Defensive role of allelopathic secondary compounds in plants: a review of data on two independent general hypotheses

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ABSTRACT

This review examines and whenever appropriate, reanalyses published literature related to two general and independent hypotheses having the underlying assumption that phytoactive secondary compounds produced by plants evolved primarily as plant defences against competitor plant species. The first hypothesis is that production and the main way of release of phytoactive compounds reflect an adaptive response to climate conditions. Thus, higher phytoactivity by volatile-compounds should prevail in plants of hot, dry environments whereas higher phytoactivity by water- solubles should be preponderant in plants from wetter environments. The second hypothesis is that the synergy between phytoactive compounds of plants should be widespread while antagonism or absence of interaction of effects should be rare because of the higher efficiency of energy and use of resources provided by synergy. Published literature does not support either hypotheses. We found no pattern of association between higher phytoactivity in volatile compounds in plants from drier environments or in water-soluble compounds in plants from wetter environments. Neither did we found evidences for the predominance of synergy. On the contrary, antagonism or no interaction of effects among allelopathic compounds largely prevailed.

Keywords: allelopathy, antagonism, interaction of effects, synergy, volatile compounds, water-soluble compounds.

Introduction

In the late 1930s Hans Molisch defined allelopathy as the mutual influence of plants by means of gaseous compounds (Molisch, 1937, 2001) and some sixty years later the newly created International Allelopathy Society provided an all-encompassing definition of allelopathy as any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of agricultural and biological systems (Reigosa *et al.*, 2013); secondary metabolites in the sense that they have no known role in fundamental processes of plants producing them either as final or as intermediate products (Bell, 1981). Molisch was not the first to notice or study this phenomenon. Augustin Pyramus de Candolle noticed that what would later be termed allelopathy might play an important role in soil sickness (de Candolle, 1832; Willis, 1996, 2002). Reviews of reports that might qualify as allelopathy, including works from ancient Greece and Rome can also be found in (Rice, 1984; Willis, 1985).

However, studies on allelopathy did not blossom until the 1950s and 1960s and focused primarily on ecological and plant community work. Rice (Rice, 1974, 1984) described this work thoroughly and investigated the role of allelopathy in old-field successions, the nitrogen cycle and bacterial inhibition. Rice also helped to develop and establish approaches and techniques still in use in allelopathic studies (Rice *et al.*, 1960; Abdul-Wahab & Rice, 1967; Wilson & Rice, 1968; Rice, 1996). In addition to Rice's reviews, a detailed eco-historical account of the research on the role of allelopathy in vegetation patterning in Southern California can be found in Halsey (2004).

As stressed by the authors elsewhere (Pereira *et al.*, 2016) the reality and ecological relevance of allelopathy has been questioned since very early. However it has been argued that allelopathy has been unfairly subjected to a more stringent and heavier burden of proof than other processes of plant-plant interference, like plant resource competition (Williamson, 1990), the latter being frequently misunderstood, overrated (Campbell *et*

al., 1991; Grubb, 1992) or lacking a comprehensive explanation or even a sound scientific basis (Zimdahl, 1993). Despite the criticism, research on allelopathy continued. In 1994 *Allelopathy Journal* was launched and a total of 1081 review papers, research papers or short communications were published by the end of 2016, while in 2015 *Journal of Allelochemical Interactions* was launched with the support of University of Vigo (Spain) and International Allelopathy Society.

New research emerged that frequently did not mention allelopathy but focused on secondary metabolites as putative means of short- or long-distance signalling and communication between plants above ground by volatile compounds (Karban *et al.*, 2000; Dicke & Bruin, 2001; Farmer, 2001; Pieterse *et al.*, 2001; Prieston *et al.*, 2001; Weber, 2002; Ninkovic, 2003; Mescher *et al.*, 2006; Runyon *et al.*, 2006; Pierik *et al.*, 2013; Poelman, 2013) or below ground by volatile or non-volatile compounds (Song *et al.*, 2010; Barto *et al.*, 2011, 2012; Babikova *et al.*, 2013; Pierik *et al.*, 2013; Achatz *et al.*, 2014).

Recently we reported (Pereira *et al.*, 2016) the results of the experimental test of two independent hypotheses derived from the assumption that selection for a defensive role against neighbouring competitors played a major role in the evolution leading to the actual profile of secondary metabolites of plants. The first hypothesis is that the volatile way of release of allelopathic compounds (allelopathins) would largely prevail in plants thriving in hot, dry climates whereas water-soluble allelopathins would predominate in wetter climates. The second hypothesis is that the phytochemical composition resulting from plant secondary metabolism would reflect the higher economy and efficiency provided by the synergy of effects among allelopathins as compared with antagonism or no interaction of effects. Our experimental data provided no support for any of the hypotheses being tested, thus leading to the conclusion that defence against competitor was not a driving force in the evolution of allelopathins. However, our study had only two experiments. One conducted using four strongly aromatic Mediterranean-type shrubs, the other using one tree and three strongly aromatic Mediterranean-type shrubs, *Rosmarinus officinalis* being common to both. Therefore it can be argued that our conclusion was based upon too few data.

Thus we set out to review allelopathic literature, implicitly or explicitly dealing with the two hypotheses described above and whenever appropriate we reanalysed published data. Insects, nematodes, microbes and non-vascular plants will not be examined in the review. Like in the experimental test of the two hypotheses presented

above (Pereira *et al.*, 2016), this review is not aimed at ascertaining the occurrence and ecological relevance of allelopathy, or lack, under natural conditions. Its objective is only to evaluate the stated hypotheses in the light of a comprehensive review of published literature. All statistics were done using Excel®2010 (Microsoft Corporation) and Statgraphics 4.2 (STSC, Inc., Rockville, MD, USA).

