



Plant cell responses to allelopathy: from oxidative stress to programmed cell death

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Abstract

Allelopathy is a plant–plant interaction in which one plant releases biologically active compounds that have negative effects on the fitness of the target plant. The most pronounced effects are inhibition of seed germination and growth of neighboring plants. The roots of these plants are in contact with the allelochemicals released into the soil, as the primary target of the allelopathic action. To date, the best documented allelopathic activities relate to some weeds and invasive alien plants that show rapid spread and successful growth. A better understanding of the mechanisms of allelopathy will help to improve crop production and to manage and prevent plant invasions. At the cellular level, allelochemicals induce a burst of reactive oxygen species in the target plants, which leads to oxidative stress, and can promote programmed cell death. Lipid peroxidation and cell membrane changes, protein modifications, and increased protease activities are the early signs of cell damage. When enzymatic and nonenzymatic antioxidants cannot scavenge reactive oxidants, this can result in hydrolytic or necrotic degradation of the protoplast. Cell organelles then lose their integrity and function. In roots, the structure and activity of the apical meristem are changed, which affects root growth and water absorption. Such allelopathically active compounds might thus be applied to control and manage weeds and invasive plants in a more sustainable way, to reduce chemical pollution.

Keywords Allelopathy · Oxidative stress · Plant proteases · Programmed cell death · Reactive oxygen species · Ultrastructure

Introduction

Allelopathy is a biochemical interaction between plants in which an allelopathic plant produces secondary compounds (allelochemicals) and releases them mainly into the rhizosphere, which leads to the suppression of germination, growth and development of neighboring plants (Gniazdowska and Bogatek 2005; Mushtaq et al. 2020; Schandry and Becker 2020). Allelopathic compounds can be stored in a variety of plant tissues, including roots, rhizomes, bark, stems, leaves, and flowers. These bioactive secondary compounds can be

released from allelopathic plants into the environment as root exudates, volatile compounds, or leaf decomposition material (Weir et al. 2004; Farooq et al. 2020). The concentration of allelochemicals in plant tissues can change seasonally (Chen et al. 2013; Frantík et al. 2013) and according to geographic region (Fan et al. 2010; Chen et al. 2013), due to different ecological conditions.

The phytotoxic impact of one plant on another plant that is growing in the same soil has been observed since ancient times (Patni et al. 2018). It was soon recognized that some plants can alter the physical properties of the soil, which consequently affects crop productivity. Therefore, crop rotation was developed to minimize the impact of phytotoxic substances released into the soil by some plants (Shahzad et al. 2016). However, it was not until the early twentieth century that the term allelopathy was first used, by the Austrian scientist Hans Molisch (Gniazdowska and Bogatek 2005; Patni et al. 2018). The term comes from two Greek words: *allelon* meaning “to each other”, and *pathos* meaning “to suffer”. Molisch described allelopathy only as a negative effect of one

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plant on another plant via chemical compounds released into the environment. In 1984, the American scientist Elroy Leon Rice extended the definition of allelopathy to both the positive and negative effects one plant can have on another plant or on microorganisms in the vicinity (Gniazdowska and Bogatek 2005; Patni et al. 2018). However, today, allelopathic studies mainly focus on its negative aspects.

Over the last few decades, the study of allelopathy has become of great interest in agronomy. Allelopathic weed control can serve as a useful “green” alternative to the widespread use of large quantities of chemical herbicides in agriculture, to thus contribute to the reduction of soil and water pollution (Cheng and Cheng 2015; Patni et al. 2018; Farooq et al. 2020). Various allelopathic crop species can be effectively used to control weeds in the field. Rye (*Secale cereale*), sunflower (*Helianthus annuus*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), alfalfa (*Medicago sativa*), maize (*Zea mays*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), soybean (*Glycine max*), and sorghum (*Sorghum bicolor*) have shown strong allelopathic potential for the suppression of weeds (Farooq et al. 2020).

In addition to planting allelopathic crops in the field, allelochemicals can be isolated from these plants and used commercially as bioherbicides (Cheng and Cheng 2015; Ghimire et al. 2019). One example is the highly active allelochemical coumarin, which was reported to significantly alter the root system of *Arabidopsis thaliana* at the concentration 10^{-4} M: primary root was more than 50% shorter, while the number of lateral roots increased for 85% (Lupini et al. 2014). On the other hand, many weeds also have such “allelopathic weapons” that can inhibit the growth of nearby weeds (Farooq et al. 2020), as well as that of some crop species (Majeed et al. 2012; Mitić et al. 2018; Mustafa et al. 2019).

To date, little is known about the structural, physiological, and molecular mechanisms behind allelopathic interactions. Even less is known about the relationship between allelopathy and programmed cell death (PCD). The plant response to allelopathic stress is not (always) “survive or die”, as it involves multiple defense mechanisms that allow plants to respond in this complex network of plant–plant allelopathic interactions. Therefore, in this review, we describe the initial plant responses to allelopathic compounds that lead to the disruption of the reactive oxygen species (ROS)–antioxidant balance, and that are accompanied by changes related to the physiological status of the plant (Fig. 1). When allelopathic stress is too high or the exposure to allelopathic compounds is too long, the activation of proteases in plant cells begins, and PCD can occur. This mechanism results in the affected cells or tissues being “sacrificed”, so that the rest of the plant can survive, and it gradually leads to changes in the root anatomy and the plant growth. In this review, we attempt to

