Chapter 11
Root Exudation: The Role of Secondary Metabolites, Their Localisation in Roots and Transport into the Rhizosphere

Leslie A. Weston and Ulrike Mathesius

11.1 Introduction

In addition to provision of mechanical support, water and nutrients, roots perform more specialised roles in the rhizosphere including the ability to synthesise and secrete a multitude of metabolites (Bertin et al. 2003; Brigham et al. 1995; Walker et al. 2003). Metabolites or secondary products are not just released passively over time, but also serve active and important roles in plant defence and communication. The processes of root exudation and rhizodeposition clearly influence plant growth and soil microbial dynamics in the rhizosphere. We now understand that living plant roots can repel or attract herbivores and microbes, stimulate symbiotic relationships, alter soil textural properties and inhibit the growth of competing species (Mathesius and Watt 2011; Nardi et al. 2000; Watt and Weston 2009). This review describes the roles of two families of secondary products in the rhizosphere, the flavonoids and long-chain hydroquinones produced by *Sorghum* spp., their production and transport in roots or root hairs and their subsequent release into the soil rhizosphere. We also discuss the need for additional research to detail the fate of these compounds in the environment and their role in important physiological processes in both plants and microbes.

L.A. Weston (✉)
EH Graham Centre, Charles Sturt University, Wagga Wagga, NSW 2678, Australia
e-mail: lewestonn@csu.edu.au

U. Mathesius
Division of Plant Science, Research School of Biology, Australian National University, Canberra, ACT 0200, Australia

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11.2 Root Exudation

Root exudation is estimated to release anywhere from approximately 5 to 20 % of all photosynthetically fixed carbon from higher plants (Marshner 1995). Carbon loss is assumed to present some cost to plants performing active exudation from their roots, but relatively little research has actually occurred to document this in taxonomically diverse plants (Bais et al. 2004; Mathesius and Watt 2011). We now know that low molecular weight constituents such as amino acids, organic acids, sugars, phenolics and other secondary metabolites tend to comprise the majority of root secretions (Bertin et al. 2003). In addition high molecular weight root constituents consisting primarily of mucilage (high molecular weight polysaccharides) and proteins are also present in many exudates (Walker et al. 2003).

In terms of ecological interactions, the rooting zone and rhizosphere is a very competitive environment where roots compete with roots of neighbouring species for space, water, nutrients and gases. Soil macro- and microbiota also compete for the aforementioned organic materials, many of which serve as metabolic substrates (McCully 2005; Ryan and Delhaize 2001). In order to facilitate successful competition, organisms have evolved chemical communication systems that assist in regulation of interactions between roots and soil organisms and mediate processes in response to environmental stressors; root exudates therefore serve important roles in facilitation of this communication in the rhizosphere.

When roots are under stress or encounter challenges in the rhizosphere, they react by releasing increasing amounts of small molecular weight compounds, including amino acids, organic acids, phenolics and proteins. These secretions are thought to play important protective roles for the plant, thus initiating a negative form of communication in the rhizosphere (Bertin et al. 2003). Alternatively, they may elicit symbiotic responses, such as the signals that initiate legume rhizobium N fixation, a positive form of communication (Mathesius and Watt 2011; Peters et al. 1986) and serve as attractants to common soil microbes (Shi et al. 2011).

In comparison to the symbiotic associations studied, the negative forms of signal communication have received less attention in recent years, likely due to the difficulty in studying complex interactions in a diverse soil matrix (Inderjit et al. 2005; Weston and Duke 2003). Recent findings suggest that the chemical diversity of plant-derived natural products could be the result of adaptive evolution or niche colonisation, which has occurred through natural selection, and these compounds may function in plant defence as well as defence-related signalling processes (Bednarek and Osbourne 2009).

Plant-derived natural products associated with plant defence are commonly referred to as allelochemicals, and allelopathy is defined as the process mediated by the production and release of bioactive secondary products by plant parts that negatively impacts the establishment of neighbouring plant species (Rice 1984). Allelopathy has been studied in the context of its effects upon agricultural systems (Weston 2005), and its effects can be positive or negative in terms of crop establishment and performance (Weston and Duke 2003). Allelopathic crops may

be used to effectively suppress weeds, and invasive weeds may also successfully become established as successful competitors in part due to their allelopathic tendencies (Callaway and Aschehoug 2000; Gurevitch et al. 2011).

Allelopathic interactions in the rhizosphere have been less well-characterised than interactions observed above ground (Inderjit et al. 2005). Root-produced allelochemicals are generally associated with plant growth reduction and resistance to or suppression of plant pathogens, soil microbes and other herbivores. We now have the capacity to characterise production and release of minute quantities of bioactive secondary products in the rhizosphere and study their release and metabolism in soil settings over time (Weidenhamer et al. 2009); as a result we are now able to report on these interactions with greater understanding of the chemical constituents involved in these below-ground interactions.

Although plant exudates contain many constituents, some roots produce large quantities of specific allelochemicals, which function in plant defence roles in the rhizosphere (Hassan and Mathesius 2012; Watt and Weston 2009; Weston et al. 2012). Plants which produce copious quantities of phytotoxins or allelochemicals often employ mechanisms to prevent autotoxicity to these self-generated secondary products. These protective mechanisms have sometimes been evaluated in living plant cells and root cell suspension cultures (Yazaki 2005; Yazaki et al. 2008), but have not been well characterised in living root systems to date. However, it is now apparent that plant cells which produce bioactive secondary products have typically developed highly specific transport mechanisms within specialised plant cells to move these compounds around and out of the cell (Weston et al. 2012).

Currently, molecular approaches are being utilised to study both active and passive means of transport of secondary plant products. A review of current literature suggests that specialised transport mechanisms are very important in plant roots and living root cells for transport of secondary products into the rhizosphere or their overtime release by root exudation or rhizodeposition. This chapter will thus focus on the role of secondary products and allelochemicals in the rhizosphere and the known mechanisms that plants use to transport these products within the plant and from the cells producing these compounds to facilitate their release into the rhizosphere.

11.3 Example of Flavonoids: Important Bioactive Secondary Products

Flavonoids are low molecular weight compounds that are produced by plants and are generally described as non-essential for plant survival, unlike primary metabolites. Secondary products are biologically active in many ways, and over 10,000 structural variants of flavonoids have been reported (Ferrer et al. 2008; Williams and Grayer 2004). Flavonoid synthesis appears to be ubiquitous in plants and has

likely evolved early during land plant evolution for plant defence and chemical signalling (Delaux et al. 2012; Pollastri and Tattini 2011). Due to the specific physical and biochemical properties of flavonoids, they are potentially able to interact with diverse targets in subcellular locations and elicit various activities in microbes, plants and animals (Buer et al. 2010; Taylor and Grotewold 2005). Although flavonoids play important roles in higher plants, including their influence on plant development through auxin transport and root and shoot development (Brown et al. 2001; Buer and Djordjevic 2009; Peer and Murphy 2007; Wasson et al. 2006), they also play important roles in modulating the levels of reactive oxygen species (ROS) in plant tissues (Agati et al. 2012; Taylor and Grotewold 2005) and provide colouring to various plant tissues including flowers (Davies et al. 2012). In addition, they are required for signalling symbiotic bacteria in the legume rhizobium symbiosis (Djordjevic et al. 1987; Zhang et al. 2009).