Allelopathic effects of water-soluble and volatile phytochemicals

Despite the large effort of research on phytoactive or allelopathic effects of secondary metabolites of plants, such research deals mostly with water-soluble modes of release and delivery and as far as we know there are comparatively very few papers reporting simultaneous and comparable bioassays where the effects of water-solubles and volatiles are simultaneously investigated.

In their pioneer study of comparative phytoactivity of water-soluble and volatile ways of release of secondary products of plants, Moral and Cates (1971) investigated whether the partitioning of water-solubles and volatiles differed between species of humid regions and species of more arid regions. Water-solubles and volatiles produced by 40 species common in western Washington (USA) were bioassayed and according to the authors, data suggest that species from predominantly arid zones produce more volatile compounds than species from humid zones. Re-examining only the comparable data of radicle elongation of *Hordeum vulgare* in volatile and sponge bioassays using leaf extracts but without pooling the data, volatiles appear to be more phytoactive than water-solubles in eight out of nine species of arid zones, in nine out of 18 species of humid zones, and in 11 out of the 13 remainder species not classified either as arid or humid species. We assumed that errors in the equations presented by Moral and Cates are no more than typing errors and levels of inhibition presented by the authors were rightly determined. Otherwise, those levels were under-estimated in variable and unknown amounts and no comparisons can safely be made.

Ballester *et al.* (1977) studied the effects of water-soluble and volatile compounds produced by *Erica scoparia* in the humid north-western corner of Galicia (Spain). Water-solubles inhibited germination and radicle growth of *Trifolium pratense* and induced morphological alterations of rootlets while volatiles (data not shown by the authors) were said to drastically suppress seed germination and radicle growth, thus suggesting that volatiles might be more phytoactive than water-solubles.

Friedman *et al.* (1977) investigated the phytoactivity of water-solubles and volatiles of *Artemisia herba-alba* and of *Zygophyllum dumosum* growing in northern areas of Negev desert (Israel, dry Mediterranean type of climate). Germination of *Medicago laciniata* was always unaffected and *Z. dumosum* was ineffective as donor. According to the authors, who did not provide evidences to support their statement, the major inhibitors of *A. herba-alba* were volatiles because of the similar inhibition of germination of *Stipa capensis*, *Helianthemum ledifolium* and *Zygophyllum dumosum* by volatiles and water-solubilized compounds.

Despite that experiments described in Hussain *et al.* (1979) are not fully comparable a trend for higher inhibition of seed germination and of root growth seemed to be present when *Brassica campestris*, *Lactuca sativa*, *Pennisetum americanum* and *Setaria italica* were treated with water extracts of *Daturia inoxia* growing in Peshawar (Pakistan) in comparison with treatments by volatiles.

Squires & Trollope (1979) investigated the effects of water-solubles and volatiles of *Chrysocoma tenuifolia* (Easter Cape Province of South Africa) on germination and radicle growth of *Cucumis sativus*, *Lolium multiflorum* and *Lycopersicon esculentum*. The authors did not present statistical analyses but assuming that the experimental design always involved two replicates (as in the sole experiment for which this information was provided) we reanalysed the data by exact or approximate two-tailed Student's *t* tests after checking for homoscedasticity using the two-tailed *F* distribution, both at a significance level of *P*=0.05. There is no evidence that water-solubles affected germination while only the higher concentration of leaves placed above the seeds affected the germination of recipient species. As for radicle length, statistical reanalyses could not be done because no information on sample sizes was provided.

Nevertheless, water-solubles seem to be less inhibitory than volatiles on radicle growth of *C. sativus*, approximately equally inhibitory on *L. multiflorum* and more inhibitory than volatiles on *L. esculentum*.

In a survey designed to compare the release of water-extractable and volatile allelopathins in northern California (USA) plants (Heisey & Delwiche, 1983) the effects of a large number of species on the root growth of *Bromus mollis* and *Hordeum vulgare*. were investigated. Re-examining only the comparable data of *H. vulgare*, water extracts were clearly more phytoactive than volatiles. In fact, 30 out of 55 species inhibited the root length of *H. vulgare* by water extracts alone, 12 by both ways of delivery, but none by volatiles alone. In the 12 species showing phytoactivity by volatiles and water-solubles, one

(*Trichostema lanceolatum*) completely suppressed the root length both by water extracts and volatiles, eight were more inhibitory by solubilization, and only three by volatilization.

AlSaadawi & AlRubeaa (1985) investigated the role of allelopathy in patterning of vegetation under the canopy of *Citrus aurantium* growing in central Iraq. Re-examining only comparable data, water extracts of non-decaying senescent leaves of *C. aurantium* slightly inhibited the germination of seeds of *Amaranthus retroflexus*, water extracts of decaying senescent and water extracts of non-senescent leaves were intermediate, whereas the higher concentration of volatiles released by non-decaying senescent and non-senescent leaves more drastically reduced the germination of *A. retroflexus*.

Volatiles from intact plants of *Helenium amarum* harvested from pastures in central Georgia (USA) reduced the length of seedlings of *Medicago sativa* to 70% while leaf water extracts reduced the length only to 30% (Smith, 1989). In a later paper (Smith, 1990) volatiles from intact plants of *Athemis cotula* and *Eupatorium capillifolium* harvested from the same areas were shown to have no activity against germination and growth of *M. sativa* but water extracts of leaves were shown to markedly reduce the growth of seedlings.