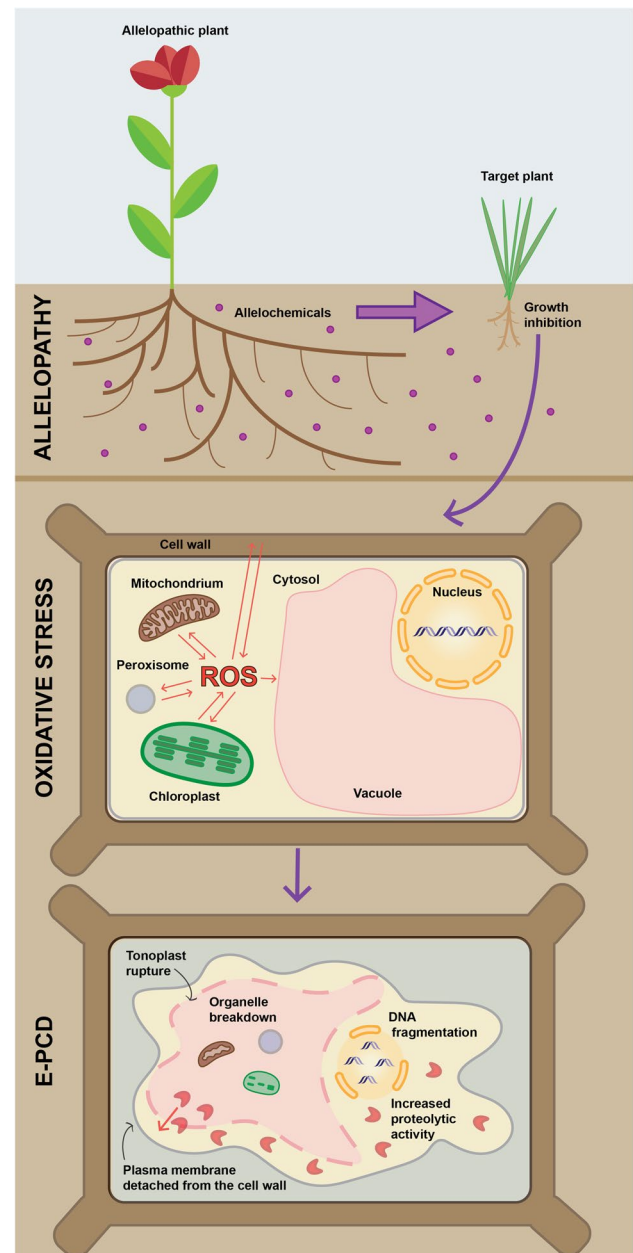


Fig. 1 Allelopathy is a plant–plant interaction in which an allelopathic plant releases allelochemicals into the rhizosphere to suppress the growth of neighboring plants. At the cellular level, allelochemicals can induce the production and accumulation of reactive oxygen species (ROS), which leads to oxidative stress, degradation of macromolecules, and disruption of organelles through increased proteolytic and nucleolytic activities, and finally to environmentally induced programmed cell death (E-PCD). Figure designed by K. Šoln

bridge the gap between the current knowledge of allelopathic stress and PCD. In addition, we suggest some guidelines for further studies in the field of allelopathy.

Allelopathy: inhibition of germination and growth

Inhibition of germination and early growth of seedlings is commonly observed as the main allelopathic effect (for review, see Gniazdowska and Bogatek 2005) (Tables 1 and 2). Many studies have focused on seedlings exposed to allelopathic compounds over short periods of time, as usually between 2 and 14 days (Lara-Núñez et al. 2006; Soltys et al. 2011, 2012; Ciniglia et al. 2015; Tucker Serniak 2016; Yan et al. 2016; Araniti et al. 2018). Fewer studies have focused on growth over more than 30 days (Murrell et al. 2011; Qu et al. 2021). Indeed, seedlings are more susceptible to the deleterious effects of allelopathic compounds than older plants (Dolenc Koce and Šoln 2018), as they are more sensitive to various biotic and abiotic stressors (Weir et al. 2004).

Although some allelopathic compounds can completely inhibit the germination of various weeds (e.g., sorgoleone; Uddin et al. 2014), exposure of germinating seeds to extracts that contain mixtures of allelochemicals only delay germination, as was shown for a rhizome extract of knotweed (*Fallopia*) that contained epicatechin, emodin, and resveratrol. After 3 days, the germination of radish seeds treated with the 10% extract of *F. japonica* was inhibited for 5% and of *F. ×bohemica* for 9%, while after 7 days their germination rate was in the control range (Šoln et al. 2021a). Allelopathic compounds can also affect the growth of seedlings. Shorter roots have been observed in almost all short-term treatments. For example, treatment with coumarin umbelliferone from *Stellaria chamaejasme* inhibited root growth of lettuce (*Lactuca sativa*) seedlings for 71% after 48 h of exposure (Yan et al. 2016). In addition, the study of Araniti et al. (2018) demonstrated that 14 days of exposure to rosmarinic acid suppressed the primary root length of *Arabidopsis* seedlings ($ED_{50} = 175 \mu\text{M}$) and reduced the formation of lateral roots ($ED_{50} = \text{about } 53 \mu\text{M}$). Also, 14-day exposure of radish seedlings to (–)-epicatechin resulted in more than 30% shorter roots compared to the untreated seedlings (Tucker Serniak 2016). Similarly, plant extracts suppress root growth, as showed for 7-day old radish seedlings exposed to aqueous extracts of *Fallopia japonica* and *F. ×bohemica* rhizomes, which at 10% concentration had up to 65% shorter primary roots (Šoln et al. 2021a). On the other hand, the root width was for 85% and 72% wider, respectively (Šoln et al. 2021b).

The effect of shorter roots can be the consequence not only of direct inhibition of root growth and development due to the allelochemicals, but also of delayed germination and postponed growth. For this reason, Soltys et al. (2012) first germinated *Arabidopsis* seeds for 36 h, and

then transferred only equally sized seedlings with a 5-mm-long radicle to the medium containing the allelochemical cyanamide. This experimental set-up successfully divided the effects on germination and on seedling growth. Furthermore, extreme caution must be exercised when designing experiments for short-term exposure, as some physiological changes (e.g., changes in enzymatic activities) might be part of the developmental processes, and thus might not be related to plant defense mechanisms. All of the biochemical and physiological changes should, therefore, be interpreted in relation to the control group.

However, by longer duration of growth and exposure to the extracts, the inhibitory effects are not so strong, especially if the test plants grow in the soil. African marigold (*Tagetes erecta*) plants, which grew for 60 days in pots with soil and were watered daily with 10 mg/mL *Rhus typina* root extract had at the end for 13% shorter primary roots when compared to control plants (Qu et al. 2021).