In relation to their role in allelopathy and the inhibition of seedling root growth, the activity of flavonoids as regulators of auxin transport and degradation is potentially very important. Depending on their structure, flavonoids can regulate the breakdown of auxin by IAA oxidases and peroxidases (Furuya et al. 1962; Mathesius 2001; Stenlid 1963) and also affect polar auxin transport (Jacobs and Rubery 1988; Peer and Murphy 2007; Stenlid 1976), thereby impacting root growth of target species. Some isoflavonoid phytoalexins act as cofactors to auxin in adventitious root development, although the mode of action of these molecules remains unknown (Yoshikawa et al. 1986). In addition, flavonoids show affinity for many enzymes and other proteins in plants and animals, including those required for mitochondrial respiration. In this case, certain flavonoids contribute to inhibition of NADH oxidase and the balance of reactive oxygen species (Hodnick et al. 1988, 1994), thereby impacting respiration. This remains to be investigated in root tissues, however.

In animal systems, flavonoids are important dietary components and are known to possess a broad range of properties including antibacterial, antifungal, antiviral and anticancer activity (Soto-Vaca et al. 2012; Taylor and Grotewold 2005). Many flavonoids have also served as templates in the development of new pharmaceuticals (Cutler et al. 2007). Interestingly, flavonoids can be transported within and between tissues and cells and are often released into the rhizosphere where they are involved in plant-to-plant interactions, specifically allelopathic interference (Hassan and Mathesius 2012). They can be released by root exudation or through tissue degradation over time, and although both aglycones and glycosides of flavonoids are found in root exudates, their relative role in allelopathic interference, their specific activity and selectivity and their mode(s) of action still remain less well characterised (Berhow and Vaughn 1999; Hassan and Mathesius 2012; Levizou et al. 2004; Weston and Duke 2003).

11.4 Flavonoid Structure, Function and Biosynthesis

Flavone ring structures are found in all plant parts and are ubiquitous throughout nature, playing an integral role in plant growth and development (Harborne 1973). The term flavonoid is generally used to describe those natural products possessing a C6–C3–C6 skeleton, or specifically a phenylbenzopyran function (Marais et al. 2007). The typical flavone ring is the backbone of flavonoid structure or the nucleus of more diverse molecules. The flavonoid biosynthetic pathway is now well elucidated (Dixon and Steele 1999; Winkel-Shirley 2001). Typically, flavonoids are synthesised through the phenylpropanoid or acetate-malonate metabolic pathway. Interestingly, in *Arabidopsis* chalcone reductase is lacking and also the related isoflavone synthase enzymes, so therefore, it cannot produce one subset of flavonoids, the isoflavonoids, which are produced in many legumes (Buer et al. 2007, 2010).

Arabidopsis mutants (Peer et al. 2001) and transgenic legumes with modified branches of the flavonoid pathway (e.g. Subramanian et al. 2005, 2006; Wasson et al. 2006; Yu et al. 2003) currently provide a unique tool for studying the role of flavonoids in rhizosphere interactions. Although flavonoids have similar precursors to those utilised for lignin biosynthesis, they exhibit a number of basal structures that result in generation of a series of diverse compounds including flavones, flavonols, flavan-3-ols, flavanones, isoflavanones, isoflavans and pterocarpans. Substitution by glycosylation, malonylation, methylation, hydroxylation, acylation, prenylation, polymerisation or other modifications leads additional diversity in this family and impacts their function, solubility and degradation (Dixon and Steele 1999; Winkel-Shirley 2001; Zhang et al. 2009).

In higher plants, flavonoid synthesis begins when enzyme complexes form on the cytosolic side of the endoplasmic reticulum (Jorgensen et al. 2005). Complexes may subsequently localise to the tonoplast for glycosylation and storage in the vacuole (Winkel 2004). In many plant tissues, flavonoid synthesis and accumulation is located in distinct cells (Fig 11.1). Subcellularly, flavonoids have been found in the nucleus, vacuole, cell wall, cell membranes and cytoplasm (Erlejman et al. 2004; Hutzler et al. 1998; Naoumkina and Dixon 2008; Saslowsky et al. 2005). While flavonoid glycosides stored in the vacuole may not be active in the cell, their released aglycone counterparts likely have functions in the plant cytoplasm, e.g. in regulation of enzyme activity, in formation of reactive oxygen species and in auxin transport (Naoumkina and Dixon 2008; Taylor and Grotewold 2005). Flavonoid glycosides have also been found to have active roles in regulation of IAA oxidase, which could lead to changes in auxin accumulation (Furuya et al. 1962; Stenlid 1968). An accumulation of flavanols (catechins) has been observed in nuclei, especially in gymnosperm species. Their roles could include the regulation of gene expression through chromatin remodelling and effects on enzymes and protein complexes that regulate gene expression (Feucht et al. 2012).

In contrast to other plant tissues, flavonoids in roots can be accumulated at the root tip and in cap cells from where they can be exuded or sloughed off into the soil