Qasem (1999) reported the effects of water-solubles (extracts or leachates), of volatiles present in shoot extracts or foliage leachates, and of volatiles from intact or chopped shoots of *Ranunculus arvensis* presumably collected in Jordan, on germination and early growth of wheat. To our knowledge this is the only paper in which volatiles dissolved in water were separately tested in addition to volatiles emitted directly by plant materials. However the author investigated differences among treatments using the notoriously liberal least significant difference (LSD) test (Jones, 1984) and failed to provide any data on the variability of samples, be it standard deviations or standard errors, thus preventing the probabilistic reanalyses of results. To complicate matters further, the quantity of plants materials and number of seeds used in bioassays were not uniform, which prompted us to express concentrations of all treatments in terms of fresh weight of plant materials used and, because of possible effects of seed density on phytochemical activity (Weidenhamer *et al.*, 1987) concentrations per fresh weight were further divided by the number of seeds used, the final result being presented in fig. 1. Taking concentration per seed into account, water extracts of *R. arvensis* shoots were the most effective inhibitors of wheat because not only seed germination was completely inhibited but also such complete inhibition was attained at the lowest concentrations
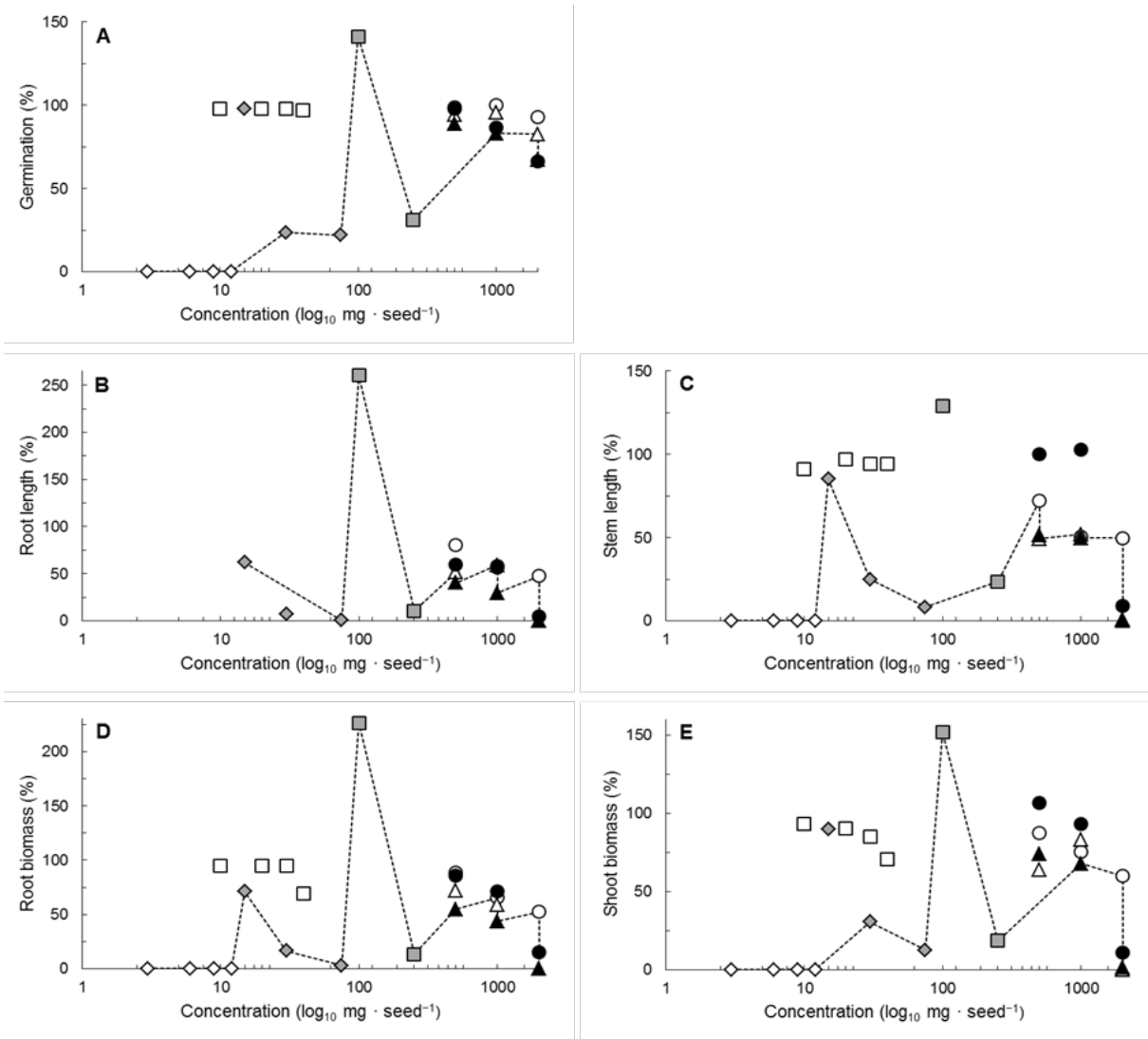
Figure 1.
Effects of water-solubles and volatiles of *Ranunculus repens* on germination, root and stem length, and root and shoot biomass of wheat expressed as percentage of controls. Open diamonds, water extracts of fresh shoots; grey diamonds, volatiles present in water extracts of fresh shoots; open squares, water leachates of fresh shoots; grey squares, volatiles present in water leachates of fresh shoots; open circles and open triangles, volatiles of intact or chopped shoots applied to seeds for 60 h; closed circles and closed triangles, the same but applied for one week. Treatments in (Qasem, 1999) found to be significantly different from controls using the least significant difference (LSD) test at *P*=0.05 are connected with dashed lines. Figure drawn by the authors using the original data presented in tables in (Qasem, 1999).

