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Defensive role of allelopathic secondary compounds in plants I: testing two independent general hypotheses

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This study tests two general and independent hypotheses with the basic assumption that phytoactive secondary compounds produced by plants evolved primarily as plant defences against competitor plant species. The first hypothesis is that the production and main way of release of phytoactive compounds reflect an adaptive response to climatic conditions. Thus, higher phytoactivity by volatile compounds prevails in plants of hot, dry environments, whereas higher phytoactivity by water-soluble compounds is preponderant in plants from wetter environments. The second hypothesis is that synergy between plant phytoactive compounds is widespread, due to the resulting higher energy efficiency and economy of resources. The first hypothesis was tested on germination and early growth of cucumber treated with either water extracts or volatiles from leaves or vegetative shoot tops of four Mediterranean-type shrubs. The second hypothesis was tested on germination of subterranean clover treated with either water extracts of leaves or vegetative shoot tops of one tree and of three Mediterranean-type shrubs or with each of the three fractions obtained from water extracts. Our data do not support either hypotheses. We found no evidence for higher phytoactivity in volatile compounds released by plants that thrive in hot, dry Mediterranean-type environments. We also found no evidence for the predominance of synergy among the constituents of fractions. To the contrary, we found either antagonism or no interaction of effects among allelopathic compounds.

Introduction

The term allelopathy, coined in the late 1930s by Hans Molisch (Molisch 1937, 2001) to refer to the mutual influence of plants by means of gaseous compounds, may encompass all types of effects, direct or indirect, of plant chemical compounds produced in one plant on other plants or on the same plant.

Contrary to plant competition, in which neighbouring plants use the same environmental resource — be it quantum of light, ion of mineral nutrient, molecule of water or volume of space (Grime 2001) — and also contrary to plant allelomediation, in which a plant interferes with neighbours through a third organism without exhausting or enriching the environment in any way (Szczepanski 1977), allelopathy is a type of interference between plants in which molecules produced by one plant are released to the environment, where they may or may not be transformed, and are taken by another plant of

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the same or a different species, which is affected in some discernible way. Chemicals involved, ways of release, transformation in soil, uptake by neighbouring plants, and physiological modes of action may be highly variable, and reviews are available in Tukey (1969, 1970), Horsley (1977), and Rice (1974, 1984).

Chemicals acting as allelopathic agents, hereafter referred to as allelopathins, are secondary metabolites of plants, in the sense that they have no known role in fundamental processes of organisms producing them, either as final or as intermediate products (Bell 1981). Secondary plant metabolites have been considered to be metabolic waste products (Whittaker & Feeny 1971), involved in self-regulation (Robinson 1974), or as part of a plant's defence against herbivores (Ehrlich & Raven 1964) or competitors (Rice 1984). Although the defensive or protective functions of most, if not all, secondary products are unknown, a reasonable assumption is that they are advantageous to the plant producing them (Bell 1980).

The reality of allelopathy as a natural and meaningful phenomenon has been questioned because, (1) vascular plants rapidly evolve tolerance to herbicides and environmental toxins like metals, and (2) allelopathins would be quickly broken down and inactivated by microbes in soil (Harper 1977). The lack of correspondence between laboratory and field results adds to this criticism, and the significance of bioassays as a means to investigate allelopathy have been questioned (Stowe 1979). Vegetation patterning was attributed to other effects, namely herbivory (Bartholomew 1970, Halligan 1974, Christensen & Muller 1975), and methodological deficiencies may have been involved in some reports (Stowe 1979, Qasem & Hill 1989, Hershey 1996, Romeo 2000, Inderjit &Weston 2000, Inderjit & Callaway 2003, Lau *et al*. 2008). This led to proposals to adapt Koch's postulates to allelopathic studies, especially their emphasis on 'symptoms' (Harper 1977, Ballester & Vieitez 1978, Putnam 1985, Willis 1985, Hale & Orcutt 1987, Williamson 1990, Halbrendt 1996, Weir *et al*. 2004). Thus, proof of allelopathy would require that compounds suspected of being allelopathic were applied under natural field conditions when the plant that produces them is absent or is removed, to make sure that symptoms were still found (Williamson 1990). However allelopathic protocols seldom fully adhere to them (Willis 1985) and, thus, the unequivocal demonstration of ecologically meaningful allelopathic effects is rare (Duke 2010).

If allelopathy is more than an episodic and coincidental phenomenon, secondary chemicals should have been selected for their phytoactivity, thus allowing the formulation of general ecological hypotheses to be tested experimentally. Therefore we set out to test two general hypotheses for the defensive role of allelopathins: the first involved a comparison of the phytoactivity of water-soluble and of volatile compounds, the second involved a comparison of separate and joint effects of naturally-occurring allelopathins.

The rationale for the first hypothesis is that the mechanisms through which secondary compounds are released is an evolved response of plants to climate. Thus in hot, dry climates, highly phytoactive compounds would be easily volatilized, while in wet climates they would be preferentially released by leaching (Whittaker 1970, Muller 1974). Almost all plants produce and release volatile organic compounds, most of which are terpenoids (Went 1970). Increased production and release of volatile allelopathins is to be expected in response to dry environmental conditions and should be detectable in bioassays. If increased phytoactivity is associated with exclusively volatile delivery in dry conditions, and decreased or nil phytoactivity associated with exclusive water-soluble delivery, this would support the hypothesis under test.