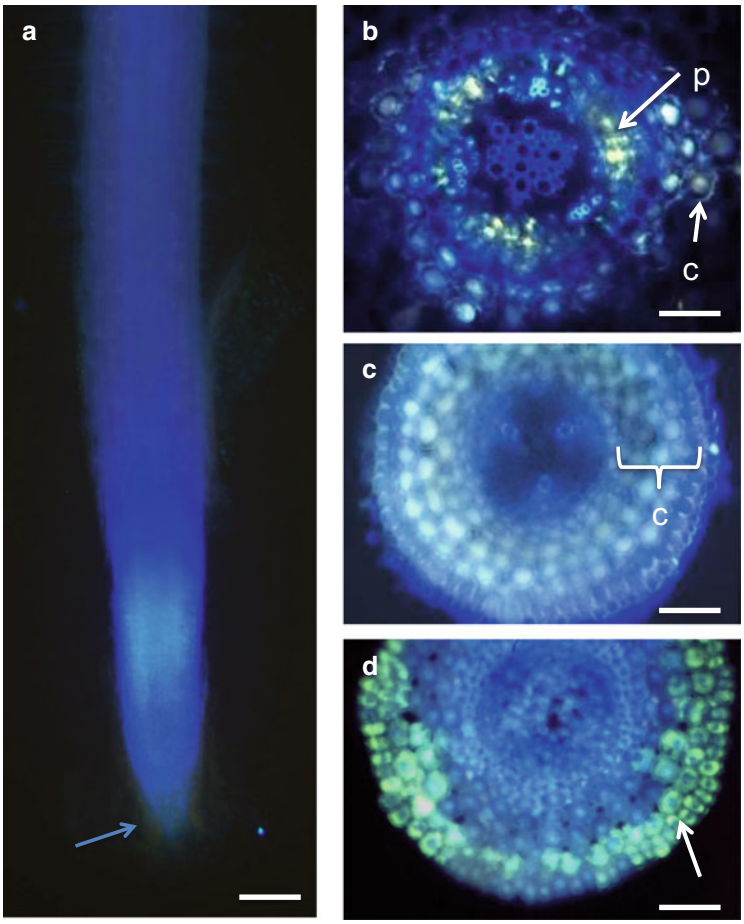

Fig. 11.1 Flavonoid accumulation in roots is cell type specific. (**a**) Flavonoid accumulation in root tips of *Medicago truncatula*. *Blue* and *orange* autofluorescence is due to the presence of flavonoids. Note the high accumulation of flavonoids in the root tip and in root cap cells (*orange*, *arrow*). (**b**) Flavonoid accumulation in a mature white clover (*Trifolium repens* L.) root. Note the accumulation of different flavonoids exhibiting different emission wavelengths in different cell types, e.g. pericycle (p) and cortex (c). (**c**) Flavonoid accumulation in a young but differentiated root section of white clover in cortex cells (c). (**d**) Flavonoid accumulation in a section through the root tip of white clover showing flavonoid accumulation in nuclei of meristematic cells (*light blue*) and in the cytoplasm of epidermal and outer cortical cells (*yellow*, *arrow*). All images were taken using fluorescence microscopy with UV excitation. Magnification bars are 500 μm in (**a**) and 100 μm in (**b**), (**c**) and (**d**)

(see below). Interestingly, flavonoids are also localised in specific cell types of the root (Fig. 11.1) and can be readily studied by use of fluorescent imaging due to their autofluorescence (Bayliss et al. 1997; Hutzler et al. 1998; Mathesius et al. 1998). Plant roots produce a diversity of flavonoids that are stored as glycosides or aglycones and are released both by root exudation or tissue decomposition and

leaching (Rao 1990). Their accumulation in roots is highly dependent on biotic and abiotic environmental conditions (Rao 1990), but we now understand that root flavonoids play highly specific roles in signalling to microbes and other plants as well as in protection from soil pathogens.

11.5 Flavonoid Transport, Exudation and Activity in Plants and the Rhizosphere

While flavonoid accumulation is often cell- and tissue-specific, there is evidence for intra- and intercellular flavonoid movement, specifically through active transport. Intracellular movement is most likely to occur via vesicle-mediated transport or through membrane-bound transporters of the ABC (ATP binding cassette) or MATE (multidrug and toxic extrusion compound) families (Sugiyama et al. 2007; Zhao and Dixon 2009). Flavonoid transport occurs across the tonoplast as well as the plasma membrane (Fig. 11.2), and it is generally not well understood how the directional transport of flavonoids is regulated. Conjugation of flavonoids to other molecules, e.g. sugars and glutathione seems one determinant of transport direction (Zhao and Dixon 2010).

Vesicle-mediated transport has been observed in flowers for anthocyanins, which are synthesised in the cytoplasm and then surrounded by a membrane. These so-called anthocyanoplasts then fuse to form larger vesicles and can fuse with the vacuole inside which they can form anthocyanic vacuolar inclusion bodies (Grotewold and Davies 2008). Vesicle transport of 3-deoxyanthocyanidin phytoalexins has been demonstrated in sorghum, where anthocyanin-containing vesicles accumulate at sites of attack by pathogenic fungi on leaf epidermal cells (Snyder and Nicholson 1990). Whether vesicle-mediated transport of flavonoids occurs in roots during flavonoid exudation is currently unknown.

Evidence for transporter-mediated exudation of flavonoids and other metabolites from roots is starting to emerge (Fig. 11.2). The ATP transporter family is diverse and the many members of this transporter family transport metabolites ranging from auxins, organic acids, lipids, waxes, terpenoids and alkaloids to flavonoids (Rea 2007). As expected, ABC transporter mutants of *Arabidopsis* show altered root exudate profiles, although the mutations affected not only flavonoid transport but also that of other constituents in the exudates (Badri et al. 2008, 2009). It is likely that each ABC transporter has several possible substrates and that different substrates can be transported by different transporters, although the regulation of this process has not been determined. An ATP-dependent ABC transporter was specifically shown to be involved in the exudation of genistein, an isoflavonoid, from soybean root plasma membrane vesicles (Sugiyama et al. 2007). This ABC transporter was also shown to transport other isoflavonoids and is the first characterised flavonoid transporter from plants. Interestingly, even though genistein acts as a *nod* gene inducer in the rhizobial symbionts of soybean, nitrogen

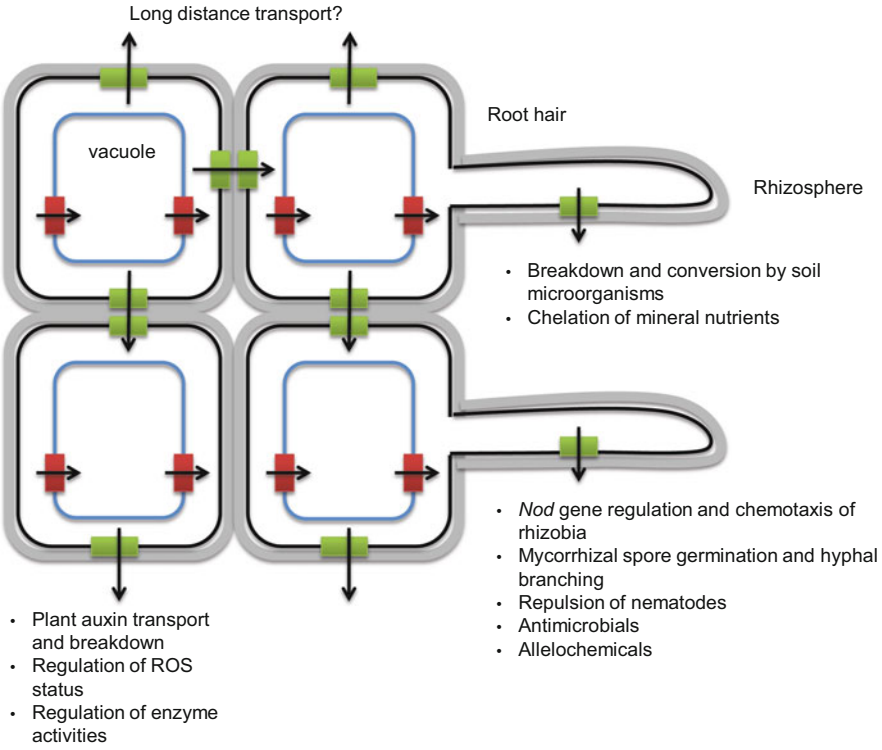

Fig. 11.2 Model for the transport of flavonoids within, between and out of root cells. Flavonoids are likely to be transported across the plasma membrane (*black line*) and tonoplast (*blue line*) by ABC-type or MATE transporters (*red* and *green boxes*). It is not known whether different transporters of each family reside on the tonoplast or plasma membrane and what their specificity is in most cases. Exudation into the apoplast (*grey line*) and rhizosphere is also likely via ABC transporters

nutrition only had minor effects on the expression of the flavonoid transporter (Sugiyama et al. 2007).