(≤ 3 mg of fresh shoots per wheat seed). The bioassay of volatiles present in water extracts shows that an important fraction of the inhibitory activity of water-solubles should probably be attributed to volatile compounds solubilized in water. These findings diverge from our results with perennial aromatic shrubs in which hypocotyl growth was inhibited only by water-solubles, volatiles alone being ineffective (Pereira *et al.*, 2016), while the annual *R. arvensis* is always inhibitory to wheat by volatiles.

Water-solubles and volatiles from shoots of *Artemisia herba-alba* growing in semiarid areas of central Spain were investigated and the former were found to completely inhibit the germination of seeds of *Helianthemum squamatum* while volatiles were partly or completely ineffective (Escudero *et al.*, 2000).

In a study of interactions of effects of water-solubles and volatiles from *Cistus ladanifer* growing in a semiarid site of southern Portugal (Dias & Moreira, 2002) it was found that water-solubles alone could affect the germination and root growth of *Trifolium subterraneum* but volatiles were active against seed germination only when applied simultaneously with water-solubles and were completely ineffective against root growth even in the presence of water-solubles.

As part of a wider screening of herbicidal activity of medicinal plants in Jordan (Qasem, 2002), it was shown that nine species completely inhibited the seed germination of *Amaranthus retroflexus* with water extracts of shoots, and that four species had the same effect on *Chenopodium murale*. Volatiles of eight of those nine species, of the four species effective against *C. murale*, and also of *Ricinus communis* were investigated for their effects on *A. retroflexus* and *C. murale* but none could completely prevent the germination of the target species. Apparently, only volatiles from *Origanum syriacum* and *Rosmarinus officinalis* were able to partly inhibit the seed germination of *A. retroflexus* and *C. murale*.

Moradshahi *et al.* (2003) studied the effects of water extracts and crude volatile oil from leaves of *Eucalyptus camaldulensis* growing in Shiraz (Iran) on a number of crop and weed species. Water-solubles and volatiles reduced or similarly blocked the cell division of root apical meristems of *Allium cepa*, while Hill reaction evaluated by the reduction of 2,6-dichlorophenolindophenol in isolated chloroplasts of *Spinacea oleracea* was less inhibited by water-solubles than by volatiles. However volatiles failed to significantly affect the root peroxidase activity in *Avena fatua*, *Echinochloa crus-galli*, *Lepidium sativum*, *Lycopersicon esculentum*, *Rumex acetosella* and *Zea mays* whereas water-solubles effectively decreased the root peroxidase activity of all tested species. Finally,

water-solubles from *E. camaldulensis* were equally or less effective than volatiles in reducing the radicle length of those six species.

Peng *et al.* (2004) extracted water-solubles from leaves and stems, and volatile organic compounds from leaves of the trees *Castanopsis chinensis*, *Castanopsis fissa*, *Cryptocarya chinensis*, *Cryptocarya concinna* and *Schima superba*, species characteristic of the climax stage in Dinghu Mountain (China). Water-solubles and volatiles were separately bioassayed to assess their effects on root length and shoot height of cabbage, cucumber and radish. With only one exception, growth was always reduced in absolute numbers by treatments, but no probabilistic assessment of the differences between treatments and controls was provided. In spite of that, a consistent trend can be detected for greater reductions of growth by water-solubles than by volatiles, the exception being shoot height of cabbage.

Onen (2007) investigated the auto-toxicity of *Artemisia vulgaris* growing in Tokat (Turkey) using water extracts of shoots and essential oil extracted by hydro-distillation from leaves and flowers. The two treatments inhibited germination, radicle and hypocotyl growth. Considering only the higher concentration used, water extracts and essential oil were similarly inhibitory except for hypocotyl growth which was much more inhibited by essential oil than by water extract.

The activity of water-soluble and volatile compounds of the two umbelliferous herbs *Laserpitium siler* and *Grafia golaka* growing in southwestern Slovenia was investigated for their effects on germination and early growth of the herbs *Lepidium sativum and Festuca rubra*, and of the tree *Pinus sylvestris* (Kaligaric *et al.*, 2011). Water-soluble compounds from *L. siler* or *G. golaka* presumably could inhibit the germination of the herbs as well as hypocotyl and radicle growth of *F. rubra*. However the scattered way of data presentation and the almost complete absence of results of bioassays in which volatiles alone were tested prevent us to compare the effectiveness of the two ways of delivery of secondary compounds.

The auto-toxicity of water-solubles and volatiles of seeds, roots and stems of *Brassica napus* presumably growing in Tsukuba City, Ibaraki, Japan was investigated for their effects on germination and early root or hypocotyl growth (Yasumoto *et al.*, 2011). With few exceptions, always involving stems and water-solubles, germination and early growth was said to be inhibited by the treatments. A reanalysis of data by exact two-tailed Student's *t* tests after checking for homoscedasticity using the two-tailed *F* distribution, both at a significance level of *P*=0.05,

generally showed no significant differences between water-solubles and volatiles treatments or, when differences were present, mean values in water-solubles treatments were significantly higher than in volatiles treatments.

Chen *et al.* (2013) separately bioassayed water extracts and volatiles of *Rosmarinus officinalis* to evaluate their effects on root length and shoot height of three weed species, *Cynodon dactylon*, *Digitaria sanguinalis* and *Eleusine indica*. Growth was always reduced. Considering only the highest concentration used in the two treatments, water extracts were clearly more inhibitory than volatiles to root growth of *C. dactylon* and to root and shoot growth of *D. sanguinalis*; volatiles clearly showed more inhibition to shoot growth of *E. indica* compared to water extracts, while root growth of *E. indica* and shoot growth of *C. dactylon* were similarly inhibited by water extracts or volatiles.

