased. Also, the betaine level was lowered by the treatments, while, among sugars, glucose was increased whereas sucrose was not affected.

A. geniculata responded in the same way to the treatment with both *Cistus* spp. and with *M. communis* extracts: they caused a response analogous to that of *A. unedo*, but milder, at least for the amino acids. An exception was observed for asparagine, as its increase was more drastic. Among organic acids, only *cis*-aconitic acid was significantly affected, while malic acid was differently affected in the three treatments. The main difference was observed for sugars: glucose was increased, but to a lower extent compared to *A. unedo* extract treated plants and also a decrease of sucrose (around 70%) was observed. The *P. lentiscus* extracts triggered a response similar to that observed with *Cistus* spp. and *M. communis* extracts, but sucrose was slightly affected.

M. littoralis caused a different response with a massive accumulation of asparagine (more than 500%). The responses at the amino acid (excluding asparagine) and organic acid levels were similar to that following the treatment with *A. unedo*, but concerning sugar levels only mild effects were detected on sucrose and no effect was seen for glucose.

The *P. angustifolia* extracts caused a drastic increase of amino acids and sucrose, while, concerning organic acids, an accumulation of citric acid and a marked decrease of malic acid (along with the decrease of *cis*-aconitic acid) was observed.

The *M. minima* extracts had only mild effects, while *M. neapolitana* caused an effect on citric acid cycle intermediates with particularly a significant increase of citric acid. Mild effects were observed for amino acids.

Finally, plants belonging to the Lamiaceae family triggered similar responses, with differences in the intensity of the response: a decrease of *cis*-aconitic acid and an increase of asparagine, other amino acids and sucrose was observed.

2.7. Visual inspection of NMR spectra

The aromatic region of spectra of *A. geniculata* plants treated with donor plant extracts appeared richer in signals if compared to controls (Fig. 5). From the visual inspection, it was possible to determine which metabolites were present.

In the aromatic region tryptophan, tyrosine, phenylalanine, adenine, and *p*-hydroxybenzoic acid were detected.

Tryptophan was identified thanks to a triplet ($J = 7.5$) at δ 7.21, a singlet at δ 7.29 and two coupled doublets ($J = 8.4$) at δ 7.47 and 7.72. Tyrosine was identified through two coupled doublet ($J = 8.4$) at δ 6.85 and 7.19, while a multiplet between 7.33 and 7.52 ppm was attributed to phenylalanine. Adenine signals showed two singlets at δ 8.19 and 8.21. Finally, *p*-hydroxybenzoic acid was identified thanks to two doublets ($J = 8.4$) at δ 7.04 and 7.92.

Some signals, like some doublets ($J = 1.8$) in the region 6.45–6.60 ppm, could not be assigned as they were present in too small amounts. Furthermore, a singlet whose chemical shift varied around 8 ppm, was not attributed to any metabolite (due to the changeable chemical shift, probably it is an acid).

In the extracts of the receiving plant treated with *P. saxifraga* extracts (Fig. 6a), γ-pyrone derivatives were detected (Fig. 6b and c). Two doublets at δ 6.53 and 8.05 ($J = 5.4$) and a methyl singlet at δ 2.46, very likely to be due to dianthoside coming from the donor plant extract.

In the extract of the receiving plant treated with *A. unedo* two doublets ($J = 2.1$) at δ 6.31 and 6.50, diagnostic for an A ring of flavonoids, were observed. Unfortunately, the region where ring B protons resonate overlapped with the signals of aromatic amino acids. However, the presence of a singlet at δ 5.27 and the methyl doublet at δ 0.91 (attributable to the rhamnosyl moiety), in ratio with the aromatic signals, suggested the presence of quercitrin. This metabolite was present in the donor plant extract (Table 2). The uptake of arbutin by the receiving plant was also hypothesized based on the presence of two doublets ($J = 8.4$) at δ 6.80 and 7.02 in

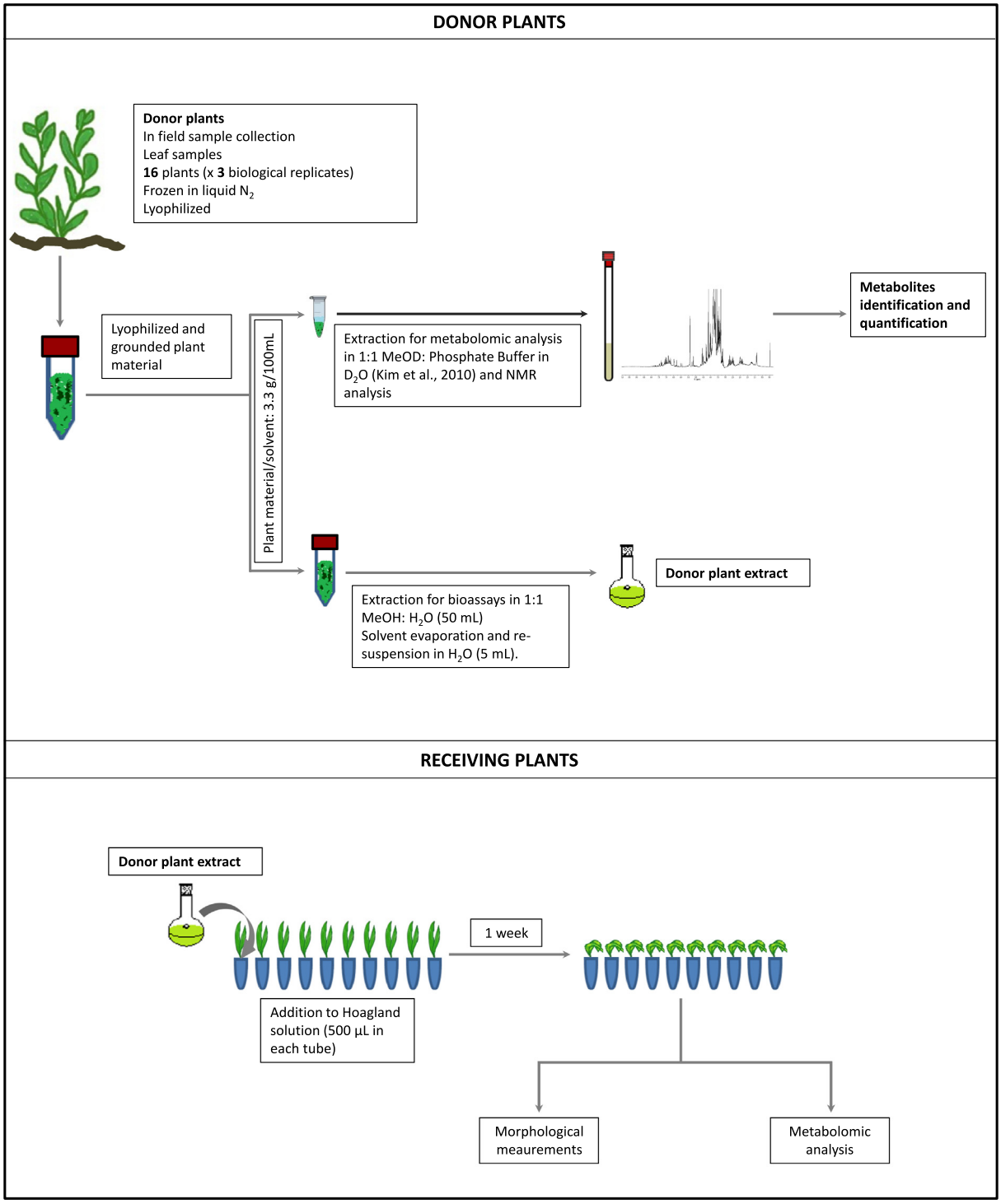

Fig. 1. Method scheme.

A. geniculata extracts obtained from plants treated with *A. unedo* extract.

Based on NMR of the receiving plants, verbascoside and teucrioside uptake was hypothesized for plants treated with *T. chamaedrys* and *T. polium* respectively, because of characteristic signals in the aromatic region of the NMR of the receiving plant extracts. Their presence was also confirmed by the detection of signals attributable to the sugar moieties of these compounds and by comparison with NMR spectra of donor plant extracts.

Gallic acid was detected in receiving plants treated with *M. communis*, *Cistus* spp. and *P. lentiscus*. Plants treated with *P. lentiscus* also showed the presence of shikimic acid, identified thanks to the characteristic olefinic proton. Finally, in *M. neapolitana* treated plants, the uptake and transport to leaf of melilotoside (but not of coumarin) in the receiving plant was observed.

Table 2
Main metabolites detected in donor plant extracts. ^{1}H-NMR data are measured in ppm and coupling constants (J) in Hertz. Relative amount is expressed as the mean value (n = 3) ± SD. For secondary metabolites, also % of each secondary metabolite in the donor plants has been calculated (Supplementary data).

