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Inhibition of *Orobanche crenata* Seed Germination and Radicle Growth by Allelochemicals Identified in Cereals

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Supporting Information

ABSTRACT: *Orobanche crenata* is a parasitic weed that causes severe yield losses in important grain and forage legume crops. Cereals have been reported to inhibit *O. crenata* parasitism when grown intercropped with susceptible legumes, but the responsible metabolites have not been identified. A number of metabolites have been reported in cereals that have allelopathic properties against weeds, pests, and pathogens. We tested the effect of several allelochemicals identified in cereals on *O. crenata* seed germination and radicle development. We found that 2-benzoxazolinone, its derivative 6-chloroacetyl-2-benzoxazolinone, and scopoletin significantly inhibited *O. crenata* seed germination. Benzoxazolinones, L-tryptophan, and coumalic acid caused the stronger inhibition of radicle growth. Also, other metabolites reduced radicle length, this inhibition being dose-dependent. Only scopoletin caused cell necrotic-like darkening in the young radicles. Prospects for their application to parasitic weed management are discussed.

KEYWORDS: *allelopathy, broomrape, benzoxazolinones, parasitic weed management*

INTRODUCTION

Cereals, family Poaceae (syn. Gramineae) include the most important crops in the world for human and animal food such as wheat, barley, oat, rice, maize, shorgum or rye, among others. In addition to this economic interest, cereals are known for their allelopathic effects that can be exploited for weed management.[1] Several metabolites released by various cereals have been suggested as responsible for the allelopathic effects.[2−5]

The broomrapes (*Orobanche* and *Phelipanche* spp.) are root parasitic plants widespread in Mediterranean areas. Several broomrape species have specialized to survive in agricultural ecosystems as weeds feeding on the roots of important crops.[6] A wide variety of approaches including physical, cultural, chemical, and biological control measures have been explored against broomrapes, but most of them are not effective or not selective to the majority of susceptible crops. The methods currently available to farmers are the use of resistant cultivars and chemical control with systemic herbicides such as glyphosate or imidazolinones at low rates or soil applications of sulfonylurea,[7−9] but all have drawbacks and new options need to be explored.

The early stages of parasitic weed life, from seed germination to parasitic radicle contact with the host, are major targets for their control due to their complete dependence on the host for both nutrient and water supply. The germination of broomrapes is triggered upon recognition of host root exuded metabolites.[10] Once germination is initiated, they rapidly develop a radicle of a few millimeters with a terminal haustorium whose only purpose is to penetrate the host root and feed on it.[11] If the host contact and penetration is not established within few days, the parasitic seedling dies. Hence, measures targeting parasitic germination or radicle growth might efficiently control infection. It has also been shown that intercrops with cereals can reduce *Orobanche crenata* parasitism on legumes, allelopathy being a major component for the reduction.[12] It is thus suggested that cereal roots might be exuding substances that reduce *O. crenata* development, thus preventing parasitism. These allelochemicals might have a direct application for broomrape control that should be explored.

Cereals are a source of allelochemicals such as hydroxamic acids, caffeic acid, *p*-coumaric acid, ferulic acid, benzoic acid, gramine, L-tryptophan, and scopoletin.[4,5,13] Hydroxamic acids are plant secondary metabolites present in both cultivated and wild Gramineae.[14,15] They decompose to 2-benzoxazolinone,[15] which is known to interfere with the germination and early growth of several vegetable crops,[16] inducing biochemical changes such as decreased activity of hydrolytic amylases enzymes, interfering with vital metabolism. Caffeic acid is an inhibitor of seed germination and root elongation of several plants. A disruption of plant water relations was the suggested primary mechanism of plant growth inhibition, the water channel aquaporin being the suggestive target.[17] Other enzymatic alterations have been described, such as inhibition of phosphorylase, decreased activity of proteases, generation of reactive oxygen species, and significant change in the activities