The effects of volatiles or leachates of fresh stems and leaves of *Solidago canadensis* growing in wasteland in Nanjing (eastern coastal China) were investigated for their effects on germination and early root and shoot growth of wheat and radish (Zhang *et al.*, 2014). Volatiles applied alone had no effect on the test species which were generally inhibited in water-solubles treatments.

Single and combined allelopathic effects of phytochemicals

Interaction can be defined as "an effect involving a number of bodies, particles, or systems as a result of which some physical or chemical change takes place to one or more of them" (Isaacs *et al.*, 1999) and can include the effects of one plant on another by means of allelochemicals produced by the first and taken by the second, thus allelochemicals interactions. However, and perhaps unfortunately because of the confusion it can generate, "interaction" is also applied to the effects produced by the agents involved in the interaction among bodies, particles or systems. In the cases examined here "interaction" is used in relation to the effects produced by the allelochemicals that mediate the interaction between the plants involved. Interaction of effects, or the less-mechanistic term "combined action" (Greco *et al.*, 1995) is usually divided into synergism, additivity and antagonism, the latter having been studied for almost 150 years (Fraser, 1870-1871, 1872).

As happened with phytoactivity by water-solubles or volatiles, the number of papers dealing with interactions of allelopathic effects is relatively low, and often based upon unproven and rarely stated assumptions on the shape of dose-response curves to compounds under investigation, and thus on the underlying mechanisms of action of the allelopathins involved. As discussed with greater detail elsewhere (Pereira *et al.*, 2016) it would be preferable to determine isoeffective concentrations of single and combined application of compounds thus making possible a mechanism-free approach. However when isoeffective concentrations are unknown or impossible to determine, 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 effective, (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, or (4) one of the constituents does not produce the effect of the combination and simultaneously 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) reflect antagonism, while (2) and (3) reflect synergy (Berenbaum, 1989).

Rasmussen & Einhellig (1977) investigated the single and combined effects of *p*-coumaric and ferulic acids on grain sorghum. Despite that no data is provided on significance levels in germination experiments it appears that synergistic effects on seed germination might have unequivocally occurred. Conversely, unequivocal decisions cannot be taken in relation to the occurrence of interaction of effects on shoot length and dry weight or when they can be reached their meaning is at least puzzling. Results fit outcome 1 and thus antagonism was unequivocal. However it occurred in an unusual way because the two phenolic acids when applied separately significantly increased the biomass of sorghum seedlings but significantly reduced it when applied jointly.

Einhellig & Rasmussen (1978) investigated the single and combined effects of vanillic acid and *p*-hydroxybenzoic acid on radish and grain sorghum. Here again despite that no data is provided on significance levels in germination experiments it appears that synergistic effects on seed germination might have unequivocally occurred. Conversely unequivocal decisions can hardly be reached in relation to the combined effects of the two phenolic acids on root and shoot elongation of the two target species and on dry weight of sorghum. However, synergy was clearly in place in diffusive resistance of sorghum leaves up to the 3rd day after treatment, but unequivocal decisions cannot be made when later days are considered.

The effects of four phenolic acids in single and combined applications of two, three or four phenolics on germination and early growth of grain sorghum were studied in (Einhellig *et al.*, 1982) and synergistic effects were almost always found. However, the authors assumed that dose-response curves followed the model proposed by Colby (1967) which is nothing more than the Bliss Multiplicative Model described elsewhere (Pereira *et al.*, 2016). Therefore we reanalysed data for which means, standard errors and sample sizes were provided. Reanalysis was done by analysis of variance (ANOVA) followed by exact or approximate two-tailed Student's *t* tests and an experiment-wise type I error rate (α_E) of 0.05 calculated using the Dunn-Šidák method (Ury, 1976) after checking for homoscedasticity at a significance level of *P*=0.05 using the two-tailed *F* distribution. Unequivocal decisions could be reached for germination where the outcome 2 and thus synergy, as described above always occurred. When seedling growth was examined interactions of effects were almost always absent. Exceptions were the combination of the four phenolic acids and the combination of three (provided that cafeic acid and *p*-coumaric acid were not simultaneously present) which resulted in outcome 3 described above and thus in synergy.

The effects of single and combined effects of ferulic, vanillic and caffeic acids were investigated in root length of cucumber (Blum *et al.*, 1984) and almost without exception either no interaction of effects occurred or if present it was antagonic.

Blum *et al.* (1985a) investigated the single effects of eight phenolic acids and the combined effects of ferulic acid and each one of the other seven phenolics (caffeic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, and vanillic acids) on growth of cucumber seedlings growing in nutrient culture, with special emphasis on leaf expansion. According to the authors either no interaction of effects occurred or when it did it was antagonic.

The same authors (Blum *et al.*, 1985b) investigated the effects of single and combined applications of two phenolic acids, ferulic and *p*-coumaric acids, on the relative rates of leaf expansion of cucumber seedlings. No information was provided on the statistical significance of single and combined applications compared with control which prevents unequivocal conclusions but no interaction of effects seems to be generalized as stated by the authors or, if interaction of effects was present antagonism would be involved.

In a paper intended to establish an efficient design for investigating joint effects of similar compounds (Gerig *et al.*, 1989) it was concluded that ferulic and vanillic acids acted antagonistically against mean absolute rate of leaf expansion of cucumber seedlings under the unsubstantiated assumption that the biological response to the joint action of the two phenolic acids could be described by a logistic function and thus that the response was assumed to be sigmoidal and symmetric.

Using the model presented by Gerig *et al.* (1989) for the joint action of chemically similar compounds cited in the preceding paragraph, it was found that interactions of effects between pairs of phenolic acids on leaf growth of cucumber were only present when ferulic acid was bioassayed and only at pH 5.2 and were always antagonistic. Conversely, at pH 6.0 or when vanillic and *p*-hydroxybenzoic acids were present in mixtures no interactions of effects were detectable (Blum *et al.*, 1989).