Strongly aromatic species thriving in dry environments are especially suited to investigate the hypothesis of an adaptive response of volatile vs. water-soluble phytochemicals. Four highly aromatic Mediterranean-type species producing large amounts of volatiles, and occurring together in dry areas of southern Portugal, were used to compare the activity of their volatile and water-soluble compounds on seed germination and early growth of a standard receiver species.

The rationale for the second hypothesis is that individual plants produce many secondary metabolites, and their allelopathic activity probably results from the combined effects of several different chemical agents—frequently

from more than one chemical class (Einhellig 1989, 1999). Presumably, under natural conditions allelopathic activity requires fine-tuned regulation processes in which compounds act simultaneously, rather than separately or sequentially (Rizvi *et al*. 1992), which implies that interactions can be expected to occur. If exogenous pressures have selected for the bouquet of metabolites produced today, synergy should be widespread because a lesser investment will achieve the same result (Berenbaum 1985, Nelson & Kursar 1999), while antagonism or lack of interactions should be the exception.

Therefore we selected three Mediterranean-type shrubs and one tree, and tested both their complete and fractioned water-extracts on germination of a standard test species to determine if synergy of effects prevailed over antagonism or no interaction, which would support the hypothesis that exogenous pressure selected for the bouquet of metabolites.

The design of the experiments was not intended to ascertain the occurrence and relevance of allelopathy, or its lack, under natural conditions, but only to evaluate the stated hypotheses under our experimental conditions.

Methods

Effects of water-soluble and volatile phytochemicals

Origin of plants used for source of phytochemicals

Plant material was collected in early summer from individuals growing on south-facing slopes of Serra da Arrábida, southern Portugal (approximately 38°28´N, 08°58´W). Cover was dense and accompanying vegetation included typical Mediterranean-type shrubs like *Arbutus unedo*, *Cistus albidus*, *C. crispus*, *Daphne gnidium* and *Quercus coccifera*. The climate is Mediterranean with almost all rainfall occurring during the autumn and winter, and dry hot weather in the late spring and summer. A detailed characterization of the area can be found in Catarino *et al*. (1982).

Plant material

Leaves of *Cistus salvifolius* (*Cistaceae*), *Myrtus communis* (*Myrtaceae*), *Foeniculum vulgare* (*Apiaceae*) and vegetative shoot tops of *Rosmarinus officinalis* (*Lamiaceae*) were harvested, stored in a portable thermal box equipped with frozen ice packs, brought to the laboratory and extracted. Seeds of the receiver species, cucumber (*Cucumis sativus* cv. 'Pepino Inglês Comprido') were purchased at a commercial seed provider (Icarpedome, Lda., Pedome, Portugal).

Plant extractions

Plant extracts were prepared by soaking 32.5 g of intact leaves or shoot tops in 130 ml of distilled water for 70 h at 20 °C under constant light. Extracts were filtered through Whatman no. 1 paper and adjusted with distilled water to a concentration of 0.25 g ml^{-1} (fresh weight:volume).

Bioassays

Two bioassays were conducted. The bioassay for volatile phytochemicals followed the technique described in Moral and Cates (1971), while the bioassay for water extracts followed standard techniques in allelopathic research (Reinhardt *et al*. 1999, Dias *et al*. 2016). For volatile phytochemicals, cylindrical plastic tubes (4.8 cm high, 1.5 cm diameter) were filled with 5 g of intact leaves or shoot tops freshly collected as described above (~6.6 mg cm^{-3}). Two tubes were placed at opposite corners of transparent plastic boxes (11.0 × 11.0 × 6.3 cm), two per treatment, fitted with 5-mm thick sponge covered by Whatman no. 4 paper, sown with 20 cucumber seeds, and wetted with 32.5 ml of distilled water. Bioassays for water soluble phytochemicals did not include the plastic tubes, and 32.5 ml of the appropriate extract was used instead of distilled water. Controls (n = 4) were prepared in the same manner except that distilled water was used. All boxes were sealed and seeds incubated under 25 °C, constant dark. Seeds were considered germinated when the radicle was at least as

long as the length of the seed (Rietveld 1975). After 70 h, germinated seeds were counted, roots and hypocotyls measured to the nearest millimetre, and secondary roots counted.

Statistical analyses

As stated above, increased phytoactivity associated with exclusively volatile delivery under dry conditions has to be found to support the hypothesis under test. Thus either only volatiles are phytoactive or, if volatiles and water extracts are phytoactive, the activity of the former has to be significantly higher than the activity of the latter. Therefore a two-step procedure was adopted. First we compared each treatment with the appropriate control using a two-tailed test and whenever the two treatments were significantly different from their control and phytoactivity of volatiles exceeded that of water extracts, we compared the two using a one-tailed test. Otherwise, phytoactivity by volatiles could never be significantly larger than phytoactivity by water extracts and no statistical test was necessary. Comparisons between treatments and controls or between treatments were made by exact or approximate two- or one-tailed Student's *t* tests after checking for homoscedasticity, at a probability level of $p = 0.05$ using the two-tailed *F* distribution. For means comparisons of germination and growth parameters between treatments and controls, an experiment-wise type I error rate (α_E) of 0.05 was adopted and calculated using the Dunn-Šidák method (Ury 1976). All statistical analyses were done with MS Excel®2010. Data are presented as mean ± SE.

Joint and separate effects of phytochemicals

Origin of plants used for source of phytochemicals

Plant material was collected in mid-spring from plants growing in a relatively dense stand of *Eucalyptus globulus* near Évora, southern Portugal (approximately 38°32´N, 08°01´W). The climate is Mediterranean, with almost all rainfall occurring during autumn and winter, and dry hot weather during late spring and summer.