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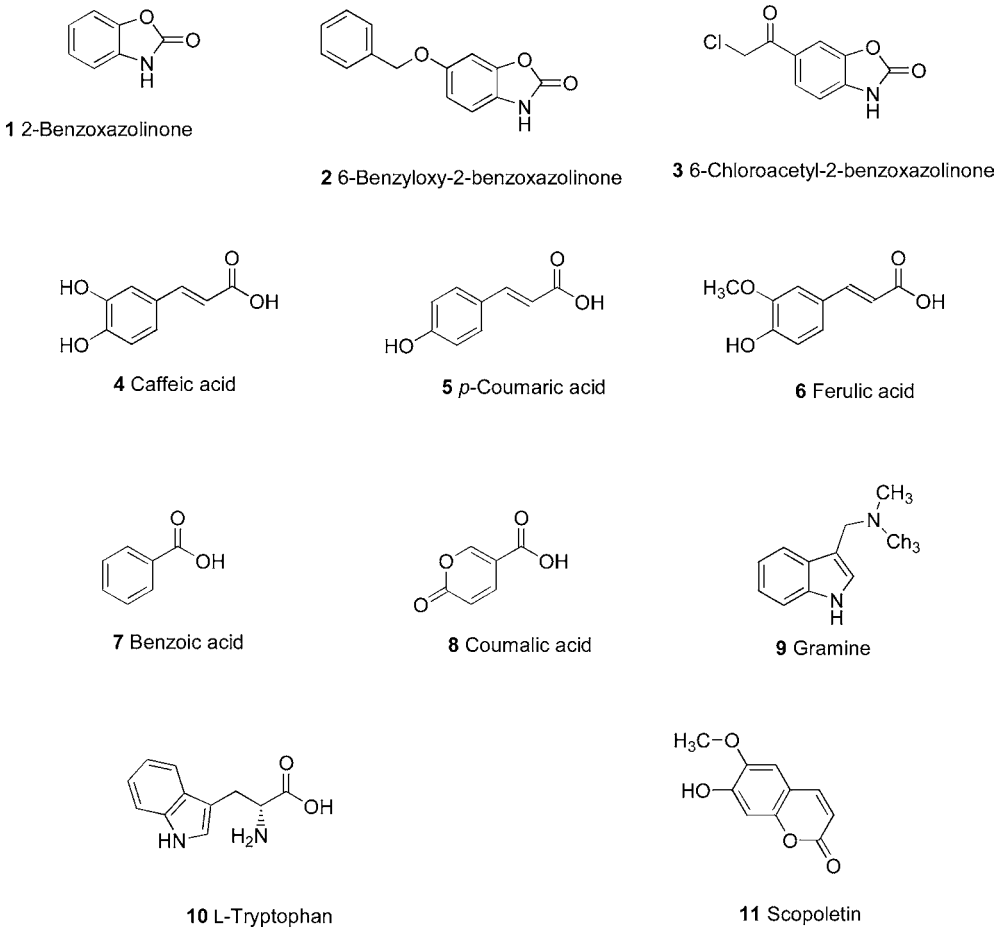

Figure 1. Structure of allelochemicals tested.

of peroxidase.[18] Ferulic and *p*-coumaric acid are allelochemicals that inhibit the growth of plants[19] by inhibiting acetolactate synthase. Herbicides such as sulfonylureas induce both ferulic and *p*-coumaric acid accumulation.[20] Benzoic acid inhibits auxin activity, which is responsible for plant growth, and has been used following chlorination as Dicamba, which is an auxinic herbicide of foliar use.[21] Coumalic acid is an acid pyrone with potential as a phytotoxin, widely implicated in allelopathic studies.[22,23] Gramine is an alkaloid reported in barley with inhibitory activity on other plants.[24,25] L-Tryptophan is realeased to the rhizosphere by many plants, including oats,[3] having an inhibitory effect on root growth of a number of weeds.[3,26] Scopoletin is known to be exuded by cereal roots, being considered responsible for the allelopathic effects on weeds.[2,4,27]

The objective of this work was to determine their potential to inhibit the first stages in the parasitic life cycle by inhibiting *O. crenata* germination and/or radicle growth toward the host root.