Using the same model, Gerig and Blum (1991) found that whenever *p*-hydroxybenzoic acid was present in phenolic mixtures, antagonistic effects occurred in the growth of cucumber seedlings while interactions of effects were undetectable when it was absent but ferulic, *p*-coumaric or vanillic acids were in the mixture.

The single and combined effects of three benzoxazinones on germination and early root and shoot growth of *Echinochloa crus-galli*, *Cucumis sativus*, *Lepidium sativum* and *Phaseolus vulgaris* were evaluated in (Chase *et al.*, 1991). The authors assumed that dose-response curves followed the model proposed by Colby (1967), which equates to the Bliss Multiplicative Model, but no reanalysis is possible because only means are provided and comparisons between treatments were done by LSD, known to be one of the most liberal simultaneous test procedures (Jones, 1984). It is highly unlikely that Bliss Multiplicative Model fitted the dose-response curves, but if it did fit then interactions of effects would always be antagonistic in *E. crus-galli*, antagonistic or synergistic to root and shoot growth of *C. sativus*, *L. sativum* and *P. vulgaris*, and always synergistic to germination of *L. sativum*.

Weidenhammer *et al.* (1994) individually bioassayed five menthofuran monoterpenes present in leaves of *Calamintha ashei* for evaluating their effects on germination and early root growth of four species. In addition the two most abundant and active monoterpenes, desacetylcalaminthone and (+)-evodone were also investigated jointly. Synergy was clearly found on germination of *Schizachyrium scoparium* (0.25 mM and 0.50 mM equimolar mixtures), *Leptochloa dubia* and *Lactuca sativa* (both at 0.50 mM equimolar mixture), and on root growth of *L. dubia* and *Rudbeckia hirta* (both at 0.25 mM equimolar mixture). Antagonism was clearly found on germination of *L. sativa* (0.05 mM and 0.25 mM

equimolar mixtures), on root growth of *L. dubia* (0.05 mM equimolar mixture) and of *L. sativa* (0.25 mM and 0.50 mM equimolar mixtures). In the remaining cases either no significant effects of single or mixed applications were found or no unequivocal decisions could be taken about interactions of effects.

Reigosa *et al*. (1999) bioassayed six phenolic acids, *p*-coumaric acid, ferulic acid, vanillic acid, *p*-hydroxybenzoic acid, gallic acid and *p*-vanillin individually and jointly in a series of concentrations and their effects on germination and early root growth of *Amaranthus retroflexus*, *Chenopodium album*, *Cirsium* sp., *Plantago lanceolata*, *Rumex crispus* and *Solanum nigrum* were evaluated. Reanalysis of data of germination and root length as percentage of control by forward stepwise regression without replication to concentration was possible only for individual effects of phenolic acids and was done with an experiment-wise error rate (α_E) for coefficients of 0.05 calculated using the Dunn-Šidák method (Ury, 1976). Candidate models included up to the second degree of concentration, plus qualitative independent variables, namely the different phenolic acids applied individually, binary coded as 0, 1 and all interactions between variables. Conversely, regression models could not be fitted to joint effects of phenolic acids, except for *R. crispus* and therefore isoeffective analyses were not possible. Nevertheless a look at data provided in (Reigosa *et al.,* 1999) reveals that interaction of effects is almost always present regardless of the species and concentrations tested. Antagonism was largely prevalent (34 out of 40 possible cases), while synergy could be identified only in four cases (three of them in germination) and no interaction of effects in two.

Dias & Moreira (2002) investigated the interaction of effects between water-solubles and volatiles of *Cistus ladanifer* relying upon the interaction term of factorial ANOVA, meaning that the authors assumed dose-response curves to follow the Linear Additive Model described elsewhere (Pereira *et al.,* 2016) which is clearly an inadequate method to search for interactions of effects (Williams & Hoagland, 1982; Caudle & Williams, 1993). Reanalysis of data based upon the equations published by the authors makes clear that outcome 1 and thus antagonism unequivocally occurred in total germination and in root length. However lag of germination and rate of germination were significantly affected by water-solubles and volatiles applied separately or jointly, a result that prevents unequivocal decisions about interactions of effects. Nevertheless, in lag and in rate of germination isoeffective concentrations could be determined from the equations published by the authors, making it possible to perform isobolographics

as described in Berenbaum (1989) and Nelson & Kursar (1999) which clearly shows that antagonistic interactions of effects were in place.

Isobolographics were used to investigate interactions of effects resulting from the joint action of the three paired combinations of the phenolic acids *p*-hydroxybenzoic acid, *p*-coumaric acid and ferulic acid on root growth of *Lolium perenne* (Inderjit *et al.*, 2002). Synergy was found for the mixture of *p*-hydroxybenzoic acid and *p*-coumaric acid. Synergy was also found for the dose required for 20% reduction, ED_{20}. Besides that, antagonism was largely prevalent, its frequency increasing with the increase of isoeffective concentrations from ED_{20} to ED_{80}.

Vokou *et al*. (2003) investigated the response of germination and maximum seedling length of *Lactuca sativa* tested individually with 47 monoterpenoids and with a selection of 17 monoterpenoids arranged in 11 pairs in different proportions. Interactions of effects were examined extending the model proposed in An *et al.* (1993) for a single compound. Such method appears to result in an isobolographic-like approach based upon a variation of the general equation for zero interaction of effects presented in Berenbaum (1989) for mechanism-independent analyses. Interactions of effects were absent in approximately half of the 21 cases examined. Synergy was found in four pairs of compounds (three in germination, one in seedling length), and antagonism in six pairs (three in germination and three in seedling length).