Plant material

Vegetative shoot tops including the three upper nodes of *Cistus ladanifer* (*Cistaceae*), juvenile leaves of *Eucalyptus globulus* (*Myrtaceae*) and leaves of *Lavandula stoechas* and *Rosmarinus officinalis* (both *Lamiaceae*) were harvested, stored in a portable thermal box equipped with frozen ice packs, brought to the laboratory and extracted. Seeds of the receiver species, subterranean clover cucumber (*Trifolium subterraneum* cv. 'Clare') were provided by Estação Nacional de Melhoramento de Plantas (Elvas, Portugal).

Plant extraction

Intact shoot tops of *C. ladanifer* or intact leaves of *E. globulus*, *L. stoechas* and *R. officinalis* were soaked in 250 ml of distilled water for 18 h at 30 °C in darkness, filtered through Whatman no. 1 paper, and stored at 2 °C. After extraction, plant material was oven dried at 60 °C, weighed, and water extracts were adjusted to a concentration of 20 mg ml^{-1} (dry weight after extraction:volume), hereafter referred to as complete extracts.

For each species, 60 ml of the complete extract was mixed with 10% $NaHCO_3$ to pH 8–9, and extracted 10 times with 30 ml-portions of diethyl ether. The organic layer was washed with distilled water and the solvent evaporated under reduced pressure, giving fraction A. The aqueous fraction was acidified to pH 1 with HCl, and extracted 10 times with 30 ml-portions of diethyl ether. The resulting organic layer was washed with distilled water, and the solvent evaporated under reduced pressure, giving fraction B. The remaining aqueous fraction was evaporated under reduced pressure, giving fraction C. All fractions were re-dissolved in 60 ml of distilled water. All reagents were of analytical grade from E. Merck.

Bioassays

Bioassays followed standard techniques in allel-

opathic research (Reinhardt *et al*. 1999, Dias *et al*. 2016). There were four treatments for each donor species: one for the complete extract and one for each of the three fractions. In each bioassay, four replicated 10-cm glass Petri dishes were fitted with Whatman no. 1 paper, sown with 25 seeds of subterranean clover, and wetted with 5 ml of either the complete extract or one of the fractions. Controls ($n = 16$) were prepared with distilled water. Seeds were incubated under constant dark and a 20 °C/30 °C, 16/8-h temperature cycle, and were considered germinated when the root was at least as long as the length of the seed (Rietveld 1975). Germinated seeds were regularly counted and discarded, and the experiment was finished after a 72-h period without any seed germination.

Determination of germination parameters

The time-course of germination was determined separately for each treatment using the Weibull function (Weibull 1951), a highly flexible and useful equation to describe germination in phytotoxic and allelopathic studies (Dias 2001). The three-term Weibull function can be expressed as:

$$G = 1 - \exp - \{[(T - l)/k]^c\} \qquad (1)$$

where G is the cumulative germination at time T as a proportion of total germination (number of seeds germinated in the end of bioassay), l (lag of germination) is a location parameter that represents the last moment at which germination is strictly zero (Bonner & Dell 1976) and for all practical purposes estimates the time at which the first seed germinates, k (rate of germination) is a scale parameter with $l + k$ estimating the time for approximately 63% of cumulative germination (Bonner & Dell 1976) and c is a shape parameter with Weibull functions of $3.25 \leq c \leq 3.61$ reflecting symmetric distributions and representing a good approximation to normally distributed data, $c < 3.25$ reflecting positive, $c > 3.61$ negative asymmetry (Dubey 1967, Bonner & Dell 1976). Weibull equations were fitted by non-linear least squares without replication using the Marquardt method (Marquardt 1963). Fitted equations were only accepted after a consistency check of parameter estimates against original data.

Statistical analyses

Comparisons between treatments and controls were performed after checking for homoscedasticity at a probability level of $p = 0.05$ using the two-tailed F distribution. When heteroscedasticity occurred, data were transformed using the Box-Cox transformation (Box & Cox 1964). Simultaneous comparisons among all means were done using a least squares linear regression approach with dummy variables to prevent the ambiguity that might result from lack of 'transitivity'. For example, when mean A is not significantly different from mean B, mean B is not significantly different from mean C either, but mean A and C are significantly different (Chew 1976, Penas *et al*. 2002). Forward stepwise selection with replication was used and complete candidate models included only qualitative independent variables, namely the treatment levels (control, complete extract, fractions A, B and C), binary coded as 0, 1. An experiment-wise type I error rate of 0.05 was adopted and calculated using the Dunn-Šidák method (Ury 1976). Coefficients of determination (R^2) are presented as proportions of the maximum R^2 possible (Draper & Smith 1998).

Unequivocal occurrence of mechanism-free interactions of effects was followed by the determination of expected sample-values under the null hypothesis of interaction of effects (*see* Appendix), which were compared with normed-values of complete extract samples by exact or approximate one-tailed Student's t tests after checking for homoscedasticity at a probability level of $p = 0.05$ using the two-tailed F distribution.

All statistical analyses were performed with Statgraphics 4.2 (STSC, Inc., Rockville, MD, USA), except the Box-Cox transformations were done with BIOM (Applied Biostatistics, Inc., New York, NY, USA) and homoscedasticity tests, Student's t-test and lack of fit tests were done with MS Excel®2010. Data are presented as mean ± SE.

Results

Effects of water-soluble and volatile phytochemicals

Cucumber germination ranged from 87.5% ± 2.50% in seeds treated with volatiles from *M. communis* and *R. officinalis*, to 100% in seeds treated with water-soluble extracts of *R. officinalis*. No significant differences were found between treatments and control ($p \geq 0.256$) and the germination mean, pooled across control and treatments was 93.0% ± 1.11%.