MATERIALS AND METHODS

Allelochemicals. The allelopathic potential against *O. crenata* seed germination and seedling development was evaluated on eight commercially available metabolites reported in cereals: 2-benzoxazolinone (**1**), caffeic acid (**4**), *p*-coumaric acid (**5**), ferulic acid (**6**), benzoic acid (**7**), gramine (**9**), L-tryptophan (**10**), and scopoletin (**11**). They were selected on the basis of an allelopathy-based literature survey.[4,5,13] In addition, we selected one acid pyrone (coumalic acid (**8**)) and two hemisynthetic derivatives of 2-benzoxazolinone, one with a benzyloxy ring (**2**) and another with a cloroacetyl substituent at the C-6 position (**3**) (Figure 1). All chemicals tested were obtained from Sigma-Aldrich (St. Louis, MO), except for benzoic acid, which was obtained from Merck (Merck, Germany).

Plant Material. The *O. crenata* seeds used in this work were collected in a heavily infested faba bean field in Cordoba, Spain. Seeds were collected from mature, dry inflorescences using a 0.6 mm mesh-size sieve. Seeds were stored dry in the dark at 4 °C in the parasitic plant germplasm bank of the IAS-CSIC, Córdoba, Spain.

Prior to starting the experiments, viability of the seeds was estimated on 3 replicates of 50 seeds per species according to the 2,3,5-triphenyltetrazolium chloride (TTC) method.[28] Seeds were imbibed in a solution of 1% TTC (Sigma) and incubated at 37 °C for 3 days to stain the living embryos. After that, the TTC solution was eliminated and seeds were immersed in a solution of 50% sodium hypochlorite for 2 min to bleach the seed testa. Seeds were observed under stereoscopic microscope at 30× magnification to determine the percentage of living seeds. Living seeds were considered when embryos were stained from pale pink to red. Seeds were considered to be dead when embryos were completely white.

Conditioning of *Orobanche crenata* Seeds. The parasitic seeds were surface sterilized with 0.2% (w/v) formaldehyde and 0.02% (v/v) Tween 20, rinsed thoroughly with sterile distilled water, and dried for 60 min in a laminar air flow

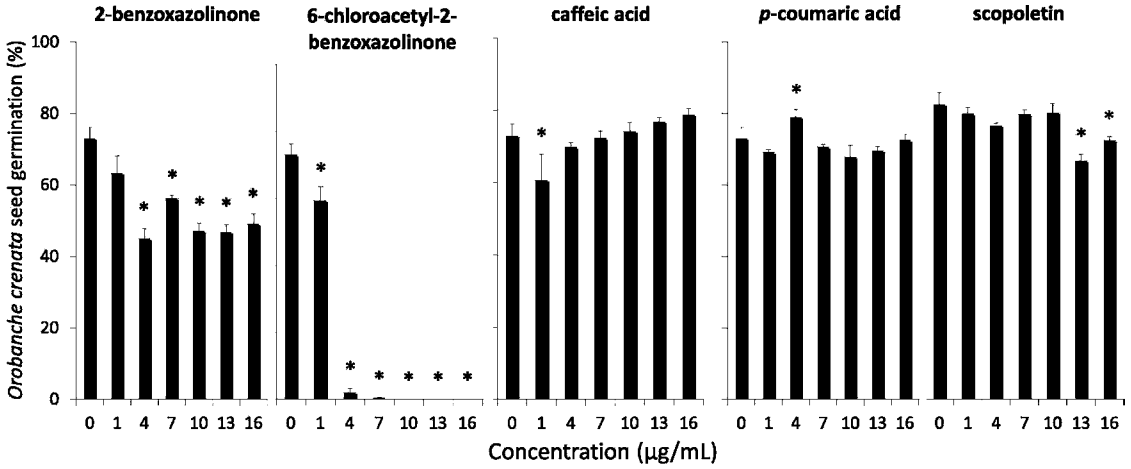

Figure 2. Inhibition of *Orobanche crenata* seed germination by 2-benzoxazolinone (**1**), 6-chloroacetyl-2-benzoxazolinonce (**3**), caffeic acid (**4**), *p*-coumaric acid (**5**), and scopoletin (**11**). *Indicates differences at the 0.05 level compared with the control (concentration 0; seeds induced to germinate by GR24) by Dunnet tests. 6-Benzyloxy-2-benzoxazolinone (**2**), ferulic acid (**6**), benzoic acid (**7**), coumalic acid (**8**), gramine (**9**), and L-tryptophan (**10**) had no significant effect on the germination (therefore, data is not shown).