Sensitivities to allelochemicals often vary depending on the concentrations of the allelochemicals or the dilutions of the extracts used. In nature, the concentrations of allelochemicals in the rhizosphere are low, as usually range from 10^{-5} to 10^{-6} M (for review, see Gniazdowska and Bogatek 2005). Under laboratory conditions, several concentrations of allelochemicals and/or extract dilutions are used to evaluate concentration-dependent effects and to determine minimal inhibition concentrations. The most pronounced (negative) effects are often seen for treatments with the highest concentrations, as was shown by the exposure of Mexican marigold (*Tagetes erecta*) to root extracts of the invasive stag horn (*Rhus typina*), where the marigold developed significantly shorter roots in treatments with 10-mg/mL extract (Qu et al. 2021). Inhibition of root growth is also associated with various ultrastructural changes in the root tip and in cell division, as was shown for radish seedlings treated with rhizome extracts of Japanese (*Fallopia japonica*) and Bohemian (*F. ×bohemica*) knotweed (Šoln et al. 2021b). Soltys et al. (2011) showed that the high coumarin concentration of 10 mM resulted in the disappearance of the mitotic spindle and phragmoplast in the root tip cells of onion (*Allium cepa*), thus blocking root growth and elongation.

Unintended changes in concentrations of allelochemicals can also result from adding more medium or water volume during experiments to prevent the drying of roots on filter paper due to evaporation. Indeed, Tucker Serniak (2016) grew radish seedlings for 14 days while applying the allelochemicals every 4 days, and Qu et al. (2021) irrigated plants with 20 mL of extracts every day for 60 days. In these cases, the total concentrations of the allelochemicals in the experimental substrate will most likely have increased, and thus this needs to be monitored. In contrast, when only the control solution (i.e., distilled water) is used for watering, a hormesis effect can be observed (Dolenc Koce and Šoln 2018) that might result in

Table 1 Allelopathic compounds involved in oxidative stress and programmed cell death, and their effects on their target plant species

Allelopathic compound	Plant species	Effect	Target tissue	Reference
Oxidative stress				
Benzoic acid	<i>Arabidopsis thaliana</i>	↑ ROS production	Root	Zhang et al. (2018)
2-Benzoxazolinone	Mung bean (<i>Phaseolus aureus</i>)	↑ H ₂ O ₂ level; ↑ lipid peroxidation; ↑ catalase activity; ↑ ascorbate peroxidase activity; ↑ guaiacol peroxidase activity; ↑ glutathione reductase activity	Root	Batish et al. (2006)
Cinnamic acid	Cucumber (<i>Cucumis sativus</i>)	↑ H ₂ O ₂ and O ₂ ^{•-} levels; ↑ lipid peroxidation; ↓ activity of H ⁺ -ATPase; impact on NADPH oxidase; ↑ catalase activity; ↑ guaiacol peroxidase activity; ↑ superoxide dismutase activity	Root	Ding et al. (2007)
Cyanamide	Onion (<i>Allium cepa</i>)	↑ H ₂ O ₂ and O ₂ ^{•-} levels	Root	Soltys et al. (2011)
Diallyl disulfide	Tomato (<i>Lycopersicon esculentum</i>)	↑ Catalase activity; ↑ peroxidase activity; ↑ superoxide dismutase activity; upregulation of genes involved in glutathione metabolism	Root	Cheng et al. (2016)
Juglone, plumbagin	Tobacco BY-2 cell cultures	↑ ROS production; impact on dehydrogenases and oxidoreductases; disruption of mitochondrial electron transport	Cell culture	Babula et al. (2009)
Juglone	Lettuce (<i>Lactuca sativa</i>)	↑ ROS production; ↑ lipid peroxidation	Root	Babula et al. (2014)
Pyrogalllic acid	Cyanobacteria <i>Microcystis aereginosa</i>	↑ ROS production	/	Lu et al. (2017)
Rosmarinic acid	<i>Arabidopsis thaliana</i>	↑ H ₂ O ₂ and O ₂ ^{•-} level; ↑ lipid peroxidation; disruption of mitochondrial membrane potential; ↓ catalase activity; ↓ superoxide dismutase activity	Root	Araniti et al. (2018)
Umbelliferone	Lettuce (<i>Lactuca sativa</i>)	↑ H ₂ O ₂ level; ↑ lipid peroxidation; ↑ proline concentration	Root	Yan et al. (2016)
Biochemical changes during programmed cell death				
H ₂ O ₂	Rice (<i>Oryza sativa</i>)	Upregulation of <i>OsVPE2</i> and <i>OsVPE3</i>	Leaf	Deng et al. (2011)
Juglone	Lettuce (<i>Lactuca sativa</i>)	↑ Intracellular Ca ²⁺ ions	Root tip	Babula et al. (2014)
Pyrogalllic acid	Cyanobacteria <i>Microcystis aereginosa</i>	↑ Caspase-3-like activity	/	Lu et al. (2017)
Structural changes				
Benzoic acid	<i>Arabidopsis thaliana</i>	↓ Size of meristem zone; ↓ cell number	Root apical meristem	Zhang et al. (2018)
2(3 <i>H</i>)-Benzoxazolinone; 2,4-dihydroxy-1,4(2 <i>H</i>)-benzoxazin-3-one	Cucumber (<i>Cucumis sativus</i>)	Vacuole enlargement	Root cap	Burgos et al. (2004)
Cinnamic acid	Cucumber (<i>Cucumis sativus</i>)	↓ Cell viability	Root	Ding et al. (2007)
Cyanamide	Onion (<i>Allium cepa</i>)	↓ Mitotic index; ↓ cell viability; modification of the cell cycle; cytoskeleton alterations	Root tip	Soltys et al. (2011)

Table 1 (continued)

Allelopathic compound	Plant species	Effect	Target tissue	Reference
Cyanamide	Tomato (<i>Lycopersicon esculentum</i>)	↓ Mitotic index	Root tip	Soltys et al. (2012)
Coumarin	Radish (<i>Raphanus sativus</i>)	↑ Vacuolization	Root apical meristem	Aliotta et al. (1993)
Farnesene	<i>Arabidopsis thaliana</i>	↓ Size of meristem zone; ↓ cell number	Root apical meristem	Araniti et al. (2017)
H ₂ O ₂	Rice (<i>Oryza sativa</i>)	Destroyed tonoplast	Leaf	Deng et al. (2011)
Juglone	Lettuce (<i>Lactuca sativa</i>)	↓ Mitotic index	Root tip	Babula et al. (2014)
Juglone; plumbagin	Cultures of tobacco BY-2 cells	↓ Cell viability; ↓ cell number	Cell culture	Babula et al. (2009)
Pyrogallol acid	Cyanobacteria <i>Microcystis aureginosa</i>	↑ Cell death	/	Lu et al. (2017)
Rosmarinic acid	<i>Arabidopsis thaliana</i>	↑ Cell death	Root	Araniti et al. (2018)
Umbelliferone	Lettuce (<i>Lactuca sativa</i>)	↓ Cell viability	Root	Yan et al. (2016)