Flavonoids are also passively released from decomposing root cap and root border cells directly into the rhizosphere (Hawes et al. 1998; Shaw et al. 2006). Flavonoid exudation has also been shown to respond to various microbial signal molecules of symbionts and pathogens (Armero et al. 2001; Schmidt et al. 1994) and to abiotic conditions (Coronado et al. 1995; Dixon and Paiva 1995; Juszczuk et al. 2004). There are still large gaps in our knowledge of the exact transporters for various flavonoids, whether they require flavonoid conjugation and how their expression is regulated.

In addition, to flavonoid transport into and out of cells, flavonoids also appear to move over longer distances in *Arabidopsis*, although the extent and importance of this transport are currently not understood (Buer et al. 2007). This long-distance transport is likely to be catalysed by members of the ABC transporter families

because application of ABC transporter inhibitors reduced long-distance auxin transport (Buer et al. 2007). However, the molecular mechanisms of inter- and intracellular flavonoid transport require further study in higher plants.

Once exuded, flavonoid persistence in the soil also varies with environmental conditions and is influenced strongly by the presence of soil microbes, some of which can metabolise or modify flavonoids (Hartwig and Phillips 1991; Rao and Cooper 1994, 1995). Flavonoids also can become unavailable due to absorption to soil particles and organic matter (Shaw and Hooker 2008). Their mobility in soil varies greatly with chemical composition, e.g. glycosylation, which determines their water solubility. In turn, the presence of flavonoids in the soil can alter soil composition and nutrient availability through their activity as antioxidants and metal chelators. Chelation and reduction of metals in the soil impact nutrient availability, especially phosphorus and iron. For example, an isoflavonoid identified from *Medicago sativa* root exudates dissolved ferric phosphate, enhancing phosphate and iron availability (Masaoka et al. 1993). The flavonoids genistein, quercetin and kaempferol were shown to alter iron availability by reducing Fe(III) to Fe(II) and by chelating unavailable iron from iron oxides (Cesco et al. 2010).

Some of the more well-known biological roles of flavonoids in the rhizosphere include the activation of *nod* genes from symbiotic rhizobia, chemoattraction of rhizobia and nematodes, inhibition of pathogens and activation of mycorrhizal spore germination and hyphal branching. These functions can indirectly act on the growth of conspecifics through the regulation of nitrogen fixation and mycorrhization, as well as their ability to be infected by pathogens.

Legume root exudates were shown to contain species-specific flavonoids, specifically flavones, that activate the nodulation genes of their respective rhizobial symbionts by binding to the transcriptional activator NodD (Cooper 2004; Peck et al. 2006; Peters and Long 1988; Redmond et al. 1986). This leads to Nod factor synthesis and subsequent infection and nodulation of the legume host. However, some flavonoids, especially isoflavonoids, also inhibit *nod* gene induction (Zuanazzi et al. 1998). Some flavonoids that induce *nod* genes, specifically luteolin and apigenin, have dual actions as chemoattractants, with different flavonoids attracting different *Rhizobium* species (Aguilar et al. 1988; Dharmatilake and Bauer 1992). Flavonoid exudation by the host changes during different stages of the symbiosis, presumably fine-tuning Nod factor synthesis during nodule development and colonisation (Dakora et al. 1993). Flavonoid composition in the rhizosphere around legume roots can also be altered by rhizobia, which metabolise and alter the structure of flavonoids over time (Rao and Cooper 1994, 1995).

Flavonoids and other phenolic compounds also specifically repel soil-dwelling plant parasitic nematodes and affect hatching and migration. For example, the flavonols kaempferol, quercetin and myricetin acted as repellants for the root lesion nematode *Radopholus similis* and the root knot nematode *Meloidogyne incognita*, whereas the isoflavonoids genistein and daidzein and the flavone luteolin acted only on *R. similis* (Wuyts et al. 2006). Kaempferol, quercetin and myricetin also inhibited motility of *M. incognita*, and kaempferol inhibited egg hatching of *R. similis*, whereas other nematodes were not affected by any of these compounds.

It was demonstrated that nematode-resistant plant cultivars contained increased amounts of flavonoids, specifically the isoflavonoids and the pterocarpan medicarpin in alfalfa (*Medicago sativa*) (Baldridge et al. 1998; Edwards et al. 1995).

Flavonoids and other phenolics can also inhibit a range of root pathogens, especially fungi (Makoi and Ndakidemi 2007). Generally, isoflavonoids, flavans or flavanones have been found as the most potent antimicrobials. These compounds can either be induced upon pathogen attack (phytoalexins) or be preformed (phytoanticipins), while others are exuded into the soil (Armero et al. 2001). In this role, flavonoids were shown to act as antimicrobial toxins (Cushnie and Lamb 2011) and anti- or pro-oxidants (Jia et al. 2010). Pterocarpans, end products of the isoflavonoid pathway, including medicarpin, pisatin and maackiain, also have antimicrobial properties (Naoumkina et al. 2010). For example, pisatin from pea provided protection from pathogenic fungi and oomycetes (Pueppke and Vanetten 1974). The likely mechanism of action against fungi is through inhibition of elongation of fungal germ tubes and mycelial hyphae (Blount et al. 1992; Higgins 1978).

During pathogen attack in a resistant plant species, phytoalexins are thought to become oxidised, leading to formation of toxic free radicals that could stimulate cell death during a hypersensitive response (Heath 2000). Flavonols also contribute to resistance against pathogens. For example, quercetin is an antimicrobial compound that inhibits the ATPase activity of DNA gyrase in bacteria (Naoumkina et al. 2010; Plaper et al. 2003). Carnation (*Dianthus caryophyllus*) was also shown to mount a significant defence against *Fusarium oxysporum* by formation of the fungitoxic flavonol triglycoside of kaempferide (Curir et al. 2005).

Other known roles of flavonoids in the rhizosphere include effects on arbuscular mycorrhizal fungi, which form a beneficial symbiosis with the majority of land plants under conditions of phosphorus deficiency (Harrison 2005). Hyphae of the mycorrhizal fungi are attracted to root exudates, and in some cases this has been attributed to the presence of flavonoids, which stimulated hyphal branching and presymbiotic growth towards the host (Scervino et al. 2005a,b 2006, 2007; Siqueira et al. 1991; Steinkellner et al. 2007). In *Medicago* spp., the hyperaccumulation of coumestrol, a potent hyphal stimulator, was correlated with hyperinfection by the symbiont (Morandi et al. 2009). However, both hosts and non-hosts have also been reported to exude flavonoids that inhibit hyphal branching (Akiyama et al. 2010; Tsai and Phillips 1991). Exudation of flavonoids from the host is also phosphorus-regulated (Akiyama et al. 2002), similar to the dependence of flavonoid accumulation on nitrogen availability in legumes forming nitrogen-fixing symbioses (Coronado et al. 1995). As one can see from review of the plant literature, the role of flavonoids in plant defence in the rhizosphere and other physiological processes is diverse and will no doubt be the subject of additional study as we continue to evaluate regulation of these processes from a molecular perspective.