Conversely, both water-soluble and volatile compounds of the four aromatic species significantly affected growth (Table 1). Significant differences between treatments and controls were found in root length ($p \leq 0.003$, $\alpha_E = 0.025$), number of secondary roots ($p \leq 0.0003$, $\alpha_E = 0.003$) and hypocotyl length ($p \leq 0.0004$, $\alpha_E = 0.001$). Both water extracts and volatiles affected root length and the number of secondary roots (Fig. 1A and B), while only water extracts affected hypocotyl length (with the exception of *F. vulgare*; Fig. 1C).

Whenever water extracts and volatiles affected subterranean clover growth, the value of inhibition by the former was almost always greater than the corresponding value by the latter. Therefore, by definition, phytoactivity by volatiles cannot be significantly larger than phytoactivity by water extracts and no further testing is needed. However, two exceptions were found, both involving the number of secondary roots, treated with *C. salvifolius* or with *M. communis* (Table 1 and Fig. 1B). One-tailed comparisons revealed no significant differences between water extracts and volatiles, with $t_{74} = 1.122$, $p = 0.133$ in the former donor species, $t_{70} = 0.169$, $p = 0.433$ in the latter.

Hypocotyl length was the more intensely affected growth parameter, with inhibition ranging from 33.2% ± 3.95% to 75.1% ± 5.91%, and a mean inhibition value of 57.9% ± 12.69%. The inhibition of secondary roots was intermediate, ranging from 26.6% ± 3.57% to 78.1% ± 4.55%, and a mean inhibition value of 64.0% ± 5.64%. Root length ranked last in inhibition intensity, which ranged from 67.1% ± 5.89% to 87.4% ± 3.47%, and a mean inhibition value of 78.7% ± 2.69%.

With only one exception, water extracts were always inhibitory. The intensity of inhibition of root length and of number of secondary roots by water extracts was similar or larger than the inhibition by volatiles (mean inhibition values of 66.2% ± 6.15% and 76.5% ± 3.03% respectively) but almost never smaller, while only water extracts inhibited hypocotyl length.

As for donors, *R. officinalis* was clearly the more inhibitory species (mean inhibition 54.6% ± 10.61%), while *C. salvifolius*, *F. vulgare*, and *M. communis* had similar levels of mean inhibition (76.9% ± 1.80%, 74.5% ± 5.32% and 72.1% ± 3.93%, respectively).

Joint and separate effects of phytochemicals

Weibull equations could be fitted to germination

Table 1. Effects of water-solubles and volatiles (mean ± SE, sample size inside parentheses) on growth of cucumber seedlings.

Species	Treatment	Root length (mm)	Number of secondary roots	Hypocotyl length (mm)
	Control	86.3 ± 2.07 (75)	18.9 ± 0.59 (75)	57.0 ± 1.98 (75)
Cistus salvifolius	Water-solubles	67.4 ± 3.01 (38)	14.7 ± 0.86 (38)	42.8 ± 3.37 (38)
	Volatiles	70.6 ± 2.40 (38)	13.4 ± 0.80 (38)	55.2 ± 2.34 (37)
Foeniculum vulgare	Water-solubles	69.8 ± 4.36 (36)	11.5 ± 0.86 (36)	60.6 ± 3.09 (33)
	Volatiles	73.2 ± 3.71 (38)	13.4 ± 0.84 (38)	50.3 ± 3.19 (37)
Myrtus communis	Water-solubles	57.9 ± 5.08 (35)	13.4 ± 1.30 (35)	37.4 ± 4.71 (35)
	Volatiles	75.4 ± 3.00 (37)	13.1 ± 0.50 (37)	60.8 ± 3.50 (37)
Rosmarinus officinalis	Water-solubles	58.0 ± 2.60 (40)	5.0 ± 0.67 (40)	18.9 ± 2.25 (38)
	Volatiles	70.9 ± 2.82 (35)	12.1 ± 0.63 (35)	59.3 ± 2.76 (34)