cabinet. Approximately 100 broomrape seeds were placed separately on each of 264 discs of 1 cm diameter glass fiber filter paper (GFFP) moistened with 120 μL of sterile distilled water and incubated in 10 cm sterile Petri dishes in the dark at 20 °C for 11 days to promote warm stratification, which is a conditioning period necessary for the seeds to become sensitive to the posterior chemical signal that triggers the germination.[10]

Germination Induction and Allelophatic Treatments of *Orobanche crenata* Seeds. After 11 days of conditioning, the 1 cm diameter GFFP discs were transferred inside a laminar air flow cabinet to a sterile sheet of paper for a few seconds to remove the excess of moisture used in the conditioning in order to increase the perception of the chemicals by the seeds. Then, they were transferred again to new 10-cm sterile Petri dishes. Stock solutions of the synthetic germination stimulant GR24[29] and of the allelochemicals were prepared with organic solvents and kept in the freezer until use. Inmediately before use, each treatment was prepared by mixing GR24 with each allelochemical in sterile distilled water. First, GR24 was diluted at 3 μg/mL and aliquoted into 77 tubes of 2 mL each. This constitutes the inductor component of the mixture. In addition, different aliquots of the stock solutions of 11 allelochemicals were diluted individually in the tubes containing 2 mL of GR24 to reach allelochemical concentrations of 0 (positive control), 1, 4, 7, 10, 13, 16 μg/mL but constant GR24 concentration. Immediately after mixing the GR24 and each allelochemical, triplicated aliquots of 100 μL for each of the 77 treatments were applied in three replicated GFFP discs containing the conditioned *O. crenata* seeds. Immediately, GFFP-treated discs were placed in Petri dishes, sealed with Parafilm, and incubated in the dark at 20 °C for 9 days to promote the germination and radicle growth. Dilutions carrying decreasing concentrations of allelochemical (including the positive control GR24 mixed with 0 μg/mL of allelochemical) carried the same amount of organic solvent contained in the highest allelopathic concentration. This was done in order to allow comparisons between treatments and to discard the possibility of mistaking the allelopathic effect with a toxic effect of the solvent at the highest concentrations. The negative control consisted of sterile distilled water containing the equivalent amount of solvent without GR24 and allelochemical.

Measurements. After 9 days of treatment, the percentage of seed germination, radicle length, and the percentage of radicles that developed necrosis were established for each GFFP disc by using a stereoscopic microscope at 30× magnification. The germination percentage was determined by scoring the number of seeds with an emerged radicle through the seed coat as germinated in a total of 100 seeds per disc. Radicle length was measured in 15 randomly selected germinated seeds from each disc.[30] Presence of necrotic areas was assessed in 50 randomly selected radicles in each disc.

Statistical Analysis. Percentage data on *Orobanche* germination and necrosis of radicle was approximated to normal frequency distribution by means of angular transformation and submitted to analysis of variance (ANOVA). Parasitic radicle growth was adjusted to the dose response curves caused by each allelopathic metabolite by means of regression analysis (linear, quadratic, cubic, and exponential) on the basis of determination coefficient and ANOVA regression significance (Supporting Information Table S1). All analyses were conducted using SPSS 21.0.

RESULTS AND DISCUSSION

Inhibition of *O. crenata* Seed Germination. No germination was observed in the *O. crenata* seeds treated with the negative control. High levels of *O. crenata* seed germination (72.9%) were achieved in the positive control GR24. Moderate but significant *O. crenata* seed inhibition (around 33%) was caused by 2-benzoxazolinone (**1**) when applied at rates higher than 1 μg/mL. No effect was observed for 6-benzyloxy-2-benzoxazolinone (**2**) at any doses applied. On the contrary, germination was markedly inhibited in presence of 6-chloroacetyl-2-benzoxazolinone (**3**), with a moderate but significant inhibition when applied at 1 μg/mL and a complete inhibition of germination at concentrations higher than 4 μg/mL (Figure 2). All other allelochemicals tested had little or no inhibitory effect at any dose, except for scopoletin that caused slight inhibition (19%) at concentrations ≥13 μg/mL (Figures 2B and 2C).