Flavonoid production in roots of perennial legumes has been shown to be strongly regulated by nitrogen supply. Under nitrogen-limiting conditions, flavonoid biosynthesis genes such as chalcone synthase and isoflavone reductase are

upregulated and show enhanced expression, indicating the nitrogen nutrition status of the plant plays a role in impacting secondary product production (Coronado et al. 1995). This is also the case for other secondary plant products such as the hydroxamic acids BOA and DIMBOA produced by *Secale cereale* (Mwaja et al. 1995). The bioavailability of soil nitrogen could thus also play a critical role in the regulation of allelopathy or autoallelopathic interactions in established legume stands.

11.6 Alfalfa and Clover Autotoxicity

The perennial legumes alfalfa (*Medicago sativa* L.) and red or white clover (*Trifolium pratense* or *Trifolium repens* L.) are widely used in temperate regions as high quality pastures and fodder plants containing substantial levels of protein (Hancock 2005; Oleszek and Jurzysta 1987). These crops are also important for their contributions of large quantities of organic matter to the soil, improvement of soil structure and enhanced water infiltration following establishment. Alfalfa generally contributes about twofold higher levels of organic dry matter in comparison to the forage crops of red or white clover. Most alfalfa and certain clovers are typically established as perennials, and as such they tend to be fairly resistant to weed infestation over time. However, as pastures age, both established alfalfa and clover pasture stands often exhibit significant reductions in plant counts and productivity. This phenomenon, known as autotoxicity, severely limits the ability of producers to renovate declining pastures (Hancock 2005; Tesar 1993). When renovating these pastures, the planting of successive crops also frequently leads to poor stands in crops immediately following established clover or alfalfa. In the past, the cause of this phenomenon was thought to be depletion of soil moisture and nutrients or build-up of soil pathogens during perennial crop growth, but now there is strong evidence that these crops also exhibit phytotoxicity or allelopathy, through production and release of toxic secondary metabolites. In the case of alfalfa or lucerne, autotoxicity can limit the development and productivity of the crop itself (Cosgrove and Undersander 2003; Hancock 2005) and result in permanent morphological reductions in root development and shoot growth (Jennings 2001; Jennings and Nelson 2002).

Hancock has speculated on the evolutionary role or purpose of autotoxicity in alfalfa (also known as lucerne) or clover. Alfalfa, along with other perennial pasture legumes, is believed to have developed and evolved in the northern and eastern coastal regions of the Mediterranean. During the period in which evolution was thought to have occurred, these areas likely experienced hot dry conditions and resource limitations (Hancock 2005). Under conditions such as these, Hancock and others postulated a competitive advantage would arise if other plants, including alfalfa seedlings, could be prevented from establishing near mature plants, specifically through autotoxicity (Jennings 2001) (Fig. 11.3).

Fig. 11.3 Established stand of alfalfa (*Medicago sativa* L.) in Australian paddock. Note the concentric space around each alfalfa plant, with little to no other vegetation in this concentric ring. Older alfalfa stands often exhibit autotoxicity and allelopathy over time and individual plant growth becomes limited by presence of adjacent plants

Since these perennial legumes are known to be both autotoxic and allelopathic (Hedge and Miller 1992) (Fig. 11.3), numerous investigators have attempted to identify the allelochemicals responsible for phytotoxicity, with limited success. Oleszek and Jurzysta (1987) reported the release of water-soluble allelochemicals from alfalfa and red clover roots, which inhibited fungal and seedling growth, in a variety of soils with different textural properties over time. They concluded that although the extracts contained numerous phytoinhibitors, including saponins, that were soluble in water or alcohol, the presence of mediagenic acid along with other unidentified water-soluble inhibitors was associated with inhibition.

In perhaps the most interesting field study outlining the fate of flavonoids in the soil over time, Fomsgaard and colleagues used sensitive LC-MS/MS techniques to profile a diverse group of over 20 flavonoids released from living and decomposing white clover stands in Denmark, in situ and after soil incorporation of the clover as a green manure (Carlsen et al. 2012). As the authors report, numerous studies have implicated allelochemicals produced by white clover with weed suppression, as well as negative interactions associated with allelopathy or replant/pathogenesis problems following white clover establishment. This ground-breaking study evaluated the pattern of flavonoid release from living clover grown under field conditions and also from leachates following incorporation of green cover crops into field soil. Their results help to explain the potential for allelopathy and autoallelopathic interactions associated with established white clover stands. Specifically, the flavonoid aglycones formononetin, medicarpin and kaempferol predominated in soil analyses, with glycosides of kaempferol and quercetin also present at relatively high concentrations. Kaempferol persisted for days in field soil surrounding living or incorporated clover stands. These aglycones and related constituents have specifically been noted to possess substantial phytoinhibitory activity (Rice 1984). Kaempferol and kaempferol-3-*O*-L-arabinofuranoside stimulated seed germination at low concentrations, but inhibited seedling growth at higher concentrations (Hai et al. 2008); these compounds are also present in walnut (*Juglans regia* L.) leaf extracts. The Carlsen study (2012) also noted that highest concentrations of flavonoids in clover crops were associated with presence of clover flowers, in comparison to leaves, stems or roots in soil degradation studies. Several of the

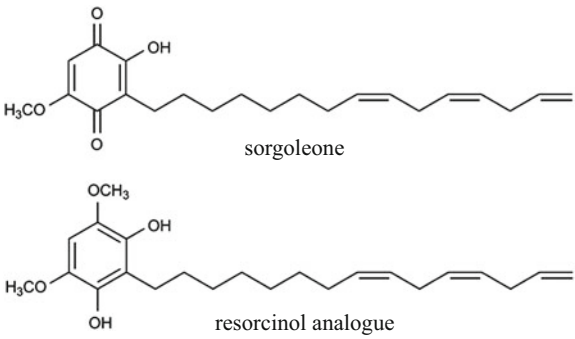

Fig. 11.4 Structure of sorgoleone and its resorcinol analogue

flavonoids identified are also known inhibitors of fungal growth, while others are associated with stimulation of microbial growth in the rhizosphere (Mandal et al. 2010).

Based on these interesting findings, we would suggest that additional studies are required to determine (1) mobility of flavonoids in various soil types and profiles, (2) location of maximal concentrations in the rhizosphere (likely to be nearest living roots, for example), and (3) the relative half-life(s) of major flavonoids and their glycosides in living soils. The application of comprehensive metabolic and proteomic profiling performed from similarly designed experimentation with legumes growing in a field setting will most certainly aid in further defining the roles of simple phenolics as well as flavonoids and their related degradation products in the rhizosphere. Although many flavonoids have been implicated in allelopathic inhibition of seedling growth and radicle elongation such as kaempferol and 6-methoxy-kaempferol and rhamnetin and isorhamnetin (Levizou et al. 2004), the mode of action of these inhibitors has not often been carefully examined in recent research (Berhow and Vaughn 1999).