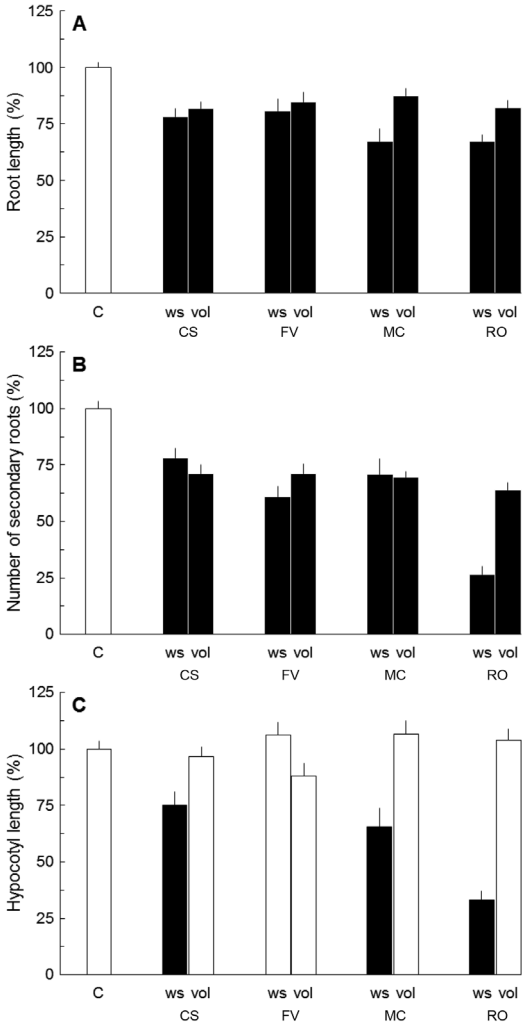

Fig. 1. Effects of water-soluble (ws) and volatile (vol) compounds of *Cistus salvifolius* (CS), *Foeniculum vulgare* (FV), *Myrtus communis* (MC) and *Rosmarinus officinalis* (RO) on (**A)** root length, (**B**) number of secondary roots and (**C**) hypocotyl length of cucumber seedlings expressed in percentage of controls (C) as mean + SE. Treatments with open bars do not differ significantly from controls, treatments with closed bars significantly differ from controls for an experiment-wise error rate $\alpha_E = 0.05$.

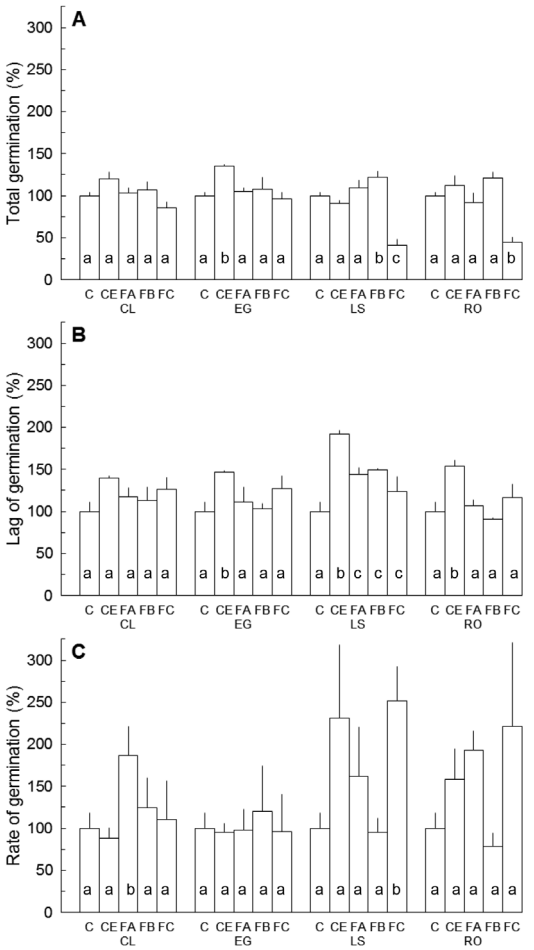

Fig. 2. Effects of complete extracts (CE), and of fractions A, B and C (FA, FB, FC respectively) of *Cistus ladanifer* (CL), *Eucalyptus globulus* (EG), *Lavandula stoechas* (LS) and *Rosmarinus officinalis* (RO) on (**A**) total germination, (**B**) lag of germination and (**C**) rate of germination of subterranean clover seeds in percentage of controls (C) as mean + SE. For each donor species, treatments with the same low case letter are not significantly different for an experiment-wise error rate $\alpha_E = 0.05$.

data, except in 7 samples out of 80. When equations were fitted, R^2 ranged between 0.786 and ~1 with a mean value of 0.970 ± 0.007. Total germination, germination lag and germination rate are summarized in Table 2.

No significant differences were found in the values of shape of germination *c* among treatments of the four donor species ($p \geq 0.142$). Values of *c* ranged from 0.62 ± 0.156 (*C. ladanifer*, fraction C) to 1.78 ± 0.362 (*C. ladanifer*, fraction A) and the mean of *c* pooled across control and treatments was 1.01 ± 0.069.

Conversely, significant differences among treatments were found in total germination (Fig. 2A), lag of germination *l* (Fig. 2B) and rate of germination *k* (Fig. 2C).

Total germination of subterranean clover was significantly affected when seeds were treated

with *E. globulus* (coefficients at $p \leq 10^{-4}$, lack of fit at $p = 0.359$; $R^2 = 0.842$), *L. stoechas* (coefficients at $p \leq 0.017$, lack of fit at $p = 0.280$; $R^2 = 0.957$) or *R. officinalis* (coefficients at $p \leq 10^{-4}$, lack of fit at $p = 0.101$; $R^2 = 0.844$). Synergy, corresponding to outcome 2 (*see* Appendix), occurred in total germination of seeds treated with *E. globulus,* which was only affected by the complete extract (Fig. 2A). Antagonism, corresponding to outcome 4, occurred in seeds treated by *L. stoechas* and *R. officinalis*; in both cases inhibition by one fraction was not found in the complete extract.

Lag of germination was significantly affected by *E. globulus* (coefficients at $p \leq 0.032$, lack of fit at $p = 0.446$; $R^2 = 0.466$), *L. stoechas* (coefficients at $p \leq 0.008$, lack of fit at $p = 0.439$; $R^2 = 0.969$) and *R. officinalis* (coefficients at $p \leq 0.0004$, lack of fit at $p = 0.780$; $R^2 = 0.856$). Synergy, corresponding to outcome 2, occurred in seeds treated by *E. globulus* and *R. officinalis*; the complete extract of both increased the time needed for germination to start (Fig. 2B). No unambiguous conclusion can be drawn for the response of seeds treated with *L. stoechas*.