Plant species	Metabolites	NMR	Relative amount
Arbutus unedo	Arbutin	7.02 (H2/H6, d, J = 8.4); 6.80 (H3/H5, d, J = 8.4); 4.80 (H1′, ov)	6.81 ± 1.44
	Catechin	2.52 (H4b, dd); 2.82 (H4a, dd); 5.95 (H6, d, J = 2.1 Hz); 6.03 (H8, d, J = 2.1); 6.94–6.96 (ring B, ov)	3.27 ± 0.60
	Myricitrin	5.45 (H1″, d, J = 1.8); 6.36 (H6, d, J = 2.1); 6.39 (H8, d, J = 2.1); 7.30 (H2′/H6′, s)	1.61 ± 0.67
	Quercitrin	0.91 (H6″, d, J = 6.0); 5.27 (H1″, d, J = 1.8); 6.31 (H6, d, J = 2.1); 6.50 (H8, d, J = 2.1); 7.00 (H5′, d, J = 8.4); 7.32 (H6′, dd, J = 8.7, 2.1); 7.37 (H2′, d, J = 2.1)	7.29 ± 1.52
	Malic acid	2.39 (H3a, dd, J = 15.6, 9.3); 2.78 (H3a, dd, J = 15.6, 3.6); 4.31 (H2, dd, J = 9.3, 3.6)	33.14 ± 5.09
	Quinic acid	1.87 (H2a, m); 1.96 (H6a, m); 2.01 (H2b, m); 2.02 (H6b, m), 3.40 (H4, ov); 4.00 (H3, ov); 4.11 (H5, ov);	25.62 ± 0.50
	Glucose	4.59 (H1 β, d, J = 7.8); 5.19 (H1 α, d, J = 3.8)	17.29 ± 1.86
	Sucrose	4.15 (H5′, d, J = 8.4); 5.38 (H1, d, J = 3.6)	63.50 ± 7.38
Bellis sylvestris	Chlorogenic acid	1.84–2.20 (H2 and H6 quinic acid, m); 5.45 (H5, m); 6.37 (H8′, d, J = 15.9); 6.90 (H5′, d, J = 8.1); 7.07 (H6′, dd, J = 8.4, 2.1); 7.15 (H2′, d, J = 2.1); 7.62 (H7′, d, J = 15.9)	14.25 ± 10.14
	Dicaffeoylquinic acid	6.30 (H8′, d, J = 16.2); 6.48 (H8″, d, J = 15.6); 7.65 (H7′, d, J = 16.2); 7.66 (H7″, d, J = 15.6)	2.00 ± 2.46
	Neochlorogenic acid	6.39 (H8′, d, J = 15.9); 7.52 (H7′, d, J = 15.9)	5.19 ± 2.96
	Besylvosides	0.68 (H24, s); 0.70 (H25, s); 0.90 (H29, s); 0.94 (H28, s); 1.01 (H26, s); 1.14 (H25, s); 5.50 (H12, tl); (5.20–5.50, ov, anomeric protons)	2.38 ± 0.54
	Citric acid	2.59 (H2a, d, J = 17.6); 2.72 (H2b, d, J = 17.6)	30.23 ± 11.91
	Malic acid	*see A. unedo*	45.24 ± 12.67
	Glucose	*see A. unedo*	11.15 ± 10.63
Carex distachya	Pallidol	6.19 (H12/ H12′, d, J = 2.1); 6.64 (H10/H10′, d, J = 2.1); 6.74 (H3/H5/H3′/H5′, d, J = 8.7); 6.96 (H2/H6/H2′/H6′, d, J = 8.7)	1.19 ± 0.12
	Malic acid	*see A. unedo*	49.48 ± 2.88
	Quinic acid	*see A. unedo*	9.25 ± 2.36
	Alanine	1.48 (H3, d J = 7.2)	1.25 ± 0.28
	Glucose	see *A. unedo*	6.15 ± 1.86
	Sucrose	see *A. unedo*	35.96 ± 8.34
Cistus incanus	Flavonoids[a]		3.63 ± 0.60[a]
	Apigenin[a]	6.31 (H6, d, J = 2.1); 6.52 (H8, d, J = 2.1); 6.63 (H3, s); 7.07 (H3′/H5′, d J = 9.0); 7.92 (H2′/H6′, d J = 9.0)	
	Luteolin[a]	6.31 (H6, d, J = 2.1); 6.52 (H8, d, J = 2.1); 6.62 (H3, s); 7.48 (H2′, d J = 2.1); 7.51 (H6′, d J = 8.7, 2.1)	
	Myricetin[a]	6.26 (H6, d, J = 2.1); 6.48 (H8, d, J = 2.1)7.34 (H2′/H6′, s)	
	Quercetin[a]	6.27 (H6, d, J = 2.1); 6.48 (H8, d, J = 2.1); 6.99 (H5′, d J = 8.5); 7.59 (H6′, d J = 8.5, 2.1); 7.75 (H2′, d J = 2.1)	
	Rutin[a]	0.91 (H6‴, d, J = 6.0); 4.50 (H1‴, d, J = 2.1); 4.68 (H1″, d, J = 7.2); 6.27 (H6, d, J = 2.1); 6.48 (H8, d, J = 2.1); 7.58 (H6′, d J = 8.5, 2.1); 7.70 (H2′, d J = 2.1)	
	Gallic acid	7.03 (H2/H6, s)	4.31 ± 0.34
	Galloyl derivative 1	6.76 (H2/H6, s)	4.89 ± 0.61
	Galloyl derivative 2	6.80 (H2/H6, s)	4.51 ± 0.67
	Galloyl derivative 3[b]	6.95 (H2/H6, s)	5.12 ± 0.93 [b]
	Galloyl derivative 4[b]	6.96 (H2/H6, s)	
	Quinic acid	*see A. unedo*	12.50 ± 1.47
	Shikimic acid	2.19 (H6a, dd, J = 18.0, 5.1); 2.74 (H6b, dd, J = 18.0, 5.1); 3.99 (H5, m); 4.40 (H3, dd, J = 4.2, 4.2); 6.71 (H2, m)	33.08 ± 1.80
	Alanine	*see C. distachya*	1.19 ± 0.15
	Glucose	*see A. unedo*	9.89 ± 1.15
	Sucrose	*see A. unedo*	10.19 ± 1.97
Cistus salviifolius	Flavonoids [a]	*see C. incanus*	3.84 ± 0.73[a]
	Apigenin [a]	*see C. incanus*	
	Luteolin [a]	*see C. incanus*	
	Myricetin [a]	*see C. incanus*	
	Quercetin [a]	*see C. incanus*	
	Rutin [a]	*see C. incanus*	
	Gallic acid	*see C. incanus*	3.99 ± 0.70
	Galloyl derivative 1	*see C. incanus*	4.53 ± 0.50
	Galloyl derivative 2	*see C. incanus*	4.19 ± 0.53
	Galloyl derivative 3[b]	*see C. incanus*	4.85 ± 0.30 [b]
	Galloyl derivative 4[b]	*see C. incanus*	
	Quinic acid	*see A. unedo*	9.76 ± 1.46
	Shikimic acid	*see C. incanus*	26.04 ± 7.58

(*continued on next page*)

Table 2 (*continued*)

Plant species	Metabolites	NMR	Relative amount
	Alanine	*see C. distachya*	1.22 ± 0.38
	Glucose	*see A. unedo*	8.94 ± 1.77
	Sucrose	*see A. unedo*	9.34 ± 1.96
Lobularia maritima	Kaempferol	6.35 (H6, d, *J* = 2.1); 6.52 (H8, d, *J* = 2.1); 7.00 (H2′/H6′, d, *J* = 8.4); 8.09 (H3′/H5′, d, *J* = 8.4)	1.77 ± 0.46
	Malic Acid	*see A. unedo*	23.19 ± 2.76
	GABA	1.92 (H3, m); 2.36 (H2, t, *J* = 7.5); 3.01 (H4, t, *J* = 7.5)	11.64 ± 2.77
	Alanine	*see C. distachya*	2.41 ± 0.61
	Glutamic Acid	2.01 (H4, m); 2.11 (H3, m)	18.30 ± 3.00
	Glutamine	2.21 (H3, m); 2.43 (H4, m)	13.32 ± 1.95
	Threonine	1.32 (H4, d, *J* = 6.6)	4.48 ± 0.70
	Valine	1.05 (H4, d, *J* = 6.9)	1.46 ± 0.28
	Glucose	*see A. unedo*	14.32 ± 1.93
	Sucrose	*see A. unedo*	34.80 ± 3.42
Medicago littoralis	Apigenin[c]	*see C. incanus*	
	Catechin[c]	*see A. unedo*	
	Kaempferol[c]	*see L. maritima*	
	Coumestrol [c]	6.90 (H4, d, *J* = 2.1); 7.00 (H2, dd, *J* = 8.1, 2.1); 7.03 (H8, d, *J* = dd, *J* = 8.4, 2.1); 7.16 (H10, d, *J* = 2.1); 7.92 (H1, d, *J* = 8.1), 8.11 (H7, d, *J* = 8.4)	
	Citric Acid	*see L. maritima*	27.83 ± 8.60
	Malic Acid	*see A. unedo*	30.68 ± 5.73
	GABA	*see L. maritima*	15.35 ± 1.96
	Alanine	*see C. distachya*	3.59 ± 0.63
	Asparagine	2.88 (H2a, dd *J* = 17.1, 8.1); 2.96 (H2b, dd *J* = 8.1, 3.9)	14.71 ± 2.72
	Glucose	*see A. unedo*	3.68 ± 0.82
	Sucrose	*see A. unedo*	4.81 ± 2.99
	Betaine	3.26 (s)	7.08 ± 1.33
	Choline	3.20 (s)	4.95 ± 0.54
Medicago minima	Flavones[a]		2.19 ± 1.22[a]
	Daidzein[a]	6.96 (H3′/H5′, d *J* = 9.0); 6.97 (H8, d *J* = 2.0); 7.04 (H6, dd *J* = 9.0, 2.0); 7.43 (H2′/H6′, d *J* = 9.0); 8.06 (H5, d *J* = 9.0); 8.21 (H2, s)	
	Daidzin[a]	5.24 (H1″, d, *J* = 7.8); 6.96 (H3′/H5′, d *J* = 9.0); 7.28 (H8, d *J* = 2.0); 7.25 (H6, dd *J* = 9.0, 2.0); 7.41 (H2′/H6′, d *J* = 9.0); 8.17 (H5, d *J* = 9.0); 8.29 (H2, s)	
	Genistein[c]	6.31 (H6, d *J* = 2.0), 6.47 (H8, d *J* = 2.0), 7.05 (H3′/H5′, d *J* = 9.0); 7.44 (H2′/H6′, d *J* = 9.0); 8.21 (H2, s)	
	Quercetin-7-*O*-glucoside[c]	5.23 (H1″, d, *J* = 7.8); 6.54 (H6, d, *J* = 2.1); 6.90 (H8, d, *J* = 2.1); 7.06 (H5′, d, *J* = 8.4); 7.66 (H6′, dd, *J* = 8.7, 2.1); 7.83 (H2′, d, *J* = 2.1)	
	Phenylpropanoid	6.42 (H8, d, *J* = 15.9) 7.60 (H7, d, *J* = 15.9)	0.87 ± 0.94
	Malic acid	*see A. unedo*	41.39 ± 8.21
	GABA	*see L. maritima*	12.84 ± 4.26
	Alanine	*see C. distachya*	2.39 ± 0.45
	Asparagine	*see M. littoralis*	15.84 ± 5.48
	Glucose	*see A. unedo*	4.18 ± 1.95
	Sucrose	*see A. unedo*	7.29 ± 1.76
	Choline	*see M. littoralis*	3.81 ± 1.25
Melilotus neapolitana	Coumarin	6.51 (H3, d *J* = 9.6); 8.08 (H4, d *J* = 9.6); 6.89 (H6/H8, m); 7.40 (H5/H7, m)	11.65 ± 2.15
	cis-Melilotoside	5.03 (H1′, d *J* = 7.8);6.06 (H3, d *J* = 12.6); 6.86 (H4, d *J* = 12.6)	2.08 ± 3.50
	trans-Melilotoside	5.01 (H1′, d *J* = 7.5); 6.84(H3, d *J* = 16.2);7.86 (H4, d *J* = 16.2);	2.62 ± 0.40
	Alanine	*see C. distachya*	2.46 ± 0.71
	Malic acid	*see A. unedo*	165.89 ± 42.16
	Glucose	*see A. unedo*	11.27 ± 3.64
	Sucrose	*see A. unedo*	13.25 ± 5.84
Myrtus communis	Gallic acid	*see C. incanus*	7.46 ± 1.70
	Galloyl derivative 5	6.73 (H2/H6, s)	1.79 ± 0.38
	Galloyl derivative 6	7.24 (H2/H6, s)	1.58 ± 0.27
	Myricetin	*see A. unedo*	2.93 ± 0.52
	Quinic acid	*see A. unedo*	53.52 ± 31.82
	Glucose	*see A. unedo*	12.36 ± 4.50
	Sucrose	*see A. unedo*	27.20 ± 1.70