11.7 Biosynthesis and Role of Sorgoleone and Long-Chain Hydroquinones in the Soil Rhizosphere

Sorgoleone, typically described as 2-hydroxy-5-methoxy-3[(8′Z.11′Z)-8′,11′, 14′-pentadecatriene]-*p*-benzoquinone, is the major component of sorghum root exudates (Fig. 11.4). In the literature, the name sorgoleone also often refers to the reduced form of this compound and a number of structurally related *p*-benzoquinones which are present in small quantities in the root exudates and extracts of sorghum species, making up the exudates as a whole (Dayan et al. 2010). Sorgoleone was first discovered in 1986 by investigators searching for secondary metabolites involved in triggering the germination of the parasitic weed, *Striga asiatica* (witchweed) (Chang et al. 1996). Sorgoleone in its quinone form was not involved in this communication between parasitic plants and their

hosts, but the reduced form of sorgoleone known as dihydrosorgoleone is associated with the germination of witchweed (Dayan et al. 2010). Sorgoleone, along with its resorcinol analogue (Fig. 11.4), occurs in a 1:1 ratio as an exudate from the root hairs of sorghum (Czarnota et al. 2001; Dayan et al. 2010). Both of these molecules are phytotoxic to plant growth, along with some of the other congeners in the root exudates of sorghum species (Kagan et al. 2003; Rimando et al. 1998).

Structurally, sorgoleone is a long chain benzoquinone and has a unique and interesting partially unsaturated side chain that renders it non-polar. It is contained in the oily golden-coloured droplets produced by living roots of sorghum (Fig. 11.5) along with its resorcinol analogue (Chang et al. 1996; Czarnota et al. 2001, 2003a; Weston et al. 2012). Sorgoleone has several interesting modes of action in both young and older plant tissue, which may be responsible for the inhibition of plant growth in the laboratory or field settings (Czarnota et al. 2001; Dayan et al. 2009; Hejl and Koster 2004). The multiple sites of action of sorgoleone in the plant include photosynthetic and mitochondrial electron transport (Czarnota et al. 2001; Dayan et al. 2009; Einhellig et al. 1993; Rimando et al. 1998) and the enzyme HPPD, *p*-hydroxyphenylpyruvate dioxygenase, an enzyme involved in the formation of plastoquinone and subsequently photosynthesis (Meazza et al. 2002). Hejl and Koster (2004) also found that sorgoleone inhibits root H^+-ATPase activity and subsequent water uptake in sensitive species.

Due to sorgoleone's lipophilicity, there has been some doubt about its ability to be taken up and translocated by mature plants (Hejl and Koster 2004). Recently Dayan et al. (2009) showed that younger seedlings are able to translocate radiolabelled sorgoleone effectively, whereas older seedlings do not translocate sorgoleone acropetally, thereby leading to reduction in photosynthesis mainly in younger or germinating seedlings. This is in agreement with the findings of Hejl and Koster (2004) and Czarnota and Weston (2001) and suggests that the primary mode of action may be inhibition of photosynthesis or respiration in young seedlings, along with its activity at other molecular target sites in older plants.

Recent molecular investigations have shed significant light on the genes and corresponding enzymes associated with sorgoleone biosynthesis (Baerson et al. 2010; Dayan et al. 2010; Pan et al. 2007; Yang et al. 2004b). Sorgoleone belongs to a family of compounds referred to as phenolic lipids, which have been identified in numerous plant, fungal and bacterial taxa, but relatively few animal species. Among the major classes of phenolic lipids, which include alkylphenols, alkylresorcinols, anacardic acids and alkyl catechols, the alkylresorcinols are the most prevalent in nature (Baerson et al. 2010). The synthesis of sorgoleone and other phenolic lipids occurs via the action of specialised type III polyketide synthase (PKS) enzymes utilising atypical fatty acyl-CoA starter units. In vivo labelling studies performed by Fate and Lynn (1996) provided the first evidence of this, although definitive proof for this concept was finally obtained following the isolation of type III PKSs from *S. bicolor* (designated ARS1 and ARS2; alkylresorcinol synthase) possessing alkylresorcinol-forming activity. Additionally, gene knockdown experiments using RNA interference targeting *ARS1* and *ARS2* in transgenic sorghum plants resulted in multiple independent transformation

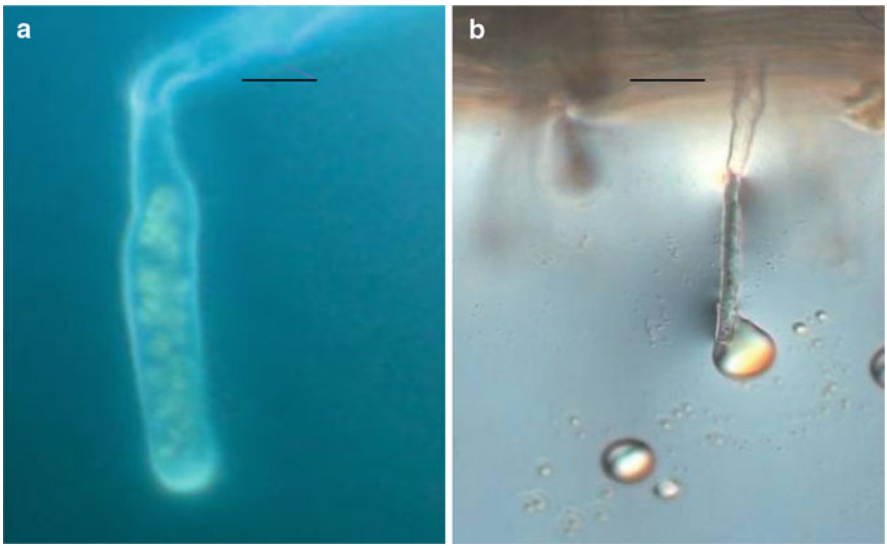

Fig. 11.5 (**a**) *Sorghum bicolor* root hair containing large numbers of vesicles containing sorgoleone, light microscopy. (**b**) Sorghum root hair exuding a droplet of sorgoleone, insoluble in water, light microscopy. Taken by Michelle Watt, CSIRO Plant Industries, Black Mountain, ACT, Australia and Leslie Weston. Published with permission of Oxford Journals. Magnification bars are 20 μm in (**a**) and 500 μm in (**b**)

events exhibiting dramatically reduced or undetectable levels of sorgoleone, thus providing unambiguous proof for the involvement of ARS1 and ARS2 in sorgoleone biosynthesis (Baerson et al. 2010; Cook et al. 2010). Evidently, sorgoleone biosynthesis occurs only in sorghum root hair cells, and this is supported by the fact that the 5-*n*-pentadecatrienyl resorcinol biosynthetic intermediate as well as the $\Delta^{9,12,15}$-C16:3 fatty acid used to generate the starter acyl-CoA used for its production accumulates only within this cell type (Cook et al. 2010; Pan et al. 2007; Yang et al. 2004b).