Legend: ↑, increased; ↓, decreased; /, no data

gradually decreasing concentrations of allelochemicals during an experiment. For this reason, we propose short exposure periods of 3 to 5 days when studying direct effect of allelochemical, such that only a single application of the allelopathic substance at the beginning can be carried out without the risk of drying of the plants. In addition, with a single application the concentration remains the same throughout the experiment, without any dramatic decreases or increases. For longer allelopathy studies, the use of vermiculite or soil is mandatory, as these substrates are better at retaining water and preventing evaporation than filter paper. The final concentration of allelochemicals would also provide valuable data in the interpretation of results.

The last important point before setting up an experiment is the selection of the plants to be tested as the sensitivities to allelochemicals often vary according to the plant species. The 1% aqueous leachate of *Callicarpa acuminata* suppressed root growth of tomato (*Lycopersicon esculentum*) seedlings for 47% but had no effect on the root of bean (*Phaseolus vulgaris*) and maize (*Zea mays*) seedlings (Cruz-Ortega et al. 2002). Exposure to 0.25-mM cinnamic acid decreased root and shoot biomass of cucumber (*Cucumis sativus*) seedlings for 53% and 43%, respectively, but had only little effect on figleaf gourd (*Cucurbita ficifolia*) seedlings (Ding et al. 2007). In addition to *Arabidopsis thaliana* as a standard model plant (Araniti et al. 2018; Zhang et al. 2018), we recommend radish, not only for its sensitivity to allelochemicals, but also for its rapid growth and robust primary root. Unlike monocot maize, which takes 3 to 4 days to germinate, radish germination is faster, as most seeds germinate at around 24 h (Dolenc Koce and Šoln 2018).

Oxidative stress: an initiation of allelopathy-triggered response

Reactive oxygen species: a bridge between allelopathy and programmed cell death

Reactive oxygen species are highly reactive toxic by-products of aerobic metabolism. The main ROS are the superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂), singlet oxygen (¹O₂), and the hydroxyl radical (OH[•]) (Gechev et al. 2006; Das and Roychoudhury 2014; Gniazdowska et al. 2015). ROS have dual functions in plants. At low concentrations, they act as signaling molecules to regulate growth and developmental processes (Gechev et al. 2006), such as the size of the root meristem (Yamada et al. 2020), and to activate plant defense mechanisms, and, therefore to increase, plant tolerance (Hayat et al. 2016). However, at high concentration, ROS can oxidize macromolecules in plant cells, and thus contribute to their degradation (Gechev et al. 2006; Das and Roychoudhury 2014). The ROS levels in cells can increase dramatically during different conditions of environmental stress, such as drought, high temperatures (Liu et al. 2019), heavy metals (Tamás et al. 2017), and pathogen infections (Rossi et al. 2017).

Over the last decade, many studies have suggested that exposure to allelochemicals can lead to excessive bursts of ROS in the target plants (Gniazdowska et al. 2015). Also, oxidative damage might be crucial for allelopathic toxicity to suppress the growth of the target plant (Araniti et al. 2018). For example, it was shown with dihydroethidium

Table 2 Allelopathic extracts involved in oxidative stress and programmed cell death and their effects on their target plant species

Allelopathic extract	Plant species	Effect	Target tissue	Reference
Oxidative stress				
Aqueous leachate of cucurbit (<i>Sicyos deppelii</i>)	Tomato (<i>Lycopersicon esculentum</i>)	↑ H ₂ O ₂ and O ₂ ^{•-} level; ↑ lipid peroxidation; impact on NADPH oxidase; ↑ catalase activity; ↑ superoxide dismutase activity	Root	Lara-Núñez et al. (2006)
Extract of coffee senna (<i>Senna occidentales</i>)	Native Brazilian plants	↑ H ₂ O ₂ level; ↑ lipid peroxidation	Root	da Silva and Vieira (2019)
Extract of deadly carrot (<i>Thapsia garganica</i>)	Lettuce (<i>Lactuca sativa</i>)	↑ Proline concentration; ↑ flavonoids; ↑ flavanols; ↑ carotenoids; ↑ tannins; ↑ proanthocyanidins	Leaf, root	Jmii et al. (2020)
Extract of Japanese knotweed (<i>Fallopia japonica</i>); extract of Bohemian knotweed (<i>Fallopia × bohémica</i>)	Radish (<i>Raphanus sativus</i>)	↑ Total antioxidative capacity	Root	Dolenc Koce and Šoln (2018)
Extract of nettle leaf goosefoot (<i>Chenopodium murale</i>)	Wheat (<i>Triticum aestivum</i>)	↑ H ₂ O ₂ level	Root	Mitić et al. (2018)
Extract of peppermint (<i>Mentha × peppermint</i>)	Radish (<i>Raphanus sativus</i>)	↑ Catalase activity; ↑ ascorbate peroxidase activity; ↑ peroxidase activity	Seedling	Mahdavikia et al. (2017)
Eurasian watermilfoil (<i>Myriophyllum spicatum</i>)	Cyanobacterium <i>Microcystis aureginosa</i>	↑ ROS production	/	He et al. (2016)
Walnut (<i>Juglans regia</i>) husk washing water	Maize (<i>Zea mays</i>)	↑ H ₂ O ₂ level; ↑ lipid peroxidation; ↑ superoxide dismutase activity	Root	Cimiglia et al. (2015)
Biochemical changes during programmed cell death				
Eucalyptus (<i>Eucalyptus globulus</i>) trees litter	Soybean (<i>Glycine max</i>)	Upregulation of five papain-like cysteine protease genes and their inhibitors	Leaf	Abdelmigid and Morsi (2017)
Eurasian watermilfoil (<i>Myriophyllum spicatum</i>) co-culture	Cyanobacteria <i>Microcystis aureginosa</i>	↑ Caspase-3-like activity	/	He et al. (2016)
Walnut (<i>Juglans regia</i>) husk washing water	Maize (<i>Zea mays</i>)	DNA fragmentation; ↑ DNase activity; upregulation of <i>metacaspase type II gene</i>	Root tip	Cimiglia et al. (2015)
Structural changes				
Extract of cagaíta (<i>Eugenia dysenterica</i>)	Radish (<i>Raphanus sativus</i>)	↑ Vacuolization	Root cap	Pereira et al. (2017)
Extract of cucurbit (<i>Sicyos deppelii</i>)	Bottle gourd (<i>Cucurbita ficifolia</i>); bean (<i>Phaseolus vulgaris</i>)	Vacuole enlargement; swollen mitochondria with dilated cristae; plasma membrane detached from the cell wall; irregular shape of nucleus	Root cap	Cruz-Ortega et al. (1998)
Extract of nettle leaf goosefoot (<i>Chenopodium murale</i>)	Wheat (<i>Triticum aestivum</i>)	Thickening of the cell wall	Root cap	Mitić et al. (2018)
Eurasian watermilfoil (<i>Myriophyllum spicatum</i>) co-culture	Cyanobacteria <i>Microcystis aureginosa</i>	↑ Cell death	/	He et al. (2016)