Petrorhagia saxifraga	Maltol	2.34 (H7, s); 6.47 (H6, d, *J* = 5.4); 8.00 (H5, d, *J* = 5.4)	5.92 ± 4.38
	Dianthoside	2.46 (H7, s); 6.53 (H6, d, *J* = 5.4); 8.05(H5, d, *J* = 5.4)	6.40 ± 2.78
	Glucose	*see A. unedo*	1.73 ± 1.01
	Sucrose	*see A. unedo*	10.78 ± 2.68
Phillyrea angustifolia	DHPEA	1.94 (H10, d *J* = 7.2); 2.77 (H7′, ov); 6.58 (H6′, dd *J* = 8.4, 2.1); 6.70 (H2′, d *J* = 2.1); 6.79 (H5′, ov); 9.09 (H1, s)	6.20 ± 3.44
	Ligstroside[c]	1.50 (H10, d); 5.88 (H1, s)	
	Oleuropein	1.57 (H10, q *J* = 7.2); 2.78 (H7′, ov); 5.85 (H1, s); 6.06 (H8, q *J* = 7.2); 6.64 (H6′, dd *J* = 8.4, 2.1); 6.75 (H2′, d *J* = 2.1); 6.79 (H5′, d *J* = 8.4); 7.54 (H3, s)	11.16 ± 4.63
	Verbascoside	1.05 (H6‴, d, *J* = 6.3); 2.83 (H7′, t, *J* = 6.9); 4.48 (H1″, d, *J* = 7.8), 5.34 (H1‴, d, *J* = 1.2); 6.36 (H8, d, *J* = 15.9); 6.68 (H6′, dd, *J* = 7.8, 2.1); 6.77 (H5, d, *J* = 8.1); 6.79 (H2′, d, *J* = 2.1); 6.80 (H5′, d, *J* = 7.8); 7.08 (H6, dd, *J* = 8.1, 1.8); 7.16 (H2, d, *J* = 1.8); 7.66 (H7, d, *J* = 15.9)	6.56 ± 2.44
	Malic acid	*see A. unedo*	41.04 ± 6.76
	Glucose	*see A. unedo*	13.97 ± 5.06
Pistacia lentiscus	Galloyl derivative 7	7.14 (H2/H6, s)	6.67 ± 3.90
	Galloyl derivative 8	7.18 (H2/H6, s)	5.39 ± 2.35
	Galloyl derivative 9	7.22 (H2/H6, s)	8.62 ± 3.75
	Catechin	*see A. unedo*	1.01 ± 0.59
	Myricetin	*see A. unedo*	3.12 ± 0.98
	Quinic acid	*see A. unedo*	30.18 ± 2.98
	Shikimic acid	*see C. incanus*	64.67 ± 26.80
	Glucose	*see A. unedo*	17.21 ± 1.77
	Sucrose	*see A. unedo*	14.14 ± 4.00
Rosmarinus officinalis	Rosmanol	0.84 (H18/H19, s); 1.18 (H16/H17, d, *J* = 6.0)	5.44 ± 1.27
	Rosmarinic Acid	3.00 (H7′a, dd, *J* = 14.1, 9.6); 3.15 (H7′b, dd, *J* = 14.1, 3.6); 6.30 (H8, d, *J* = 15.9); 6.71 (H6′, dd, *J* = 7.8, 2.1); 6.81 (H5′, d, *J* = 7.8); 6.82 (H5, d, *J* = 8.1); 6.89 (H2′, d, *J* = 2.1); 7.00 (H6, dd, *J* = 8.1, 1.8); 7.11 (H2, d, *J* = 1.8); 7.50 (H7, d, *J* = 15.9)	26.34 ± 2.17
	Glucose	*see A. unedo*	13.37 ± 0.44
	Sucrose	*see A. unedo*	18.50 ± 3.70
Teucrium chamaedrys	Verbascoside	*see P. angustifolia*	43.00 ± 7.08
	Clerodanes[d]	0.93 (H17, d, *J* = 6.9)[d]; 0.96 (H17, d, *J* = 6.9)[d]; 1.14 (H17, d, *J* = 6.9)[d]; 7.58 (H16, m); 7.45 (H15, m); 6.48 (H14, m); 5.42, (H12, t, *J* = 3.9)	5.34 ± 1.07
	Malic acid	*see A. unedo*	14.77 ± 2.91
Teucrium polium	Teucardoside	2.30 (H10, s); 4.56 (H1″, d, *J* = 7.8); 4.90 (H4 d, *J* = 6.6); 5.34 (H1″, d, *J* = 1.5); 5.92 (H6, d, *J* = 1.5); 6.03 (H7, d, *J* = 1.5); 6.44 (H3, d, *J* = 6.6)	3.07 ± 0.91
	Poliumoside	1.06 (H6‴, d, *J* = 6.3); 1.21 (H6⁗, d, *J* = 6.3); 2.83 (H7′, t, *J* = 6.9); 4.48 (H1″, d, *J* = 7.8); 4.64 (H1⁗, d, *J* = 1.2); 5.12 (H1‴, d, *J* = 1.2); 6.36 (H8, d, *J* = 15.9); 6.68 (H6′, dd, *J* = 7.8, 2.1); 6.77 (H5, d, *J* = 8.1); 6.79 (H2′, d, *J* = 2.1); 6.80 (H5′, d, *J* = 7.8); 7.08 (H6, dd, *J* = 8.1, 1.8); 7.16 (H2, d, *J* = 1.8); 7.66 (H7, d, *J* = 15.9)	23.87 ± 5.77
	Clerodanes[d]	0.91 (H17, d, *J* = 6.9)[d]; 0.97 (H17, d, *J* = 6.6)[d]; 1.00 (H17, d, *J* = 6.9)[d]; 1.16 (H17, d, *J* = 6.9)[d]; 7.58 (H16, m); 7.45 (H15, m); 6.48 (H14, m); 5.42, (H12, t, *J* = 3.9)	4.18 ± 1.68
	Malic acid	*see A. unedo*	46.40 ± 7.25

Signal multiplicity indicated as: d = doublet, dd = doublet of doublets, m = multiplet, ov = overlapped, q = quartet, s = singlet, t = triplet.

[a] Due to many overlapping signals, the amount was calculated for total flavonoids based on ring A protons. NMR data are reported for each flavonoid.

[b] Signals are partially overlapped, hence the total amount of both is given.

[c] Signal to noise ratio too low to allow quantification.

[d] Slightly different compounds, quantified as a whole based on the common signals attributable to the furan ring.

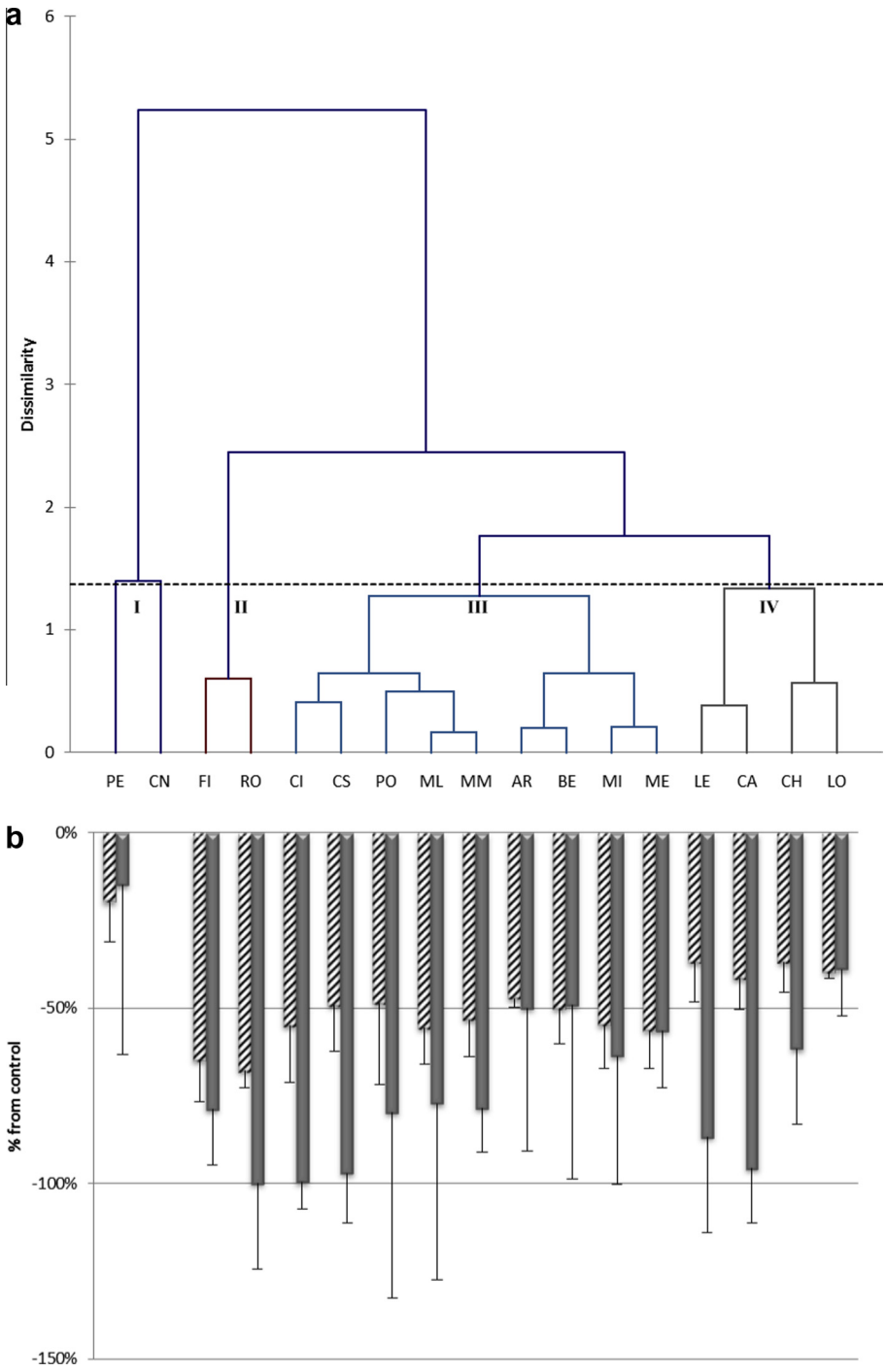

Fig. 2. Morphological analysis of *A. geniculata* plants treated with plant extracts: (a) HCA dendrogram calculated with Euclidean distance and complete linkage aggregation (variables = root and shoot lengths); (b) inhibition of root (plain grey) and aerial part (stripes) growth as % variation from control (±SD; $n = 3$) on data normalized against 7 days old root and leaf length. Variations were significant according to *t*-test ($P \leqslant 0.05$).