In laboratory and soil-based experiments, sorgoleone was shown to act similarly to preplant incorporated soil herbicides such as trifluralin, in terms of its lipophilicity, movement in soils and ability to suppress the growth of germinating seeds or seedlings (Czarnota et al. 2001; Dayan et al. 2009; Weston et al. 1997). As long as sorghum remained actively growing and produced ample root hairs, sorgoleone was continually produced and released into the rhizosphere (Czarnota et al. 2001, 2003a) (Fig. 11.5). This continual de novo synthesis and deposition of sorgoleone likely accounts for its ability to inhibit sensitive plant growth in the rhizosphere, particularly during the cropping season as weeds and germinating seedlings contact the zone of deposition. Sorgoleone also was shown to persist in laboratory soils for a number of weeks, after initial rapid degradation, suggesting again strong potential for allelopathic activity up to and immediately following sorghum harvest (Weston et al. 1997). In a study conducted in the Weidenhamer

laboratory, sorgoleone could actually be detected in significant quantities when exuded by living root hairs through the use of PVC tubing or coated stir bars to trap this non-polar compound in the rhizosphere in a controlled growth environment (Loi et al. 2007; Weidenhamer et al. 2009).

What is the exact role of sorgoleone in the rhizosphere? This question still does not have a specific answer, based on the research conducted to date, but we are closer to understanding the diverse roles of secondary plant products in plant defence. It is likely that secondary compounds like sorgoleone may have multiple roles in the rhizosphere, involving general phenomena such as chemical signalling to germinating seedlings and mature plants, as well as bacteria or other microbes (Weston et al. 2012). Sorgoleone is also selectively metabolised by certain microbes as a carbon source, whereas other microorganisms are inhibited by its presence (Weston, personal communication). Sorghum cover crops are also strong general suppressants of nematode activity (Weston 2005), potentially through release of phenolics, sorgoleone and other long-chain hydroquinones. The resorcinol analogue of sorgoleone has been shown more specifically to stimulate germination of *Striga asiatica* (Chang et al. 1996). Sorgoleone itself appears to function in a more specific manner as a preemergent soil herbicide, to inhibit the growth of competing seedlings (Czarnota et al. 2001; Dayan et al. 2009; Weston and Czarnota 2001). In addition, sorgoleone may modify the soil rhizosphere by altering textural and chemical properties of soil particles in the vicinity of sorghum roots and also potentially by modifying water and nutrient uptake in the rhizosphere by sensitive species (Hejl and Koster 2004). As plant species exhibit differential sensitivity to sorgoleone, the impact of sorgoleone is thus likely to be dependent upon plant species encountered, the rhizosphere environment and the quantity of sorgoleone released by living roots over time (Dayan et al. 2009).

11.8 Transport, Release and Fate of Sorgoleone in the Rhizosphere

Sorgoleone production has now been observed and compared in a number of related sorghum species, and it was found that *Sorghum bicolor*, *Sorghum bicolor* x *Sorghum sudanense* and *Sorghum halepense* seedlings produce substantial amounts of sorgoleone in the early stages of root growth (Czarnota et al. 2003b). In addition, a large screening study showed that all of the cultivated sorghum genotypes screened produced a significant amount of sorgoleone (Nimbal et al. 1996). Production occurs shortly after germination and is associated with the formation of functional root hair cells (Czarnota et al. 2003a). Living root hairs transport sorgoleone in vesicles in the root hair cell, and these are eventually deposited between the plasmalemma and the cell wall at the root hair tip (Czarnota et al. 2001, 2003a; Weston et al. 2012) (Fig. 11.5). The porous root hairs exude sorgoleone in copious amounts through their tips, producing up to 1 mg sorgoleone/g

fresh weight of roots (Czarnota et al. 2003a, b; Rimando et al. 1998). This excretion or extrusion is believed to be a passive process and driven by continued production and accumulation of sorgoleone in the root hair cell (Dayan et al. 2009; Weston et al. 2012).

Current models propose that small organelles or vesicles transport newly synthesised secondary metabolites such as sorgoleone to other storage compartments or to the plasma membrane for efflux (Battey and Blackbourn 1993; Grotewold 2004). This is also the case with *Sorghum* spp. producing sorgoleone. However, as sorgoleone is cytotoxic due to its capacity to inhibit cellular processes such as respiration, its separation from the symplast by transportation using membrane-bound vesicles allows for safe transport around the cell (Bertin et al. 2003). Like other toxins produced by plant roots (Grotewold 2004; Grotewold and Davies 2008; Weston et al. 2012), sorgoleone is synthesised in a similar region from which vesicles originate in the cell, so careful coordination of vesicle loading can occur. Microscopic studies using light, SEM and TEM techniques indicate deposition of large quantities of these globules in the apoplast and extrusion through the plasmalemma of the root hair itself (Fig. 11.6). Since sorgoleone and related hydroquinones are largely non-polar, transport through lipid bilayers in the plasmalemma is facilitated. Root hairs typically exude these droplets throughout their lifetime, which generally consists of several days to several weeks. Exudation has been noted as early as 3–4 h following seed germination and radical elongation in sorghum seedlings (Czarnota et al. 2003a; Weston et al. 2012), and rate of exudation is dependent on environmental factors including plant stressors (Dayan et al. 2009).

Dayan et al. (2009) showed that removal of sorgoleone droplets resulted in the production of additional sorgoleone and continued exudation, suggesting that production of sorgoleone is a dynamic process, as long as the root hair is functional. Not unexpectedly, temperature and environment played a role in sorgoleone production (Dayan et al. 2010) and root hair formation (Yang et al. 2004a, b). Moderate temperatures of 25–35 °C were optimal for maximal production, and high relative humidity coupled with ample oxygen led to functional root hair formation. Root hair formation could be prevented in conditions of very high humidity, at which point low oxygen and higher CO_2 and/or ethylene may result in limited formation of root hairs and thus negligible sorgoleone production. Yang et al. (2004b) also found that sorgoleone production was constitutive and limited to root hairs of sorghum species.