Legend: ↑, increased; ↓, decreased; /, no data

staining that the allelochemicals juglone and plumbagin increased ROS production in cultured tobacco BY-2 cells in the concentration-dependent doses at time course (Babula et al. 2009). Similar effects were reported for lettuce roots treated with juglone (Babula et al. 2014) and in *Arabidopsis* roots treated with benzoic acid (Zhang et al. 2018). Many allelopathic compounds can increase H₂O₂ levels in target plants: 0.5-mM 2-benzoxazolinone (BOA) increased the H₂O₂ levels in the roots of mung bean (*Phaseolus aureus*) for 25%, while 5 mM increased H₂O₂ accumulation for nearly two-fold over their representative control (Batish et al. 2006); an extract of coffee senna (*Senna occidentalis*) increased H₂O₂ levels in the roots and leaves of several native Brazilian plants (da Silva and Vieira 2019); an extract of nettle leaf goosefoot (*Cenopodium murale*) increased H₂O₂ levels in wheat roots by > 60% (Mitić et al. 2018); and rosmarinic acid induced synthesis of O₂^{•-} and H₂O₂ in the roots of *Arabidopsis* seedlings (Araniti et al. 2018). Increased levels of these last two ROS here were also detected in the roots of onion (*Allium cepa*) after exposure to cyanamide: interestingly exposure to 10-mM concentration for 1 day resulted in more than 2-times higher concentration of H₂O₂ (153-mM H₂O₂) in roots than after 6 days (65 mM) (Soltys et al. 2011). In tomato (*Lycopersicon esculentum*) treated with an aqueous leachate of cucurbit (*Sicyos deppei*), the H₂O₂ concentration in seeds increased for 77% after 24 h (Lara-Nuñez et al. 2006), and in cucumber (*Cucumis sativus*) treated with 0.25-mM cinnamic acid for 3 days the production of O₂^{•-} and H₂O₂ in roots increased by 179% and 74%, respectively (Ding et al. 2007).

Antioxidative system: adaptive strategy for allelopathic stress

The plant cell defense mechanism against the overproduction of ROS is the complex ROS-scavenging system of antioxidants that is responsible for redox homeostasis in cells. As soon as the level of ROS increases, they are quickly detoxified by the antioxidants (Gechev et al. 2006; Das and Roychoudhury 2014). Therefore, an increase in antioxidants indicates that a plant is in a stress situation; i.e., in an allelopathic interaction (Lara-Nuñez et al. 2006). For example, an extract of peppermint (*Mentha × peppermint*) increased the activity of the antioxidative enzyme catalase, ascorbate peroxidase, and peroxidase in radish seedlings (Mahdavi et al. 2017). These enzymes are involved in H₂O₂ detoxification (Das and Roychoudhury 2014). The catalase is only active at high concentrations of H₂O₂ in the cell, while low H₂O₂ concentrations are detoxified by other peroxidases (Gechev et al. 2006). The activities of catalase and ascorbate peroxidase increased for nearly 6.6-fold and 8.5-fold in the roots of mung bean treated with 5-mM BOA (Batish

et al. 2006), as for catalase and peroxidase in tomato roots exposed to the garlic allelochemical diallyl disulfide after 24-h and 48 h-exposure (Cheng et al. 2016), and the increase of catalase activity for 40% and guaiacol peroxidase activity for 38% in cucumber roots treated with 0.25-mM cinnamic acid (Ding et al. 2007). The antioxidative response is often delayed according to the ROS production (Gechev et al. 2006; Das and Roychoudhury 2014). Exposure to an aqueous leachate of cucurbit first increased H₂O₂ levels in the roots of tomato, with the maximum after 24 h of exposure. At this time, the activity of catalase was low. However, the early production of H₂O₂ stimulated the catalase activity, which then reached its maximum 72 h after the treatment (Lara-Nuñez et al. 2006).

A similar response was detected in maize roots treated with walnut husk washing waters, where the enzyme activity of superoxide dismutase was low after 3 h, and then increased slightly after 6 and 12 h of exposure, reaching an increase of threefold after 24 h (Ciniglia et al. 2015). Superoxide dismutase is responsible for the degradation of toxic O₂^{•-} to H₂O₂ and water (Das and Roychoudhury 2014). An increased activity of superoxide dismutase (for 58%) was also observed in tomato roots in the first 24 h after the treatment with a cucurbit leachate, which correlated to lower O₂^{•-} concentration in the roots (Lara-Nuñez et al. 2006). In cucumber roots exposed for 3 days to 0.25-mM cinnamic acid the activity of superoxide dismutase increased for 16% (Ding et al. 2007) and for 17% in roots of tomato seedlings treated with diallyl disulfide for 24 h (Cheng et al. 2016).

Other antioxidative enzymes that have crucial roles in ROS homeostasis include monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase, which react with O₂^{•-} and OH[•] (Das and Roychoudhury 2014). For the roots of 7-day-old mung bean seedlings, exposure to 0.5-mM BOA nearly doubled the activity of glutathione reductase, while 5-mM concentration increased the activity for 22-fold compared to control (Batish et al. 2006).