3. Discussion

Already a number of plant extracts have been screened for their activity as plant growth regulator (Farooq et al., 2011; Imatomi et al., 2013; Jafariehyazdi and Javidfar 2011; Omezzine et al., 2014; Seal et al., 2010; Silva et al., 2012), but few information is available on the active compounds and on the molecular mechanisms involved.

The experimental design proposed in the present study (Fig. 1) encompasses both the determination of the effects of plant extracts on the growth and the metabolome of the receiving plant and the chemical characterization of the plant extracts.

Primary metabolites present in the extracts have been taken into account (Table 2): although they do not act as allelopathic agents, they can modify the activity of secondary metabolites (Inderjit and Duke, 2003). Some primary metabolites have been identified in plant root exudates (Inderjit and Duke, 2003). Furthermore, it has been demonstrated that sugars can act as signaling molecules (Aranjuelo et al., 2011).

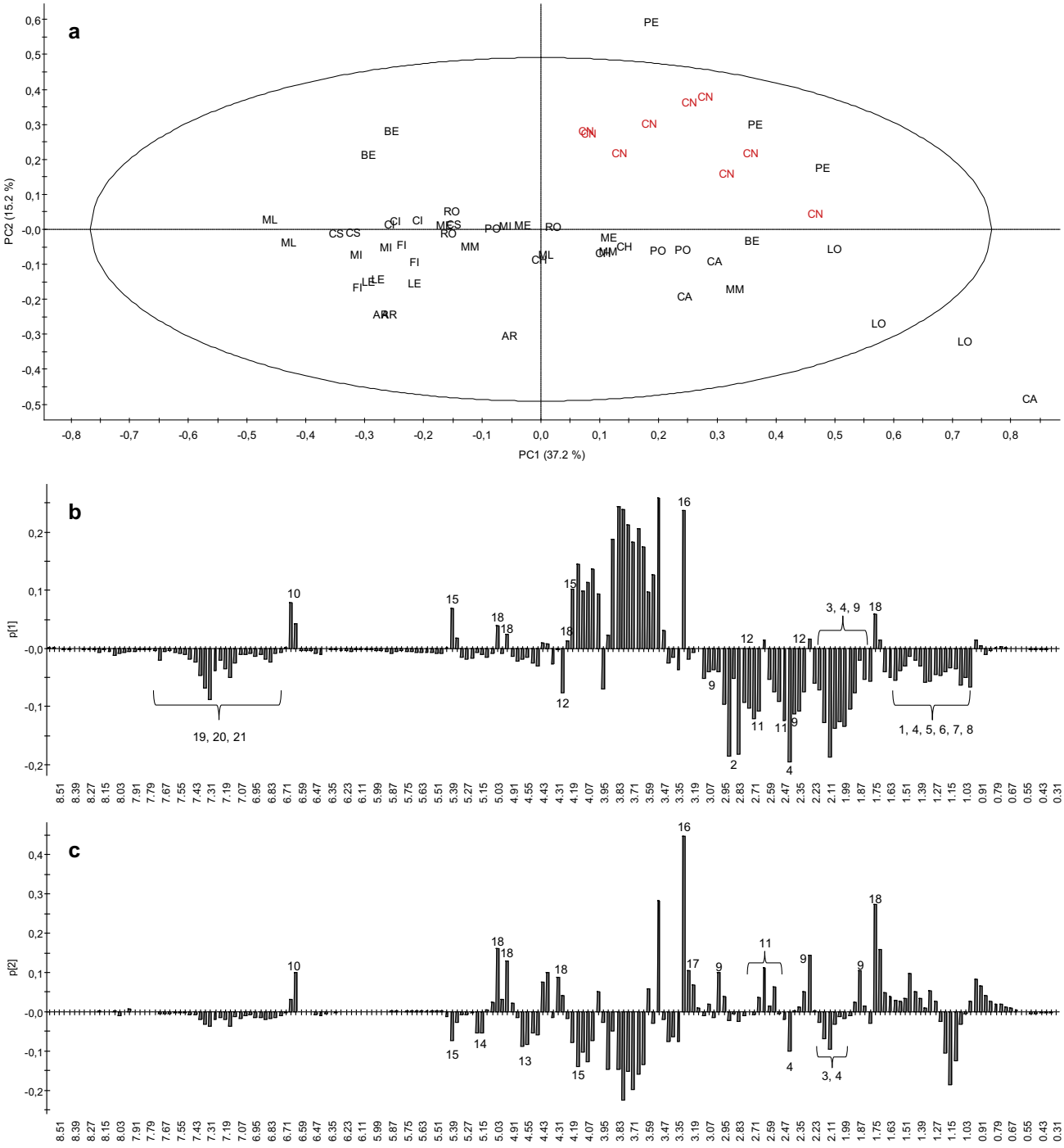

Fig. 3. Principal component analysis of ^{1}H-NMR data of treated receiving plants: score scatter plot of PC1 versus PC2 (a), PC1 loading column plot (b), PC2 loading column plot (c). The ellipse represents the Hotelling T2 with 95% confidence in score plots. The metabolites important for separation have been identified in the loading plots by the following numbers: 1 Alanine; 2 Asparagine; 3 Glutamic Acid; 4 Glutamine; 5 Isoleucine; 6 Leucine; 7 Threonine; 8 Valine; 9 GABA; 10 *cis*-Aconitic Acid; 11 Citric Acid; 12 Malic Acid; 13 α-glucose; 14 β-glucose; 15 Sucrose; 16 Betaine; 17 Choline; 18 Oblongaroside A; 19 Phenylalanine; 20 Tyrosine; 21 Tryptophan.

3.1. Receiving plant growth and metabolome perturbations: correlations with donor plant metabolic profiles

The addition of plant extracts to the culture medium always caused perturbations at morphological level on *A. geniculata* (Fig. 2), with the exception of *P. saxifraga* extracts, whose lack of activity was evidenced also at the metabolic level of the receiving plant (Figs. 3 and 4).

The final concentration of each metabolite during the tests was the same as the concentration of metabolites analyzed by NMR (Table 2), as assured by the experimental design (Fig. 1). The concentrations of extracts used in the bioassays are in the range of that usually reported for phytotoxicity assessment of plant extracts (Farooq et al., 2011; Imatomi et al., 2013; Jafariehyazdi and Javidfar, 2011; Omezzine et al., 2014; Seal et al., 2010; Silva et al., 2012).

Two groups, Ha and Hb, were observed among the active plants (Figs. 2 and 3). The most active plants at metabolic level shared common features concerning their content of secondary metabolites: all of them (with the only exception of *P. angustifolia*) were characterized by the presence of flavonoids (Table 2).

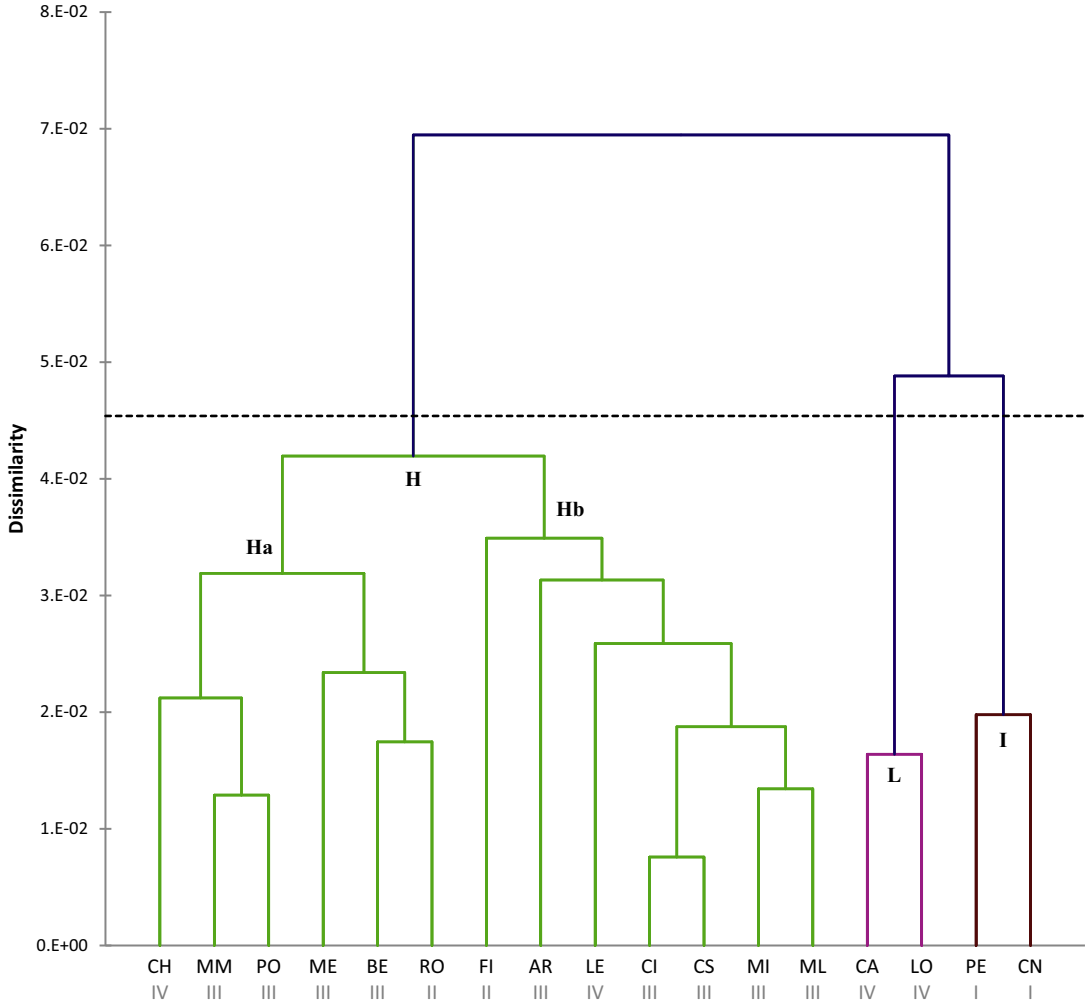

Fig. 4. HCA dendrogram calculated with Euclidean distance and complete linkage aggregation on metabolomics data of receiving plant. The number under the extract name represents the groups evidenced in Fig. 2.

L. maritima, clustered with the other plants, also contained flavonoids, but with very low amount; furthermore, all of the other plants contained flavonoids characterized by a poly-hydroxylated B ring, while this plant was reported to contain only kaempferol derivatives (Table 2). Analogously, *M. minima*, clustered in a different group, contained flavonoids, but belonging to the class of isoflavonoids (the only flavonoid detected was traces of quercetin 7-O-glucoside). Many of the plants belonging to this group also exhibited high quantities of quinic acid.