Rate of germination was significantly affected by *C. ladanifer* (coefficients at $p \leq 0.034$, lack of fit at $p = 0.939$; $R^2 = 0.846$) and *L. stoechas* (coefficients at $p \leq 0.016$, lack of fit at $p = 0.096$; $R^2 = 0.517$). Antagonism, corresponding to outcome 4, occurred in rate of germination of seeds treated by *C. ladanifer* and *L. steochas*; the complete extract of both donor species increased the rate by approximately 63% (Fig. 2C).

Whenever interactions of effects among fractions were unequivocally established, observed values of effects of complete extracts were compared (Table 3), with expected values for the combinations of fraction effects under the null hypothesis for the Bliss Multiplicative Model (E_{BMM}) or the Linear Additive Model (E_{LAM}). In total germination, E_{BMM} and E_{LAM} were always significantly different from the complete extracts. The opposite occurred with lag of germination, in which significant differences were never found, while an intermediate pattern was found in rate of germination with significant differences present in seeds treated with *C. ladanifer* and absent in seeds treated with *L. stoechas*. However because in all cases the null hypothesis for E_{BMM} and E_{LAM} was simultaneously accepted or rejected, no conclusion could be reached on the models that best describe the interactions of observed effects.

Table 2. Effects of complete extracts and fractions (mean ± SE, sample size inside parentheses) on total germination, lag of germination, and rate of germination of subterranean clover seeds.

Species	Treatment	Total germination (%)	Lag of germination (days)	Rate of germination (days)
	Control	58.5 ± 2.58 (16)	1.2 ± 0.13 (14)	0.5 ± 0.10 (14)
Cistus ladanifer	Complete extract	70.5 ± 4.27 (4)	1.6 ± 0.03 (4)	0.5 ± 0.06 (4)
	Fraction A	60.1 ± 3.94 (4)	1.4 ± 0.12 (4)	1.0 ± 0.18 (4)
	Fraction B	62.5 ± 5.85 (4)	1.3 ± 0.18 (4)	0.7 ± 0.18 (4)
	Fraction C	50.0 ± 4.16 (4)	1.5 ± 0.16 (4)	0.6 ± 0.25 (4)
Eucalyptus globulus	Complete extract	79.0 ± 1.00 (4)	1.7 ± 0.02 (4)	0.5 ± 0.05 (4)
	Fraction A	61.6 ± 2.51 (4)	1.3 ± 0.21 (3)	0.5 ± 0.13 (3)
	Fraction B	63.0 ± 8.06 (4)	1.2 ± 0.07 (3)	0.6 ± 0.28 (3)
	Fraction C	56.0 ± 4.90 (4)	1.5 ± 0.17 (3)	0.5 ± 0.23 (3)
Lavandula stoechas	Complete extract	53.3 ± 1.88 (4)	2.2 ± 0.05 (3)	1.2 ± 0.46 (3)
	Fraction A	64.0 ± 4.96 (4)	1.7 ± 0.09 (4)	0.9 ± 0.31 (4)
	Fraction B	71.5 ± 3.93 (4)	1.7 ± 0.02 (4)	0.5 ± 0.09 (4)
	Fraction C	24.0 ± 4.32 (4)	1.4 ± 0.20 (4)	1.3 ± 0.22 (4)
Rosmarinus officinalis	Complete extract	65.6 ± 6.70 (4)	1.8 ± 0.08 (4)	0.8 ± 0.20 (4)
	Fraction A	53.5 ± 6.60 (4)	1.2 ± 0.08 (4)	1.0 ± 0.12 (4)
	Fraction B	70.6 ± 4.35 (4)	1.0 ± 0.02 (4)	0.4 ± 0.08 (4)
	Fraction C	26.0 ± 3.46 (4)	1.3 ± 0.19 (3)	1.2 ± 0.52 (3)

Discussion

Effects of water-soluble and volatile phytochemicals

Phytoactivity of volatile allelopathins in drought-adapted plants was investigated, but the hypothesized increased activity associated with exclusive volatile delivery, and lower activity associated with exclusive water-soluble delivery, was not observed. On the contrary, water extracts inhibited root length and the number of secondary roots as effectively, or more effectively, than volatiles. With one exception, water extracts also inhibited hypocotyl growth, which was not affected by volatiles.

Similar to previous findings on the allelopathic potential of *C. salvifolius*, *F. vulgare*, *M. communis* or *R. officinalis* (Heisey & Delwiche 1983, Dias *et al*. 1995, Qasem 2002, Colvin & Gliessman 2011, 2012, Chen *et al*. 2013), all donor species were phytoactive by water-soluble delivery alone.

Likewise, all tested species were phytoactive by volatile delivery alone, as might be expected from their known richness in volatile compounds (e.g. Demetzos *et al*. 2002 for *C. salvifolius*; Ravid *et al*. 1983, Garcia-Jimenez *et al*. 2000 for *F. vulgare*; Vanhaelen & Vanhaelen-Fastré 1980, Romani *et al*. 1999, Asllani 2000 for *M. communis*; Porte *et al*. 2000, Pintore *et al*. 2002, Serrano *et al*. 2002, Salido *et al*. 2003 for *R. officinalis*). Phytoactivity by volatiles was shown in *R. officinalis* (Qasem 2002, Angelini *et al*. 2003, Chen *et al*. 2013), while absence (Heisey & Delwiche 1983) or contradictory evidence (Azirak & Karaman 2008) of volatile phytoactivity exist for *F. vulgare*. To our knowledge, this is the first report of phytoactivity against vascular plants by volatile compounds of *C. salvifolius* and *M. communis*.