As well as the enzymatic antioxidants, there are the non-enzymatic antioxidants, the most common of which are ascorbic acid, reduced glutathione, α-tocopherol, carotenoids, flavonoids, and proline (Gechev et al. 2006; Das and Roychoudhury 2014). A leaf extract of deadly carrot (*Thapsia garganica*) increased the activity of antioxidative enzymes in leaves and roots of lettuce as well as the levels of the non-enzymatic antioxidants proline, flavonoids, flavanols, carotenoids, and other protective secondary compounds (e.g., tannins, proanthocyanidins) (Jmii et al. 2020). Cheng et al. (2016) showed that exposure to the garlic allelochemical diallyl disulfide favors upregulation of several genes involved in glutathione metabolism in tomato roots. In addition, a 5% aqueous extract of the invasive Japanese knotweed (*Fallopia japonica*) leaves increased the total antioxidative capacity in the roots of a week-old radish seedlings

for about 3% (Dolenc Koce and Šoln 2018), and 400- μ M coumarin umbelliferone increased the proline levels in lettuce seedlings from 49 to 98 μ M/g FW after 24 h of exposure (Yan et al. 2016).

Successful detoxification of ROS by the antioxidative system results in the decrease of ROS levels in a time-dependent manner. As an example, benzoic acid increased ROS levels in the roots of *Arabidopsis* seedlings over the first 12 h of exposure, but later, these ROS levels decreased due to the activation of the antioxidative system (Zhang et al. 2018). This system then scavenged the majority of ROS in the cells to thus prevent further cell damage (Gechev et al. 2006; Das and Roychoudhury 2014; Gniazdowska et al. 2015). A similar response was seen in maize roots treated with walnut husk washing water (Ciniglia et al. 2015). This adaptive strategy can enable the target plant to survive despite the allelopathic stress (Gniazdowska et al. 2015). However, intense or long allelopathic exposure can lead to over production and accumulation of ROS in the target plants; therefore, once the ROS levels surpass a threshold level, the antioxidative system cannot detoxify ROS anymore, resulting in oxidative stress (Das and Roychoudhury 2014). Inhibition of the activity of ROS scavengers was observed in *Arabidopsis* seedlings, where the activity of catalase and superoxide dismutase decreased after 7 days for 40% and 35% and after 14 days of exposure to rosmarinic acid for 69% and 55% (Araniti et al. 2018). At this time, the ROS levels increased dramatically and initiated a hypersensitive response, which led to PCD (Gechev et al. 2006).

Allelochemicals and oxidative damage

Excessive production and accumulation of ROS in plant cells can damage their macromolecules, such as lipids, proteins, and DNA. ROS often interact with the lipids of the plasma membrane, which can result in a radical chain reaction known as lipid peroxidation, which leads to the destruction of the plasma membrane (Das and Roychoudhury 2014). Lipid peroxidation is often accompanied by allelopathic stress, as has been shown by exposure to BOA (Batish et al. 2006), rosmarinic acid (Araniti et al. 2018), cinnamic acid (Ding et al. 2007), juglone (Babula et al. 2014), and umbelliferone (Yan et al. 2016). Lipid peroxidation in treated plants can also be increased by various plant preparations, such as an extract of coffee senna (da Silva and Vieira 2019), a leachate from cucurbit (Lara-Nuñez et al. 2006), and walnut (*Juglans regia*) husk washing water, as a by-product of walnut processing (Ciniglia et al. 2015). This is especially seen in the roots, as these are the first tissue to come into contact with the allelochemicals (Batish et al. 2006). Increased membrane peroxidation decreases the activity of the plasma membrane H^+ -ATPase, and, therefore, leads to cell death, as was shown for cucumber roots exposed

to cinnamic acid (Ding et al. 2007). The mitochondrial membranes can also undergo ROS attack, as the mitochondria are one of the sites of ROS generation (Das and Roychoudhury 2014). An example here is exposure to rosmarinic acid, which strongly inhibited mitochondrial membrane potential in roots of 7-day-old and 14-day-old *Arabidopsis* seedlings (Araniti et al. 2018).

ROS can also result in the oxidation of proteins, which will affect their various modifications, such as carboxylation, nitrosylation, glutathionylation, and formation of disulfide bonds, thus preventing their correct functioning in plant cells (Das and Roychoudhury 2014). As an example here, the naphthoquinones juglone and plumbagin have effects on the enzymatic activities of dehydrogenases and oxidoreductases of tobacco BY-2 cells (Babula et al. 2009). Both of these enzymes have important roles in mitochondrial electron transport. However, naphthoquinones can enter plant cells, and as electron acceptors, they can disrupt mitochondrial electron transport (Babula et al. 2009). Treatment with the allelochemical cinnamic acid increased the activity of plasma membrane enzyme NADPH oxidase in cucumber seedlings (Ding et al. 2007), while a cucurbit extract inhibited its activity in tomato roots for more than half after 72 h, which may be related to membrane damage caused by the formation of ROS (Lara-Nuñez et al. 2006). The oxidized proteins quickly become targets for proteolytic enzymes (Das and Roychoudhury 2014).

Overaccumulation of ROS can also result in DNA damage (Das and Roychoudhury 2014), DNA fragmentation (Babula et al. 2014), and other nucleolytic activities which are further discussed below. Allelopathy-triggered oxidative stress also results in DBA decreased cell viability (Ding et al. 2007; Babula et al. 2009; Yan et al. 2016) and cell number (Šoln et al. 2021b) in target plants (Babula et al. 2009), thus reducing plant growth (Soltys et al. 2012; Araniti et al. 2018).

Allelopathy and cell death

Types of plant cell death

Just like in animals, cells in plants also die. This death, however, can be a consequence of either “accidental cell death,” which is caused by severe damage, or “regulated cell death,” which occurs as a controlled response that eliminates specific cells under developmental or environmental stimuli (Galluzzi et al. 2018). Although the term regulated cell death was suggested to be used where cell death is controlled at genetic and biochemical levels, in the plant field, the term “programmed cell death” is generally used, irrespective of its developmental or environmental context (Locato and De Gara 2018). Developmental PCD is activated by internal

stimuli, and occurs, for example, during tracheary element differentiation, trichome formation, and female gametophyte differentiation (for review, see Van Hautegeem et al. 2015). Instead, environmental PCD occurs as a response to external stimuli, such as biotic invaders and abiotic stressors (e.g., heat, UV light, drought, and others). One of the best explored cases of environmental PCD in plants is the hypersensitive response. This is a cell death mechanism that can be triggered by pathogens and that functions as a defense strategy to limit the location of an infection, and thus to prevent it from spreading through the whole plant (Salguero-Linares and Coll 2019). Allelopathic substances were also implied to induce cell death, and due to the nature of this trigger, which is not of developmental nature, it can be classified within the subtype of environmental PCD. The current knowledge of the intracellular mechanisms that lead to PCD as a result of allelopathy are briefly discussed below.