However, from the analysis of qualitative and quantitative data, it was clear that the responses both at metabolic and at morphological level did not depend on the concentration of a single compound, hence the occurrence of synergistic effects is very probable.

The active plants belonging to group Hb (Fig. 4) showed similar but some slightly different effects on the metabolome of *A. geniculata*: indeed, *Cistus* spp., *P. lentiscus* and *M. communis* caused the same pattern of metabolites that increase or decrease. This could be ascribed to the presence of flavonoids along with a number of galloyl derivatives (not detected in *A. unedo* and *M. littoralis*).

Concerning quinic acid, although it was present almost in all of the metabolomes, activity cannot be linked to its presence. Indeed, *A. unedo*, *P. angustifolia* and *M. littoralis* caused the strongest effects at metabolic level (Figs. 3 and 4; Table 3), but only *A. unedo* extract contained quinic acid (Table 2). Concerning the effects on growth inhibition, the plants belonging to group Hb which contained the highest amount of quinic acid (Table 2) showed the mildest effects (Fig. 2). On the other hand, the amount of quinic acid observed in *Cistus* spp. was comparable to that of *C. distachya* (a mildly active plant).

Concerning the effects on growth, the plants are spread over three different clusters (Fig. 2), but these differences are mainly due to effects at the root level, as the inhibition of leaf growth was comparable for all the treated plants (around 50% inhibition), with the exception of *P. lentiscus*.

The group Ha of active plants, which differently affected the *A. geniculata* metabolome, was characterized by the presence of hydroxycinnamate derivatives (Table 2). Among them, *M. neapolitana* was differentiated for the response observed: while for the other plants belonging to this group the main effect at Krebs cycle intermediates was the decrease of *cis*-aconitic acid, receiving plants treated with *M. neapolitana* extract showed effects also on other organic acids. Although this extract was characterized by the presence of coumarin and coumarin like compounds, a known group of compounds with allelochemical activity, the observed effects were different from those observed for pure coumarin (D'Abrosca et al., 2013).

The other donor plants belonging to group Ha induced mild changes on the receiving plant metabolome (Table 3, Fig. 5).

From the results one might conclude that, although some classes of putative active compounds were identified, the effects

Table 3
Quantitative analysis of the compounds of *A. geniculata* metabolome influenced by plant extract treatments. Each (+) stands for 1–25% increase, (∗) stands for 100% increase, (#) 500% increase; each (-) stands for 1–25% decrease. Differences for control are significant according to *t*-test ($P < 0.05$).

	Citric acid	*cis*-Aconitic acid	Malic acid	Alanine	Asparagine[a]	GABA	Glutamic acid	Glutamine	Threonine	Valine	Glucose	Sucrose	Choline	Betaine	Oblongaroside	Aromatic compounds
Arbutus unedo	+	- - -	+++		∗ ++++	++	∗ ++++	**** ++	∗ ++++	** +	++++		+	- -		**** +
Cistus incanus		- - -			** +++	++	++++	** +++	∗ ++	∗ +	+	- - -	+	-	-	∗ +
Cistus salvifolius		- - -	+		** ++++	++	∗ +	** ++++	++++	∗ +	++	- - -	+	-	-	** +
Medicago littoralis	++	- - - -	++	+	# +++	+++	∗ ++++	*** ++	∗ ++++	∗ ++		-	+	- -		*** +
Myrtus communis		- - -	++		**** ++	++	++++	∗ ++++	+++	++++	++	- -	+	-	-	∗ ++++
Phillyrea angustifolia	+++	- - - -	+	∗ ++++	*** +	++++	** +	*** +	** +	∗ +	-	∗ ++		- -	- -	# ∗ +++
Pistacia lentiscus		- - - -	+	-	** ++	++	∗ +	∗ ++++	++++	++++	++++	-		- -	- -	# +
Medicago minima		- -			∗ ++++				∗ +		∗ +	++		-	-	++++
Melilotus neapolitana	∗ +	- -	+		∗ +		∗ ++	∗ +	∗ +				-	-		# +++
Rosmarinus officinalis		- -			∗ ++++	++	∗ ++	∗ +		++++	+++					# ** ++++
Teucrium chamaedrys		-			∗ ++	++	∗ +	++++		** +	+++	+++				# ** +
Teucrium polium		- -			∗ +	+		+++			+++		+		-	

[a] Might be overlapped with aspartic acid.

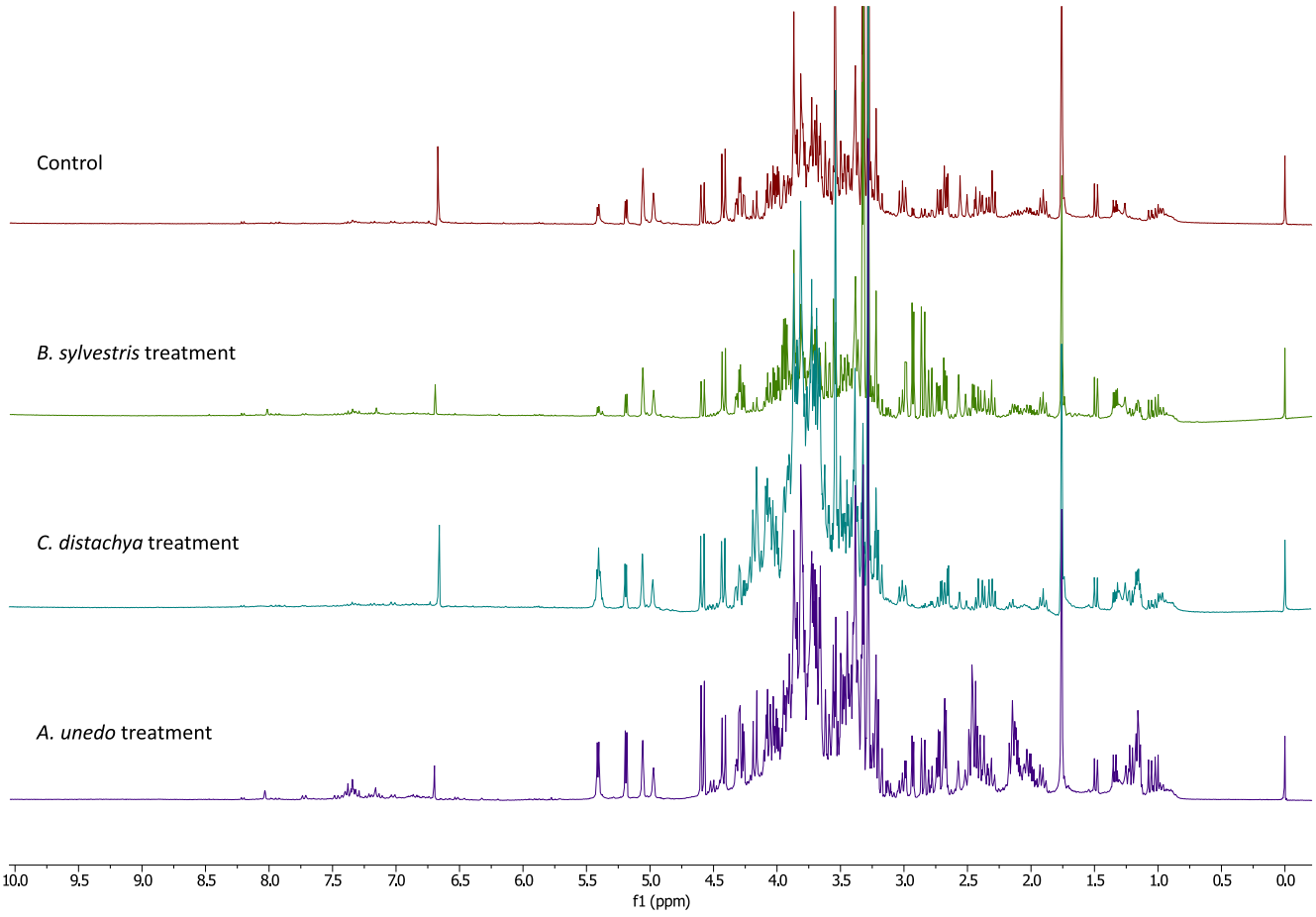

Fig. 5. ^{1}H-NMR spectra of control and treatments representative of groups evidenced in Fig. 4. *B. sylvestris* = group Ha; *A. unedo* = group Hb; *C. distachya* = group L.

observed are not related to a single compound, but rather to mixtures of compounds. For several of the compounds detected allelopathic activities have been reported, whereas other may contribute in e.g. improving the uptake or inhibit catabolism, or any other form of possible synergism.

Although phenolics were the main responsible for the observed activity, in the case of *B. sylvestris*, the contribution of saponins is very likely, as these compounds have already been demonstrated to have phytotoxic potential (Scognamiglio et al., 2012). Further studies will be needed in order to demonstrate the involvement of these compounds or the occurrence of synergistic effects.

3.2. Growth inhibition vs. metabolic changes

There is no direct correlation among the effects at morphological level and the effects at metabolic level.

The apparent incongruence observed between the effects at morphological and metabolic level (Figs. 2–4) (the extracts causing the highest perturbation at metabolic level were not always the ones affecting growth most) might be explained by the fact that the morphological measurements considered only the elongation of root and leaf. These are good parameters, but they do not necessarily correlate to other defense related changes, whereas photosynthesis is still functioning, in absolute amount at a lower level, but relatively at the same level as in bigger plants. Drastic changes at metabolic level are usually the result of an adaptation of the plant to the new condition, a new threat, the carbon fluxes are not channeled to growth but to defense related pathways.

3.3. Receiving plant metabolome: fate of allelochemicals

Changes in the metabolome of the receiving plant can be due to a response to external stimuli, but also due to the uptake of compounds from the extracts of the donor plants. In fact the uptake and translocation to the aerial part was observed for many metabolites, with very different physico-chemical features and with very different molecular weights. It has also to be stressed that some (active) metabolites might not be detected because of catabolism in the receiving plant (Hachinohe et al., 2004) or because of these compounds and their metabolites being under the limit of detection. This can be true for the flavonoids of *Cistus* spp. or of *A. unedo*: their amount in the donor extracts was lower than the amount of quercitrin in *A. geniculata* extract treated with *A. unedo*. The same applies to verbascoside, which uptake was detected in plants treated with *T. chamaedrys* and not in those treated with *P. angustifolia* (containing a much lower concentration of verbascoside).