The effects of volatiles from leaves of *Ageratum conyzoides* and of their major components were tested on fresh weight of *Bidens pilosa*, *Digitaria sanguinalis* and *Cyperus difformis* growing during one week in Petri plates with quartz sand and Hoagland's solution (Kong *et al.*, 2004). Some evidences exist that synergy might have been present but the absence of data for the single effects of precocene II, the most abundant component of the volatile mixture, prevents substantiated conclusions on the effective occurrence and type of interaction of effects.

The effects of abietic acid, *trans*-ferulic acid and 5-hydroxyindole-3-acetic acid applied individually or combined with a mixture of five other phenolic acids were tested on root growth of *Sagittaria montevidensis* (Seal *et al.*, 2004). Dose-response curves clearly show that either there were no interactions of effects or when present they were always antagonistic.

The single and combined effects of 3,4-dimetoxybenzoic acid and 3,4,5-trimetoxybenzoic acid from *Parkia pendula* leaves were investigated for their effects on germination and early growth of *Mimosa pudica* and *Senna obtusifolia* (Souza Filho, 2006). A modification of the isobolographic approach in which no isoeffective concentrations can be calculated

was used by the author. The combined effects of the two phenolic acids were clearly antagonistic to germination, root and hypocotyl growth of the two weed species.

The effects of water extracts of leaves and stems of *Raphanus raphanistrum* applied separately or jointly on germination and early growth of *Avena sativa* and *Triticum aestivum* were evaluated in Dias & Dias (2007). It is doubtful whether interactions of effects on *A. sativa* were present and if they were they would be synergic, but unequivocally only in leaf/root length ratio. Unequivocal interactions of effects were also found in germination, root length and leaf/root length ratio of *T. aestivum*, antagonistic in the first two, synergic in the last.

The phytoactivity of several ecotypes of two *Vetiveria* species was reported in (Laosinwattana *et al.* 2007). Dried leaves of the most active ecotype were extracted with methanol, the extract sequentially fractioned and the methanol extract and its three fractions were bioassayed for determining their effects on germination and early root and shoot growth of *Raphanus sativus*. Bioassays were done at various concentrations, apparently at the same levels regardless of using the methanol extract or its fractions. Thus the correspondence between the amount of compounds present in the methanol extract and in its fractions can only be guessed and comparisons can only be made with a high degree of uncertainty. Nevertheless, graphical data provided by the authors strongly suggest that interactions of effects among fractions were present and were always antagonistic.

Lobo *et al.* (2010) investigated the effects of three stilbenes isolated from leaves of *Deguelia rufescens* var. *urucu* on germination and early growth of the weed *Mimosa pudica*. Stilbenes 4-methoxylonchocarpene, 3,5-dimethoxy-4'-hydroxy-3'-prenil-*trans*-stilbene and 3,5-dimethoxy-4'-O-prenil-*trans*-stilbene were bioassayed singly or in pairs. No information was provided on the statistical significance of single applications in comparison with control. Thus unequivocal conclusions on the occurrence of synergic effects on radicle and hypocotyl early growth cannot be reached. However, antagonic effects on germination were clearly present whenever 3,5-dimethoxy-4'-hydroxy-3'-prenil-*trans*-stilbene was involved.

The triterpenoids lupenone and lupeol produced by *Acacia mangium* were bioassayed separately and in combination for assessing their effects on germination and early growth of the weeds *Mimosa pudica* and *Senna obtusifolia* (Luz *et al.*, 2010). In general, no interactions of effects were found between the two triterpenoids but when they occurred (radicle growth of *M. pudica*) they were clearly antagonistic.

Kato-Noguchi *et al.* (2011) determined isoeffective concentrations of 3-hydroxy-β-ionone and 9-hydroxy-4-megastigmen-3-one, the two main inhibitors isolated from the aqueous methanol extract of rice cultivar Kartikshail. Those compounds were bioassayed separately or jointly for evaluating their effects on root and shoot growth of *Lepidium sativum* or *Echinochloa crus-galli*. At the concentration required for 50% growth inhibition clear and very intense synergistic effects were found between the two nor-isoprenoids.

The effects of single and combined applications of cinnamic and ferulic acids (CIN and FER respectively) plus piperonylic acid (PIP) and 3,4-(methylenedioxy) cinnamic acid (MDCA) on growth, and on the activity of phenylalanine ammonia-lyase and of tyrosine ammonia-lyase, and on lignin content and on the composition of *p*-hydroxyphenyl, guaiacyl and syringyl monomers in maize roots were investigated (Ferro *et al.*, 2015). In the two cases in which data of experimental controls are provided (effects of CIN and PIP or effects of FER and MDCA on lignin content), antagonism through outcome 4 is clearly present. Except in these two cases either combined effects are not presented or comparisons are made in relation to one of the treatments instead of a control which prevents an informed assessment of the occurrence of interaction of effects. Even so, a closer look suggests no interaction of effects between CIN and PIP on *p*-hydroxyphenyl and guaiacyl content. Conversely, synergy on syringyl content occurs through outcome 2, while antagonism between FER and MDCA on the content of the three monomers occurs, always through outcome 1.

The effects of ferulic, *p*-hydroxybenzoic and vanillic acids applied singly or in combination on germination, radicle growth and hypocotyl growth of tomato, *Erythrina verna* and *Mimosa bimucronata* were evaluated in (Jose *et al.* 2016). Interactions of effects were unequivocally found in radicle growth of tomato (antagonism through outcome 1) and *E. verna* (synergy through outcome 2), and in hypocotyl growth of tomato (again antagonism through outcome 1) and *M. bimucronata* (synergy through outcome 3), while no interaction of effects were recognizable in root growth of *M. bimucronata*.