The general higher incidence of phytoactivity by water-soluble compounds found in this study is not completely inconsistent with a preferred release of volatile phytoactive compounds in aromatic plants thriving in dry, hot environments; some volatile compounds are soluble in water either individually (Weidenhamer *et al*. 1993, 1994, Fischer *et al*. 1994, Li *et al*. 1998) or as mixtures (Heisey & Delwiche 1985, Smith 1989, 1990). Volatile compounds could therefore dissolve in water, but also affect germination and early growth of test plants by aerial contact alone (Qasem 1999), vastly extending the model proposed by Muller (1970) to describe the uptake of volatiles by seeds and seedlings.

Water extraction results in solutions consisting of compounds that are easily soluble in water and are essentially absent in volatiles, but some volatile compounds can be present in the water extracts due to their solubility in water. Only volatile compounds would be responsible for phytoactivity in the volatile bioassays, but they could also contribute to the phytoactivity of water extracts. Clearly this was not the case when hypocotyl growth was examined. Volatiles from the four test species, all strongly aromatic, had no effect on hypocotyl growth, but water extracts from three of them inhibited hypocotyl growth with the same or greater intensity than

Table 3. Observed (complete extract) and expected effects (means ± SE in percentage of controls) for the combination of effects of fractions A, B and C under the null hypothesis of interaction of effects for the Bliss Multiplicative Model (E_{BMM}) or for the Linear Additive Model (E_{LAM}). Inside parentheses are significance levels of comparisons between each observed and expected effect by exact or approximate one-tailed Student's *t*-test.

Parameter	Species	Complete extract (%)	E_{BMM} (%)	E_{LAM} (%)
Total germination	*Eucalyptus globulus*	135.1 ± 1.71	108.6 ± 4.80 (3.3×10^{-5})	108.8 ± 4.80 (3.6×10^{-5})
	Lavandula stoechas	91.1 ± 3.22	54.9 ± 2.42 (7.6×10^{-7})	72.7 ± 3.21 (0.007)
	Rosmarinus officinalis	112.1 ± 11.45	49.1 ± 2.17 (0.005)	56.7 ± 2.51 (0.007)
Lag of germination	*Eucalyptus globulus*	146.5 ± 1.61	146.6 ± 15.95 (0.500)	142.1 ± 15.46 (0.389)
	Rosmarinus officinalis	153.9 ± 6.74	113.1 ± 12.32 (0.053)	114.2 ± 12.43 (0.059)
Rate of germination	*Cistus ladanifer*	88.1 ± 12.29	256.6 ± 47.71 (0.002)	221.7 ± 41.23 (0.004)
	Lavandula stoechas	231.3 ± 87.01	386.4 ± 71.86 (0.178)	308.0 ± 57.27 (0.285)

they inhibited root growth and secondary root formation (Fig. 1). In no case were volatiles more phytoactive than water-extracts as they should be to accept (or not reject) the hypothesis of preferential ways of release of allelopathins dependent upon the prevailing dry/wet conditions where plants live.

Joint and separate effects of phytochemicals

Because of the large number of secondary metabolites produced by plants and accepting that evolution selected for lesser expenditure to achieve the same objective, then synergy should be widespread while antagonism or no interaction of effects should be the exception. However, this hypothesis, which derives from the assumed defensive role of allelopathins against neighbouring plants, was not supported by our data.

Water extracts of all donor species investigated in this experiment had compounds active on seed germination, which was expected based on allelopathic investigation of these species (Dias *et al*. 1995, 2004, Dias 2001, Dias & Moreira 2002, Bagavathy & Xavier 2007, Yamagushi *et al*. 2011, Chen *et al*. 2013).

Leaves of Mediterranean-type vegetation are known to produce high amounts of secondary metabolites at a high cost (Margaris 1981, Mooney 1981). As for the species used in this study, more than 370 compounds have been identified in the essential oil or in leaves of *C. ladanifer* (Dias *et al*. 2005), at least 50 compounds in *E. globulus* (Asefa & Dagne 1997, Silvestre *et al*. 1997, Benayache *et al*. 2001, Mandal *et al*. 2001, Viturro *et al*. 2003), about 80 secondary compounds in *L. stoechas* (Granger *et al*. 1973, Kokkalou 1988, Valentini *et al*. 1993) and more than 150 compounds in *R. officinalis* (Porte *et al*. 2000, Pintore *et al*. 2002, Serrano *et al*. 2002, Salido *et al*. 2003).

Although an ecological role for the majority of these compounds is not clearly nor fully established, it is unlikely that biochemical selection acted upon individual compounds alone. To the contrary, and given the high number of secondary metabolites produced by plant species, selection might also have resulted in mixtures of compounds that benefit the plant producing them at the minimum possible cost. Therefore, if plant-plant interactions are a driving force for the production and selection of secondary metabolites, synergy of effects should be expected to be widespread.

According to our data, all species tested possess compounds active against subterranean clover, either when complete water extracts or when fractions were separately tested. *L. stoechas* and *R. officinalis* were the most effective species, affecting total germination, lag, and rate, especially by fractions B and C. *C. ladanifer* showed activity on rate of germination with fraction A, while *E. globulus* affected total germination and lag of germination only with complete water extracts. Also, all parameters of subterranean clover germination were affected, with the exception of shape of germination. Values of shape of germination were low, between 0.62 and 1.78, and are consistent with values found in other studies involving subterranean clover as recipient species (Dias 2001). It also means that the distribution of germination over time is strongly positively asymmetric and thus, in all likelihood factors that govern germination, whichever they are, act multiplicatively (Limpert *et al*. 2001) and are insensitive to differences in chemical composition of treatments.