Proteolytic activities in allelopathy-driven programmed cell death

Proteases are enzymes that can catalyze the irreversible cleavage of a peptide bond in a substrate protein. The MEROPs database (<http://merops.sanger.ac.uk>) is an integrated information resource of proteases, where they are classified into five main types based on their catalytic mechanisms: cysteine, aspartate, threonine, serine, and metalloproteases (Rawlings et al. 2018). Representatives of all five types of proteases have been shown to be involved in developmental PCD and environmental PCD processes in plants (for review, see Stael et al. 2019). However, only a few allelopathy-related studies have examined proteases at the level of either mRNA or their enzymatic activities. Furthermore, due to the strong historic link between animal caspases and PCD, proteases other than those with caspase-like structures, or caspase-like activities have rarely been chosen as targets for analysis. As such, the literature is unfortunately relatively poor in this field, and is strongly biased. For this reason, no general overview regarding the involvement of total proteolytic processes during allelopathy-driven PCD in plants is available yet.

Apoptosis is the best-known form of PCD in animals, and it is dependent on the activity of caspases, which are cysteine proteases that cleave their substrates after negatively charged amino-acid residues (Van Doorn et al. 2011). Importantly, caspases are not found in any other organisms. Instead, bacteria, fungi, and plants all contain their structural homologs, which are termed orthocaspases in prokaryotes (Klemenčič et al. 2015) and metacaspases in all other organisms (Uren et al. 2000). These are also cysteine proteases, but they show contrasting substrate specificities in comparison to caspases, as they cleave their substrates after

positively charged amino-acid residues (Vercammen et al. 2004; Machado et al. 2013; Klemenčič and Funk 2018).

Two studies have examined the expression of one of the maize (*Zea mays*) metacaspase II genes using quantitative PCR, with treatments with *Chenopodium ambrosioides* volatile oil (Li et al. 2018) and walnut husk washing waters (Ciniglia et al. 2015). In both cases, these allelochemicals induced increased expression of the metacaspase II gene. However, no data are given for either study in terms of other maize type I and additional type II metacaspase genes. Monitoring of protease gene expression was also carried out in another semi-quantitative PCR study, where soybean (*Glycine max*) was grown in soil contaminated with eucalyptus tree (*Eucalyptus globulus*) litter (Abdelmigid and Morsi 2017). In this case, the transcript levels of all five of the papain-like cysteine proteases (PLCPs for short) were monitored. Results show that their expression levels decreased with the allelopathic treatment.

Although no genes that encode caspase homologs have been found in plant genomes, many studies have reported increased caspase-like protease activities in higher plants, either during developmental PCD (Bonneau et al. 2008; Xu and Zhang 2009) or after exposure to various external stimuli (Stael et al. 2019). To date, proteases from three protease groups have been reported to be responsible for these activities. The first group is the vacuolar processing enzymes (VPEs for short), which are cysteine proteases (Hatsugai et al. 2004; Vorster et al. 2019) that have caspase-1-like activities (i.e., with peptide YVAD specificity). The second group is the serine proteases, which belong to the subtilisin-like proteases (subtilases), known as phytaspases and saspases (Chichkova et al. 2010; Vartapetian et al. 2011; Figueiredo et al. 2018). The phytaspases mainly show caspase-6-like activities (i.e., with peptide VEID specificity), while the saspases show their greatest affinity for the short peptide VKMD (Coffeen and Wolpert 2004). The third group of plant proteases with caspase-like activities are represented by the threonine protease proteasome subunit PBA1, and these generally have caspase-3 like activities (i.e., with peptide DEVD specificity) (Hatsugai et al. 2009). To date, only in the study by Abdelmigid and Morsi (2017) using soybean grown in soil contaminated with eucalyptus litter were the expression levels of the three genes for vacuolar processing enzymes monitored. Here, as for the papain-like cysteine proteases, the expression levels of all of these genes decreased with the allelopathic treatment.

According to the data available, it is not yet possible to establish any link between the allelopathy-driven cell death phenotypes and the changes in proteolytic activities. However, we strongly advise further research to be directed into the monitoring of the protease activities using broad-spectrum non-specific protease substrates (e.g., FITC-casein) or

specific caspase-like/metacaspase-like substrates (Minina et al. 2017), rather than relying solely on their mRNA levels.

Nucleolytic activities in allelopathy-driven programmed cell death

Despite little evidence of proteolytic activities, increased DNA degradation does appear to correlate with increased presence of allelopathic compounds. This can mainly be attributed to the overaccumulation of ROS, which can result in DNA damage, such as alterations to nucleotide bases, loss of nucleotides, oxidation of deoxyribose sugar residues, and breaks in the DNA strands and cross-linking (Das and Roychoudhury 2014). Allelochemicals can reduce DNA integrity in the target plants, as was shown by Ciniglia et al. (2015), who reported that the exposure of maize to walnut husk washing waters increased the DNase activity in the maize roots and induced DNA fragmentation. DNA fragmentation was also observed in the root tips of lettuce seedlings treated with juglone (Babula et al. 2014), and in soybean in response to *Eucalyptus* tree litter (Abdelmigid and Morsi 2017). These effects were often associated with decreased mitotic index in the root meristem cells of the target plants, as demonstrated by treatments with juglone (Babula et al. 2014), cyanamide (Soltys et al. 2011, 2012), and umbelliferone (Yan et al. 2016).

Morphological changes induced by allelopathy

The morphological characterization of PCD in plants is complex, and it is often contradictory in the literature. In this review, it is classified according to the morphological (i.e., ultrastructural) characteristics described by Van Doorn et al. (2011), and it is divided into two classes: vacuolar cell death and necrotic cell death. Studies on allelopathy-induced ultrastructural changes related to PCD are rare; therefore, we will discuss the most relevant from the last 20 years.