The observation that many metabolites are transported into leaves raises some doubts of the quantities of the primary metabolites which were measured in *A. geniculata*, some may originate from the extract added to the culture medium. This experimental set up does not allow to exclude their uptake and translocation to leaves. However, for some of them, a decrease instead of an increase was observed in the treated plants and, anyway, their presence in the extracts has been taken under consideration when evaluating the data. Sucrose, for example, was present in high amounts in several donor plant extracts: *A. unedo*, *Cistus* spp., *P. lentiscus*, *M. neapolitana*, *R. officinalis* and also in the mildly active plants *L. maritima* and *C. distachya*, but these plant extracts did

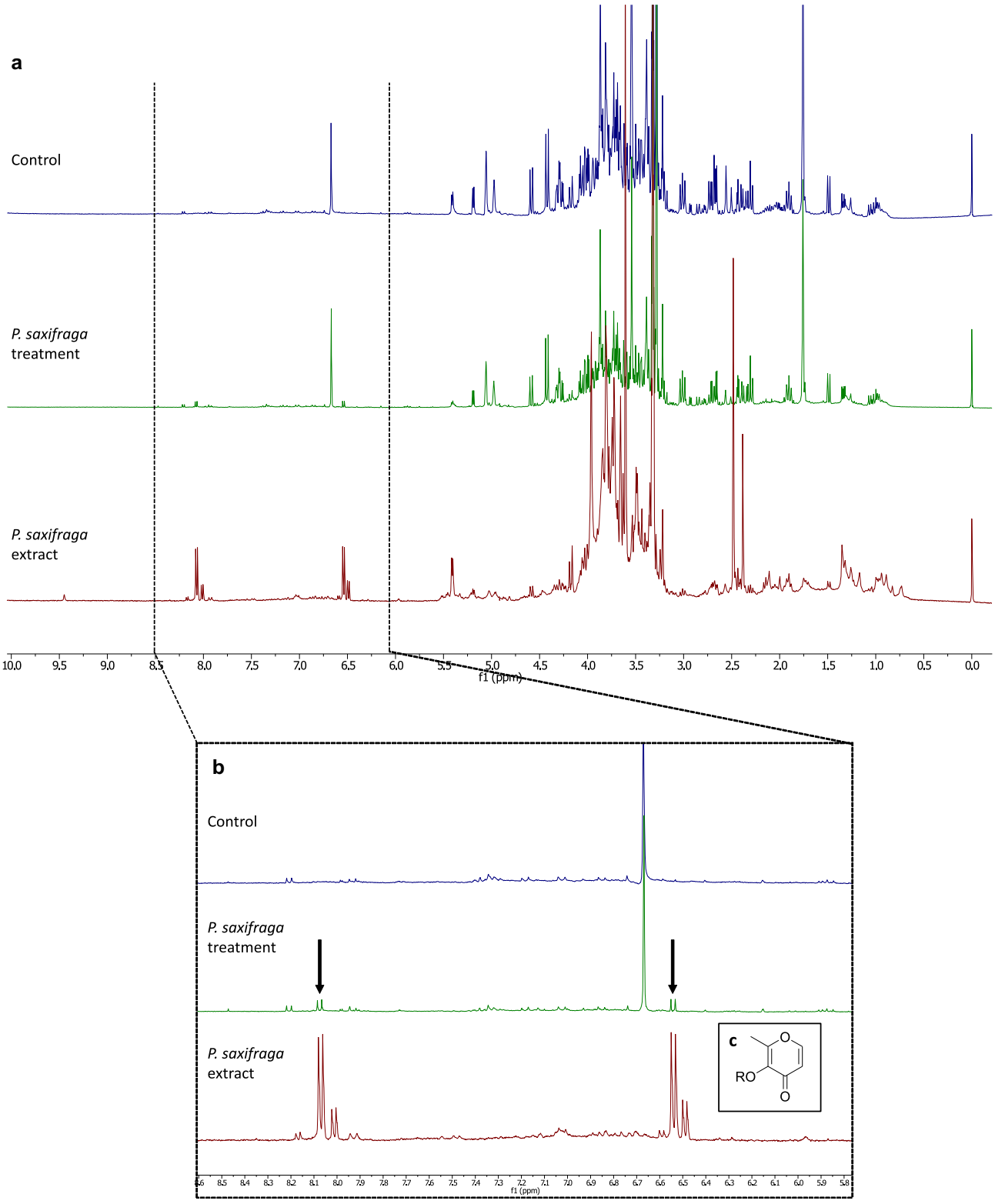

Fig. 6. (a) ^{1}H-NMR spectra of *A. geniculata* (control), *A. geniculata* treated with *P. saxifraga* extract, and *P. saxifraga* extract; (b) expansion of the aromatic region (arrows indicate γ-pyrone derivative signals in *A. geniculata* extract); (c) γ-pyrone derivative structure.

not affect sugar levels in the receiving plants or even caused a lower level of sugars.

Although malic acid was increased upon some treatments with donor plant extracts containing this acid, this increase was not dose-dependent, as malic acid was detected in high amounts in *M. neapolitana* extract, but caused only a slight increase in plants treated with this extract.

It is probable that the receiving plant is able to use these primary metabolites for its own nutrition, while this is not true for some secondary metabolites. However, the primary metabolites

may also contribute to the allelopathic activity by tuning the effect of secondary metabolites. Of course, this hypothesis needs to be further explored.

One of the methodological barriers in allelopathy studies is the lack of techniques to measure the uptake of allelochemicals (Loi et al., 2008; Inderjit and Duke, 2003). Quinate has been reported to be absorbed and translocated to remote organs (Zulet et al., 2013). This uptake might also be species-specific. For example, coumarin has been demonstrated to be absorbed by tomato, but not by ragweed and purslane (Loi et al., 2008). The results of the present study show that the metabolomics approach may help to measure both effect and uptake.

3.4. Receiving plant's metabolomic changes: what they tell us

The action of allelochemicals on receiving plants affects a large number of biochemical reactions at different levels: macromolecules (e.g. DNA, RNA, proteins, polysaccharides, lignin), small molecules, cell, tissues (anatomical and morphological), physiology and ecology (Gniazdowska and Bogatek, 2005). When dealing with plant extracts it is difficult to identify the molecular targets of allelochemicals, as several allelochemicals could be present and they could be acting on different targets, and with the number of molecules present in an extract, synergy cannot be found by a reductionist approach, as separation of an extract will result in loss of activity. Thus rather than taking a reductionist approach, one should take a systemic approach. In such an approach one observes on the one hand metabolic changes in the receiving plants, which may give insight in the possible targets for the allelopathic compounds in extracts of the donor plants. At the same time by fractionation of the extracts or using different extracts from a donor plant one may find compounds that correlate with the intensity of the allelopathic effect of the extracts or fractions. The changes in plant metabolism has been monitored under different stress conditions in many plants and these changes may lead to the identification of the mode of action (Lehmann et al., 2012).

Flavonoid containing extracts caused in *A. geniculata* perturbations in the citric acid (TCA) cycle, as the levels of the intermediates appeared to be changed. The depletion of *cis*-aconitic acid and the accumulation of citric acid might be linked to Radical Oxygen Species (ROS) formation that has been demonstrated to be induced by several flavonoids (Bais et al., 2003). Indeed, *cis*-aconitase is very sensitive to oxidative stress (Baxter et al., 2007; Nunes-Nesi et al., 2013). The increase of GABA is a further sign of TCA cycle depletion, as demonstrated by Araújo et al. (2012), with the GABA shunt operating as an alternative source of mitochondrial succinate. When this process takes place, a depletion of malate is usually observed, along with the decrease of the amino acids linked to the TCA cycle (D'Abrosca et al., 2013; Nunes-Nesi et al., 2013), but this was not observed in this study. The increased accumulation of malate could be due to two different mechanisms: the uptake and translocation from the culture medium; or the activation of different pathways based on the activation of C4 enzymes in C3 plants under stress, as demonstrated by Doubnerová and Ryšlavá (2011). However, the hypothesis that malic acid comes from the culture medium can be rejected based on the observation that in *M. communis* this increase was observed even if malic acid was not detected in the extracts, while in other extracts (e.g. *T. chamaedrys*) higher amounts of malic acid were detected, but no increase was observed in the treated plants.

Concerning the increase in the amino acids linked to the TCA cycle, protein degradation might still go on as reported in the case of juglone induced oxidative stress (Sytykiewicz, 2011). Only in plants treated with *P. angustifolia* extract an increase of sucrose was observed which might be linked with stress (Lehmann et al., 2012). Further studies are needed to explore possible other modes of actions.

The donor plant extracts containing hydroxycinnamate also showed a decrease of *cis*-aconitic acid, possibly as a consequence of oxidative stress (see above), which could mean that phenolic compounds may cause a similar response in the receiving plant. But also here further studies are required to see if a highly specific or a very general molecular mechanism is involved (Fig. 4).

Finally, an increase in aromatic compounds in the receiving plant was caused by all the plant extracts and is only partially explained by the uptake of aromatic metabolites from the growth medium.

The differentiation into two groups of activity (Fig. 4) is probably due to more drastic effects observed at metabolic level for flavonoid containing extracts, but, as can be seen from table 3, each extract triggered different responses.

Due to the highly changed metabolome, it is fair to assume effects at different levels of metabolism. Some allelochemicals act by inhibiting photosynthesis (through interaction with photosystem II) (Weir et al., 2004), while some allelopathic compounds are thought to interact with the mitochondrial membranes. Among these is uncouple oxidative phosphorylation. Also many other mechanisms have been proposed (Zhuo and Yu, 2006). For sure, allelochemicals disrupt the normal metabolic processes, altering plant growth and development, possibly as a consequence of channeling ATP away from growth to other energy demanding processes, such as secondary metabolite production and toxin catabolism as response to the allelochemicals (Gniazdowska and Bogatek, 2005).

In the present study, although different responses were observed for each treatment, oxidative stress seems to be induced by all the donor plant extracts, but further studies are needed in order to elucidate the molecular mechanisms involved. Oxidative stress has been reported as a consequence of the imbalance between the production and the scavenging of ROS, no matter the initial stress (Schutzendubel and Polle, 2002), as part of the defense system. It has been demonstrated that ROS exert various effects on plant defense response at different levels of cell organization and different elicitors have been shown to induce the so-called "oxidative burst" (Zhao et al., 2005). Abiotic stresses have been shown to induce ROS production, with the consequence of damaging biomolecules or activate programs leading to cell death (Baxter et al., 2007) and in some studies also allelochemicals have been demonstrated to trigger this response (Weir et al., 2004).

4. Conclusions

Selected plants of the Mediterranean vegetation were studied for their allelopathic effect on a model plant by an NMR-based metabolomics approach. With the only exception of *P. saxifraga*, all of the tested donor plants caused some effects both at morphological and at molecular level in the model receiving plant.

Although specific responses were observed for each donor plant extract, all the active extracts seem to induce the production of ROS in the receiving plants, which could be part of the common general non-specific stress response, e.g. stress caused by toxins or infections. All plant species have similar signal transduction pathways involved in stress responses, some of these responses are general, like rapid changes in pH and immediate formation of ROS, but differ in the biosynthetic pathways induced as defense. These pathways may lead to terpenoids, alkaloids, phenolics, etc., which are specific for each species. Because plants differ in their capacity to catabolize various toxins, there will be differences in the response to different plant extracts, as also shown in this study.