Inhibition of *O. crenata* Radicle Growth. Contrary to what was observed for *O. crenata* seed germination, most of the compounds inhibited *O. crenata* radicle growth. Figure 3A

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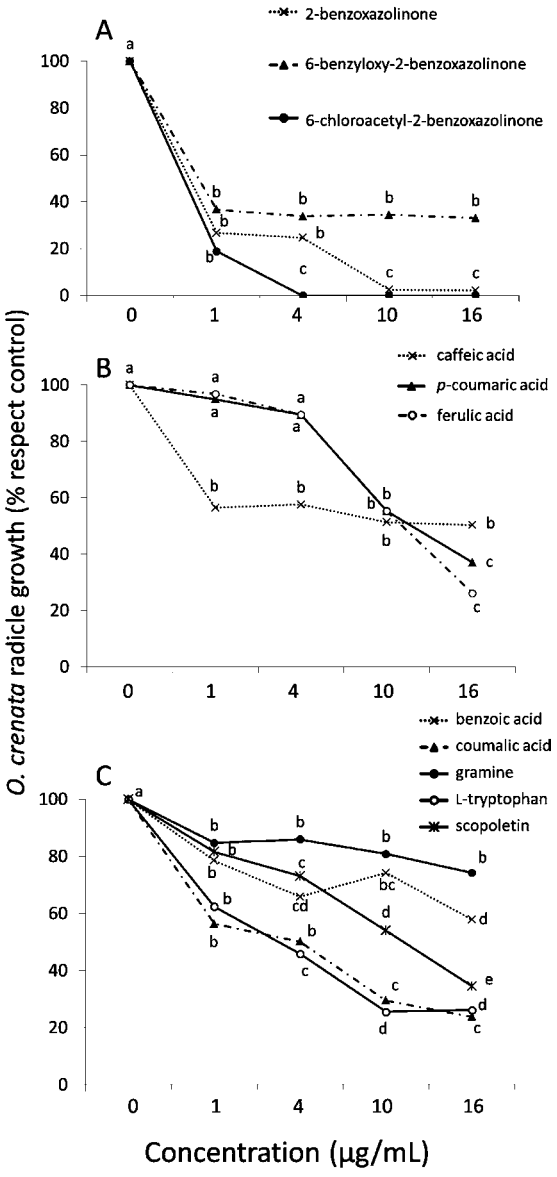

Figure 3. Inhibition of *Orobanche crenata* radicle growth by (A) benzoxazolinones, (B) hydroxycinnamic acids, and (C) other inhibitors.

shows that benzoxazolinones were very active, reducing radicle growth even at the lowest concentration (1 μg/mL). This drastic growth inhibition was increased with an increase in the dose of 6-chloroacetyl-2-benzoxazolinone (**3**) and 2-benzoxazolinone (**1**) but not with an increase in the doses tested for 6-benzyloxy-2-benzoxazolinone (**2**). No radicle was visible through the seed coat in seeds treated at doses greater than 4 μg/mL with 6-chloroacetyl-2-benzoxazolinone (**3**), which was the most effective compound. The length of *O. crenata* radicle treated with 2-benzoxazolinone (**1**) at doses ≥10 μg/mL was reduced by more than 97% of the radicle length in the positive controls, having barely emerged through the seed coat (Figure 3). The hydroxycinnamic acids inhibition followed a linear equation in the case of *p*-coumaric and ferulic acid; however, caffeic acid induction followed an exponential equation in which the strongest reduction (44% radicle length redution) was achieved by the lowest concentration of 1 μg/mL, but further increases in the doses did not lead to further growth inhibition levels. Benzoic and coumalic acids, gramine, L-tryptophan, and scopoletin also caused a significant inhibition best fitted to linear equations. The regression equations predicted 1.8, 1.5, and 1.3 μg/mL as the respective concentration required for (**1**), (**2**), and (**3**) to inhibit the growth of parasitic radicle by 50% (IC_{50}). In the cases of coumalic acid and L-tryptophan, IC_{50} was predicted to be a concentration of 2.9 μg/mL by their respective regression equations. The IC_{50} for *p*-coumaric acid, ferulic acid, and scopoletin were 13, 11, and 11 μg/mL, respectively. Caffeic acid, benzoic acid, and gramine did not reach the IC_{50} with the experimental doses used in this work, but the maximum growth inhibition observed was 49, 42, and 26% of the maximimum growth reached with their respective controls.