Whenever active against subterranean clover, either fractions or complete extracts increased lag (l) and rate (k) of germination of subterranean clover. Given the meaning of l and k, this implies that treatments with fractions and complete extracts of donor species increased the time that subterranean clover seeds took to start germinating but reduced the speed at which germination proceeded afterwards.

However, for total germination the effects of fractions and complete extracts were not always the same and, with the exception of fraction C, whenever fractions and complete extracts were active the result was an increase in total germination. It can be hypothesised that the increase of total germination does not necessarily equate with an advantage to the recipient species, for example if coupled with delays in the beginning of germination or reductions in the speed at which germination proceeds thereafter, as happens in seeds treated with *E. globulus*. In addi-

tion, in subterranean clover, significant increases in total germination by water-solubles and volatiles of *C. ladanifer* were followed by a strong inhibition of root growth, in a two-step process that might lead to a faster depletion of seedbanks (Dias & Moreira 2002).

In relation to interaction of effects, there is no clear evidence of prevalence of synergy among the constituents of fractions A, B, and C of *C. ladanifer*, *E. globulus*, *L. stoechas* and *R. officinalis*. Synergy was only found in three cases and never on rate of germination, antagonism was found in four cases and zero interaction in four (eight if the shape of germination is also counted). This prevalence of antagonism or of zero interactions is not entirely new either in plant-plant (Dias & Moreira 2002) or in plant-insect relationships (Diawara *et al*. 1993). Thus, assuming that exogenous pressures by competitors drove the selection for the actual blend of secondary metabolites of plants, our data fail to provide clear evidence for the prevalence of synergy as hypothesised, in spite of its presumed advantage for the efficient use of matter and energy by plants.

Concluding remarks

Using "preponderance of evidence", or its absence, instead of "proof beyond a reasonable doubt", an approach viewed as appropriate for the consideration and evaluation of allelopathy (Romeo 2000), our results agree with published literature and do not support the two hypotheses under test: 1) that volatilization is preferred over water-solubility for the release of highly phytoactive secondary metabolites by plants in hot, dry environments, and 2) that phytoactive secondary compounds produced by plants act synergistically in mixtures.

A possible consequence might be that evolution essentially did not select secondary metabolites for direct defence against other plants but that, in general, secondary compounds of plants might be primarily involved in the self-regulation of plants producing them or, as was recently argued (Zeng 2014), that their role and therefore their selection was essentially aimed not at provoking direct, but indirect effects, on competitors.

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Appendix

Very frequently, and not restricted to allelopathy, investigation of interactions of effects is based on unproven assumptions, implicitly or explicitly stated, about the shape of dose-response curves of compounds under investigation, thus on their mechanisms of action. These assumptions may or may not be correct but usually no evidence on its correctness is provided or possible to guess. A more appropriate approach would be investigating interactions of effects by strictly mechanism-free methods, which in general can be done by determining isoeffective concentrations of single and combined application of compounds followed by the construction of isoboles and plotting the response of mixtures on isobolographs of individual components of the combinations (Berenbaum 1989, Nelson & Kursar 1999).

When isoeffective concentrations are unknown or impossible to determine, as is usually the case, mechanism-free assessment of interactions of effects can still be done and unequivocal decisions can be reached provided that one of the following outcomes happens: (1) the combination is less effective than one or more of its constituents, (2) all constituents are ineffective but the combination is, (3) one of the constituents does not produce the effect of the combination and simultaneously the effect of the combination exceeds the effect of the active constituent, (4) the same but the effect of the combination is less than the effect of the active constituent. Interactions of effects occur in all these outcomes, (1) and (4) reflecting antagonism, (2) and (3) reflecting synergy (Berenbaum 1989).

After the occurrence of interactions of effects has been unequivocally established, expected mean values under the null hypothesis of interaction of effects of components of mixtures can be calculated

for mechanism-dependent Bliss Multiplicative Model (BMM) using the equation (Greco *et al*. 1995)

$$E_{\mathrm{BMM}} = \frac{\prod \bar{Y}_{\mathrm{T}}}{100^{z-1}} \tag{A1}$$

and separately for the mechanism-dependent Linear Additive Model (LAM) using the equation (Berenbaum 1989):

$$E_{\mathrm{LAM}} = 100 - \sum \left(100 - \bar{Y}_{\mathrm{T}}\right) \tag{A2}$$

where E_{BMM} and E_{LAM} are the expected means under the null hypothesis of no interaction of effects for BMM and for LAM, respectively, $\bar{Y}_{\mathrm{T}}$ is the mean effect of each treatment in percentage of control and z is the number of treatments considered in the product. Expected means can be calculated for each model (BMM or LAM) from sample expected values (EV_i) using the following equation:

$$\mathrm{EV}_i = \bar{Y}_{\mathrm{E}} \frac{Y_{\mathrm{C}}}{\bar{Y}_{\mathrm{C}}} \tag{A3}$$

where EV_i are sample normed expected items, $\bar{Y}_{\mathrm{E}}$ is the sample normed expected mean value which is equal to E_{BMM} of Eq. A1 or to E_{LAM} of Eq. A2 under the null hypothesis of interaction of effects for BMM or LAM, respectively, Y_{C} are non-normed values of control and $\bar{Y}_{\mathrm{C}}$ is the non-normed mean of control (Dias & Dias 2007).

Whenever interactions of effects were established according to the four possible outcomes described above, the determination of expected values for mechanism-dependent models like the Bliss Multiplicative Model, the Linear Additive Model or others can be investigated by comparing observed effects of combinations with expected effects under the null hypothesis of interaction of effects provided by mechanism-dependent models (Dias & Dias 2007).