Unraveling the mechanism of the allelopathic activities has two aspects. One is the identification of the compounds responsible for the allelopathic activity of an extract. This requires a systemic approach in which the allelopathic effect of different extracts from the same donor plant, or fractions of these extracts is determined and subsequently the metabolome of all these samples is measured. Multivariate data analysis can then be applied to identify the compounds that correlate with activity.

Knowing the active compounds is also important for determining the mode of action, which in fact is the major challenge, as it involves many different processes, starting with the uptake (and excretion) and subsequent transport through the plant of the allelochemicals. Different cell types and tissues may have different constitutive or inducible detoxification mechanisms, the toxic effect of allelochemicals and their metabolites may be connected with different target receptors, transporters or enzymes present in specific tissues or cells. That means that both on macro- (e.g. plant part, tissue) and micro-level (e.g. cells) changes must be measured. The integration of transcriptomics, proteomics and metabolomics will be needed on both macro- and micro-scale. Obviously working with pure compounds identified as putative allelochemicals is needed not to further complicate such studies.

Finally, the effects observed with pure compounds, partially purified fractions and extracts could be integrated by multivariate data analysis in order to determine the occurrence of synergistic or additive effects.

The present study shows that apparently the interaction between plants is a very complex system. Metabolomics allowed us to see some general points which can serve as basis for more in depth studies. These points include the correlation of the induction of ROS and high allelopathic activity; each donor plant causes different effects on the receiving plant's metabolome; and phenolic compounds seem to play a role in the allelopathic activity. The experiments used in our studies is complex, a mixture of compounds (an extract) is added to the growth medium of the model plant, in the field there will be many plants excreting different allelochemicals all at the same time affecting the model plant, in an environment were also numerous microorganims are present. No wonder that the allelopathic research has only been developing slowly in the past decades. However, with the advent of very sensitive omics technologies there are now exciting new systemic approaches possible to unravel the allelochemistry. Some of the plants found to be very active in our test system would be quite interesting for such in depth studies.

5. Experimental

5.1. Donor plant analysis

5.1.1. Plant collection

Donor plants were collected at "Castel Volturno" Nature Reserve, (40°57′N, 13°33′E; southern Italy), a flat coastal area with a maximum elevation of 9 m above the sea level, characterized by stabilized dunes of alluvial deposits and loose siliceous-calcareous sand and a typically Mediterranean climate (Rutigliano et al., 2004) with landscape dominated by a Mediterranean *macchia* vegetation of mixed shrub and scattered herbaceous community (Esposito et al., 1999).

Sampling was carried out on selected plants (Table 1) in March or April 2011. Leaf samples (three biological replicates) of each plant were harvested and immediately frozen in liquid N_2 in order to avoid unwanted enzymatic reactions and stored at −80 °C up to the freeze drying process. Once freeze dried, they were powdered in liquid nitrogen and stored at −20 °C. Each sample was extracted and analyzed by ^{1}H NMR.

Voucher specimens for all the plants were deposited at the herbarium of the Second University of Naples (herbarium numbers are reported in Table 1).

5.1.2. Extraction procedure for metabolomic analysis

Freeze-dried and powdered plant material (50 mg) was transferred to a 2 mL microtube. The NMR samples were prepared by mixing the plant sample with 1.5 mL of the NMR solvent consisting of a phosphate buffer (Fluka Chemika, Buchs, Switzerland; 90 mM; pH 6.0) in D_2O (Cambridge Isotope Laboratories, Andover, MA, USA) - containing 0.1% w/w trimethylsilylpropionic-2,2,3,3-*d*4 acid sodium salt (TMSP, Sigma–Aldrich, St. Louis, MO, USA)- and CD_3OD (Sigma–Aldrich, St. Louis, MO, USA) (1:1). The mixture was vortexed at room temperature for 1 min, ultrasonicated (Elma® Transsonic Digital, Hohentwiel, Germanys) for 40 min, and centrifuged (Beckman Allegra™ 64R, F2402H rotor; Beckman Coulter, Fullerton, CA, USA) at 13,000 rpm for 10 min. An aliquot of 0.65 mL was transferred to an 5-mm NMR tube and analyzed by NMR (Kim et al., 2010).

5.1.3. NMR experiments

NMR spectra were recorded at 25 °C on a 300.03 MHz for ^{1}H and 75.45 MHz for ^{13}C on a Varian Mercury Plus 300 Fourier transform NMR. CD_3OD was used as the internal lock.

Each ^{1}H NMR spectrum consisted of 256 scans with the following parameters: 0.16 Hz/point, acquisition time (AQ) = 1.0 s, relaxation delay (RD) = 1.5 s, 90° pulse width (PW) = 13.8 μs. A presaturation sequence was used to suppress the residual H_2O signal. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline-corrected and calibrated to TMSP at 0.0 ppm.

2D-NMR spectra were useful for the identification of metabolites in the extracts. ^{1}H–^{1}H correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra were recorded. COSY spectra were acquired with a 1.0 s relaxation delay and 2514 Hz spectral width in both dimensions. The window function for COSY spectra was sine-bell (SSB = 0). HSQC and HMBC spectra were obtained with a 1.0 s relaxation delay and 3140 Hz spectral width in f2 and 18116 Hz in f1. Qsine (SSB = 2.0) was used for the window function of the HMBC. The optimized coupling constants were 140 Hz for HSQC and 8 Hz for HMBC.

Free induction decays (FIDs) were Fourier transformed and the resulting spectra were manually phased and baseline-corrected and calibrated to TMSP at 0.0 ppm, using ^{1}H NMR processor (ACDLABS 12.0, Toronto, Canada).

5.1.4. Quantitative analysis

The main metabolites identified in donor plant extracts were analyzed by quantitative analysis. ^{1}H-NMR spectra were bucketed, reducing it to integral segments with a width of 0.02 ppm with ACDLABS 12.0 ^{1}H NMR processor (ACDLABS 12.0, Toronto, Canada). Spectra were scaled to the internal standard (whose area, from −0.01 to 0.01 ppm, was set equal to 1). For each metabolite, buckets corresponding to non-overlapping signals were used to calculate the relative amount, as follows:

$$\text{Metabolite relative amount} = \frac{SA \times n_{HTMSP}}{n_s}$$

where SA is the metabolite signal area, but it is also equal to the signal area/standard area ratio, as standard area is equal to 1; n_{HTMSP} is a constant equal to 9 (the number of protons responsible for the signal between −0.01 and 0.01 ppm) and n_s is the number of protons of the metabolite signal area.

5.2. Receiving plant: bioassays and metabolomic analysis

5.2.1. Seed collection

Seeds of *A. geniculata* Roth. (Poaceae) (syn. *Triticum ovatum* L.) were randomly collected in June 2010 at "Castel Volturno" Nature Reserve. Voucher specimens (CE0125) were deposited at the Herbarium of Department of Environmental, Biological and Pharmaceutical Sciences and Technologies of the Second University of Naples. Yellow caryopses of *A. geniculata* were selected according to Onnis et al. (1995) on the basis of their uniformity, by observing them under a binocular microscope Zeiss Stemi 2000 (Oberkochen, Germany), by discarding the undersized and damaged ones.

5.2.2. Plant growth

Seeds were germinated in *Petri* dishes and transferred, after 24 h, to the hydroponic system made up by a tube filled with 5 mL of Hoagland solution (KH_2PO_4 0.50 mM, K_2HPO_4 0.50 mM, K_2SO_4 1.25 mM, $MgSO_4$ 2.05 mM, $CaCl_2$ 1.00 mM, KNO_3 5.00 mM, KCl 25.0 μM, H_3BO_3 12.5 μM, $CuSO_4$ 0.25 μM, $ZnSO_4$ 1.78 μM, Na_2MoO_4 82 nM, $FeCl_3$ 25 μM, Na_2EDTA 28 μM) and with a 3 mm layer of perlite, in order to assure mechanical support. Plants were placed in a growth chamber with controlled temperature and relative humidity (27 °C and 60%, respectively), under photoperiod 16:8 (light:darkness).

5.2.3. Plant extracts for the bioassays

Plant extracts were prepared mixing together 1.67 g of lyophilized plant material and 50 mL of a mixture H_2O/MeOH (1:1). This mixture was extracted by ultrasound assisted extraction for 40 min, and then centrifuged at 13,000 rpm for 10 min. The supernatant was completely removed and the solvent distilled by a rotary evaporator giving crude extracts.

5.2.4. Plant treatments

Seven days after sowing, at the two leaves stage, *A. geniculata* plants were treated with the extracts. The extracts were dissolved in 5 mL of distilled water, and 500 μL of this solution were added to each tube. The Hoagland volume was adjusted to 5 mL. Controls were made up in the same way, adding only 500 μL of distilled water to the tubes.

Hoagland solution was added daily. Analyses were carried out in triplicate, each sample consisted of ten seedlings. The plants were harvested 1 week after treatment and root and shoot lengths were determined as explained below. Plants were immediately frozen, and then lyophilized.

5.2.5. Morphological measurements and metabolomic analysis

The elongation of root and aerial part was measured. As the plantlets were already grown when treatment was carried out, measures of both controls and treated plants were corrected against root and leaf length of 7-days old plants.

Metabolomic analysis was carried out as described above.

5.2.6. Multivariate data analysis

^{1}H-NMR spectra were scaled to total intensity and the region between δ 0.31–9.99 was bucketed, reducing it to integral segments with a width of 0.04 ppm with ACDLABS 12.0 ^{1}H NMR processor (ACDLABS). The regions at δ 4.67–4.91 and 3.31–3.35 were excluded from the analysis because of residual solvents signals. Principal component analysis (PCA) was performed with the SIMCA-P+ software (version 12.0, Umetrics, Umeå Sweden) with scaling based on Pareto method.

Hierarchical cluster analysis (HCA) was performed on the same dataset using XLSTAT plug-in (XLSTAT 2013, Addinsoft, New York, NY, USA) for Microsoft Office Excel 2010 (Microsoft Corporation; Redmond, WA, USA). Instead of the single replicate, mean values for each bucket were used, in order to have a single sample for each plant. The method used was based on Euclidean distance (dissimilarity) with complete linkage as the agglomeration method.

Hierarchical cluster analysis was performed (with the same method) also on morphological measurements by using root and leaf measures.

5.2.7. Quantitative analysis

The *A. geniculata* metabolites were subjected to quantitative analysis. ^{1}H-NMR spectra were bucketed, (bucket width = 0.02 ppm) with ACDLABS 12.0 ^{1}H NMR processor. Spectra were normalized to 1 of internal standard. For each metabolite in each sample, integral values of buckets corresponding to non-overlapping signals were used to calculate the variation of that metabolite from control. Data were reported as mean % variation from control (n = 3).