Discussion

The number of secondary metabolites produced by plants is known to be very large. In the late 1970s more than 10,000 were already known in vascular plants and fungi (Swain, 1977) and it was admitted that the total number of secondary metabolites of plants might equal the number of known plant species

put by then at 400,000 (Raven *et al.*, 1976). By the late 1980s the number of secondary metabolites with known structure exceeded 15,000 (Wink, 1988), and in the early 2000s about 47,000 secondary metabolites were known (Luca & Pierre, 2000) or just exceeded 24,000 according to others (Rao & Ravishankar, 2002). More recently the number of secondary metabolites of plants with known structures exceeded 54,000 (Wink, 2013). Numbers can be equally large when individual species are considered. For example in *Cistus ladanifer*, one of the species used for testing the hypothesis of prevalence of synergy over antagonism or over no interaction of effects (Pereira *et al.*, 2016), more than 370 secondary compounds have been identified just in the essential oil or in leaves (Dias *et al.*, 2005).

Several roles have been attributed to secondary metabolites of plants. These include excretion (Whittaker & Feeny, 1971), self-regulation (Robinson, 1974), and defence against phytophagous (Ehrlich & Raven, 1964) or against neighbouring plants (Rice, 1984). Recently it was argued that coevolution between plants and herbivores could explain increases in the diversity of secondary metabolites and their persistence even after they apparently lacked their anti-herbivory activity because of the resistance developed by the herbivores towards them (Speed *et al.*, 2015). However the roles attributed to secondary metabolites are not necessarily mutually exclusive and the acceptance of the argument of anti-herbivory as an evolutionary driving force does not prevent the possibility that other roles might be simultaneously played by secondary metabolites or even that some of them might also have primary functions in plants (Seigler & Price, 1976).

Whichever their roles are, the production of secondary metabolites necessarily demands a high investment from plants, and might require between 15% and 25% of plants genome (Pichersky & Gang, 2000). Therefore it is reasonable to admit that bouquets of secondary metabolites produced nowadays are those that in a way or another are or have been advantageous to the plants producing them (Bell, 1980). Two hypotheses derived from this assumption were experimentally tested (Pereira *et al.*, 2016) and the existing literature was reviewed and reanalysed. The first hypothesis is that allelopathic activity would be predominantly by volatile compounds in dry environments, and by water-soluble compounds in humid environments. The second hypothesis is that synergy would be vastly predominant whenever mixtures of allelopathic compounds were considered.

None of the hypotheses was supported either by our bioassays (Pereira *et al.*, 2016) or by published literature reported here. More often than not,

the correspondence between volatiles and dry environments or between water-solubles and wet environments not only did not happened but the opposite occurred, while the frequency of synergy was largely surpassed by the frequency of antagonism and even more by antagonism and absence of interaction of effects together.

As advanced by a number of authors, 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). Not only they can be soluble in water but those that are soluble do not lose their phytoactivity (Qasem, 1999). Therefore it could be argued in defence of the first hypothesis that in the more demanding dry habitats, the volatile release of allelopathins represents an additional way of defence against neighbouring plants acting almost exclusively in dry periods, and adding to leaching during rainy periods. However our data on the absence of effects of volatiles on hypocotyl growth while water-solubles were inhibitory (Pereira *et al.*, 2016) and similar results found in a number of studies (Hussain *et al.*, 1979; Heisey & Delwiche, 1983; Escudero *et al.*, 2000; Dias & Moreira, 2002; Qasem, 2002) clearly contradicts such hypothesis.

Published data on interactions of effects shows that synergy is not the prevalent type of interaction of phytotoxic effects of secondary metabolites of plants. Instead, antagonism or zero interaction of effects is more frequent. It could be argued that in large part this happens because most part of research involved 1) a selection of compounds wherein their number is much lower than what might be expected to be found in plants, and 2) compounds belonged to the same chemical family, phenolic acids being the most frequent case.

The first argument is contradicted either by the prevalence of antagonism or no interaction of effects over synergy found in studies in which the whole chemical composition of plant parts was bioassayed (Dias & Moreira, 2002; Laosinwattana *et al.*, 2007; Dias & Dias, 2007; Pereira *et al.*, 2016).

In more than two thirds of studies reviewed in which only a limited number of compounds were investigated, phenolics were the group of choice. Phenolic compounds are known to act on a variety of processes including plasma transmembrane electrochemical potential, retention of ions and their flux across cell membranes, indoleacetic acid oxidase system or net photosynthesis through the reduction of stomatal conductance and chlorophyll reduction (Einhellig, 2004). However, despite the variety of processes they can affect, phenolics are thought to have essentially the same sites of action which

implies that their combined effects could never be synergic (Inderjit *et al.*, 2002). This review supports the argument but only in what concerns plant growth. In fact, almost without exception the combined effects of phenolics on seedlings' growth were either antagonic or no interaction of effects was present while synergy was frequently found in combined effects of phenolic acids when seed germination was involved.

Conclusion

In short, the review of published literature fails to support the hypotheses under investigation, namely the hypothesis that volatilization is the preferred way of release of phytoactive secondary compounds in dry, hot environments and water-solubility in wetter areas, and the hypothesis that phytoactive compounds act synergistically when applied in mixtures. As a consequence it can be hypothesized that selection did not result in secondary metabolites of plants acting primarily as direct defence agents against other plants but might be primarily involved in self-regulation of plants that produce them. Alternatively their role might be more aimed at inducing indirect effects on competitors rather than at provoking direct effects (Zeng, 2014).

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