Inhibitory effects on *Orobanche* development by allelopathic crops have been suggested before, but little was known about the responsible metabolites. Inhibition of *O. ramosa* germination was described in the wild tomato accession *Lycopersicum pennellii*, suggesting that it was due to an excess of unidentified stimulatory substances.[31] Allelopathy by inhibition of *O. crenata* seed germination was suggested as the mechanism responsible for the reduction of parasitism in legumes intercropped with fenugreek.[32] Trigoxazonane was identified in fenugreek as an active metabolite involved in the allelopathy against *O. crenata*.[33] Intercrops with berseem clover[34] or with cereals[12] reduced *O. crenata* parasitism in legumes, but the responsible metabolites have not been studied so far. Inhibition of development of *Striga* haustoria, although not of seed germination, has been reported in intercropping with *Desmodium uncinatum*.[35] Both germination stimulants (uncinanone B) and some inhibitory metabolites (uncinanone C and isoschaftoside) have been identified in *D. uncinatum*.[36,37]

2-Benzoxazolinone (**1**) is known to interfere with germination and early growth of several vegetable crops,[16] which is in agreement with our findings that (**1**) inhibits (over 30%) *O. crenata* seed germination. In addition, we tested two metabolites showing two different substituent groups at C-6 of (**1**). When a benzyloxy group was located at the C-6 position as in (**2**), the inhibitory effect of (**1**) in *O. crenata* germination was lost; however, when at the same position of the benzene ring was substituted by a chloroacetyl group as in (**3**), the inhibitory effect of (**1**) was greatly enhanced. The comparison between the resonance structures of these two 6-substituted derivatives of (**1**) (Supporting Information Figure S1) showed a negative charge on the aromatic ring and a positive charge on the oxazolinone nitrogen. In particular, the structure is greatly stabilized by the presence of the electron-withdrawing chloroacetyl group attached to the negatively charged carbon of the aromatic ring. The same structure is destabilized by the presence of the electron-donating benzyloxy group attached to the same carbon.

O. crenata germination was not influenced by the rest of compounds tested in this work except for scopoletin, which also had minor inhibitory effect. Scopoletin and other coumarins excreted by sunflower roots have already been reported to have an inhibitory effect on *O. cumana* seed germination.[38]

The view of Hedge and Miller[39] that the inhibitory effect associated with allelopathic plants is often more pronounced on growth of an indicator plant than on its germination was confirmed in this work for *O. crenata*. All the metabolites tested, including those that were ineffective in seed germination, inhibited the growth of *O. crenata* radicle to some degree, although there were large differences in the dose response

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curves. Benzoxazolinones were again very active metabolites interfering with *O. crenata* radicle growth. Their growth inhibitory effect on other plant species has been attributed to the ability to modify auxin action[40] as well as the enhancement of cell wall peroxidase activity, which leads to increased production of H_2O_2 and premature lignification of cell walls.[41] We observed differences in inhibitory effects in the three benzoxazolinones studied, suggesting their influence on the selectivity toward the inhibition of *O. crenata*. Similarly, inhibition of the auxin-induced growth of etiolated coleoptile in maize with benzoxazolinones carrying an alkoxy group at the C-6 position has been reported,[42] but (**1**) without a side chain and (**2**) with benzyloxy ring at C-6 did not show the inhibitory effect.

The three hydroxycinnamic acids tested in this work, caffeic, *p*-coumaric, and ferulic acids, induced reduction of *O. crenata* radicle growth, although clear differences were observed in the dose response. Caffeic acid has been reported to inhibit seed germination and root elongation of several plants, such as *Euphorbia esula*.[17] We observed a strong reduction of *O. crenata* radicle length with the lowest doses of caffeic acid applied, but further increases in the doses were inefficient to achieve further inhibition of *O. crenata* radicle growth. Ferulic acid treatments inhibited the root growth of maize seedlings associated with the inhibition of amylase and maltase. This would reduce the glucose supply from the starch via the amylolytic pathway, influencing respiration and growth.[43] In our work, ferulic and *p*-coumaric acid induced very similar effects in *O. crenata* radicle growth. The exogenous applications of ferulic and *p*-coumaric acid to pea plants caused growth inhibition, carbohydrate and quinate accumulation in leaves, and induction of ethanolic fermentation, effects that are similar to those physiological effects after herbicides that inhibit branched chain amino acid biosynthesis. Ferulic and *p*-coumaric acids could play a role in the mode of action of these herbicides.[44]