5.2.8. Statistical analysis

All the analyses were carried out using three biological replicates. Statistical analyses of the quantitative data were performed using Excel 2010 (Microsoft Corporation; Redmond, WA, USA). ANOVA (Analysis of variance) test was used to test the significance of the observed changes ($P < 0.001$). Student's t test was used to determine the significance of differences from controls ($P < 0.05$).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.07.006.

References

Aranjuelo, I., Molero, G., Erice, G., Avice, J.C., Nogué, S., 2011. Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.). J. Exp. Bot. 62, 111–123.

Araújo, W.L., Nunes-Nesi, A., Nikoloski, Z., Sweetlove, L.J., Fernie, A.R., 2012. Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant, Cell Environ. 35, 1–21.

Bais, H.P., Vepachedu, R., Gilroy, S., Callaway, R.M., Vivanco, J.M., 2003. Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Science 301, 1377–1380.

Baxter, C.J., Redestig, H., Schauer, N., Repsilber, D., Patil, K.R., Nielsen, J., Selbig, J., Liu, J., Fernie, A.R., Sweetlove, L., 2007. The metabolic response of heterotrophic *Arabidopsis* cells to oxidative stress. Plant Physiol. 143, 312–325.

Bonanomi, G., Esposito, A., Mazzoleni, S., 2012. Plant-soil feedback in herbaceous species of Mediterranean coastal dunes. Biol. Lett. 49, 53–62.

Callaway, R.M., Ridenour, W.M., 2004. Novel weapons: invasive success and the evolution of increased competitive ability. Front. Ecol. Environ. 2, 436–443.

Callaway, R.M., Walker, L.R., 1997. Competition and facilitation: a synthetic approach to interactions in plant communities. Ecology 78, 1958–1965.

Chou, C.H., 1999. Roles of Allelopathy in plant biodiversity and sustainable agriculture. Crit. Rev. Plant Sci. 18, 609–636.

Cipollini, D., Stevenson, R., Enright, S., Eyles, A., Bonello, P., 2008. Phenolic metabolites in leaves of the invasive shrub, *Lonicera maackii*, and their potential phytotoxic and anti-herbivore effects. J. Chem. Ecol. 34, 144–152.

Cui, Q., Lewis, I.A., Hegeman, A.D., Anderson, M.E., Li, J., Schulte, C.F., Westler, W.M., Eghbalnia, H.R., Sussman, M.R., Markley, J.L., 2008. Metabolite identification via the madison metabolomics consortium database. Nat. Biotechnol. 26, 162–164.

D'Abrosca, B., Scognamiglio, M., Fiumano, V., Esposito, A., Choi, Y.H., Verpoorte, R., Fiorentino, A., 2013. Plant bioassay to assess the effects of allelochemicals on the metabolome of the target species *Aegilops geniculata* by an NMR-based approach. Phytochemistry 93, 27–40.

De Albuquerque, M.B., Dos Santos, R.C., Lima, L.M., Melo Filho, P.A., Nogueira, R.J.M.C., Da Camara, C.A.G., Ramos, A.R., 2011. Allelopathy, an alternative tool to improve cropping systems. A review. Agron. Sustain. Dev. 31, 379–395.

Doubnerová, V., Ryšlavá, H., 2011. What can enzymes of C_4 photosynthesis do for C_3 plants under stress? Plant Sci. 180, 575–583.

Esposito, A., Fiorentino, A., D'Abrosca, B., Izzo, A., Cefarelli, G., Golino, A., Monaco, P., 2008. Potential allelopathic interference of *Melilotus neapolitana* metabolites on three coexisting species of Mediterranean herbaceous plant community. J. Plant. Interact. 3, 199–210.

Esposito, A., Mazzoleni, S., Strumia, S., 1999. Post-fire bryophyte dynamics in Mediterranean vegetation. J. Veg. Sci. 10, 261–268.

Esposito, A., Strumia, S., Caporaso, S., Mazzoleni, S., 2006. The effect of fire intensity on soil seed bank in Mediterranean macchia. Forest. Ecol. Manag. 234, 207–214.

Farooq, M., Habib, M., Atique-Ur-Rehman, Wahid, A., Munir, R., 2011. Employing aqueous allelopathic extracts of sunflower in improving salinity tolerance of rice. Agric. Soc. Sci. 7, 75–80.
Fernandez, C., Lelong, B., Vila, B., Mévy, J.P., Robles, C., Greff, S., Dupouyet, S., Bousquet-Melou, A., 2006. Potential allelopathic effect of *Pinus halepensis* in the secondary succession: an experimental approach. Chemoecology 16, 97–105.
Gniazdowska, A., Bogatek, R., 2005. Allelopathic interactions between plants. Multisite action of allelochemicals. Acta Physiol. Plant. 27, 395–407.
Golisz, A., Sugano, M., Fuji, Y., 2008. Microarray expression profiling of *Arabidopsis thaliana* L. in response to allelochemicals identified in buckwheat. J. Exp. Bot. 59, 3099–3109.
Hachinohe, M., Sunohara, Y., Matsumoto, H., 2004. Absorption, translocation and metabolism of L-DOPA in barnyardgrass and lettuce: their involvement in species-selective phytotoxic action. Plant Growth Reg. 43, 237–243.
Imatomi, M., Novaes, P., Matos, A.P., Gualtieri, S.C.J., Molinillo, J.M.G., Lacret, R., Varela, R.M., Macías, F.A., 2013. Phytotoxic effect of bioactive compounds isolated from *Myrcia tomentosa* (Myrtaceae) leaves. Biochem. Syst. Ecol. 46, 29–35.
Inderjit, Duke, S.O., 2003. Ecophysiological aspects of allelopathy. Planta 217, 529–539.
Inderjit, Dahl, C.C., Baldwin, I.T., 2009. Use of silenced plants in allelopathy bioassays: a novel approach. Planta 229, 569–575.
Jafariehyazdi, E., Javidfar, F., 2011. Comparison of allelopathic effects of some Brassica species in two growth stages on germination and growth of sunflower. Plant Soil. Environ. 57, 52–56.
Kim, H.K., Choi, Y.H., Verpoorte, R., 2010. NMR-based metabolomic analysis of plants. Nat. Protoc. 5, 536–549.
Lehmann, M., Laxa, M., Sweetlove, L.J., Fernie, A.R., Obata, T., 2012. Metabolic recovery of *Arabidopsis thaliana* roots following cessation of oxidative stress. Metabolomics 8, 143–153.
Loi, R.X., Solar, M.C., Weidenhamer, J.D., 2008. Solid-phase microextraction method for in vivo measurement of allelochemical uptake. J. Chem. Ecol. 34, 70–75.
Lubbe, A., Gude, H., Verpoorte, R., Choi, Y.H., 2013. Seasonal accumulation of major alkaloids in organs of pharmaceutical crop Narcissus Carlton. Phytochemistry 88, 43–53.
Macías, F.A., Molinillo, J.M.G., Varela, R.M., Galindo, J.C.G., 2007. Allelopathy – a natural alternative for weed control. Pest Manag. Sci. 63, 327–348.
Macías, F.A., Oliva, R.M., Varela, R.M., Torres, A., Molinillo, J.M.G., 1999. Allelochemicals from sunflowers leaves cv. Peredovick. Phytochemistry 53, 613–621.
Nunes-Nesi, A., Araújo, W.L., Obata, T., Fernie, A.R., 2013. Regulation of the mitochondrial tricarboxylic acid cycle. Curr. Opin. Plant Biol. 16, 335–343.
Omezzine, F., Bouaziz, M., Simmonds, M.S.J., Haouala, R., 2014. Variation in chemical composition and allelopathic potential of mixoploid *Trigonella foenum-graecum* L. with developmental stages. Food Chem. 148, 188–195.
Onnis, A., Bertacchi, A., Lombardi, T., Stefani, A., 1995. Morphology and germination of yellow and brown caryopses of *Aegilops geniculata* Roth (Graminae) population from Italy. Giorn. Bot. Ital. 129, 813–821.
Rice, E.L., 1984. Allelophathy. Academic Press, London, UK.
Rutigliano, F.A., D'Ascoli, R., Virzo De Santo, A., 2004. Soil microbial metabolism and nutrient status in a Mediterranean area as affected by plant cover. Soil Biol. Biochem. 36, 1719–1729.
Schutzendubel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53, 1351–1365.
Scognamiglio, M., D'Abrosca, B., Fiumano, V., Chambery, A., Severino, V., Tsafantakis, N., Pacifico, S., Esposito, A., Fiorentino, A., 2012. Oleanane saponins from *Bellis sylvestris* Cyr. and evaluation of their phytotoxicity on *Aegilops geniculata* Roth. Phytochemistry 84, 125–134.
Scognamiglio, M., D'Abrosca, B., Fiumano, V., Golino, M., Esposito, A., Fiorentino, A., 2014. Seasonal phytochemical changes in *Phillyrea angustifolia* L.: metabolomic analysis and phytotoxicity assessment. Phytochem. Lett. 8, 163–170.
Seal, A., Pratley, J.E., Haig, T., An, M., Wu, H., 2010. Plants with phytotoxic potential: wollemi pine (*Wollemia nobilis*). Agr. Ecosyst. Environ. 135, 52–57.
Silva, P., Piazza, L.A., López, D., López Rivilli, M.J., Turco, M.D., Cantero, J.J., Tourn, M.G., Scopel, A.L., 2012. Phytotoxic activity in *Flourensia campestris* and isolation of (-)-hamanasic acid A as its active principle compound. Phytochemistry 77, 140–148.
Sytykiewicz, H., 2011. Expression patterns of glutathione transferase gene (*GstI*) in maize seedlings under juglone-induced oxidative stress. Int. J. Mol. Sci. 12, 7982–7995.
Verpoorte, R., Choi, Y.H., Kim, H.K., 2007. NMR-based metabolomics at work in phytochemistry. Phytochem. Rev. 6, 3–14.
Weir, T.L., Park, S.W., Vivanco, J.M., 2004. Biochemical and physiological mechanisms mediated by allelochemicals. Curr. Opin. Plant Biol. 7, 472–479.
Wolfender, J.L., Rudaz, S., Choi, Y.H., Kim, H.K., 2013. Plant metabolomics: from holistic data to relevant biomarkers. Curr. Med. Chem. 20, 1056–1090.
Zhao, J., Davis, L.C., Verpoorte, R., 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. 23, 283–333.
Zhuo, Y.H., Yu, J.Q., 2006. Allelochemicals and photosynthesis. In: Reigosa, M.J., Pedrol, N., González, L. (Eds.), Allelopathy: A Physiological Process with Ecological Implications. Kluwer Academic Publishers, Netherlands, pp. 127–139.
Zulet, A., Zabalza, A., Royuela, M., 2013. Phytotoxic and metabolic effects of exogenous quinate on *Pisum sativum* L. J. Plant Growth Regul. 1007, doi: 10/s00344-013-9345-5.