In soil persistence studies performed by Weston, purified sorgoleone applied to soil was easily recovered shortly after application (1 h, 85 % recovery); however, recovery decreased over time, likely due to metabolism of sorgoleone by soil microbes. Metabolism increased in the presence of microbes in comparison to sterile soil conditions. Sorgoleone was detectable in living soils at low levels up to 7 weeks after soil incorporation, and up to two major metabolites were observed, but as yet they remain uncharacterised (Weston et al. 1997). However, preliminary studies indicate that sorgoleone can persist in the soil rhizosphere at concentrations

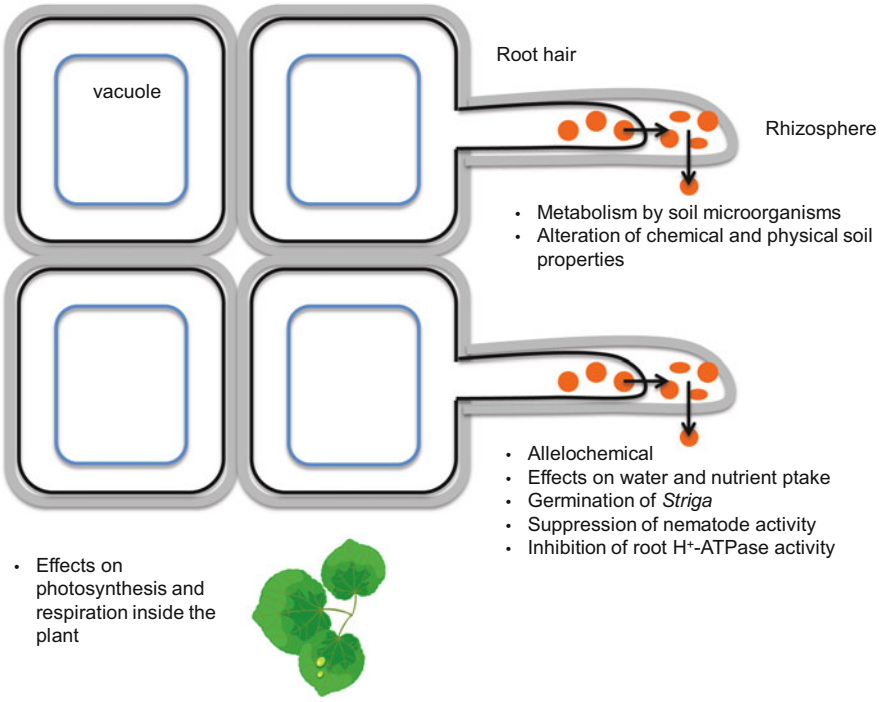

Fig. 11.6 Model for the exudation of sorgoleone from sorghum root hairs. Sorgoleone containing vesicles (*orange*) are formed in young root hairs and fuse with the plasma membrane. They accumulate in the apoplast and are exuded passively into the surrounding rhizosphere where they affect abiotic and biotic soil processes

required for biological activity for days after rhizodeposition or exudation has occurred (Dayan et al. 2009; Loi et al. 2007).

11.9 Future Research Directions

Major gaps in our knowledge of secondary metabolite exudation and function in the rhizosphere, particularly involving allelopathic interactions, include the detailed mechanisms of exudation and the identification of transport mechanisms and transporter proteins specific to secondary product transport. However, this review presents examples of two sets of secondary products, flavonoids and long-chain hydroquinones, in which detailed information regarding production, mode of action, transport and fate has been obtained. Future studies of the regulation of secondary metabolite transport by abiotic and biotic rhizosphere signals will be important to gain additional information on release rates and soil degradation of these metabolites over time. In addition, measurements of actual concentrations of

bioactive metabolites or allelochemicals in real soil environments are largely lacking. This could potentially be accomplished by solid phase root zone extraction using micro-extraction techniques in specific rhizosphere locations to determine spatial and temporal changes in flavonoid exudation (Mohney et al. 2009; Weidenhamer et al. 2009); this has been attempted with some success to measure sorgoleone release by living sorghum roots (Loi et al. 2007; Weidenhamer et al. 2009). Such an approach allows for more precise estimations of catabolism and movement in the soil and localisation around living roots. Biosensors, such as flavonoid or sorgoleone-inducible reporter genes, could also be used to estimate soil concentrations of bioactive metabolites. For example, the *Rhizobium* NodD proteins of various species might prove to be potential biosensors of specific flavonoids around roots.

Furthermore, mutants and transgenic plants with altered secondary product metabolism or exudation will continue to be used to study the effect of metabolites upon rhizosphere organisms and should continue to spawn a new flurry of research in the soil rhizosphere. Both of these approaches have been utilised to determine the biosynthetic pathways involved in production of bioactive metabolites and can be used to study mode(s) of action of these products on soil rhizosphere organisms. Mass spectrometric identification and quantification of secondary products from root exudates could also be used to screen for mutants with altered exudates profiles. Both metabolomic and proteomic profiling will undoubtedly improve our knowledge of exudation processes in higher plants in which exudation under standard laboratory conditions has been well documented, but soil profiling is now potentially feasible even when metabolites occur at ultra low concentrations.

Despite the knowledge we have garnered with specific allelochemicals, large knowledge gaps remain in our understanding of how most secondary products act as allelopathic agents. We believe that it will be important to identify molecular targets of flavonoids in plant and microbial species that are inhibited, thereby unravelling specific mechanisms of how allelochemicals work and how allelopathic plants producing these compounds are protected from autotoxicity. The mechanisms of plant uptake of sorgoleone in mature plants versus germinating seedlings have now been evaluated; however, it remains unclear how flavonoids and most secondary metabolites are taken up by target species (Buer et al. 2007), if uptake varies between species and if transporter proteins are activated for most secondary metabolites as they exit the cell. Once exuded, the modification or metabolism of allelochemicals in the rhizosphere by soil microorganisms (Rao and Cooper 1994) may result in their enhanced biological activity and is an important factor to consider when evaluating potential activity and persistence of any secondary metabolite in living soils.

11.10 Conclusion

Root exudates contain numerous biologically active low molecular weight secondary metabolites that are produced by plants. Due to their physical and biochemical properties, they are able to interact with many diverse targets in subcellular locations to elicit various activities in microbes, plants and animals. We present specific examples of two families of bioactive secondary plant products which have been well documented as allelochemicals and discuss their production and transport in the plants which produce them and their respective roles in the rhizosphere. We focus on flavonoids which play important roles in transport of auxin, root and shoot development, pollination, modulation of reactive oxygen species and signalling of symbiotic bacteria in the legume *Rhizobium* symbiosis. In addition, they possess antibacterial, antifungal, antiviral and anticancer activities. Flavonoids are transported within and between plant tissues and cells by specific transport proteins or transporters and are released into the rhizosphere by roots where they are involved in numerous interactions including allelopathy. Released by root exudation or tissue degradation over time, both aglycones and glycosides of flavonoids and other bioactive secondary metabolites are found in soil solutions and the rhizosphere. We describe their activity and fate in the soil rhizosphere in selected examples involving legumes. Long-chain hydroquinones, in contrast, are lipophilic molecules that are released by passive exudation from living *Sorghum* spp. root hairs and are involved in allelopathic interactions and in chemical signalling causing stimulation of germination by *Striga* spp. They are also thought to be involved in nematicidal activity associated with *Sorghum* haplotypes and are antibacterial to certain soil bacteria. Hydroquinones including sorgoleone are released continuously through pores in the tips of root hairs where they bind to soil particles and organic matter. Once exuded, sorgoleone and its metabolites remain bioactive in the rhizosphere for several days after introduction to living soils. We also discuss the potential for future research to further elucidate the role of secondary metabolites and their fate in the soil rhizosphere.

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