We observed a 42% *O. crenata* radicle length reduction at the highest concentration tested of benzoic acid. Benzoic acid had already been reported to reduce the root length of plants such as lettuce[45] and mustard.[21]

We found that coumalic acid did not inhibit the germination of *O. crenata* seeds, but once germinated, radicles exposed to coumalic acid were significantly shorter. Coumalic acid has been reported as having an allelochemical effect in *Ageratum conyzoides* against the early growth of rice.[22] It has been associated with autotoxicity under continuously monocropped peanuts and *Rehmannia glutinousa*.[46]

Gramine has been reported to inhibit the germination and seedling growth of white mustard[25] and to reduce the vegetative growth of chickweed.[24] In our experiments, gramine did not affect the germination of *O. crenata* seeds. The growth of the *O. crenata* radicle was slightly reduced (by 25%) at maximum concentration.

We observed a 50% reduction of *O. crenata* radicle when L-tryptophan was applied at concentrations of 2.9 μg/mL. This is in agreement with reported inhibition of root growth of bindweed when applied at $\geq$2 μg/mL[26] and of cockscomb, cress, and lettuce at $\geq$28 μg/mL.[3]

We found that scopoletin inhibits *O. crenata* radicle growth and causes a darkening of groups of radicle cells (data not shown) potentially due to necrosis. This was observed at concentrations $\geq$7 μg/mL, reaching a maximum of 50−60% of the radicles at $\geq$13 μg/mL. Scopoletin causes the same allelopathic effect, including the necrosis-like reaction of in vitro grown *Cuscuta campestris* seedlings (unpublished results). This is in agreement with earlier reports[38] of inhibitory effects of scopoletin on *O. cumana* seedling growth and on allelopathic effects on weeds[2,4,27] and tobacco, sunflower, and pigweed.[47] Such darkening of radicles was not induced by any of the other studied metabolites.

Demonstration of orobanchicidal effects of allelochemical released by cereals suggests the potential of developing a practical broomrape management strategy using cereals as green manure crops or as intercrops. However, determining the toxicity of specific compounds involved in broomrape suppression is only part of the information needed to optimize this management strategy. Further research is needed to understand the fate of allelochemicals in soil and their use for broomrape control. In addition to determining the concentration of these compounds in soil, an appropriate cultivar containing large concentrations of allelochemicals must be used. For example, the amount of hydroxamic acids varies greatly with plant age, organ, and cultivar, being affected by water stress conditions, high temperatures, and fertilization.[48] Hydroxamic acids do not persist for long periods.[49,50] This degradation in soil is interesting as this avoids the risks of accumulation of toxic compounds in soil. However, this also implies the need to adjust the timing of incorporation that should coincide with the time of germination of *O. crenata* in the soil.

Breeding programs focused on selection of host genotypes for high root exudation levels of germination or radicle growth inhibitors could identify better candidates for intercrops to be used in a control strategy. Allelopathic potential seems to be a quantitative trait of complex inheritance and highly influenced by environment and, thus, difficult to breed. Allelophatic potential of cereal crops can be enhanced by classical or marker-assisted breeding provided that genetic variation and proper screening methods exist. For instance, significant variation in the level of scopoletin has been reported within oat accessions, with accession PI-2666281 producing the highest scopoletin content and showing the highest inhibition of the growth of the tested Brassica weed.[2] Such variation in the content of various allelochemicals and in allelopathic activity exists within various cereal crops cultivars, which may allow for the selection of more allelopathic cultivars.[1] In addition, understanding the genes responsible for the biosynthesis and release of allelochemicals will allow their incorporation in high-yielding cultivars by genetic manipulation.

■ ASSOCIATED CONTENT

Supporting Information

Table S1: Regression analysis of dose response of *Orbanche crenata* radicle growth to allelochemicals tested. Figure S1: Stabilization and destabilization effects induced by electron-withdrawing or electron-donating groups linked at C-6 of 2-benzoxazolinone. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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