During vacuolar cell death, various macromolecules accumulate in the main vacuole in the cell, and its volume gradually increases (Van Doorn et al. 2011). The formation of a large vacuole has also been reported after allelopathic stress. As an example, exposure to BOA and 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one induced the formation of larger vacuoles in the root cap cells of cucumber seedlings (Burgos et al. 2004). Excessive vacuolization was also reported for the root apical meristem cells of radish (*Raphanus sativus*) exposed to the allelochemical coumarin (Aliotta et al. 1993), and in the root cap cells after treatment with an extract of cagaita (*Eugenia dysenterica*) (Pereira et al. 2017). Furthermore, a cucurbit extract initiated the formation of large vacuoles in the root cap and root apical meristem cells in bottle gourd (*Cucurbita ficifolia*) and bean (*Phaseolus vulgaris*) seedlings (Cruz-Ortega et al. 1998). As well as the minor

signs of cytoplasmic degradation, the cell turgor and the ultrastructure of the cell organelles remained intact for several days, until the last step of vacuolar PCD. This final step consisted of the collapse of the tonoplast and the release of hydrolytic enzymes into the cytoplasm (i.e., vacuolar processing enzymes), which rapidly degraded the entire protoplast. After this type of PCD, only empty cells with cell walls remain (Van Doorn et al. 2011).

Necrotic PCD is often triggered by injury or physical destruction. In contrast to vacuolar PCD, necrotic PCD occurs rapidly: from a few minutes up to 1 day after exposure. During necrotic PCD, several changes in ultrastructure can be seen, such as swelling of the cell organelles, early rupture of the plasma membrane, and shrinkage of the protoplast, which is the most recognizable sign of necrotic PCD, and which results in the loss of membrane integrity (Van Doorn et al. 2011). In the root cap cells of the bean seedlings treated with a cucurbit extract, there were swollen mitochondria with dilated cristae, the plasma membrane was detached from the cell wall, and the nuclei were of irregular shape. Similar characteristics were also reported for bottle gourd seedlings (Cruz-Ortega et al. 1998) and for radish root tip cells (Šoln et al. 2021a, b).

In addition to these cellular changes, allelopathic stress can lead to reduction in meristem size and the number of meristem cells in the root, as was shown for *Arabidopsis* seedlings treated with benzoic acid (Zhang et al. 2018) and farnesene (Araniti et al. 2017). For cyanamide, it was shown that it affected the cell cycle, altered the cytoskeleton in the root apical meristem cells, and reduced the root viability of onion (Soltys et al. 2011). An extract of the nettle leaf goosefoot (*Chenopodium murale*) induced cell wall thickening in the root cap cells of wheat (Mitić et al. 2018). In the root cap cells of radish, exposure to rhizome extracts of Japanese and Bohemian knotweed induced ring-shaped forms of mitochondria and large endoplasmic-reticulum bodies. Moreover, the root tips of the seedlings treated with Japanese knotweed extract were covered with dense layers of dead root cap cells (Šoln et al. 2021b). These effects show that allelochemicals can negatively affect the structure and growth of the roots of neighboring plants, and they are thus more successful in gaining natural resources.

The role of allelopathy in ecosystems, weed management, and the control of invasive plants

Plants compete for sunlight, space, water, and minerals (for review, see Craine and Dyzbinski 2013). An additional advantage in the competition for natural resources among plants can be provided by allelopathy (Inderjit et al. 2011). Not only do allelochemicals prevent the growth of

neighboring plants, but they also have an influence on the activity of the microbiota in the rhizosphere and the soil pH (Qu et al. 2021), and on water uptake (Hejl and Koster 2004), and nutrient availability in the soil. As an example, addition to soil of a dry powder of two allelopathic weeds camelthorn (*Alhagi maurorum*) and hoary cress (*Cardaria draba*) reduced the concentrations of macronutrients (e.g., NO_3^- , K^+ , Ca^{2+} , P) and micronutrients (e.g., Fe^{2+} , Cu^{2+}) in the roots and shoots of wheat (*Triticum aestivum*) (Mohammadkhani and Servati 2017).

Under natural conditions, plants in the same environment co-evolve and can grow in the presence of various phytochemicals. For invasive plant species, however, this balance can be disturbed. According to the Novel Weapon Hypothesis, the success of the invasive plant species in a new environment can also be based on this biochemical weapon — allelopathy (Callaway and Aschehoug 2000; Bais et al. 2003; Cappuccino and Arnason 2006; Chen et al. 2017). Allelochemicals produced by invasive plants such as Canadian goldenrod (*Solidago canadensis*) (Wang et al. 2016), tree of heaven (*Ailanthus altissima*) (Albouchi et al. 2013), and Bohemian knotweed (*Fallopia × bohemica*) (Murrell et al. 2011), often inhibit the germination and growth of the native plants, because the native species have not had time to adapt to these chemicals (Callaway and Aschehoug 2000; Hierro and Callaway 2003). Allelopathy allows invasive plants to spread more rapidly in a new environment and to form large and dense populations (Hierro and Callaway 2003; Frantík et al. 2013); these can then cast shade on the neighboring plants, and thus contribute further to the reduction of the native biodiversity (Mincheva et al. 2016). Understanding the molecular mechanisms of allelopathy will, therefore, help to manage and/or prevent new plant invasions (Chen et al. 2017).

With their broad array of biochemical weapons, plants can provide a rich source of biologically active compounds with potential applications. They can be used as isolated phytochemicals or as more complex mixtures, such as extracts and essential oils. As the awareness of the general public and the scientific community for the need to reduce pollution and enable more sustainable development is rising, the interest in such new and more “green” (i.e., consumer and nature friendly) bioprotective agents has increased. In this respect, further knowledge about the mechanisms that underlie allelopathy is needed not just to understand the interactions, but to be able to use these for the control and management of harmful plants, like weed and invasive species. To ensure selective activities of these “ioherbicides” against such plants, and not against the crops or the native plants, the defense mechanisms that prevent autotoxicity should also be studied. The main goal of allelopathy-mediated protection would be to reduce or inhibit the growth of undesirable plants without affecting

the others. As allelopathic interactions are highly specific, different combinations of plant species should be used to obtain the maximum benefits and to avoid negative effects.

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Declarations

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